RAPID *DE NOVO* EVOLUTION OF TOLERANCE TO DESICCATION AND ULTRAVIOLET-C RADIATION IN A NON-EXTREMOPHILE: IMPLICATIONS FOR POSSIBLE LIFE ON MARS AND VENUS

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ABSTRACT

Mars was once Earth-like, with habitable environments ranging from benign to extreme. Venus might also have been Earth-like, perhaps having an ocean for two billion years. But today, the surface of Mars is a dry-ice-cold, radiation-soaked, hyper-arid desert with an oxidizing regolith under a hypobaric atmosphere, while that of Venus is a lead-meltingly hot, crushingly hyperbaric desert. Understandably, most astrobiologists consider the surfaces of both Mars and Venus to be uninhabitable. However, if life ever existed on the surface of those worlds, could it have adapted to their drastic environmental change and evolved mechanisms to persist today in Mars's shallow subsurface and Venus's middle to lower clouds? If so, desiccation and, to a lesser extent, ultraviolet-C (UV-C) radiation would exert tremendous selective pressures on any such Martian and Venusian life. While the mechanisms of tolerance to those stressors in bacteria are fairly well understood, the evolutionary dynamics that can produce those tolerances have been largely unexplored. Therefore, I performed an evolution experiment in which replicated populations of a desiccation- and UV-C radiation-sensitive strain of *Escherichia coli* were exposed to daily pulses of either desiccation only, UV-C radiation only, or both stressors combined. Tolerance to those stressors, both separately and combined, evolved within a mere 500 generations. I also hypothesized that cross-tolerance would evolve, i.e., treatment with one stressor would result in a correlated gain of tolerance to the non-treatment stressor, but this hypothesis was rejected. Thus, the evolution of co-tolerance required selection with both stressors combined. My results show that a non-extremophile can readily and rapidly adapt to two Mars- and Venus-relevant stressors. The implications of my findings for our neighboring planets are that life might persist today in Mars's shallow subsurface and in Venus's clouds, provided that the drastic environmental changes that occurred on those worlds allowed adaptation to one or two stressors at a time.

This thesis is dedicated to my family and friends, both living and passed. Thank you for being there when I needed you and cheering me on the whole time. I love and will forever remember you all. And finally, a special dedication to my young kids, Thomas and Natalie. May you never stop wondering at our pale blue dot and the worlds beyond. I love you with everything I am.

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INTRODUCTION

Investigations of Mars's surface via landed spacecraft have shown that it once supported multiple habitable environments, ranging from benign to extreme (Squyres *et al.*, 2004, 2008; Grotzinger *et al.*, 2014, 2015; Hurowitz *et al.*, 2017; Arvidson and Catalano, 2018; Losa-Adams *et al.*, 2021; Vasavada, 2022). But eventually, Mars lost most of its atmosphere (Lammer *et al.*, 2018; Jakosky, 2021; Vasavada, 2022), resulting in a surface that today is a frigid, radiation-soaked, hyper-arid desert with an oxidizing regolith under a hypobaric atmosphere (Gómez-Elvira *et al.*, 2014; Hassler *et al.*, 2014; Lasne *et al.*, 2016; Martínez *et al.*, 2017; Fischer *et al.*, 2019; Lange *et al.*, 2022; Harri *et al.*, 2023; Munguira *et al.*, 2023; Polkko *et al.*, 2023). When exposed to high-fidelity simulations of Mars's present-day surface conditions, some terrestrial organisms, including *Serratia liquefaciens* and *Bacillus subtilis*, can survive for some period; however, they are unable to grow and reproduce (Schuerger and Nicholson, 2006; Hansen *et al.*, 2009; Smith *et al.*, 2009; Berry *et al.*, 2010; Kerney and Schuerger, 2011; Schuerger *et al.*, 2020; Schwendner *et al.*, 2020). Thus, many astrobiologists argue that the surface of Mars is uninhabitable (e.g., Tarnas *et al.*, 2021).

However, if life ever existed on Mars and was present near the surface, then its evolution would have been shaped by a very different environmental history than that of life on Earth (McKay and Stoker, 1989; Clark, 1998; Fairén *et al.*, 2005, 2017; Schulze-Makuch *et al.*, 2005, 2013; Dohm *et al.*, 2011; Davila and Schulze-Makuch, 2016; Cabrol, 2018, 2021; Salvatore and Levy, 2021). This historically contingent evolution would perhaps have facilitated the capacity for Martian life to not only survive, but also grow and reproduce in a specific environment — namely, the shallow subsurface — where sporadic liquid water and permissive temperatures might allow a patchy and

transitory existence (Warren-Rhodes *et al.*, 2006, 2007a, 2007b, 2019, 2022; Schulze-Makuch *et al.*, 2018; Davila *et al.*, 2020).

The environmental history of Venus is opposite that of Mars. Whereas Mars's surface went from being Earth-like to a dry-ice-cold, hypobaric desert after losing most of its atmosphere, Venus's surface went from being possibly Earth-like to a lead-meltingly hot, crushingly hyperbaric desert after its atmosphere suffered a runaway greenhouse effect (Petropoulos and Telonis, 1988; Lammer *et al.*, 2018; Gillmann *et al.*, 2022; Westall *et al.*, 2023). While the surface of Venus is now uninhabitable, its lower to middle cloud layers might be conducive to an aerial biosphere (Cockell, 1999; Schulze-Makuch *et al.*, 2004, 2013; Grinspoon and Bullock, 2007; Limaye *et al.*, 2018, 2021a, 2021b; Bains *et al.*, 2021, 2023; Schulze-Makuch, 2021; Seager *et al.*, 2021; Westall *et al.*, 2023). Therefore, as with the plausible, historically contingent evolution of hypothetical Martian life that I put forth above, Venusian life, if it ever existed, might have adapted from living on a once-habitable surface to persisting solely in the clouds.

Desiccation and, to a lesser extent, ultraviolet-C (UV-C) radiation would exert intense selective pressures on any life that might persist in Mars's shallow subsurface (Hansen *et al.*, 2009; Smith *et al.*, 2009; Berry *et al.*, 2010; Schulze-Makuch *et al.*, 2018; Warren-Rhodes *et al.*, 2019; Davila *et al.*, 2020) or Venus's middle clouds (Schulze-Makuch *et al.*, 2004, 2013; Bains *et al.*, 2021, 2023; Limaye *et al.*, 2021b; Seager *et al.*, 2021). The mechanisms of tolerance to desiccation and UV-C radiation in bacteria are fairly well understood (Krisko and Radman, 2010, 2013a, 2013b; Slade and Radman, 2011; Lebre *et al.*, 2017; Esbelin *et al.*, 2018; Greffe and Michiels, 2020; Laskowska and Kuczyńska-Wiśnik, 2020; Daly, 2023). However, the evolutionary dynamics that can produce those tolerances have been largely unexplored. I therefore carried out an evolution

experiment in which I propagated replicate populations of *Escherichia coli* under substantial stress from desiccation and UV-C radiation, both separately and in combination.

MATERIALS AND METHODS

Ancestral strain and growth conditions

I performed the evolution experiment starting from two sub-strains, REL606 and REL607, of *Escherichia coli* strain B (Lenski *et al.*, 1991; Daegelen *et al.*, 2009). These bacteria are strictly asexual, lacking plasmids or functional bacteriophages (Lenski *et al.*, 1991). Lenski *et al.* (1991) derived REL607 from REL606, which are nearly identical to each other, the key difference being that REL607 has a mutation conferring the ability to catabolize the sugar L-(+)-arabinose (Ara^+), whereas REL606 does not (Ara^-). These sub-strains are easily distinguished from one another by the color of their colonies when plated on nutrient-rich agar supplemented with arabinose and the redox indicator tetrazolium chloride (TA agar [Levin *et al.*, 1977]): colonies of REL607 (Ara^+) appear white on TA agar, while those of REL606 (Ara^-) appear red. This phenotypic marker permits direct measurement of fitness through evolved-vs-ancestor competition assays (see below, competitive fitness assays and cell density measurements). Moreover, the marker has repeatedly been shown to be selectively neutral in medium limited by glucose and other sugars, as well as in various other environmental conditions (Lenski, 1988; Bennett *et al.*, 1992; Travisano *et al.*, 1995; Travisano and Lenski, 1996; Turner *et al.*, 1998; Rozen and Lenski, 2000; Ostrowski *et al.*, 2005; Sleight and Lenski, 2007; Izutsu and Lenski, 2022).

Cells were grown at pH 7 in Davis minimal (DM) broth, which is formulated as follows: 0.7% potassium phosphate dibasic trihydrate; 0.2% potassium phosphate monobasic (anhydrous); 0.1% ammonium sulfate; 0.05% sodium citrate tribasic dihydrate; 0.01% magnesium sulfate (anhydrous); 0.0002% thiamine hydrochloride; and 0.04% D-(+)-glucose (Lenski, 1988). The magnesium sulfate, thiamine, and glucose were added to the autoclaved base medium from 0.2-µm-filtered solutions. Cells grew in 800-µL cultures in 96-deep-well plates at 37°C while orbitally

shaken at 475 rpm. The 0.04% glucose in the DM broth limited the ancestral strains' population density at stationary phase to $3.1 \times 10^8 \pm 1.7 \times 10^7$ cells mL^{-1} (95% confidence interval), and thus $2.5 \times 10^8 \pm 1.4 \times 10^7$ total cells per culture.

Treatment and control populations

Four groups of six replicate populations evolved for 500 generations (75 daily cycles) under one of four conditions: desiccation only, UV-C radiation only, both stressors combined, or no stress (control). Half of the populations comprising each group were founded by clones of REL606 and the other half by clones of REL607; each population started from a separate colony, with each colony the outgrowth of a single haploid cell. Therefore, the replicate populations were identical at the beginning of the experiment, ensuring that selection would act on *de novo* mutations and not standing genetic variation (Lenski *et al.*, 1991; Izutsu and Lenski, 2022; Lenski, 2023).

The half-and-half founding of populations allowed me to account for any effects of the arabinose-utilization marker on fitness; no such effects occurred. I archived all the evolving populations in 15% glycerol at −80°C after 50 generations, 100 generations, and every 100 generations thereafter. The REL606 and REL607 ancestral strains remained in cryogenic storage during the experiment. Aliquots of both the ancestral and evolved samples were later revived and used to assess changes in the fitness of the evolved populations relative to their ancestors (see below, competitive fitness assays and cell density measurements).

Procedures for desiccation and UV-C irradiation

I developed the following procedure for the desiccation-treated populations: I took 8-µL aliquots of cultures grown for 22 – 24 h in DM broth, spotted them onto the bottom of 5-mL borosilicate glass beakers, and dried them at 37°C for 1 h in an air-tight plastic box containing anhydrous calcium sulfate (8-mesh granules of Drierite; WA Hammond Drierite, Xenia, Ohio, USA). The

aliquots were visibly dry after 45 min; hence, the cells were in a desiccated state for at least 15 min. After desiccation, the cells were re-hydrated by adding 800 µL of fresh DM broth to each beaker and then orbitally shaken at 120 rpm for 15 – 30 min at room temperature (20 – 24°C) to re-suspend them. After re-hydration and re-suspension, I transferred the 800 µL of broth in each beaker to a well in a 96-deep-well plate, and the cultures were grown until the next day under the conditions described above. The survival rate of the ancestors under these conditions was 1.2% ± 0.3% (95% confidence interval). Figure 1(a) illustrates my desiccation procedure.

My procedure for the UV-C radiation-treated populations was similar to that for the desiccation-treated populations: 8-µL aliquots of cultures grown for 22 – 24 h in DM broth were spotted onto the bottom of 5-mL borosilicate glass beakers and then irradiated at room temperature (20 – 24°C) with 254-nm-wavelength light using a Spectroline model EF–140C lamp (Spectronics, Melville, New York, USA). After irradiation, the cells were re-suspended, without shaking, in 792 µL of fresh DM broth. I then transferred the resulting 800 µL of broth in each beaker to a well in a 96-deep-well plate, and the cultures grew alongside the desiccation-treated populations until the next day. The survival rate of the ancestors under these conditions was 2.5% ± 1.1% (95% confidence interval). Figure 1(b) shows my irradiation procedure.

Each UV-C radiation-treated population was spotted, irradiated, and then re-suspended before proceeding to the next population to prevent any evaporation of the 8-µL aliquot. Populations treated with UV-C radiation only were irradiated for 16 sec, whereas those subjected to the combined desiccation and UV-C radiation treatment were irradiated for 8 sec. The 4-Watt lamp was positioned 4 cm directly above each aliquot, giving radiation doses of ~4 and 2 J cm^{-2}, respectively. This halving of the dose was necessary to ensure adequate numbers of surviving cells in the dual-treated populations after sequential exposure to both stressors. Dual-treated populations

were first irradiated as described and then desiccated under the same conditions and for the same amount of time as those treated with desiccation only. These populations were grown in a 96-deep-well plate alongside the other stressed populations until the next day.

Compensation for a stress-induced bottleneck

During the evolution experiment, the stress-treated populations experienced an additional bottleneck when the aliquots were exposed to the stressor(s) that the non-stressed control populations did not experience. To account for those conditions, after 22 – 24 h of growth, I diluted the control populations in fresh, glucose-free DM broth such that 1% of each population would be transferred to the next round of growth. Aliquots from the dilutions were then transferred to separate wells of fresh DM broth in a 96-deep-well plate and grown alongside the stress-treated populations until the next day. This dilution ensured that the control populations' effective survival rate — and hence the impact of random drift caused by the population bottleneck — was close to that of the stress-treated populations.

Procedural controls

My experiment also included procedural controls to detect any contamination that might have occurred during the stress procedures and daily transfers to the 96-deep-well plates. I placed uninoculated DM broth in the outermost wells of the 96-deep-well plates, immediately adjacent to the wells containing the stress-treated and control populations. The procedural control wells went through the same stress procedures and daily incubations as the experimental populations. For example, aliquots from the procedural control wells next to the desiccation-treated populations underwent the same desiccation procedure and were then incubated alongside those populations. None of my procedural controls showed any contamination during the evolution experiment, as determined by the absence of colonies when I spread samples on TA agar plates. In addition, I

alternated Ara^+ and Ara^- populations in the wells of the 96-deep-well plates to monitor for possible cross-contamination events. Cross-contamination did not occur during the experiment, as verified by the lack of red colonies on TA agar plates of Ara^+ populations and the lack of white colonies on TA agar plates of Ara^- populations.

Competitive fitness assays and cell density measurements

I performed competitive fitness assays using mixtures of the evolved and ancestral cells having the opposite arabinose-marker states. Thus, evolved populations derived from REL607 (Ara^+) were mixed with the ancestral REL606 strain (Ara^-), and vice versa. Each fitness assay began by inoculating from their frozen stocks 16 µL of the evolved population in 800 µL of DM broth and 16 µL of the appropriate ancestral competitor in a separate 800 µL of broth. These cultures grew alongside each other for 24 h as described above, and they were then transferred to another pair of separate 800-µL volumes of broth for a second 24 h of growth. These two days of separate growth allowed the bacteria, after being revived from the frozen samples, to acclimate to the environment in which they would compete. After the second day's growth, aliquots of the two competitors were mixed in equal culture volumes (providing approximately equal numbers of evolved and ancestral cells) for the competition.

The two days of growth were necessary not only to allow the cells to recover from being frozen, but also to allow them to consume all the glycerol used as a cryoprotectant. The presence of glycerol could adversely impact the fitness assays and their interpretation in two ways. First, residual glycerol has been shown to alter the growth dynamics of *E. coli* (Atolia *et al.*, 2020). Second, a variety of organisms, albeit mostly eukaryotes, use glycerol and its derivatives as an osmoprotectant (Borowitzka and Brown, 1974; Adler *et al.*, 1985; Somero, 1992; Roberts, 2005; Yobi *et al.*, 2013; Raymond *et al.*, 2020; Khan *et al.*, 2023), and supplemental glycerol can be used

to alleviate stress from low water activity (e.g., Sriram *et al.*, 2011). Therefore, residual glycerol might provide some protection to *E. coli* during the desiccation process.

I performed the competitions by subjecting aliquots of the evolved-ancestor mixture to the relevant stress and then re-grew the mixed culture as described above and illustrated in Figure 1. Cells from the competition mixture were plated on TA agar at three time points to enumerate colonies for the fitness calculations described below. First, a dilution of the competition mixture was plated to determine the initial densities of the evolved and ancestral cells (diluted from N_R in Figure 1). Second, the stress was applied to an aliquot of the mixture, and the cells in that aliquot were re-suspended as described above. A dilution of the re-suspended cells was plated, again on TA agar, before placing the culture of re-suspended cells in the incubator for re-growth, to determine the densities of evolved and ancestral cells after the stress (diluted from N_S in Figure 1). Third, and lastly, a dilution of the re-grown culture was plated to determine the densities of evolved and ancestral cells after their recovery from stress (diluted from N_G' in Figure 1). I used the numbers of evolved and ancestral colonies from these three samples to calculate fitness values for the entire stress–growth cycle and for the two separate fitness components, as described next.

Fitness components and their calculations

I calculated fitness components from two non-overlapping periods of the competitions that I call "survival" and "growth". The survival component spanned the period between the first and second platings described above. The growth component spanned the period between the second and third platings. I then summed the survival and growth components to give "total" fitness.

Each fitness component was calculated as a selection-rate differential (Lenski *et al.*, 1991; Travisano *et al.*, 1995) per daily stress–growth cycle. Whereas the more commonly encountered relative fitness (W) expresses the *ratio* of the competitors' realized growth rates, the selection-rate

differential (r) is the *difference* in their realized growth rates. The selection-rate differential is more appropriate under two conditions, both of which are relevant to my study: (i) when the net growth rate of one or both competitors is negative, as it generally was during the survival phase; and (ii) when the difference in fitness between the competitors is large (Travisano *et al.*, 1995), as was the case when the ancestor competed against the stress-evolved populations in a stressful environment. I used the following three equations to calculate the selection-rate differentials for the survival (r_S) and recovery (r_G) fitness components, and then for the total fitness (r_T):

$$r_S = [\ln(E_s / E_i) - \ln(A_s / A_i)] / \text{day};$$

$$r_G = [\ln(E_g / E_s) - \ln(A_g / A_s)] / \text{day};$$

$$r_T = (r_S + r_G) / \text{day};$$

where E_i, E_s, E_g and A_i, A_s, A_g are the densities of the evolved (E) and ancestral (A) cells estimated from the three successive platings, respectively.

Statistical analyses

I performed 4 replicate competition assays for each of the 6 endpoint evolved populations in each treatment and control group, and I did so under each of three assay conditions: desiccation stress, UV-C radiation stress, and the unstressed control environment. Thus, I performed 288 competition assays (4 treatments × 6 populations × 4 replicate assays × 3 conditions), and each assay provided 3 estimates (864 total estimates): survival, growth, and total fitness. I analyzed the results as 9 sets of data, partitioned by the 3 estimates and the 3 assay conditions. For example, survival of the 24

evolved populations (4 treatments × 6 populations) when exposed to desiccation forms one set of data, their growth when exposed to that same stressor is a second dataset, and their total fitness under that same condition constitutes the third set.

I performed the statistical analyses using the SAS software, version 9.4 (SAS Institute, Cary, North Carolina, USA). This analysis involved three steps. First, I tested for outliers in each group using Cook's distance (Cook, 1977) and by visually inspecting plots of residuals. I identified a value as an outlier (i) if its Cook's distance was > 0.38 and it was evident in the plots of residuals, or (ii) if it was clearly present in the plots of residuals (e.g., a discontinuous bin in the histogram), even if its Cook's distance was < 0.38. I based the Cook's distance threshold on equation MC from Kim and Storer (1996). Only 6 of the 864 (0.7%) fitness estimates were judged to be outliers, and none of the sets of 4 replicate assays had multiple outliers. In order to maintain the balanced design, I replaced the value of each outlier with the median of the three other replicate measurements of the population to which it belonged.

Second, I checked if each group's residuals were normally distributed using Shapiro–Wilk's test (Shapiro and Wilk, 1965) and by visually inspecting plots of residuals. If a test was significant at the 95% confidence level and that result was evident in the plots of residuals, then I performed a Box–Cox power transformation (Box and Cox, 1964, 1982) of the entire fitness data set. When I performed this transformation, the group means from the transformed data were used in place of those from the untransformed data for an analysis of variance (ANOVA), which I describe next.

Third, and finally, I performed a one-way Welch's ANOVA (Welch, 1951) of group means for each of the 9 datasets using the MIXED procedure (SAS, 2015). I used Satterthwaite's approximation (Satterthwaite, 1946) to determine the denominator degrees of freedom. If the Welch's ANOVA was significant at the 95% confidence level, then I made *post hoc* comparisons

of each treatment group's mean to that of the control group using Welch's *t* test (Welch, 1938). I adjusted the *P* values of the three, two-tailed Welch's *t* tests for multiple comparisons using Dunnett's T3 correction for heterogeneous variances (Dunnett, 1980).

To determine whether the mean total fitness of the treatments varied, I performed a one-way Welch's ANOVA of the population means for each of the 12 combinations of treatment and assay condition. For example, I ran one ANOVA for the total fitness of the desiccation-treated populations when not stressed (control condition), a second ANOVA for the total fitness of the same populations when exposed to desiccation, and a third ANOVA for their total fitness when exposed to UV-C radiation. These ANOVAs were carried out in the same way as described for the ANOVAs of group means, but without *post hoc* comparisons.

Because Welch's ANOVA and Welch's *t* test are intended for unequal variances, I did not perform a preliminary test for homogeneous variances. In fact, I expected the variances between treatment groups to be heterogeneous due to the severity of the stresses from UV-C radiation and desiccation (relative to the benign control treatment) and the potential for small deviations in my procedures (e.g., slight variations in drying time) to have large effects. When there is a reasonable expectation of heterogeneous variances, an unconditional analysis is more robust against Type 1 errors, with little loss of power, than an analysis that is conditioned on whether the variances are homogeneous (Moser *et al.*, 1989; Moser and Stevens, 1992; Ruxton, 2006; Hayes and Cai, 2007; Derrick *et al.*, 2016; Delacre *et al.*, 2019).

Comparisons of treatment and control groups, but not among treatments

As I mentioned above (in the sub-section on procedures for desiccation and UV-C irradiation), the survival rates of the ancestors when stressed by desiccation and UV-C radiation were 1.2% ± 0.3% and 2.5% ± 1.1%, respectively. The lower bound of the radiation survival rate's 95% confidence

interval overlaps the upper bound for the desiccation survival rate, but it does not overlap the mean estimate of the desiccation survival rate. Thus, the difference in survival rate between the two stressors is significant.

Because of that significant difference in the ancestors' survival rate, it would not be appropriate to compare one treatment group's level of fitness to that of another treatment group. Consequently, I only compare the various stress treatments with the control group, and all my conclusions about changes in survival, growth, and total fitness are relative to the evolved control group when exposed to the same conditions as the treatment groups. Although I intended to equalize the ancestors' survival rates when stressed by UV-C radiation and desiccation, that effort was stymied by the idiosyncrasies of my irradiation apparatus and procedures, most notably the manual on/off switching of the UV lamp, which led to substantial variation in survival (as indicated by the wide 95% confidence interval).

RESULTS

Fitnesses of the evolved groups when stressed by desiccation

Figure 2(a) shows that the control populations, as a group, had no significant change in their survival when stressed by desiccation (i.e., the corresponding 95% confidence interval includes 0). Both the desiccation group and the dual-stressed group (i.e., evolved with desiccation and radiation stresses combined) achieved significant gains relative to the ancestor in their survival when exposed to desiccation. By contrast, the radiation group experienced a significant loss in survival under desiccation. In all three treatment groups, those changes are significant not only relative to the common ancestor (as shown by the confidence intervals), but also in comparison to the control group (as indicated by the asterisks).

Figure 2(b) shows that all four groups, including the control, grew faster than the common ancestor. This result is not an indication of adaptation to desiccation *per se*; rather, as I will show later, it simply reflects faster growth in the culture medium used in all the treatments. After accounting for multiple statistical comparisons, neither the desiccation group nor the dual-stressed group grew faster than the control group, although both treatment groups trended in that direction. However, the radiation group's significant loss in survival was offset by significantly faster growth than that of the control group.

Total fitness represents the sum of the survival and growth fitness components. Figure 2(c) shows that both the desiccation and dual-stressed groups had gains in total fitness that were significantly greater than the control group. The radiation group showed no significant difference in total fitness relative to the control group, as the differences in survival and growth components offset one another.

Fitnesses of the evolved groups when stressed by UV-C radiation

Figure 3(a) shows that neither the control group nor the desiccation group showed any significant change relative to the common ancestor in survival when stressed by UV-C radiation. However, the radiation and dual-stressed groups had highly significant gains in survival relative to the common ancestor as well as when compared to the control group. Moreover, as shown in Figure 3(b), only the radiation group had significantly faster growth than the control group.

Figure 3(c) shows that the radiation and dual-stressed groups had gains in total fitness that were significantly greater than those of the control group. The desiccation group had a total fitness gain relative to the ancestor that was slightly, but significantly, less than that of the evolved control group. This deficit in the desiccation group's total fitness resulted from the combination of small differences in survival and growth.

Fitnesses of the evolved groups when not stressed

Figure 4(a) shows that the evolved control group's survival did not differ from the ancestor under the control conditions. Furthermore, none of the evolved treatment groups differed from the control group in this respect. Figure 4(b) reveals that the control group grew significantly faster than the common ancestor, which demonstrates adaptation to the culture medium, as expected. Unexpectedly, both the radiation and dual-stressed groups not only grow faster in the control environment than the ancestor, but they also show significantly greater gains in this fitness component than the control group. By contrast, the desiccation group exhibited smaller gains than the control group.

In terms of total fitness, the residuals for the desiccation group were not normally distributed, perhaps in part due to heterogeneity among the replicate populations (as examined in the next sub-section), and therefore the data were transformed (as explained in the sub-section on statistical

analyses). After transformation, all four evolved groups had higher total fitness in the non-stressed environment than the common ancestor, as can be seen in Figure 4(c). The radiation group had slightly, but significantly, higher total fitness than the control group, whereas the desiccation group had significantly smaller gains than the control group. The dual-stressed group's gains were indistinguishable from the control group.

Variation among the replicate populations in each evolved group

All the analyses above examined average fitness values for a group of evolved populations; any heterogeneity among the six replicate populations within a treatment group was therefore simply a source of statistical noise. Figures 5(a) – 5(c) show the total fitness relative to the common ancestor for the 24 evolved populations (4 treatments × 6 populations) individually in each of the three assay environments. Thus, I can visualize the variation among the replicate populations in each environment and thereby assess the consistency of their evolutionary responses. Importantly, I replicated the fitness assays for each population, and therefore I could perform ANOVAs to test whether there was significant variation among the populations within a treatment group.

Overall, six of the 12 ANOVAs show significant variation among the replicate populations, as indicated by the asterisks above the set of populations in each treatment group. Thus, genetic diversity in the evolutionary responses was an important outcome of the experiment. At the same time, this visualization of the data provides compelling evidence of the specificity of adaptation to the stressful treatments. As Figure 5(a) shows, all 12 populations that experienced daily desiccation events during the evolution experiment (i.e., 6 populations in the desiccation group and 6 in the dual-stressed group) had average total fitness in the desiccation environment (direct response) that was greater than the grand mean of the control populations (shown by the dashed line). In the case

of the radiation-only-treated populations, half of them had average total fitness in the desiccation environment (correlated response) greater than the grand mean of the control group.

Like the desiccation-treated populations' direct response to desiccation, Figure 5(b) shows that all 12 populations that experienced daily exposures to UV-C radiation (i.e., 6 populations in the radiation group and 6 in the dual-stressed group) had average total fitness in the radiation environment (direct response) that was greater than the grand mean of the control group. On the other hand, all but one of the desiccation-only-treated populations had lower average total fitness in the radiation environment (correlated response) than the grand mean of the control populations.

In contrast to the populations in the stressed environments, the pattern in the unstressed control environment is less clear, as shown in Figure 5(c). The three treatment groups that evolved in stressful conditions include individual populations with average fitness values both above and below the grand mean of the evolved control populations. This ambiguity presumably reflects the fact that the culture medium was the same in all four treatments, so that adaptation to that medium was important in all cases.

In summary, the population-level analyses generally support the group-level results reported in the previous sections, which demonstrated adaptation to the specific stresses of desiccation and UV-C radiation. At the same time, the population-level analyses reveal significant variation among the independently evolved populations in most groups in their direct response to the environment where they evolved, in their correlated responses to other environments, or both.

A serendipitous finding of differences in cell pellets

Toward the end of the evolution experiment, I noticed that the cells of all 12 populations in the desiccation and dual-stressed groups formed diffuse pellets after settling overnight in their spent growth medium, as seen in the upper two rows of Figure 6. By contrast, as seen in the lower two

rows, the populations in the radiation and control groups formed condensed pellets, with the possible exception of one population in the radiation-treated group (second from left) that seems to have an intermediate phenotype. Thus, this diffuse-pellet phenotype appears to have evolved as a specific response to desiccation. I have not yet further explored this phenotype.

DISCUSSION

Hypotheses on the evolutionary dynamics of tolerance to desiccation and radiation

My experiment was designed to test four broad hypotheses concerning the evolutionary dynamics of tolerance to desiccation and radiation.

(1) The treatment groups that had daily exposures to desiccation and UV-C radiation separately would evolve increased tolerance to the corresponding stressor, while the dual-stressed group would evolve increased tolerance to both stressors. The increased tolerance would be manifest by each treatment group having greater gains in total fitness than the evolved control group, when fitness was measured by competitions against the common ancestor in the corresponding stress environment.

(2) The groups treated with only one stressor would have correlated gains in total fitness when exposed to the non-treatment stressor, and thus cross-tolerance would evolve, e.g., the desiccation group would also gain tolerance to radiation. I based this hypothesis on two observations: (i) desiccation and UV-C radiation cause similar damage to cells, primarily through oxidation of proteins (França *et al.*, 2007; Fredrickson *et al.*, 2008; Krisko and Radman, 2010, 2013a, 2013b; Slade and Radman, 2011; Santos *et al.*, 2013; Kragh and Truelstrup Hansen, 2020; Daly, 2023; Łupkowska *et al.*, 2023), and (ii) some organisms exhibit unusually high tolerance to both of these stressors (e.g., members of the bacterial genus *Deinococcus* [de Groot *et al.*, 2005; Yang *et al.*, 2009, 2010; Slade and Radman, 2011; Dong *et al.*, 2015; Liu *et al.*, 2017]).

(3) The greater gains in total fitness in the treatment groups relative to the control group would be driven by improved survival during exposure to the stressors, rather than by improved growth. All the treatment and control populations were propagated in the same culture medium, and

therefore all of them had similar opportunities to adapt to that medium. Consequently, adaptation to the medium would be evident by improvement, relative to the ancestor, in the growth component of fitness, and improvement beyond that of the evolved control group would suggest specific adaptation to the stressor. In the case of survival, any improvements in that fitness component would indicate specific adaptation to the stressor. I based this hypothesis of survival being key to evolving tolerance on previous research (cited immediately above) that suggests protective mechanisms, often mediated by the proteome during acute exposure to stressors, are central to tolerating desiccation and radiation, including UV-C, in microbes.

(4) Tolerance to desiccation and UV-C radiation would incur trade-offs, i.e., correlated losses in total fitness, when exposed to benign (control) conditions. The trade-off would be manifest in one of two ways: (i) each treatment group's total fitness grand mean would be negative relative to the ancestor, or (ii) most of each treatment group's total fitness population means would be less, but not necessarily negative, than the evolved control group's total fitness grand mean, when fitness was measured by competitions against the common ancestor in the benign environment. I derived these two trade-off criteria from concepts laid out in the works of Lenski and Travisano (1994), Novak *et al.* (2006), and Bennett and Lenski (2007). I based this hypothesis of correlated losses when not stressed on the fact that similar kinds of trade-offs have been seen in nature (Luo *et al.*, 2008; Chang and Leu, 2011; Friman *et al.*, 2013; Porter and Rice, 2013; Comont *et al.*, 2020; Yang *et al.*, 2023), and they also often arise in evolution experiments (Mongold *et al.*, 1996; Sleight *et al.*, 2006; Bennett and Lenski, 2007; Caspeta and Nielsen, 2015; Boyd *et al.*, 2022; Ballu *et al.*, 2023; Wang *et al.*, 2023).

All treatment groups evolved increased tolerance

Figures 2(c) and 3(c) show that all the treatment groups increased their total fitness more than the evolved control group when stressed, and thus they evolved tolerance to the corresponding stressor(s). This result agrees with my first hypothesis. Furthermore, this *de novo* tolerance to desiccation and UV-C radiation evolved within 500 generations, a mere blink of the eye in evolutionary time. Further still, it is important to point out that the dual-stressed group evolved tolerance to the combined stressors within the same number of generations as those groups treated with only one of the stressors. And this rapid evolution of tolerance occurred in a non-extremophile that was very sensitive to those stressors. Taken together, these observations suggest that tolerance to desiccation and UV-C radiation can readily evolve, and it is possible for a non-extremophile to evolve tolerance to more than one harsh stressor at the same time.

Figure 5(a) shows that there was significant variation among total fitness of the desiccation group's populations when stressed by desiccation, suggesting that there were multiple evolutionary paths to desiccation tolerance. Therefore, it is likely that multiple mechanisms exist between those populations for tolerance to desiccation, be it fitness components involving both direct and indirect responses or changes in the genome, physiology, biochemistry, etc. Figures 5(a) – 5(c) reveal that the heterogeneous variation among total fitness of the desiccation group's populations existed under all conditions. Future studies are needed to explore how the different types of mechanisms might be coupled. For example, one could address whether more similar paths based on fitness components are also more similar in terms of the underlying genetic and/or physiological changes.

Conversely, Figure 5(b) shows the lack of significant variation among total fitness of the radiation group's populations when stressed by UV-C radiation, suggesting that there was only one, or very limited, evolutionary path(s) to UV-C radiation tolerance. Consequently, it is likely

that a single mechanism, or very similar ones, exists between those populations for tolerance to UV-C radiation. It can be seen in Figure 5(a) that the homogeneous variation among total fitness of the radiation group's populations also existed under desiccation, but Figure 5(c) reveals it was heterogeneous under benign (control) conditions.

Regarding the dual-stressed group, Figures 5(a) – 5(c) show that its populations' total fitness did not vary significantly under any condition. When that group was stressed separately by desiccation and UV-C radiation, the lack of variation among its populations' total fitness indicates that, like evolution under radiation only, there were very limited, or perhaps a single, evolutionary path(s) to dual-tolerance. Thus, as I suggested for tolerance to UV-C radiation, dual-tolerance probably involves very similar mechanisms, or maybe just one. The dynamics of *de novo* tolerance to desiccation and UV-C radiation described immediately above leads me to suspect that the evolution of dual-tolerance was confined by the apparently limited path(s) to UV-C radiation tolerance. In other words, the multiple paths to achieving desiccation tolerance might have been more difficult hikes than the main trail leading to radiation tolerance.

To my knowledge, this is the first published study to experimentally evolve tolerance to desiccation in a prokaryote. However, desiccation tolerance has been experimentally evolved repeatedly in more than one species of fruit fly (Hoffmann and Parsons, 1989; Gibbs *et al.*, 1997; Tejeda *et al.*, 2016). Tolerance to UV-C radiation has been experimentally evolved multiple times in different bacteria (Alcántara-Díaz *et al.*, 2004; Wassmann *et al.*, 2010; Selveshwari *et al.*, 2021; Ellington *et al.*, 2023). And in one experiment, Begyn *et al.* (2020) evolved endospores under stress from UV-C radiation and found that they became even more recalcitrant, but the vegetative cells remained sensitive.

Cross-tolerance did not evolve, but an unexpected trade-off did evolve

Figures 2(c) and 3(c) show that neither the desiccation group nor the radiation group had statistically significant correlated gains in total fitness relative to the evolved control group when exposed to the non-treatment stressor, and therefore cross-tolerance did not evolve. Nonetheless, both of those groups had gains relative to the ancestor. Tolerance to both stressors required the combined treatment of the dual-stressed group. This result refutes my second hypothesis. And it is also surprising, not only because there are numerous examples of organisms that are highly tolerant of both desiccation and UV-C radiation (e.g., members of the genus Deinococcus, as cited above), but as I discuss next, an unexpected trade-off occurred between those two stressors.

Figure 3(c) reveals that the desiccation group incurred a trade-off in total fitness when stressed by UV-C radiation. I determined that this trade-off occurred based on the second criterion given above in my fourth hypothesis, whereby most of the desiccation group's population means fall below the evolved control group's grand mean when exposed to radiation, as can be seen in Figure 5(b). Five of the 6 population means are below the evolved control group's grand mean, and 4 of those 5 have 95% confidence intervals that do not include the control's mean. In the case of the radiation group under desiccation, Figure 2(c) shows that it did not incur a trade-off. This dynamic of the desiccation group incurring a trade-off while the radiation group did not, suggests that, under the conditions of my experiment, the *de novo* evolved mechanisms for tolerance to desiccation and UV-C radiation differed considerably.

Survival mechanisms were key to desiccation tolerance, but not radiation tolerance

Figure 2(a) shows that the desiccation group had a significant gain in the survival component of fitness when stressed by desiccation, both relative to the ancestor and to the evolved control group. However, Figure 2(b) shows that its gain in the growth component was significant relative to the

ancestor, but not to the evolved control group. Therefore, mechanisms for better surviving the stressor were key to evolving desiccation tolerance. Figures 2(a) and 2(b) and Figures 3(a) and 3(b) reveal that survival mechanisms were also key for the dual-stressed group's evolution of tolerance to desiccation and UV-C radiation.

When the radiation group was stressed by UV-C radiation, Figures 3(a) and 3(b) show that it had significant gains, relative to the evolved control group and the ancestor, in *both* the survival and growth components of fitness. Thus, mechanisms for better surviving the stressor *and* recovering from it contributed to the evolution of UV-C radiation tolerance. This result refutes my third hypothesis, as I thought that survival mechanisms would be key for evolving tolerance to both stressors, separately and combined.

When the radiation group was stressed by desiccation, Figure 2(a) reveals that it incurred a trade-off in survival, according to the first criterion stated above in my fourth hypothesis, whereby the group has a negative mean value relative to the ancestor. However, Figure 2(b) shows that the trade-off was offset by a trade-up in growth. Nevertheless, it can be seen in Figure 2(c) that the offsetting gain in growth was not enough to provide cross-tolerance to desiccation, though there was a gain in total fitness relative to the ancestor. Figures 3(a) and 3(b) show that this dynamic did not play out with the desiccation group when stressed by UV-C radiation, although Figure 3(a) reveals that there was a statistically non-significant loss in survival relative to the ancestor.

Desiccation, but not radiation, tolerance incurred a trade-off under benign conditions

Figure 4(c) reveals that the evolution of desiccation tolerance came with a trade-off under benign conditions, as per my second criterion. As Figure 5(c) shows, all but one of the desiccation group's total fitness population means fall below the grand mean of the evolved control group, and of those 5 populations, 4 of them have 95% confidence intervals that do not include the control's mean.

Moreover, half the populations are less fit than the ancestor, and another one's fitness is the same as that of the ancestor. Figure 4(b) shows that the desiccation group's trade-off in the benign environment was due to its growth component of fitness being significantly less than that of the evolved control group, but nonetheless slightly better than the ancestor.

Conversely, Figure 4(c) reveals that the evolution of UV-C radiation tolerance came with a trade-up under benign conditions, as its total fitness is significantly greater than that of the evolved control group. Figure 4(b) shows that this trade-up in the benign environment was due to a significant gain in the radiation group's growth component of fitness relative to the evolved control group. It can be seen in Figure 4(b) that, like the radiation group, the dual-stressed group had a significant gain in growth under benign conditions. Nevertheless, Figure 4(c) shows that this gain was insufficient to give a trade-up in that environment, perhaps due to a slight loss in survival (Figure 4(a)). And concerning survival, Figure 4(a) shows that all the treatment groups had slight losses in that fitness component relative to the ancestor, while the evolved control group's survival remained the same as the ancestor. These results partially refute my fourth, and final, hypothesis, as I thought that all stress-treated groups would incur trade-offs when not stressed.

Implications of my study for possible extant life in Mars's shallow subsurface

Researchers have experimentally evolved bacteria to tolerate 6 of the harshest stressors existing in the shallow subsurface of Mars: desiccation (this study); UV-C radiation (Alcántara-Díaz *et al.*, 2004; Wassmann *et al.*, 2010; Begyn *et al.*, 2020; Selveshwari *et al.*, 2021; Ellington *et al.*, 2023; this study); ionizing radiation (Wright and Hill, 1968; Licciardello *et al.*, 1969; Davies and Sinskey, 1973; Parisi and Antoine, 1974; Ewing, 1995; Harris *et al.*, 2009; Lim *et al.*, 2009; Byrne *et al.*, 2014; Bruckbauer *et al.*, 2019, 2020); hypobaria (Nicholson *et al.*, 2010); lowered temperature (Mongold *et al.*, 1996); and freeze–thaw–growth cycles (Sleight and Lenski, 2007;

Kwon *et al.*, 2018). Those experiments have established that non-extremophiles can easily evolve tolerance to Mars-relevant stressors, at least individually. Importantly, my evolution experiment establishes, I believe for the first time, that not only can desiccation tolerance readily evolve, but also that a non-extremophile can quickly adapt to *two* harsh stressors at the same time.

The surface of Mars is a hyper-arid desert (Martínez *et al.*, 2017; Fischer *et al.*, 2019; Polkko *et al.*, 2023), where the relative humidity can be < 5% during the day and near 100% at night, depending on the season and latitude. Nighttime highs of 10% to 30% are typical throughout most of the Martian year, except for the north polar region, where typical highs are 30% to 60%. Those levels of relative humidity and their daily fluctuation would impose severe desiccation stress on any life that might exist near Mars's surface. Nevertheless, two environments exist on Earth where life persists under extremely arid (and other) conditions that approach those of Mars, the Atacama Desert's hyper-arid core (Davila *et al.*, 2021; Azua-Bustos *et al.*, 2022; Warren-Rhodes *et al.*, 2022) and Antarctica's McMurdo Dry Valleys (McKay *et al.*, 2017; Salvatore and Levy, 2021; Warren-Rhodes *et al.*, 2022). A number of studies of those two Mars analogs have demonstrated that desiccation would be a very intense selective pressure on possible Martian life near the surface (e.g., Sun, 2013; Schulze-Makuch *et al.*, 2018).

Mars's surface is soaked in radiation (Gómez-Elvira *et al.*, 2014; Hassler *et al.*, 2014), with ultraviolet radiation ~4 times that of Earth. Yet there exist terrestrial organisms that could tolerate that level of radiation, e.g., the aforementioned *Deinococcus* species. My evolution experiment and those by Wassmann *et al.* (2010) and a few other researchers (cited above) showed that tolerance to UV-C radiation can easily evolve in radiation-sensitive microbes. Furthermore, Godin *et al.* (2023) found that *Bacillus subtilis* had a 10% survival rate under broad-spectrum UV radiation when covered by just a dusting (0.3 mm thick) of Mars's regolith, and Mancinelli and

Klovstad (2000) and Schuerger *et al.* (2003) had similar results with *B. subtilis* endospores covered by a mere 0.5 mm-thick dust layer. Other researchers have reported that a few millimeters, or even a fraction of a millimeter, of regolith or rock could be enough to attenuate UV-C and broad-spectrum UV radiation to survivable levels (Cockell *et al.*, 2005; Gómez *et al.*, 2010; Schuerger *et al.*, 2012; Mickol *et al.*, 2017; Carrier *et al.*, 2019). Further still, individual regolith grains (Osman *et al.*, 2008) and small-scale geomorphology (Moores *et al.*, 2007) could partially shield microbes from UV radiation. Thus, UV-C radiation would likely be sterilizing only in the uppermost millimeter of Mars's surface.

The evolvability of co-tolerance to desiccation and UV-C radiation, or to any two stressors, would have been highly advantageous as Mars's climate changed drastically. But today, all 6 Mars-relevant stressors to which tolerance has been experimentally evolved are concurrent in Mars's shallow subsurface (as opposed to the deep subsurface where conditions are thought to be Earth-like [Tarnas *et al.*, 2021]), and their combined effects would exert tremendous selective pressures on extant life that might exist there. However, while at first this environment, with its multitude of severe stressors, might seem inhospitable, the results of my evolution experiment and those just cited lead me to hypothesize that, if Mars's drastic climate change allowed adaptation to *one or two stressors* at a time, then Martian life, if it ever existed, might persist today in the shallow subsurface.

Implications of my study for possible extant life in Venus's clouds

The relative humidity of Venus's lower to middle cloud layers is < 0.4% (Hallsworth *et al.*, 2021). Therefore, like Mars, extreme desiccation would exert immense selective pressure on life that might exist in Venus's clouds. Ionizing and UV-C radiation do not penetrate Venus's middle cloud layer (Dartnell *et al.*, 2015; Patel *et al.*, 2022). Thus, unlike Mars, possible Venusian cloud life

would likely face only minor, intermittent selection from UV-C and ionizing radiation, as these stressors would act only when, or if, cells are lifted to the higher cloud layers.

The highly concentrated sulfuric acid droplets within Venus's potentially habitable cloud layers have a pH less than zero (Grinspoon and Bullock, 2007). Hence, severe acidity would be another intense selective pressure on possible Venusian cloud life, as it is those droplets that life would inhabit. Nevertheless, at least two chemical mechanisms that could reduce the sulfuric acid droplets' severe acidity have been proposed. Rimmer *et al.* (2021) suggested that a pH of 1 to 2 could result from the reaction of sulfur dioxide gas dissolving in acidic droplets containing hydroxide salts. The other mechanism, put forth by Bains *et al.* (2021), builds on that of Rimmer *et al.* (2021) by suggesting that dissolved sulfur dioxide gas reacts with, in particular, ammonium salts in the acidic droplets, resulting in a pH of –1 to 1.

If those chemical mechanisms indeed take place in Venus's clouds, then notwithstanding the tremendous desiccation, there exist terrestrial extreme acidophiles that could persist in the acidic droplets. For example, the archaeon, *Picrophilus torridus* grows optimally at pH 0.7 and as low as pH –0.06 (Schleper *et al.*, 1995)! Moreover, Seager *et al.* (2023) showed that nucleic acid bases were stable at sulfuric acid concentrations and temperatures that would be encountered in droplets existing in Venus's middle to lower cloud layers. Taken together, these studies indicate that the severe acidity of Venus's cloud droplets might have been an evolutionarily surmountable peak. However, the extreme desiccation of Venus's clouds would have been an even higher and more rugged peak to climb.

CONCLUSIONS

I have demonstrated that a non-extremophile can readily and rapidly adapt to two harsh, Mars- and Venus-relevant stressors simultaneously. However, co-tolerance to desiccation and UV-C radiation combined required treatment with both stressors, as populations that were treated with just one of the stressors did not evolve cross-tolerance to the other one. Furthermore, the desiccation-treated group incurred a trade-off when exposed to UV-C radiation, and when placed in the benign environment of the control group. Improvements in survival were key to evolving desiccation tolerance, while improvements in both survival and growth were significant for UV-C radiation tolerance. I also found that multiple evolutionary paths led to desiccation tolerance, whereas few paths led to UV-C radiation tolerance. Lastly, I serendipitously discovered that desiccation-treated populations form different cell pellets than those of UV-C radiation-treated and evolved control populations, whereby the former form diffuse cell pellets while the latter form typical condensed pellets.

The implications of my findings for our neighboring planets are that life might persist today in Mars's shallow subsurface and in Venus's clouds, provided that the drastic environmental changes that occurred on those worlds allowed adaptation to one or two stressors at a time. This is not to say in any way that *E. coli*, or its experimentally evolved descendants, reflect what microbes might be like on Mars or Venus. I used *E. coli* simply as a powerful and tractable model organism to explore the evolutionary dynamics of *de novo* tolerance to two Mars- and Venus-relevant stressors. To that end, future evolution experiments with non-extremophiles like *E. coli* that simultaneously combine three, or perhaps even four, stressors that are relevant to Mars and/or Venus would significantly expand our understanding of what might have been evolutionarily possible on those planets as their environments became extreme.

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APPENDIX

FIGURES

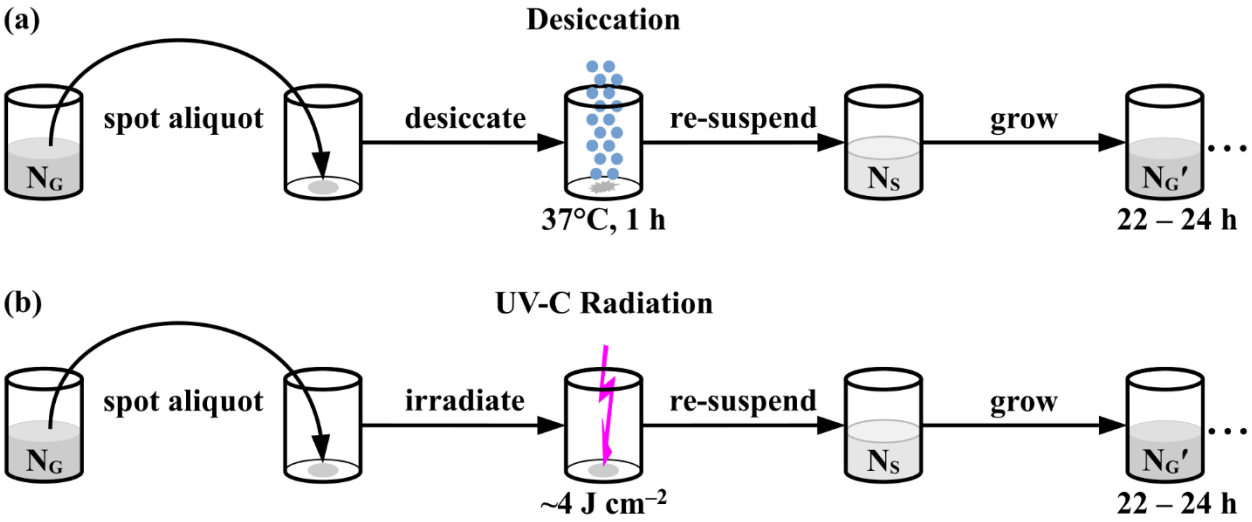

Figure 1: ***Stress–growth procedure.*** The treatment groups underwent daily pulses of stress from desiccation (a) or UV-C radiation (b), or both stressors during the evolution experiment. From left to right, an 8-µL aliquot of an 800-µL culture grown for 22 – 24 h at 37°C was spotted on the bottom of a small glass beaker, subjected to the stressor(s), re-suspended in fresh medium and then re-grown for 22 – 24 h before repeating this procedure (75 cycles total). For the group treated with both stressors, the desiccation conditions remained the same while the exposure to UV-C radiation was halved (~2 J cm^{-2}). Halving the UV-C radiation exposure ensured sufficient survival of that treatment group after being subjected to both stressors (UV-C radiation followed by desiccation). During competitive fitness assays, the cell densities used to calculate fitness were determined at the points in the procedure labeled with an "N" as explained in the text (see Materials and methods, sub-section "Fitness components and their calculations").

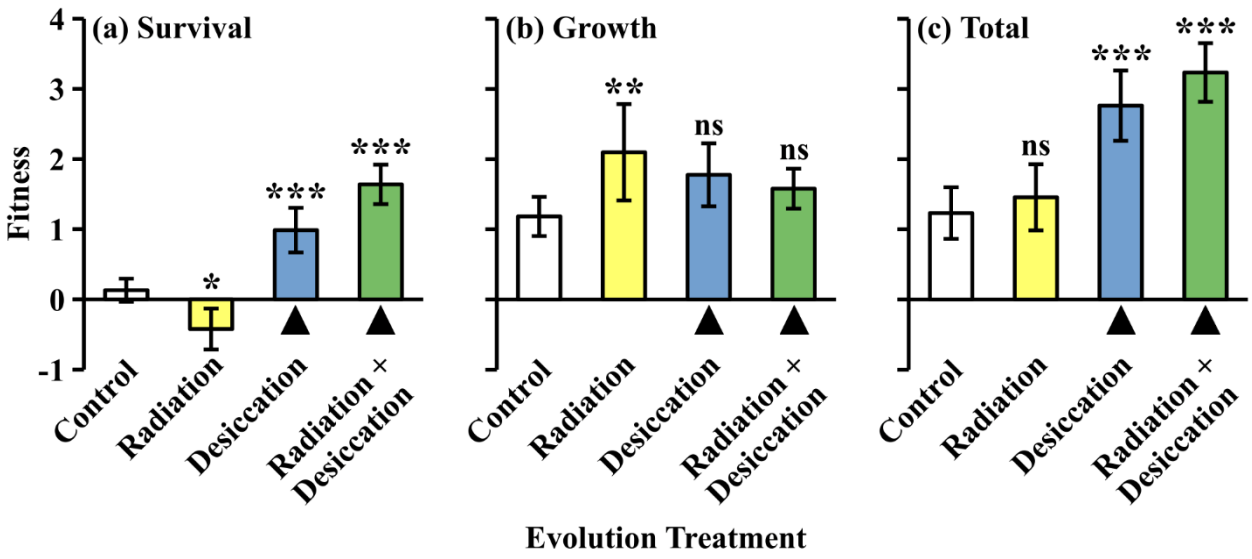

Figure 2: ***Fitness of the evolved groups when stressed by desiccation*.** The survival (a) and growth (b) fitness components were summed to give the total fitness (c). Fitness is expressed as a selection-rate differential, where a value of zero corresponds to the null hypothesis of no difference between the evolved group and ancestor. Triangles indicate that the fitness assay stress, in this case, desiccation, was also the treatment for that respective group during the evolution experiment. The error bars represent 95% confidence intervals about the group mean, where the degrees of freedom are the number of replicate populations in each group, minus one (6 – 1 = 5). Multiple two-tailed Welch's t-tests were performed to compare each treatment group mean to the evolved control group mean, with the number of asterisks signifying the following Dunnett's T3-adjusted *P*-values: *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$; "ns", no significant difference ($P > 0.05$).

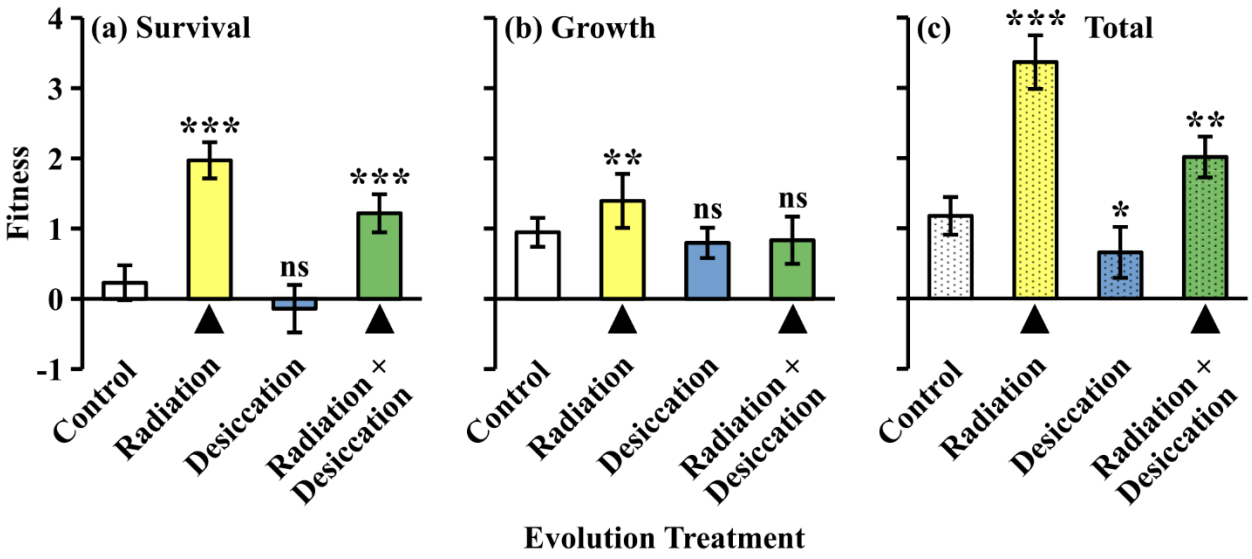

Figure 3: ***Fitness of the evolved groups when stressed by UV-C radiation*****.** The details of the charts are the same as those described in Figure 2's legend. Concerning the total fitness, the desiccation group's residuals were not normally distributed. Thus, I performed a Box-Cox power transformation of the entire total fitness data set. The stippled columns and error bars represent the untransformed data, while the asterisks represent the transformed data that were used to make comparisons of each treatment group's total fitness mean to that of the evolved control group.

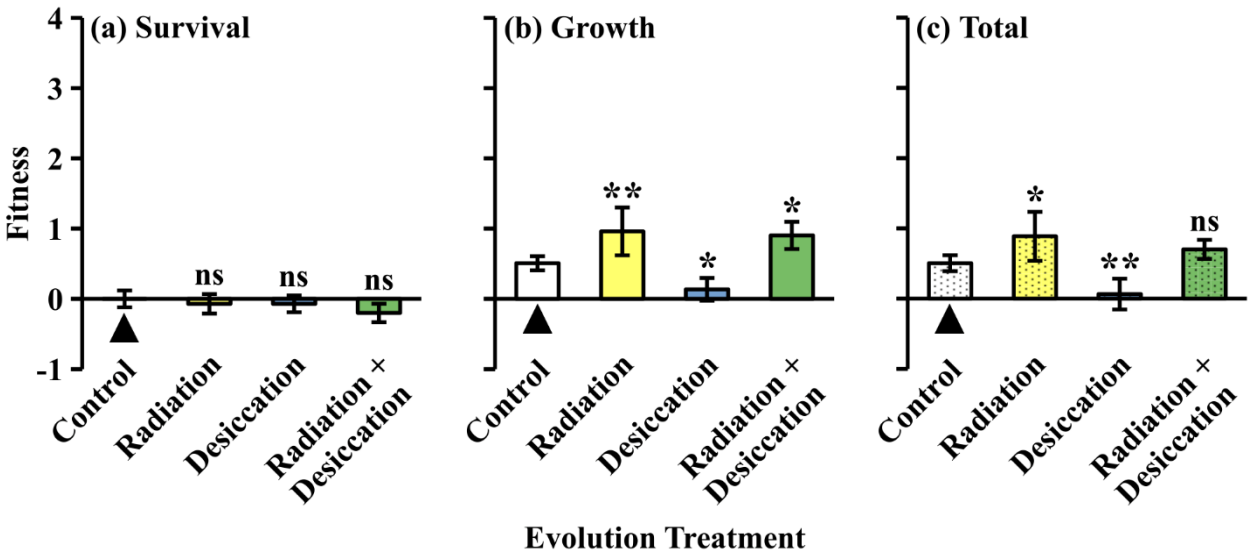

Figure 4: ***Fitness of the evolved groups when not stressed (control conditions)*****.** The details of the charts are the same as those described in Figure 2's legend. Concerning the total fitness, the desiccation group's residuals were not normally distributed. Therefore, I transformed the entire total fitness data set and made comparisons in the same way as described in Figure 3's legend.

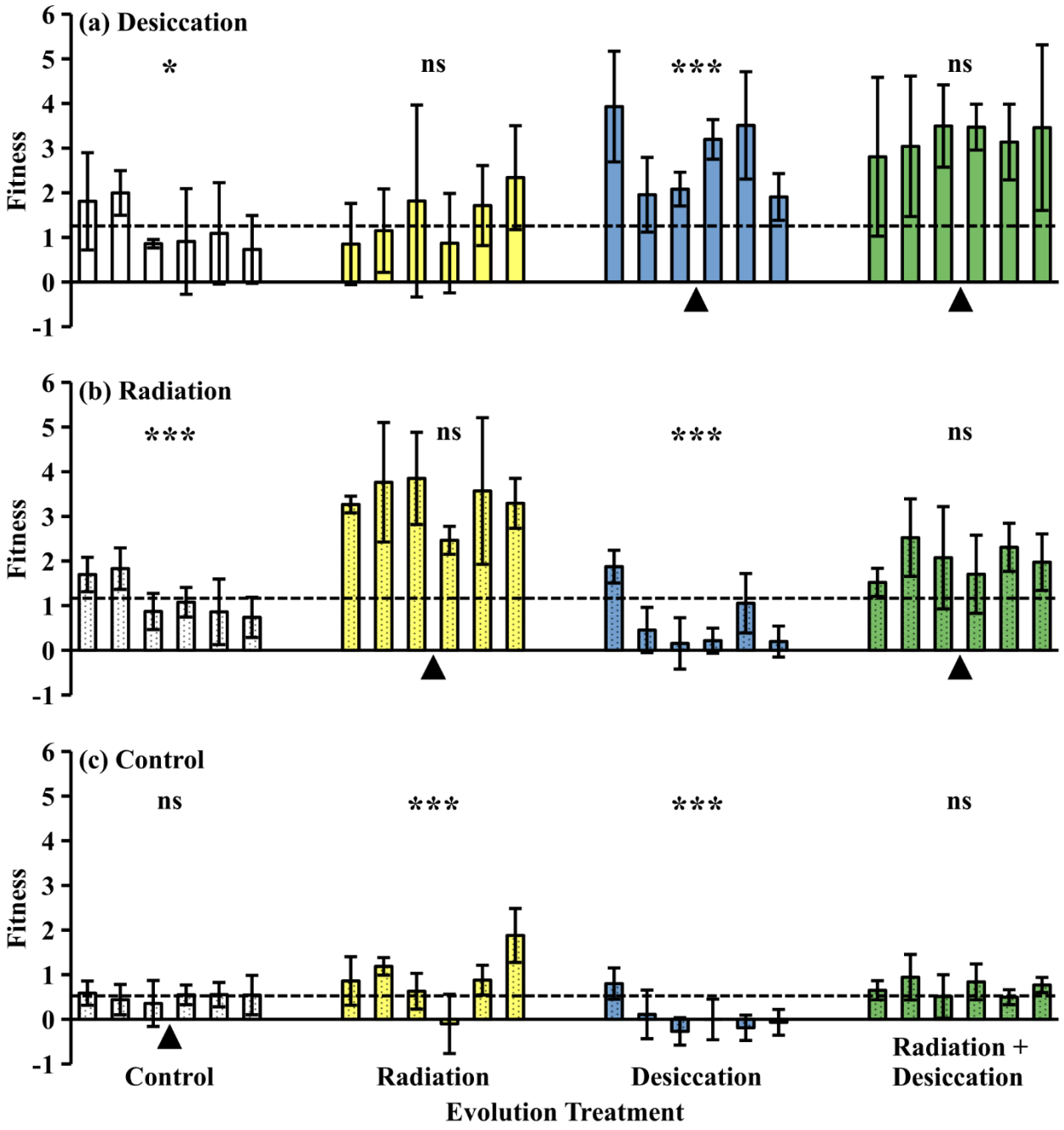

Figure 5: ***Total fitness of the evolved groups' replicate populations.*** The populations' total fitness when stressed by desiccation (a), UV-C radiation (b), and not stressed (c). Triangles indicate that the fitness assay stress was also the treatment for those respective populations during the evolution experiment. The error bars represent 95% confidence intervals about the population mean, where the degrees of freedom are the number of replicate measurements for each population, minus one (4 – 1 = 3). One-way Welch's ANOVAs were performed to determine if the means of populations within a group varied significantly, with the number of asterisks signifying the following one-tailed *P*-values: *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$; "ns", no significant difference ($P > 0.05$). Stippled columns specify that transformed data was used in the ANOVA,

Figure 5 (cont'd)
but the columns themselves and error bars represent the untransformed data. The dashed line marks the mean of the evolved control group.

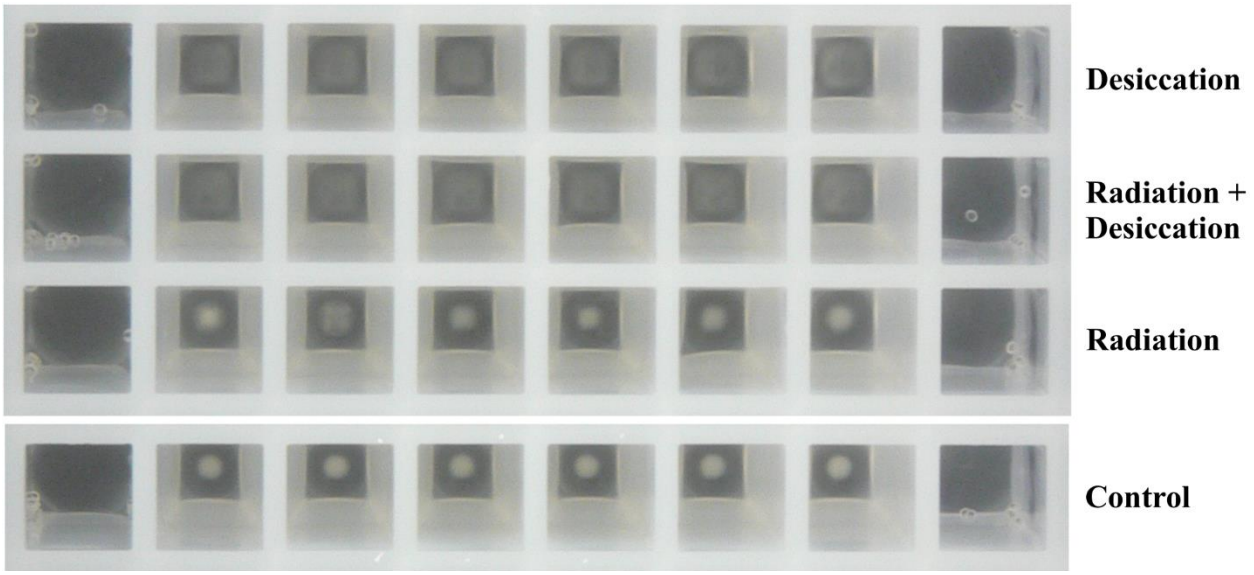

Figure 6: ***Differences in cell pellets*****.** Shown here is a composite image of a single 96-deep-well plate, seen from above, looking down into the wells. A row between the control populations and UV-C radiation-evolved populations was cropped out because that row contained cell pellets of populations not presented in this paper. The outermost wells to the left and right are negative controls (no cells). Populations that evolved under stress from desiccation only and those evolved under stress from desiccation and UV-C radiation combined formed diffuse cell pellets after settling overnight in their spent growth medium, as is evident in the upper two rows. These diffuse cell pellets were not formed by populations that evolved under stress from UV-C radiation only and those evolved under benign conditions (control), instead, those populations formed typical, condensed pellets, as can be seen in the lower two rows.