Adaptive radiation during long-term experimental evolution of the multicellular bacterium, *Streptomyces*

John T Munnoch[1*], Silja Vahtokari[1], Leena Kerr[2] & Paul A Hoskisson[1*],

[1] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow, G4 0RE, UK

[2] School of Energy, Geoscience, Infrastructure and Society, Heriot-Watt University Riccarton, Edinburgh, United Kingdom.

*** Correspondence**: paul.hoskisson@strath.ac.uk & J.t.munnoch@strath.ac.uk

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Data and code

Code and R scripts used in the analysis of the data can be found here https://github.com/PaulHoskisson/StreptomycesLTEE

Supp. data: Available on Figshare https://doi.org/10.6084/m9.figshare.29950388.v1

Abstract

The rapid diversification of a single lineage into novel ecological niches underpins evolution of biodiversity, with adaptive radiations being important drivers of ecological diversity across the tree of life. Experimental evolution has revolutionised our understanding of the fundamental ecological and evolutionary processes in adaptation. To study adaptation in the industrially and ecologically important bacterium *Streptomyces,* a long-term evolution experiment (LTEE) was undertaken in a strain lacking several antibiotic biosynthetic gene clusters, facilitating the study of epistasis in antibiotic biosynthesis. *Streptomyces coelicolor* is a filamentous soil organism that does not undergo sporulation under the LTEE conditions, affording the opportunity to study adaptation under relaxed selection on sporulation. Replicate populations showed parallel loss of sporulation and major morphological shifts in terms of hyphal growth and aggregate formation, where fragmenting hyphae became the dominant phenotype. Catabolic diversification was also evident across the replicate populations with extensive growth rate changes relative to the progenitor across several nutrient sources, where there is evidence for both antagonistic pleiotropy and mutation accumulation. Negative epistasis was predominant in antibiotic biosynthesis in strains from the LTEE. This system provides a foundation for studying diversification and adaption to a novel environment in a physiologically and morphologically complex organism.

Background

Adaptive radiation plays a central role in the emergence of biological diversity [1]. It is thought that ecological opportunity is an important means for generating the phenotypic innovations that allow adaptation into available ecological niches, but the underlying mechanisms that drive this are poorly understood [2–4]. Experimental evolution studies have revolutionised our understanding of the fundamental ecology and evolution of adaptation [1,5–9]. Where the eco-evolutionary dynamics of replicate populations can be highly informative regarding the wider process of adaptation [5,6]. Genetic adaptation to a single environment often results in rapid fitness gains but may result in the loss of fitness in other environments, resulting in ecological specialisation [10]. This adaptative process often leads to a decline in certain traits that are unimportant in the new environment. In this situation, two evolutionary processes appear to predominate: the accumulation of mutations, resulting in genetic drift of those genes not maintained by selection, and antagonistic pleiotropy, such that mutations in one environment may be deleterious in another [10–13]. The underlying evolutionary mechanisms are difficult to distinguish but are likely to be the same regardless of whether they are occurring in the natural environment or via prolonged laboratory culture [14,15]. Moreover, in the natural environment it is increasingly demonstrated that there is interdependency of mutations (epistasis) and this interdependency may act in a synergistic or antagonistic manner [16,17]. This can in part explain the commonly observed phenomenon of the decline in the rate of fitness of certain traits increase over time in natural environments and in microbial evolution experiments [10,17].

The filamentous soil bacteria of the genus *Streptomyces* are prolific producers of a range of bioactive natural products and possess a complex developmental lifecycle [18–20]. The lifecycle of *Streptomyces* begins with the germination of a spore which gives rise to an apically growing hypha, which elongate and branch during growth. In response to nutrient limitation or stress, the developmental process of *Streptomyces* is initiated, where aerial hyphae rise into the air, curl and mature into unigenomic spores through the laying down of septa in the aerial hyphae [18]. Growth in shaken liquid culture prevents sporulation in many species of *Streptomyces*, and their normal growth habit is to form aggregates of hyphae that propagate through apical growth and fragmentation

79 [21,22]. Another remarkable feature of *Streptomyces* biology is that they have large (~8-10 Mbp)
80 linear genomes, with extraordinary catabolic capability [23,24]. *Streptomyces* also possess
81 telomere-like sequences at each end of their linear chromosomes [25] and it is believed that this
82 feature leads to the genetic instability observed in these organisms and results in phenotypic
83 diversity [26–31]. Attempts to explain this genetic instability in terms of the ecology and evolution of
84 *Streptomyces* has recently demonstrated that it can, at least in part, be explained through division
85 of labour in colonies, particularly in structured, terrestrial environments [20,32–34].

86 To further understand adaptation in *Streptomyces,* a long-term evolution experiment (LTEE) was
87 undertaken using the model species *Streptomyces coelicolor* to investigate the role of adaptation to
88 a single environment. Over 3000 generations, replicate populations exhibit extensive parallel loss of
89 sporulation (antagonistic pleiotropy) and negative epistasis in antibiotic production. Major
90 morphological transitions also occurred with parallel fragmentation phenotypes emerging within the
91 populations, suggesting adaption to a homogeneous liquid environment along with widespread
92 catabolic adaptation. This LTEE in *Streptomyces* (*S*LTEE) provides a unique system to investigate
93 the role of morphology and development under conditions that are non-permissive for sporulation,
94 enabling the study of antibiotic production independent of development and in an environment
95 analogous to that used in industry to produce antibiotics.

96 **Methods**

97 **Growth and Maintenance of Bacterial Strains:** The progenitor strain used in this study was
98 *Streptomyces coelicolor* M1152 (Δ*act,* Δ*red,* Δ*cpk,* Δ*cda, rpoB* [C1298T]) [35]. All independent
99 lineages were derived from a single colony clone from a spore stock of this strain. The strain was
100 sequenced at the point the experiment was initiated to ensure exact knowledge of the progenitor
101 genotype (Bioproject: PRJNA1304580, with reads available SAMN50561540). The *Streptomyces*
102 *coelicolor* M1152 genome was constructed *in silico* manually following the publication describing its
103 construction [35] using the *S. coelicolor* M145 reference genome ([23] NCBI: NC_003888.3) and
104 Snapgene (Version 7.2.1). This reference genome was then used to generate the LTEE progenitor
105 reference genome (Bioproject: PRJNA1304580) following SNIPPY analysis
106 (https://github.com/tseemann/snippy). Final reference genome is available here
107 (https://github.com/PaulHoskisson/StreptomycesLTEE/tree/main/1_Genome_sequencing_M1152_
108 genome).

109 **Growth assays:** Each replicate population of *S. coelicolor* M1152 was grown in 250 ml Erlenmeyer
110 flasks containing 50 ml of YEME medium [36]. Cultures were propagated every 72 hours by
111 transferring 0.5 ml of culture into 49.5 ml of fresh YEME medium, using a serological pipette to avoid
112 size selection of growth aggregates. In the 72-hour growth period, cultures attained stationary phase.
113 Growth of populations and isolates was measured using a TECAN Spark plate reader with
114 populations and isolates first grown in appropriate media overnight (YEME or MM medium). Samples
115 were then washed and normalised before being placed in the plate reader in 24 well plates for 24h.
116 Growth rate was then calculated using the formula $\mu = (\ln OD_2 - \ln OD_1) / (t_2 - t_1)$.

117 **Diversity measurement:** Individual clonal samples were obtained for phenotypic examination
118 through plating on to mannitol soya flour agar and individual developmental phenotypes were scored
119 relative to the progenitor strain. Mixed population samples were obtained periodically for future study
120 through freezing each passage of cells (10 ml of culture with 5 ml of 50% glycerol) and these are
121 stored at -80 °C. The Diversity Index (H) was calculated from analysis of 100 colonies plated from
122 each independent population at the indicated generation point using the Shannon-Weaver index
123 equation [$H = (N \log N - \Sigma n \log n_i)/N$].

124 **Microscopy and cell aggregate size determination:** The area of cell aggregates was determined
125 by microscopy, where samples were visualised using a TE-2000 inverted widefield epi-fluorescence
126 microscope with a x20/0.50 numerical aperture objective lens (Nikon, Japan). Phase contrast images
127 were acquired using an ORCA-100 CCD camera (Hammamatsu, Japan) and illumination was
128 provided by a tungsten-halide lamp. Images were acquired using NIS Elements software and data
129 was processed for visualisation using FIJI. Images were scaled (2360 pixels/mm) using a 1 mm
130 graticule (Analyze/Set Scale). Areas were measured by manually drawing round pellets (using
131 freehand drawing tool), measured (Analyze/Measure) and these images flattened and saved
132 (Image/Overlay/Flatten). R scripts were used to visualise the data.

133 **Metabolic capability:** Catabolic capability of the *S*LTEE populations was determined using
134 OmniLog analysis with Biolog plates PM1 (Biolog, 12111). Strains were grown from stocks in liquid
135 culture for 24 hrs at 30°C. Biomass was collected by centrifugation, washed in PBS and added to
136 inoculation fluid IF-0a (Biolog 72268) to an $O.D_{600}$ of 0.04, to create the initial cell suspension.
137 Experimental inoculation fluid (per 24 mL) was prepared using 20 mL stock of IF-0a solution, with
138 the addition of the metal ion cocktail (1.2 mL; containing 5 mM each: $ZnCl_2$ $7H_2O$, $FeCl_2$ $6H_2O$ $MnCl_2$
139 $4H_2O$, $CaCl_2$ $2H_2O$, filter sterilised). Dye Mix D (0.24 mL; Biolog, 74224) and 1.2 mL of sterile distilled
140 water were added to 2.32 mL of cell suspension to give a final volume of 24 mL. This was inoculated
141 into PM1 plates and incubated at 30°C for 2 days in the OmniLog instrument. Data were extracted
142 using the Biolog software (conversion of D5E to OKA: D5E_OKA Data File Converter v1.1.1.15 and
143 extraction of raw kinetic data using PM analysis software: Kinetic V1.3). Data was processed using
144 BactEXTRACT [37], visualised and compared using R scripts
145 (https://github.com/PaulHoskisson/StreptomycesLTEE/tree/main/5_Biolog_populations/). In brief
146 (all files available on github), Biolog data was input into template Tecan data file for 48 hours of
147 growth (171000 seconds). This was uploaded and results generated for the whole 48 hours. All
148 'growthParameters' data was downloaded. Growth curves were visualised using R
149 (https://github.com/PaulHoskisson/StreptomycesLTEE/tree/main/5_Biolog_populations/4_BactExtr
150 act) and those exhibiting classical bacterial growth were used

151 (https://github.com/PaulHoskisson/StreptomycesLTEE/tree/main/5_Biolog_populations/3_plots).

152 Supplementary data available here https://doi.org/10.6084/m9.figshare.29950388.v1

153 **Actinorhodin assay:** The actinorhodin biosynthetic gene cluster (BGC) contained on pAH88[35] ,

154 a BAC derived from SCBAC28G1 was introduced periodically into the population via conjugation

155 from the *E. coli* strain ET12567 (*dam, dcm, hsdS*), containing the driver plasmid pUZ8002 to bypass

156 the methyl-specific restriction system of *S. coelicolor* [36,38]. Actinorhodin concentration was

157 determined according to Feeney et al., [36].

158

159 **Results**

160 **The generation of a versatile experimental evolution system for studying adaptive evolution**
161 **and epistasis in antibiotic production in *Streptomyces***

162 To investigate how adaptation and epistasis influence antibiotic production, a Long-Term Evolution
163 Experiment (LTEE) with the super host strain *Streptomyces coelicolor* M1152 was initiated (**Fig. 1A**).
164 This strain of *S. coelicolor* used is deficient in production of the blue antibiotic actinorhodin (ACT),
165 the red antibiotic undecylprodigiosin (RED), the calcium dependent antibiotic (CDA), the yellow
166 polyketide antibiotic coelimycin and has an engineered mutation in to *rpoB* to enhance production of
167 antibiotics (Δ*act,* Δ*red,* Δ*cpk,* Δ*cda, rpoB* [C1298T]) [35]. Sequencing and SNIPPY analysis of the
168 progenitor strain *Streptomyces coelicolor* M1152 strain (Bioproject: PRJNA1304580) identified 114
169 mutations compared to the published *Streptomyces coelicolor* M145 strain [23]. Analysis of the
170 mutations in *Streptomyces coelicolor* M1152 indicate that 38 are intergenic mutations, 17 are non-
171 synonymous, 39 are synonymous mutations and 15 cause frameshifts in coding sequences.

172 Serial passage of three independent parallel populations of the strain in rich medium (YEME), with
173 each passage taking 72 hours (17 generations in the progenitor strain) results in each culture
174 experiencing nutrient sufficiency and nutrient limitation over the course of each passage. This
175 resulted in the cultures reaching stationary phase during every passage, such that the physiological
176 triggers for antibiotic production are experienced by the culture. The deletion of the four main natural
177 product biosynthetic gene clusters (BGCs) results in relaxed selection on antibiotic production,
178 reducing the demand on the cellular pool of metabolites for production of antibiotics. In liquid culture
179 *S. coelicolor* cannot sporulate [39] resulting in relaxed selection on the sporulation too in this
180 experiment. At specific points in the *S*LTEE the ACT cluster was reintroduced into the evolved strains
181 to study epistasis in antibiotic biosynthesis. *Streptomyces* are normally propagated via the collection
182 of spores from mature plate grown cultures [36]. Following repeated passage of the replicate
183 populations via liquid transfer, cultures were periodically plated on to sporulation permissive medium
184 to monitor sporulation in the populations and the identification of developmental mutants.

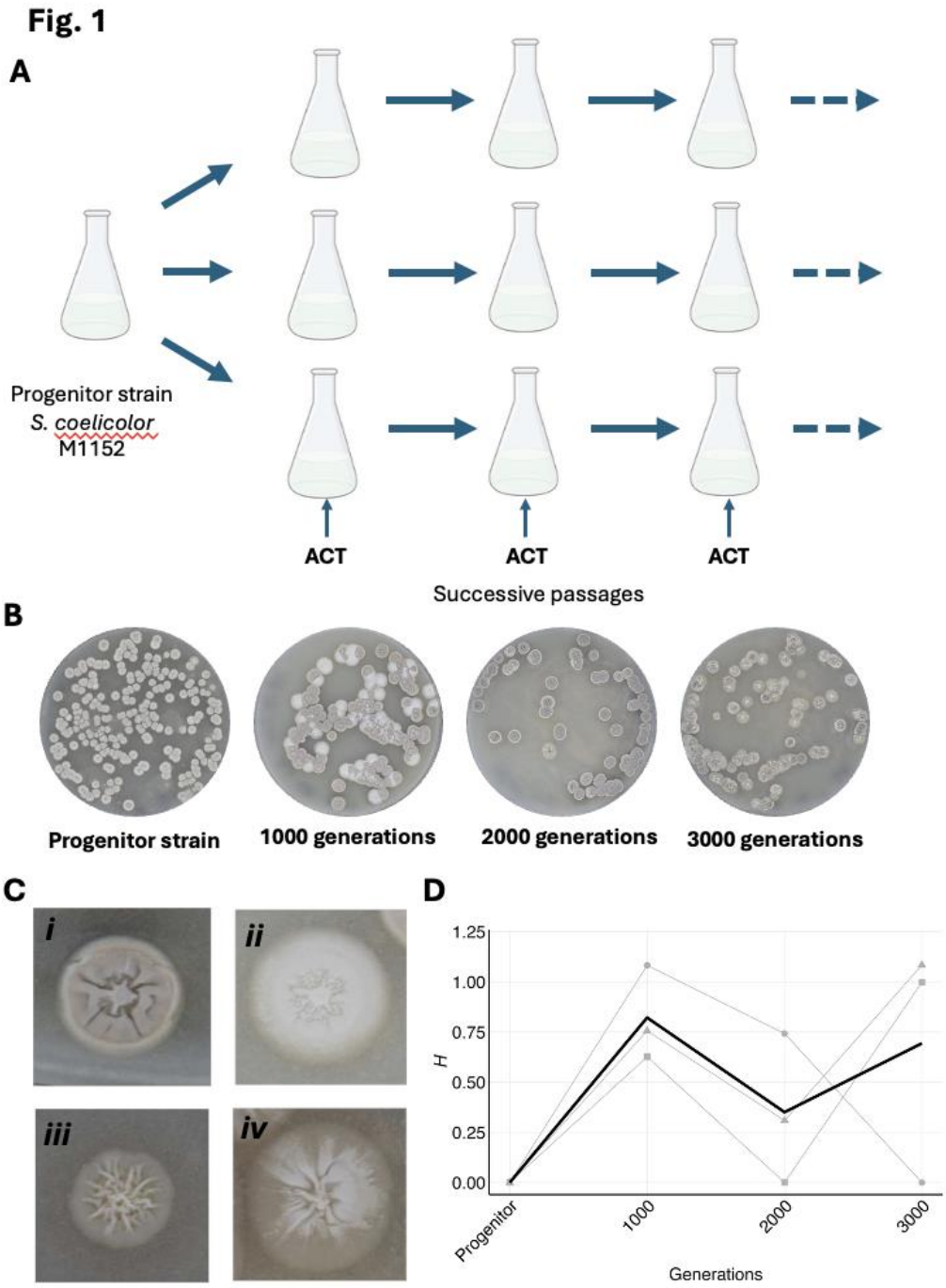

Figure 1: Adaptive radiation of *Streptomyces coelicolor* during long-term experimental evolution(LTEE). A). Experimental set-up of the LTEE showing successive passage of *S. coelicolor* M1152, followed by periodic reintroduction of the actinorhodin (ACT) biosynthetic gene cluster. **B).** Representative plates demonstrating the phenotypic variation in colony morphotypes in *S. coelicolor* M1152 strains. Replicate populations were founded from a single ancestral grey, sporulating colony and propagated over 3000 generations in YEME medium **C).** Major morphological mutation and transitions occur in the LTEE over 3000 generations and strains can be assigned to four major phenotype groups. ***i*)** Sporulating colonies, phenotypically identical to the progenitor strain ***ii*)** White-aerial hyphae formed, but no spores ***iii*)** Bald – no aerial hyphae or spores formed ***iv*)** 'Sectored-spreader' colonies **D).** Colony morphotype diversity during adaptive radiation from the parental grey sporulating morphotype. Diversity was determined through scoring the frequency of morphotypes and using the Shannon-Weaver index. Diversity *(H*) in the three independent replicate populations is indicated by the grey lines at each major generational point, and the black line indicates the mean diversity *(H*) of the three populations.

200 **Phenotypic divergence of *Streptomyces coelicolor* during long-term experimental evolution.**

201 The growth of asexual replicate populations from a single progenitor genotype results in all
202 subsequent genetic variation in the population being generated by mutation [1,40]. The
203 diversification of colony morphotypes within experimental populations of bacteria makes it easy to
204 detect the emergence of heritable genetic diversity [1,41,42]. Given sporulation in *Streptomyces*
205 *coelicolor* is not permitted in liquid cultures [39], it was hypothesised that relaxed selection on this
206 trait through liquid-to-liquid passage of replicate populations would lead to the emergence of
207 developmental mutants. Developmental mutants are easy to identify in *Streptomyces* with distinct
208 developmental stages falling into two main classes, the bald (*bld*) mutants which are unable to form
209 aerial hyphae and the white (*whi*) mutants which are unable to form mature spores and the grey
210 spore pigment [18,39].

211 Morphological mutants arise frequently in the *S*LTEE with similar morphotypes emerging rapidly and
212 in parallel across the replicate lines (**Fig. 1B)**. The morphotypes emerging in the *S*LTEE can be
213 classified into four dominant phenotypic groups *i*) Sporulating colonies, phenotypically identical to
214 the progenitor strain *ii*) White strains - where aerial hyphae are formed, but no spores *iii*) Bald strains
215 that form no aerial hyphae or spores *vi*) and colonies that sectored in appearance **(Fig. 1C-*i-iv*)**. The
216 emergence of classical *bld* and *whi* mutations likely reflect the lack of selection for sporulation in the
217 *S*LTEE and the phenotypes persist within the replicate populations.

218 To quantify the morphotypes in each replicate lineage, the Diversity (*H*) was calculated using the
219 Shannon-Weaver Index [1] **(Fig 1D)**. The homogeneous, grey, sporulating progenitor strain *S.*
220 *coelicolor* M1152 diversifies within 1000 generations to a maximum point, prior to a decline in
221 diversity as phenotypes fix through adaption to liquid culture **(Fig 1D)**. In two of the independent
222 lineages, there is a second round of diversification from 1000 to 2000 generations which is not seen
223 in the third replicate population. This adaptive radiation, followed by selective sweeps of mutants
224 likely leads to the decline in diversity observed in the replicate populations. Whilst this contrasts with
225 observations in homogeneous environments in previous studies using unicellular bacteria [1], the
226 complex hyphal nature of *Streptomyces* likely influences the emergence of diverse morphotypes in
227 liquid culture.

228 **Major morphological transitions occur in *Streptomyces* upon serial passage in liquid culture.**

229 To understand how *S. coelicolor* adapts to a liquid culture environment, the three replicate
230 populations were examined at 1000, 2000 and 3000 generations by microscopy. The progenitor
231 strain **(Fig 2A)** was found to form the large hyphal aggregates characteristic of *Streptomyces* strains
232 in liquid culture [21,22]. Subsequent serial passage of the replicate populations led to an overall
233 decline in the size of the mycelial aggregates **(Fig 2A & B)**, such that at 3000 generations much of
234 the culture consists of hyphal fragments. This fragmenting phenotype likely represents adaptation to
235 growth in liquid cultures to maximise nutrient acquisition. Hyphal aggregates are known to be nutrient
236 limited resulting in increased stress and cell death at the centre of the aggregates
237 [21,22]. Remarkably, the emergence of this fragmenting phenotype appears to be independent of
238 the developmental phenotypes observed at the same number of generations, where fragmentation
239 is not always linked to developmental phenotypes (**Fig. 1B, 1C, 1D & Fig. 2A & 2B)**.

240

Fig. 2

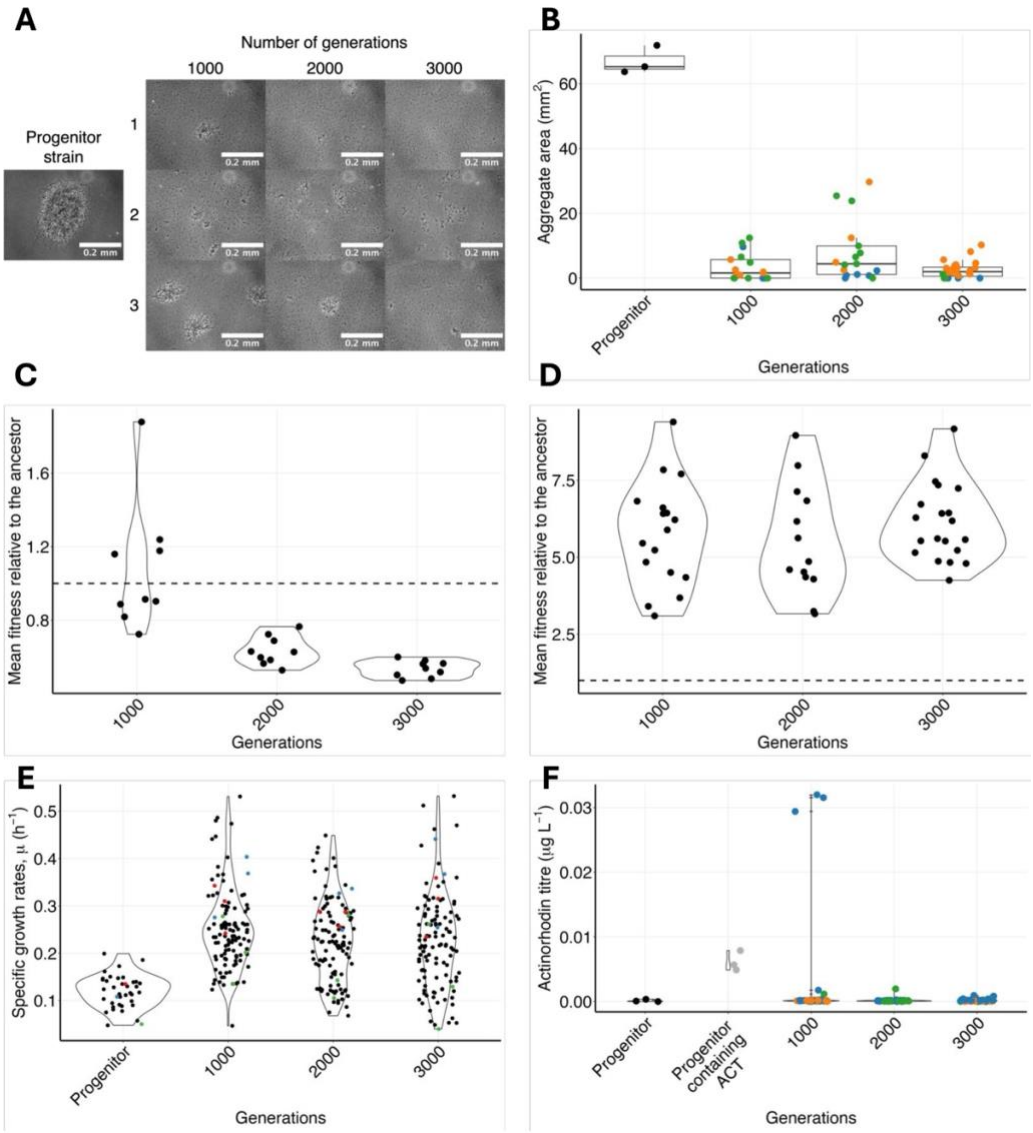

Figure 2: Major metabolic and morphological transitions occur during long-term experimental evolution (LTEE). A). Strains adapt to liquid growth by independently transitioning from an aggregating phenotype in the progenitor to a fragmenting phenotype in all replicate populations. **B).** Quantification of cell aggregate size of *S. coelicolor* M1152 replicate populations shows a decline in mean aggregate area over 3000 generations during adaptation. **C).** Fitness of the replicate populations over 3000 generations. Each point represents the ratio of the growth rate of the evolved population relative to the progenitor strain (*S. coelicolor* M1152) measured in triplicate for each of the three replicate populations. Dashed line represents equivalent fitness. **D).** Fitness of three randomly chosen independent isolates over 3000 generations from the replicate lineages. Each point represents the ratio of the growth rate of the evolved isolate relative to the progenitor strain (*S. coelicolor* M1152) measured in triplicate. Dashed line represents equivalent fitness. **E).** Diversification of carbon source utilisation in the replicate populations over 3000 generations. Each point represents the growth of one of the replicate populations on a particular carbon source in the LTEE. Three carbon sources were chosen to illustrate different usage profiles as the population adapts to the medium through changes to specific growth rate, μ (h^{-1}; Further examples in the supplementary data). • glucose; • malate; • pyruvate **E).** Negative epistasis is pervasive in ACT production in the LTEE. The progenitor strain *S. coelicolor* M1152 makes no actinorhodin, this is restored when the ACT BGC is reintroduced to the strain. The majority of strains that had the ACT BGC show reduced actinorhodin production relative to the progenitor strain containing ACT. Colours represent independent isolates – three isolates from three independent replicate populations.

262 **Relative growth rate increases in individual isolates during adaptation in *Streptomyces***

263 In previous bacterial LTEEs an increase in growth rate has been shown to account for much of the
264 fitness gains observed at the early stages of adaptation [14,43]. To determine if the specific growth
265 rate (μ) increases in the *S*LTEE the populations of *Streptomyces,* as a proxy for fitness. Strains were
266 grown in YEME medium and specific growth rates (μ) were calculated, these were then normalised
267 to the specific growth rate of the ancestor (progenitor) strain to calculate strain and population fitness
268 relative to the ancestor (**Fig. 2C & D;** [44]).

269 The mean fitness of the populations at 1000, 2000 and 3000 generations was found to decrease as
270 the populations adapt to the liquid culture conditions (**Fig. 2C**). This likely represents increased
271 competition between strains and suggests that adaptive radiation is occurring withing the cultures.
272 To investigate variation within the populations, independent isolates were selected from each
273 replicate population at 1000, 2000 and 3000 generations and the fitness relative the ancestor
274 determined. Within the isolates, when they are not competing with another strain mean fitness
275 increases steadily relative to the ancestor (**Fig. 2D**). Another notable feature of this growth is there
276 is a general convergence of relative fitness in the strains over 3000 generations.

277

278 **Adaptive diversification of carbon catabolism occurs in the *Streptomyces* LTEE**

279 To understand the process that may be responsible for the increase in growth rate in individual
280 isolates, the progenitor and each replicate population was subjected to growth in Biolog phenotypic
281 microarray PM1 plates. The specific growth rate (μ) was calculated for each population in 95 carbon
282 sources and plotted up to 3000 generations (**Fig. 2E)**. Adaptive radiation in the replicate populations
283 results in diversification of the specific growth rate (μ) in different carbon sources over 3000
284 generations. There is an overall increase in mean growth rate of the replicate populations as they
285 adapt to the medium, for example growth on glucose, malate, proline, and N-acetyl-glucosamine
286 exhibiting higher growth rates than the progenitor strain (**Fig. 2E & Supp. Data 1 & 2**). There are
287 also several examples where replicate populations show decay of catabolic function for a particular
288 carbon source such as pyruvate, tween 40, threonine and glucuronic acid (**Fig. 2E & Supp. Data 1**

289 **& 2 and Supp. Fig. 1**). These data suggest that adaptive diversification is occurring within the
290 populations as strains use different physiological pathways to consume resources exhibiting
291 sympatric diversification within the populations.

292

293 **Negative epistasis is pervasive in antibiotic production during long-term experimental**
294 **evolution.**

295 The use of the *S. coelicolor* M1152 superhost strain [33] in the *S*LTEE affords the opportunity to
296 study epistasis in antibiotic production. The replicate populations in the *S*LTEE are subjected to the
297 nutrient limiting conditions that trigger antibiotic production in cultures [45,46], but lack the metabolic
298 burden associated with biosynthesis. Serial passage of *S. coelicolor* M1152, which lacks the ACT
299 BGC enables direct comparison of ACT production in the progenitor strain and the evolved strains
300 following reintroduction of the ACT BGC. Three randomly chosen isolates from each replicate
301 population at 1000, 2000 and 3000 generations were selected and the ACT BGC was reintroduced
302 to the strains on the integrating plasmid pAH88 [35]. No ACT production was detected in the
303 progenitor strain lacking pAH88 (**Fig. 2F**). Upon the introduction of pAH88 to *S. coelicolor* M1152,
304 ACT production was restored to the strain (**Fig. 2F**). It was found that in the three replicate
305 populations at 1000, 2000 and 3000 generations that reintroduction of the ACT cluster identified
306 pervasive negative epistasis across all strains, with no ACT production apparent except for one strain
307 at the 1000 generation timepoint (**Fig. 2F**).

308

309 **Discussion**

310 Experimental evolution is a powerful tool for exploring the mechanisms and dynamics of evolution
311 [47]. In microbial experimental evolution the large populations coupled with rapid generation times
312 allow the testing of evolutionary theories on long evolutionary time scales [14,47]. *Streptomyces* are
313 excellent subjects for LTEE to study a range of adaptive behaviours given their remarkable hyphal
314 growth habit, complex developmental lifecycle which are adaptations to growth in the terrestrial and
315 ability to produce a plethora of bioactive molecules [20,48]. Growth in liquid culture results in relaxed
316 selection on morphological and developmental traits given that sporulation in *S. coelicolor* does not
317 occur in liquid culture and hyphae do not need to forage in a 3-dimensional environment for nutrients.
318 These conditions are also representative of the fermentation conditions used in industry for the
319 commercial production of antibiotics and other bioactive molecules.

320 Adaptive divergence in heterogeneous environments is well documented in bacteria [1,49,50].
321 Rainey and Travisano [1] demonstrated that a range of morphotypes emerge in *Pseudomonas* when
322 grown in a heterogenous environment, but when cultures were mixed (homogenous environments)
323 many of these morphotypes were absent. The emergence of developmental mutants during culture
324 is well-known and frequent in *Streptomyces* reflecting the pleiotropic nature of many of the
325 developmental genes identified to date, where genes associated with sporulation often also influence
326 antibiotic production [51]. Prolonged liquid culture of *Streptomyces* in chemostats results in the
327 emergence of *bld* mutants [52], although under these conditions, strains were not subjected to the
328 dynamic fluctuations between nutrient sufficiency and starvation, which are key features of batch
329 culture, which are known to trigger antibiotic biosynthesis [45,46] .

330 Reduction in the overall hyphal aggregate size likely relieves the nutrient limitation and mass transfer
331 issues associated with growth of *Streptomyces* as cell aggregates [21,22]. Initially this led to
332 increased fitness in the replicate populations, which appears to provide a trade off against hyphal
333 lifestyle and development, with the parallel emergence of independent fragmenting hyphal and
334 asporulent phenotypes. Whilst rapid growth is not always favoured in selective sweeps, the role of
335 environmental fluctuation is thought to play a major role in the fixation of major phenotypic transitions
336 [53]. The parallel emergence of fragmentation and loss of sporulation reflects phenotypes that are

337 adaptative to the liquid environment when periodic selection for sporulation on solid medium in not
338 encountered. The rapid, parallel emergence of the fragmentation phenotype would also suggest
339 that this phenotype may favour rapid cell growth, and this is observed when individual clones are
340 grown in the absence of competition. It is known that experimental lineages of *Escherichia coli* evolve
341 to grow more rapidly over successive generations through increasing the efficiency of cellular
342 metabolism [54–57]. The fragmenting phenotype of *Streptomyces* does indeed appear to favour
343 more rapid growth at the population level initially (1000 generations) along with the emergence of a
344 range of morphotypes. At the isolate level the divergence in fitness suggests that ecological
345 opportunity may be affording the emergence of the diversity (adaptive radiation) within the cultures,
346 even when strains are adapting to the same conditions [58]. If this is the case, then stochastic
347 mutational accumulation within the replicate populations would likely lead to the appearance of
348 metabolic diversity, and this was apparent in the three replicate lineages. If random mutation and
349 genetic drift was responsible for this, then carbon source utilisation would diversify in a random
350 manner in the replicate populations. If patterns of carbon utilisation displayed parallel changes in
351 replicate populations, it would suggest that antagonistic pleiotropy was occurring in the replicate
352 populations [10]. These data suggest that both forces are likely at play under these conditions, where
353 parallel increases in fitness can be seen for some carbon sources (Antagonistic pleiotropy) and non-
354 parallel declines in carbon source utilisation can also be observed (mutation accumulation).

355 The *S*LTEE was designed to examine the role of epistasis in antibiotic production. This was
356 facilitated using a strain that lacked four of the major antibiotic biosynthetic pathways [35], which
357 allowed for the reintroduction of BGCs at specific points to understand how regulation changes under
358 relaxed selection. Regulation of antibiotic biosynthesis in *Streptomyces* is multi-level, occurring at
359 the global level and at the level of BGCs [45,46]. Upon reintroduction of the ACT BGC, it was found
360 that negative epistasis was predominant, as has been observed previously for certain traits during
361 adaptation [16,59,60]. This negative epistasis was parallel across genomes evolved in the absence
362 of the ACT BGC. These data suggest that antagonistic interaction between global BGC regulation
363 and within-BGC regulation of antibiotic biosynthesis is occurring. The discovery of this antagonistic

interaction at the level of BGC regulation has not been documented previously in antibiotic biosynthesis.

This long-term evolution experiment in *Streptomyces* establishes a unique system to study how a hyphal, sporulating organism adapts to a novel environment. The experiment also facilitates the study of epistasis in antibiotic biosynthesis. The work demonstrates that adaptive radiation in *Streptomyces* results in the rapid emergence of multiple morphotypes within the population which exhibit hyphal fragmentation phenotypes as result of selection acting on hyphal aggregates, which limit nutrient uptake. Ecological opportunity facilitates the divergence of strains with antagonistic pleiotropy evident from the parallelism observed in carbon utilisation between the replicate populations. The concomitant appearance of strains with developmental defects is due to relaxed selection on sporulation suggesting that accumulation of mutations occurs in genes required for development when sporulation is not permitted. Extensive negative epistasis was observed in the control of antibiotic biosynthesis when strains evolve without the ACT BGC, suggesting that antagonistic interactions may be at play between global and cluster-situated regulators. This on-going experimental system provides an excellent, multi-faceted model for the studying adaptation in *Streptomyces* and will generate important data that will find utility in ecology, evolution and has relevance to understanding industrial antibiotic production.

CRediT (Contributor roles taxonomy).

Conceptualization – PAH.

Data curation - JTM, SV, LK.

Formal analysis - JTM, SV, PAH.

Funding acquisition – PAH.

Investigation - JTM, SV, LK.

Methodology - JTM, SV, LK, PAH.

Project administration - JTM, PAH.

Supervision - JTM, PAH.

Validation - JTM, SV, PAH.

Visualization – JTM, SV

Writing – original draft – PAH.

Writing – review & editing - JTM, SV, LK, PAH.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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564

565 **Figure Legends**

566 **Figure 1: Adaptive radiation of *Streptomyces coelicolor* during long-term experimental**
567 **evolution(LTEE). A).** Experimental set-up of the LTEE showing successive passage of *S. coelicolor*
568 M1152, followed by periodic reintroduction of the actinorhodin (ACT) biosynthetic gene cluster. **B).**
569 Representative plates demonstrating the phenotypic variation in colony morphotypes in *S. coelicolor*
570 M1152 strains. Replicate populations were founded from a single ancestral grey, sporulating colony
571 and propagated over 3000 generations in YEME medium **C).** Major morphological mutation and
572 transitions occur in the LTEE over 3000 generations and strains can be assigned to four major
573 phenotype groups. ***i***) Sporulating colonies, phenotypically identical to the progenitor strain ***ii***) White-
574 aerial hyphae formed, but no spores ***iii***) Bald – no aerial hyphae or spores formed ***iv***) 'Sectored-
575 spreader' colonies **D).** Colony morphotype diversity during adaptive radiation from the parental grey
576 sporulating morphotype. Diversity was determined through scoring the frequency of morphotypes
577 and using the Shannon-Weaver index. Diversity *(H*) in the three independent replicate populations
578 is indicated by the grey lines at each major generational point, and the black line indicates the mean
579 diversity *(H*) of the three populations.

580 **Figure 2: Major metabolic and morphological transitions occur during long-term experimental**
581 **evolution (LTEE). A).** Strains adapt to liquid growth by independently transitioning from an
582 aggregating phenotype in the progenitor to a fragmenting phenotype in all replicate populations. **B).**
583 Quantification of cell aggregate size of *S. coelicolor* M1152 replicate populations shows a decline in
584 mean aggregate area over 3000 generations during adaptation. **C).** Fitness of the replicate
585 populations over 3000 generations. Each point represents the ratio of the growth rate of the evolved
586 population relative to the progenitor strain (*S. coelicolor* M1152) measured in triplicate for each of
587 the three replicate populations. Dashed line represents equivalent fitness. **D).** Fitness of three
588 randomly chosen independent isolates over 3000 generations from the replicate lineages. Each point
589 represents the ratio of the growth rate of the evolved isolate relative to the progenitor strain (*S.*
590 *coelicolor* M1152) measured in triplicate. Dashed line represents equivalent fitness. **E).**
591 Diversification of carbon source utilisation in the replicate populations over 3000 generations. Each
592 point represents the growth of one of the replicate populations on a particular carbon source in the

593 LTEE. Three carbon sources were chosen to illustrate different usage profiles as the population
594 adapts to the medium through changes to specific growth rate, μ (h^{-1}; Further examples in the
595 supplementary data). • glucose; • malate; • pyruvate **E).** Negative epistasis is pervasive in ACT
596 production in the LTEE. The progenitor strain *S. coelicolor* M1152 makes no actinorhodin, this is
597 restored when the ACT BGC is reintroduced to the strain. The majority of strains that had the ACT
598 BGC show reduced actinorhodin production relative to the progenitor strain containing ACT. Colours
599 represent independent isolates – three isolates from three independent replicate populations.

600

601 **Supplementary data** https://doi.org/10.6084/m9.figshare.29950388.v1

602 **Supplementary data 1** – All BactEXTRACT data output (growthParameters) for all Biolog PM1 data.
603 Edited to include plate well annotations to specify carbon sources.

604 **Supplementary data 2** – Modified version **Supplementary data 1** where carbon source utilisation
605 was filtered such that only those carbon sources have at least 1 replicate exhibiting classical bacterial
606 growth profiles along with filtering columns for specific carbon sources.

607 **Supplementary figure 1** – Additional examples of diversification of carbon source utilisation in the
608 replicate populations over 3000 generations. Each point represents the growth of one of the replicate
609 populations on a particular carbon source in the LTEE. Three carbon sources were chosen to
610 illustrate different usage profiles as the population adapts to the medium through changes to specific
611 growth rate, μ (h^{-1}) Diversification of carbon source utilisation in the replicate populations over 3000
612 generations. Each point represents the growth of one of the replicate populations on a particular
613 carbon source in the LTEE. Three carbon sources were chosen to illustrate different usage profiles
614 as the population adapts to the medium through changes to specific growth rate, μ (h^{-1}) • acetic acid;
615 • 2-deoxyadenosine; • Tween 40.