Antibiotic production in *Streptomyces* is organized by a division of labour through terminal genomic differentiation

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Abstract

One of the hallmark behaviors of social groups is division of labour, where different group members become specialized to carry out complementary tasks. By dividing labour, cooperative groups of individuals increase their efficiency, thereby raising group fitness even if these specialized behaviors reduce the fitness of individual group members. Here we provide evidence that antibiotic production in colonies of the multicellular bacterium *Streptomyces coelicolor* is coordinated by a division of labour. We show that *S. coelicolor* colonies are genetically heterogeneous due to massive amplifications and deletions to the chromosome. Cells with gross chromosomal changes produce an increased diversity of secondary metabolites and secrete significantly more antibiotics; however, these changes come at the cost of dramatically reduced individual fitness, providing direct evidence for a trade-off between secondary metabolite production and fitness. Finally, we show that colonies containing mixtures of mutant strains and their parents produce significantly more antibiotics, while colony-wide spore production remains unchanged. Our work demonstrates that by generating mutants that are specialized to hyper-produce antibiotics, streptomycetes reduce the colony-wide fitness costs of secreted secondary metabolites while maximizing the yield and diversity of these products.

41 # Introduction

42 Social insects provide some of the most compelling examples of divisions of labour, with extremes in
43 morphological differentiation associated with highly specialized functions and reproductive sterility in
44 all colony members except the queen[1]. However, conditions that select for division of labour are not
45 limited to animals and it has become increasingly clear that microbes offer unique opportunities to
46 identify and study the mechanistic underpinnings of divisions of labour[2–8]. First, microbes are typically
47 clonal, which helps ensure that a division of labour is favoured by kin selection[4]. Second, microbial
48 populations are highly social, often cooperating to carry out coordinated behaviors like migration or
49 biofilm formation that require the secretion of metabolically expensive public goods that can be shared
50 among clonemates[9,10]. If these conditions are met, and investment in public good secretion trades-off
51 with fitness, divisions of labour are predicted to evolve[4,11].

52 Here we describe the cause and evolutionary benefits of a unique division of labour that has evolved
53 in colonies of the filamentous actinomycete *Streptomyces coelicolor*. After germinating from uni-
54 chromosomal spores, these bacteria establish multicellular networks of vegetative hyphae,
55 reminiscent of fungal colonies[12–14]. Vegetative hyphae secrete a broad variety of public goods, such as
56 chitinases and cellulases that are used to acquire resources, as well as a chemically diverse suite of
57 antibiotics that are used to kill or inhibit competing organisms[15–17]. Streptomycetes are prolific
58 producers of antibiotics, and are responsible for producing more than 50% of our clinically relevant
59 antibiotics[18]. Although the terminal differentiation of *Streptomyces* colonies into vegetative hyphae
60 (soma) and viable spores (germ) is well understood[19–21], no other divisions of labour in these
61 multicellular bacteria are known. However, opportunities for phenotypic differentiation are possible,
62 because even though colonies begin clonally, they can become genetically heterogeneous because of
63 unexplained high-frequency rearrangements and deletions in their large, ~9 Mb linear chromosome
64 [22–25]. The work we describe shows that these two topics are intertwined. Briefly, we find that genomic
65 instability causes irreversible genetic differentiation within a subpopulation of growing cells. This in
66 turn gives rise to a division of labour that increases the productivity and diversity of secreted antibiotics
67 and increases colony-wide fitness.

68 # Results

69 ***Genome instability and phenotypic heterogeneity are coupled***

70 Genome instability and phenotypic heterogeneity have been observed in several *Streptomyces*
71 species[26–32], but there are no explanations for the evolution or functional consequences of this extreme
72 mutability. To begin addressing this question, we quantified the phenotypic heterogeneity arising
73 within 81 random single colonies of *S. coelicolor* M145 by harvesting the spores of each of these
74 colonies and then replating the collected spores onto a new agar surface. Although most progeny are
75 morphologically homogeneous and similar to the wild-type, strikingly aberrant colonies (Figure 1a)
76 arise at high frequencies (0.79 +/- 0.06%, mean +/- SEM, ranging from 0% to 2.15%, n = 81) (Figure 1a).
77 Similarly high rates were obtained on two minimal media (MM: 2.13 +/- 0.14% and MM+CA: 5.13 +/-
78 0.37%, mean +/- SEM, n = 30 and n = 40 respectively) (Figure S1) thereby ruling out the possibility that
79 these mutations are an artifact of rapid growth on rich resources. To determine the heritability of these
80 aberrant phenotypes, we restreaked 15 random colonies from different plates onto a new agar plate
81 which revealed remarkably variability in colony morphology (Figure 1b). Rather than reverting to the

82 wild-type (WT) morphology, as would be anticipated if the initial heterogeneity were due to
83 phenotypic plasticity or another form of bistability, the colonies derived from mutant colonies are
84 themselves hypervariable, giving rise to up to nine diverse phenotypes from any single colony. Thus,
85 in the course of two cycles of colony outgrowth, an array of colony types emerged that differ in size,
86 shape and colour (Figure 1b). Because our ability to discern colony heterogeneity is limited to only a
87 few visually-distinct phenotypic characters, we assume that these estimates of diversity are lower than
88 their true level of occurrence.

89 Using whole-genome sequencing of 8 random mutants we confirmed that these isolates contained
90 profound chromosomal changes. As shown in Figure 2a, large genome deletions were observed at the
91 chromosome ends in all 8 strains. In 3 cases we found an ~297 kb amplification on the left
92 chromosomal arm flanked by the Insertion Sequence IS1649, encoced by SCO0091 and SCO0368.
93 Average sequence coverage of the amplified region suggests it contains between 2 and 15 copies of
94 this amplification (Figure 2a and S2). Sequencing results were expanded using pulsed-field gel
95 electrophoresis (PFGE) analysis of 30 mutant isolates (Figure 2b and S3). Consistent with our
96 sequencing results, this analysis revealed that mutants contained variably sized deletions of up to ~
97 240 kb or ~ 872 kb on the left chromosome arm and up to 1.6 Mb on the right chromosome arm,
98 deleting more than 1,000 genes. Additionally, 8/30 strains contained the same large amplification
99 between copies of IS1649 as noted above. Interestingly, these strains are conspicuously yellow, which
100 might be caused by the overproduction of carotenoids due to the amplification of the *crt* gene cluster
101 (SCO0185-0191;[33–35]). In addition to this and other phenotypic effects associated with these changes
102 that are discussed below, deletions to the right chromosome arm cause the loss of two loci, *argG*
103 (SCO7036) and *cmlR1*(SCO7526)*/cmlR2*(SCO7662), that result in two easily scorable phenotypes:
104 arginine auxotrophy and chloramphenicol susceptibility, respectively. Scoring these phenotypes allows
105 rapid determination of the minimal size of the deletion on the right chromosome arm in the absence
106 of molecular characterization. Chloramphenicol susceptibility indicates a deletion of at least 322 kb,
107 while the addition of arginine auxotrophy indicates a deletion of at least 843 kb (Figure 2b).

108 ***Mutants increase the production and diversity of antibiotics***

109 Mutant strains were conspicuously pigmented when compared to their parental wild-type strains
110 (Figure 1). Because several antibiotics produced by *S. coelicolor* are pigmented, namely actinorhodin,
111 prodigines and coelimycin P which are blue, red and yellow, respectively, we tested if mutant strains
112 had altered secondary metabolite and inhbitory profiles. Secreted metabolites from mutant and WT
113 strains grown on agar surfaces were analyzed using quantitative ^{1}H NMR profiling[36,37]. Principal
114 component analysis (PCA) (Figure 3a) supports the partition of strains into three well-separated groups:
115 wild-type and wild-type-like strains, and then two clusters of mutant isolates. In each case, groupings
116 corresponded to the size of genomic lesions mentioned above. More specifically, strains grouping in
117 the wild-type and wild-type-like cluster are chloramphenicol resistant (Cam^{R}) and arginine
118 prototrophic (Arg^{+}), while those clustering within the blue ellipse were chloramphenicol susceptible
119 (Cam^{S}) and protrophic for arginine (Arg^{+}), and those in the red-ellipsed cluster were susceptible to
120 chloramphenicol (Cam^{S}) and auxotrophic for arginine (Arg^{-}). To assess if genomic deletions affected
121 antibiotic biosynthesis, we used mass spectrometry-based quantitative proteomics on five
122 representative strains from the two mutant clusters. This analysis revealed that the biosynthetic
123 pathways for actinorhodin, coelimycin P1 and calcium-dependent antibiotic (CDA) were significantly
124 upregulated in all mutants (Figure 3b, c, S4 and Table S1). Because the expression level of biosynthetic

125 enzymes directly correlates with antibiotic production[38], these MS results are consistent with
126 increased antibiotic production in these strains (Figure 3b, c, d and S4). In addition to antibiotic
127 biosynthesis clusters, pathways regulating arginine and pyrimidine biosynthesis were also increased in
128 both arginine auxotrophic strains (Figure S4b and Table S1)[39]. No antibiotic-related proteins were
129 down-regulated in this analysis.

130 We next asked if these different metabolic and proteomic profiles translated to differences in
131 biological activity, specifically the ability to inhibit the growth of other bacteria. Thirty mutant strains
132 were grown on agar plates and then covered with a soft-agar overlay containing *B. subtilis*. Inhibition
133 was visualized as an absence of growth surrounding the mutant colony, and the extent of inhibition
134 was determined from the size of the inhibition zone. As shown in Figure 3d, all but three WT-like
135 mutant strains produced significantly larger zones of inhibition than the WT strain (One-tailed t-tests,
136 all $p < 0.05$). In addition, we observed significant heterogeneity among mutant strains in halo size (One-
137 Way ANOVA, $F_{29,90} = 5.45$, $p < 0.001$).

138 To test if the increased inhibition we observed against *B. subtilis* was correlated with the ^{1}H NMR
139 profiles, we used a partial least squares (PLS) regression (Figure 3e)[37]. This showed that the separation
140 into different groups significantly correlates with halo size ($Q^2 = 0.879$) which was further validated by
141 both permutation-tests and ANOVA of cross-validated residuals (CV-ANOVA, $F_{8,116} = 104.443$, $p < 0.001$).
142 To identify possible compounds that are overproduced in mutants compared to WT, we identified
143 several ^{1}H NMR signals that varied across strains and strongly correlated with the size of the zone of
144 inhibition against *B. subtilis* (Table S2); notable among these are several aromatic signals which
145 correspond to actinorhodin, consistent with our proteomic analyses (Figure 3b and c).

146 Phenotypic results indicate that mutant strains produce more antibiotics than their wild-type parent
147 when assayed against a single bacterial target, as anticipated given our NMR and proteomic results.
148 However, they do not distinguish if strains can be further partitioned on the basis of which other
149 species they inhibit. Score plots of principal component analysis based on ^{1}H NMR signals reveal clear
150 separation between mutant clones within and between clusters (Figure 3a), suggesting that their
151 inhibitory spectra may vary. In addition, quantitative proteomic data show that different strains vary
152 in their production of known antimicrobials. To test this, we measured the ability of four mutant clones
153 to inhibit 48 recently isolated *Streptomyces* strains[40]. *Streptomyces* targets were chosen because these
154 are likely to represent important competitors for other streptomycetes in soil environments. At least
155 one of the four mutant strains produced a significantly larger halo than the wild-type strain against 40
156 out of 48 targets, indicating increased inhibition (Figure 3f). More interestingly, for these 40 targets,
157 we observed significant differences between the mutant strains themselves. We found differences in
158 the size of the zone of inhibition on different target species (Two-Way ANOVA, $F_{39,117} = 21.79$, $p < 0.001$)
159 as well as a significant interaction between mutant strain and the target species (Two-Way ANOVA,
160 $F_{117,320} = 5.75$, $p < 0.001$), indicating that the inhibitory profile of each mutant strain is distinct from the
161 others. Together these results reveal that mutants arising within colonies are not only more effective
162 at inhibiting other strains, but that they are also diversified in who they can inhibit because their
163 inhibition spectra do not overlap. They also suggest that the beneficiary of diversified antibiotic
164 secretion is the parent strain, because competing bacteria are unlikely to be resistant to this
165 broadened combination of secreted antimicrobials.

166

167 ***Antibiotic production is coordinated by a division of labour***

168 Having shown that *Streptomyces* colonies differentiate into distinct subpopulations that vary in their
169 antibiotic production, we next asked how this differentiation affects colony fitness. To answer this, we
170 measured the fitness of each mutant strain by quantifying the number of spores they produce when
171 grown in isolation. As shown in Figure 4a, mutants produce significantly fewer spores than the wild-
172 type strain (all $p < 0.001$) and, in extreme cases, as much as 10,000-fold less, with significant
173 heterogeneity among strains (One-Way ANOVA: $F_{29,59} = 132.57$, $p < 0.001$). Importantly, the reduction
174 in spore production is significantly negatively correlated with antibiotic production ($F_{1,29} = 26.51$, $r^2 =$
175 0.478, $p < 0.001$) (Figure S5a). This provides evidence that antibiotic production is costly to *S. coelicolor*
176 and that there is a direct trade-off between antibiotic production and reproductive capacity, possibly
177 because energy is redirected from development to antibiotic production[41]. In addition, we observed a
178 significant negative correlation between the size of the genome deletion and CFU ($F_{1,7} = 12.32$, $r^2 =$
179 0.638, $p = 0.0099$) and a positive correlation between deletion size and bioactivity against *B. subtilis*
180 ($F_{1,7} = 37.97$, $r^2 = 0.844$, $p < 0.001$), suggesting that these phenotypes scale with the magnitude of
181 genomic changes (Figure 4b).

182 To examine the effects of mutant strains on the colony as a whole, we mixed mutant strains with their
183 parent at increasing frequencies and quantified colony-wide spore production and the ability of these
184 mixtures to kill *B. subtilis*. Results of these experiments, shown in Figure 4c, support two important
185 conclusions: 1) increasing fractions of mutants lead to increased antibiotic production; and 2) even
186 though mutant strains have individually reduced fitness (Figure S5b), their presence within colonies
187 has no effect on colony-wide spore production, until the mutant frequency exceeds > 50% of the total.
188 We carried out the same assay with 3 additional mutant clones, but at fewer frequencies to estimate
189 spore production, and observed concordant results (Figure S6): up to a frequency of ~ 50%, mutant
190 strains have no effect on colony-wide spore production, while each incremental increase in the
191 frequency of these strains enhances colony-wide antibiotic output. These results indicate that the
192 benefits of producing cells with chromosomal lesions are evident across a broad range of frequencies,
193 but that even with extremely high mutation rates the costs to colony-wide fitness are minimal or
194 entirely absent.

195 Discussion

196 Streptomycetes are prolific producers of antibiotics, with genomes typically containing more than 20
197 secondary metabolite gene clusters that comprise more than 5% of their entire genome[34,42,43]. They
198 invest heavily in these products and their biosynthesis and secretion is costly. Our results suggest that
199 by limiting antibiotic production to a fraction of the colony through division of labour, *S. coelicolor* can
200 eliminate the overall costs of biosynthesis while maximizing both the magnitude and diversity of their
201 secreted antibiotics. Although this comes at a large individual cost, it increases group fitness by
202 improving the ability for *S. coelicolor* to inhibit their competitors. Moreover, our results reveal that the
203 range of conditions that select for a division of labour are quite broad, because colony-wide fitness is
204 unaffected, even if mutant strains are as frequent as ~ 50%.

205 Division of labour is predicted to be favoured in this system for several reasons. First, *Streptomyces*
206 colonies emerge from a single spore and are clonal[19]. This, together with their filamentous mode-of-
207 growth, ensures that costly individual traits can be maintained due to their indirect fitness benefits

208 [4,5,13]. And because resistance to the diversified antibiotic profile of mutant strains is unlikely to be
209 present in competing strains, only the parent strain stands to benefit from their sacrifice. Second, the
210 costs of antibiotic production via large and dedicated multi-step biosynthetic pathways, e.g. non-
211 ribosomal peptide or polyketide synthases, are likely to be highest at the initiation of antibiotic
212 production but diminish thereafter, meaning that producing cells become more efficient at making
213 antibiotics through time[11]; furthermore, we show that antibiotic production trades-off with
214 reproduction. Finally, many antibiotics are secreted, so the entire colony, but not susceptible
215 competitors, can benefit from the protection they provide[44].

216 Even if conditions predispose to a division of labour, there must still be a process that generates
217 phenotypic heterogeneity. Our results show that in *Streptomyces*, this is caused by genomic instability
218 that creates a subpopulation of cells within colonies that contain large deletions or amplifications at
219 the termini[23,25,31]. Importantly, these mutations are severe and irreversible. Because strains, or
220 portions of colonies, containing these deletions have significantly reduced fitness, they effectively
221 behave like a sterile caste that provide direct benefits to the rest of the colony and receive little in
222 return[1]. Indeed, even when the initial frequency of mutants in mixed colonies approaches 80%, their
223 final frequency declines to less than 1% after one cycle of colony growth (Figure S7). This suggests that
224 the division labour in *S. coelicolor* is re-established independently and differently in each colony, a
225 mechanism that may help to maximize the diversity of secreted antibiotics.

226 It remains unclear if there are mechanisms regulating the size and frequency of chromosomal deletions
227 and amplifications. One possibility is that these events are induced by external environmental
228 conditions and that their rate is context dependent. Instability can be elevated by exposure to certain
229 toxicants, e.g. mitomycin C or nitrous acid[45], although no explicit stress was added in the experiments
230 we report. It may also be increased during competition with other strains, a process that is known to
231 alter the secretion of secondary metabolites[16,46]. Another possibility is that deletions, and the benefits
232 they bring for antibiotic production, are a fortuitous byproduct of the cell death that accompanies
233 development[19]. By this argument, chromosome degradation would be regulated, but wouldn't always
234 be lethal. Although we have not confirmed this experimentally, it is likely that conserved amplifications
235 result from the flanking copies of IS1649, which can facilitate intragenomic rearrangements[47]. In either
236 case, the expectation is that increased antibiotic production results from the deregulation of
237 biosynthetic clusters following the deletion of 100s of genes, many known to coordinate antibiotic
238 biosynthesis[34]. And because deletions are stochastic, especially following the removal of protective
239 telomeres at the ends of linear chromosomes, this would also cause antibiotic production to vary in
240 different sections of the colony.

241 Our preliminary surveys have found similar levels of genomic instability in streptomycete strains we
242 have freshly isolated from soil, suggesting that the division of labour we describe here is general. We
243 are limited, however, in our ability to detect polymorphisms; color changes are conspicuous and are
244 invariably associated with changes to pigmented secondary metabolites, but other secreted public
245 goods may also become modified in these multicellular bacteria. Understanding which, if any other,
246 public goods vary in the ways shown here is crucial because it will help to identify conditions that lead
247 to a genetically encoded division of labour as compared to other forms of regulation that allow
248 complex multicellular microbial systems to coordinate their behaviors and maximize their fitness.

249

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Author contributions

Z.Z., D.C. and D.R. conceptualized and planned the study. Z.Z., C. D., and F.d.B. collected data. All authors analyzed and interpreted the results. Z.Z., D.C. and D.R. drafted the manuscript with input from the other authors. All authors approved the final submitted version of this paper.

Competing interests

The authors declare there are no competing interests.

Materials and Methods

Bacterial strains and growth conditions

Streptomyces coelicolor A3(2) M145 was obtained from the John Innes centre strain collection. The strain was cultivated at 30 °C on Soy Flour Mannitol Media agar plates (SFM) for strain isolation and to quantify CFU[48]. SFM contains, per liter: 20 g mannitol, 20 g agar and 20 g soya flour. To examine antibiotic production and to extract secondary metabolites, we used minimal media (MM) supplied with 0.5% mannitol and 740 µg ml^{-1} casamino acids (CA). MM contains, per liter, 0.5 g L-asparagine, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_20$ and 10 g agar. For DNA extraction, strains were grown in liquid flasks shaken at 200 rpm at 30 °C in TSBS:YEME (1:1 v:v) supplemented with 0.5% glycine and 5mM $MgCl_2$. TSBS contains 30 g tryptic soya broth powder and 100 g sucrose/liter. And YEME contains 3 g yeast extract, 5 g peptone, 3 g malt extract, 10 g glucose and 340 g sucrose. *E. coli* and *B. subtilis* were cultivated at 37 °C in LB media with constant shaking or on LB-agar plates.

All strains were derived from a single isolate of *S. coelicolor* A3(2) M145 (designed as WT). Briefly, samples from a frozen spore stock were diluted and plated onto SFM agar to obtain single colonies. After 5-days of growth, single colonies with WT morphology were diluted and plated onto another SFM plate. From each plate single colonies with conspicuously mutant phenotypes were picked into sterile water and plated at appropriate dilutions onto SFM agar (n = 3/colony), from which we estimated the frequency of different mutant phenotype classes. Each derived type was plated to confluence on SFM agar, and after 7 days of growth, spores were harvested to generate spore stocks which were stored at -80 °C in 20% glycerol. To quantify mutation frequency, single colonies were grown for 5 days on three different media, then we picked the colonies with WT morphology, diluted and plated them onto the corresponding media. Mutatation frequency was scored based on the phenotypes after 3 to 5 days.

Phenotypic scoring

Two phenotypes that are related to the loss of loci in the right arm were scored (n = 3/strain). The arginine auxotrophs were identified by replicating 10^3 CFU of each strain on MM supplied with 0.5%

288 Mannitol with and without 37 µg ml^{-1} arginine[45]. After 5 days of growth, auxotrophs were identified as
289 those strains that only grow on the media supplied with arginine. Chloramphenicol resistance was
290 estimated by using the disk diffusion method. In detail, $2x10^5$ spores were spread onto MM
291 supplemented with 740 µg ml^{-1} casamino acids [45] in 12 mm square petri dishes, followed by placing a
292 paper disk containing 25 µg chloramphenicol on it. After 4 days, the radius of the inhibition zone
293 around the disk was measured using ImageJ [49]. Inhibition zones that were smaller than 5 mm were
294 scored as resistant while those that are larger than 5 mm were scored as susceptible.

295 ***Antibiotic production***

296 Spores of each strain were diluted to 10^5 CFU ml^{-1} in MiliQ water and 1 µl was spotted onto MM +
297 casamino acids agar plates (n = 4/strain). After growth for 5 days at 30 °C, plates were covered with
298 15 ml of LB soft agar (0.7 %) containing 300 µl of a freshly grown indicator strain (OD_{600} 0.4-0.6). After
299 overnight incubation at 30 °C, zones of inhibition around producing colonies were measured using
300 ImageJ.
301
302 The bioactivity against *Streptomyces* isolates was tested for four strains, 2H1A, 8H1B, 9H1B and 9H1A.
303 Three ml of SFM agar was poured onto each well of a 100mm square petri dish (Thermo Scientific,
304 USA), after which we spotted 1 µl of each test strain containing ~10^6 total spores in the corner of each
305 well. After 5-day growth, 500 µl of MM supplemented with 0.5% mannitol and 740 µg ml^{-1} casamino
306 acids containing ~10^5 spores of the target strain was overlaid on top. Zones of inhibition were
307 measured 2 days later using ImageJ.
308
309 ***^{1}H NMR profiling and data analysis***

310 $2x10^5$ spores were spread onto MM + casamino acids in 12 mm square petri dishes (n=3/strain, except
311 n = 2 for one WT clone). After 5-days of incubation at 30 °C, agar was chopped into small pieces using
312 a sterile metal spatula and secreted compounds were extracted in 50 ml ethyl acetate for 72h at room
313 temperature. Next, the supernatant was poured off and evaporated at 37 °C using a rotating
314 evaporator. Pellets were obtained by drying at room temperature to remove extra solvents and then
315 freeze-dried to remove remaining water. After adding 500 µl methanol-d_4 to the dried pellets, the
316 mixtures were vortexed for 30 seconds followed by a 10-minute centrifugation at 16000 rpm. The
317 supernatants were then loaded into a 3 mm- NMR tube and analyzed using 600 MHz ^{1}H NMR (Bruker,
318 Karlsruhe, Germany)[36,37].

319 Data bucketing of NMR profiles was performed using AMIX software (version 3.9.12, Bruker Biospin
320 GmbH) set to include the region from δ 10.02 to 0.2 with a bin of 0.04 ppm scaled to total intensity,
321 while the signal regions of residual HDO in methanol (δ 4.9 – 4.7) and methanol (δ 3.34 – 3.28) were
322 excluded. Multivariate data analysis was performed with the SIMCA software (version 15, Umetrics,
323 Sweden)[36].

324 **Mass spectrometry-based quantitative proteomics**
325 10^6 spores were spotted on SFM agar covered with cellophane and incubated for 5 days at 30 °C.
326 Colonies were scraped off and snap frozen in liquid N_2 in tubes, then lysed 3 times in a pre-cooled
327 TissueLyser (QIAGEN). Proteins were dissolved in lysis buffer (4% SDS, 100 mM Tris-HCl pH7.6, 50 mM
328 EDTA) and then precipitated using chloroform-methanol[50]. The dried proteins were dissolved in 0.1%
329 RapiGest SF surfactant (Waters) at 95°C. Protein digestion steps were done according to van Rooden
330 et al.[51]. After digestion, trifluoroacetic acid was added for complete degradation and removal of

331 RapiGest SF. Peptide solution containing 8 μg peptide was then cleaned and desalted using the STAGE-
332 Tipping technique[52]. Final peptide concentration was adjusted to 40 ng/μl with 3% acetonitrile, 0.5%
333 formic acid solution. 200 ng of digested peptide was injected and analysed by reverse-phase liquid
334 chromatography on a nanoAcquity UPLC system (Waters) equipped with HSS-T3 C18 1.8 μm, 75 μm X
335 250 mm column (Water). A gradient from 1% to 40% acetonitrile in 110 min was applied, [Glu[1]]-
336 fibrinopeptide B was used as lock mass compound and sampled every 30 s. Online MS/MS analysis was
337 done using Synapt G2-Si HDMS mass spectrometer (Waters) with an UDMS^E method set up as
338 described[51].

339 Mass spectrum data were generated using ProteinLynx Global SERVER (PLGS, version 3.0.3), with MS^E
340 processing parameters with charge 2 lock mass 785.8426. Reference protein database was
341 downloaded from GenBank with the accession number NC_003888.3. The resulting data were
342 imported to ISOQuant[53] for label-free quantification. TOP3 quantification result from ISOQuant was
343 used in later data processing steps.
344
345 In total, of the 7767 proteins from the database, 2261 proteins were identified across all samples. For
346 each sample, on average 1435 proteins were identified. TOP3 quantification was filtered to remove
347 identifications meeting both criteria: 1. identified in less than 70% of samples of each strain; 2. The
348 sum of TOP3 value less than 1×10^5. This led to removal of 297 protein quantification results. Proteins
349 were considered significantly altered in expression when $\log_2$ fold change $\geqq 1$ and p-value $\leqq$ 0.05.
350 Volcano plots were made from filtered data, with the four biosynthetic gene clusters color coded.

351 ***CFU production***

352 To quantify CFU, 10^4 spores of each strain were spread onto SFM agar (n = 3/strain, except n = 2 for
353 9H1B) and left to grow for 7 days to confluence. After 7 days, spores were harvested by first adding 10
354 ml MilliQ water onto the plate and then using a cotton swab to remove spores and mycelial fragments
355 from the plate surface. Next, the water suspension was filtered through an 18 gauge syringe plugged
356 with cotton wool to remove mycelial fragments. After centrifuging the filtered suspension at 4000 rpm
357 for 10 min, the supernatant was poured off and the spore pellet was dissolved in a total volume of 1ml
358 20% glycerol. CFU per plate was determined via serial dilution onto SFM agar.

359 ***DNA extraction and sequencing***

360 Nine strains, including one wild-type and eight mutants, were selected for sequencing with the Sequel
361 system from Pacific Bioscience (PacBio, USA). Roughly 10^8 spores were inoculated in 25 ml TSBS:YEME
362 (1:1 v:v) supplemented with 0.5% glycine and 5 mM $MgCl_2$ and cultivated at 30°C with 200 rpm shaking
363 speed overnight. The pellet was then collected after centrifugation and washed twice with 10.3%
364 sucrose. Samples were then resuspended in DNA/RNA shield (Zymo Research, USA) with 10x volume
365 at room temperature and sent to be commercially sequenced at Baseclear (Leiden, The Netherlands).

366 Subreads of the sequenced results shorter than 50 bp were filtered and stored in BAM format. The
367 reference alignments were performed against *Streptomyces coelicolor* A3(2) genome (NC_003888.3)
368 using BLASR (v5.3.2) [54]. Resulting BAM files were then sorted and indexed using SAMtools (v1.9) [55] .
369 For the calculation of genome rearrangements, the depths were called and exported through the
370 depth function in SAMtools. The edges of genome were identified by manually checking the break
371 point where the coverage drops to zero. The size of the amplified region was defined by the

372 dramatically higher coverage compared to the adjacent sequences. All results were further confirmed
373 by visualizing them in IGV (v2.4.15) [56,57].

374 ***Pulsed-field gel electrophoresis (PFGE)***

375 Approximately 10^8 spores were inoculated into 25 ml TSBS:YEME (1:1 v:v) supplemented with 0.5%
376 glycine and 5 mM $MgCl_2$ and cultivated overnight at 30 °C at 200 rpm. After centrifuging the culture
377 at 4000 rpm for 10 min, the pellet was resuspended in 400 µl cell suspension buffer containing 100
378 mM Tris: 100mM EDTA at pH 8.0 and 1 mg ml^{-1} lysozyme and mixed with the same volume of 1%
379 SeaKem Gold Agarose (Lonza, USA) in TE buffer containing 10 mM Tris: 1 mM EDTA at pH 8.0 with 1%
380 SDS. This mixture was immediately loaded into the PFGE plug mold (Bio-rad, USA). Next, plugs were
381 lysed in 5 ml cell lysis buffer containing 50 mM Tris: 50 mM EDTA at pH 8.0, 1% N-Lauroylsarcosine,
382 sodium salt and 4 mg ml^{-1} lysozyme incubated for 4 hours at 37 °C with gentle agitation. This was then
383 followed by a 5-hour incubation in 5 ml cell lysis buffer containing 0.1 mg ml^{-1} proteinase K at 56 °C
384 and 50 rpm. Finally, the plug was washed twice in pre-heated milliQ water and 4 times in pre-heated
385 TE buffer and incubated at 56 °C for at least 15 min with gentle mixing. Plugs were sliced into 2 mm
386 width pieces and pre-soaked in 200 µl 1x NEB 3.1 buffer for at least 30min. After replacing the buffer
387 with 200 µl 1x NEB 3.1 buffer, 2 µl of the rare-cutter AseI (New England Biolabs, UK) was added and
388 incubated at 30 °C overnight. 1% agarose was used for running fragments in 0.5x freshly prepared TBE.
389 0.225-2.2 Mb *S. cerevisiae* chromosomal DNA (Bio-rad, USA) and wild-type *S. coelicolor* DNA were used
390 as size markers for estimate fragment sizes. Two electrophoresis conditions were applied to separate
391 and visualize the smaller (<1016 kb) and larger fragments (>1016 kb): (Switch time: 2.2-75 s; Voltage:
392 200 V; Running time: 19 h) and (Switch time: 60-125 s; Voltage: 200 V; Running time: 20 h), respectively.

393 PFGE results were compared to the AseI restriction maps of the wild-type strain which contains 17
394 fragments ranging from 26bp to 1601 kb (Figure S3). Two fragments, 240 kb and 632 kb, can be easily
395 resolved if they are deleted from the left arm while one large 1601 kb fragment can be affected when
396 deletions occur in right arm.

397 ***Fitness estimates***

398 The relative fitness of four fully sequenced mutant isolates, 2H1A, 8H1B, 9H1B and 9H1A, was
399 estimated during pairwise competition with the wild-type parent. To distinguish strains, we first
400 transformed mutant and wild-type strains with the integrating plasmids PIJ82 and pSET152 which
401 confer hygromycin B and apramycin resistance, respectively. Potential fitness effects of the markers
402 were determined by generating two WT variants that were transformed with either single marker. No
403 effects were observed in these control experiments (One sample t-test, $t = 2.029$, $df = 7$, $p = 0.082$).
404 Fitness assays were initiated by normalizing each strain to a density of 10^6 spores/ml and then mixing
405 at different starting ratios of Mutant:WT. 100 µl of this mixture, containing 10^5 spores, was plated as
406 a lawn onto SFM agar and incubated at 30 °C for 5 days, while another fraction of the sample was
407 plated after serial dilution onto SFM containing either 50 µg ml^{-1} apramycin or 50 µg ml^{-1} hygromycin
408 B to precisely quantify the densities of each strain. After 5 days of growth, bacteria were harvested as
409 above and plated by serial dilution onto SFM containing either 50 µg ml^{-1} apramycin or 50 µg ml^{-1}
410 hygromycin B. Fitness was quantified, following Lenski et al [58], by calculating the ratio of the Malthusian
411 parameters of both strains. Values below 1 indicate that mutant strains have lower fitness than the
412 WT strain. More detailed assays were carried out with strain 9H1A, where we simultaneously
413 estimated the fitness of this strain at a broader range of frequencies from 10-99% and determined

how the frequency of the mutant strain influenced antibiotic production, as measured by the size of the zone of inhibition against a *B. subtilis* indicator in a soft agar overlay.

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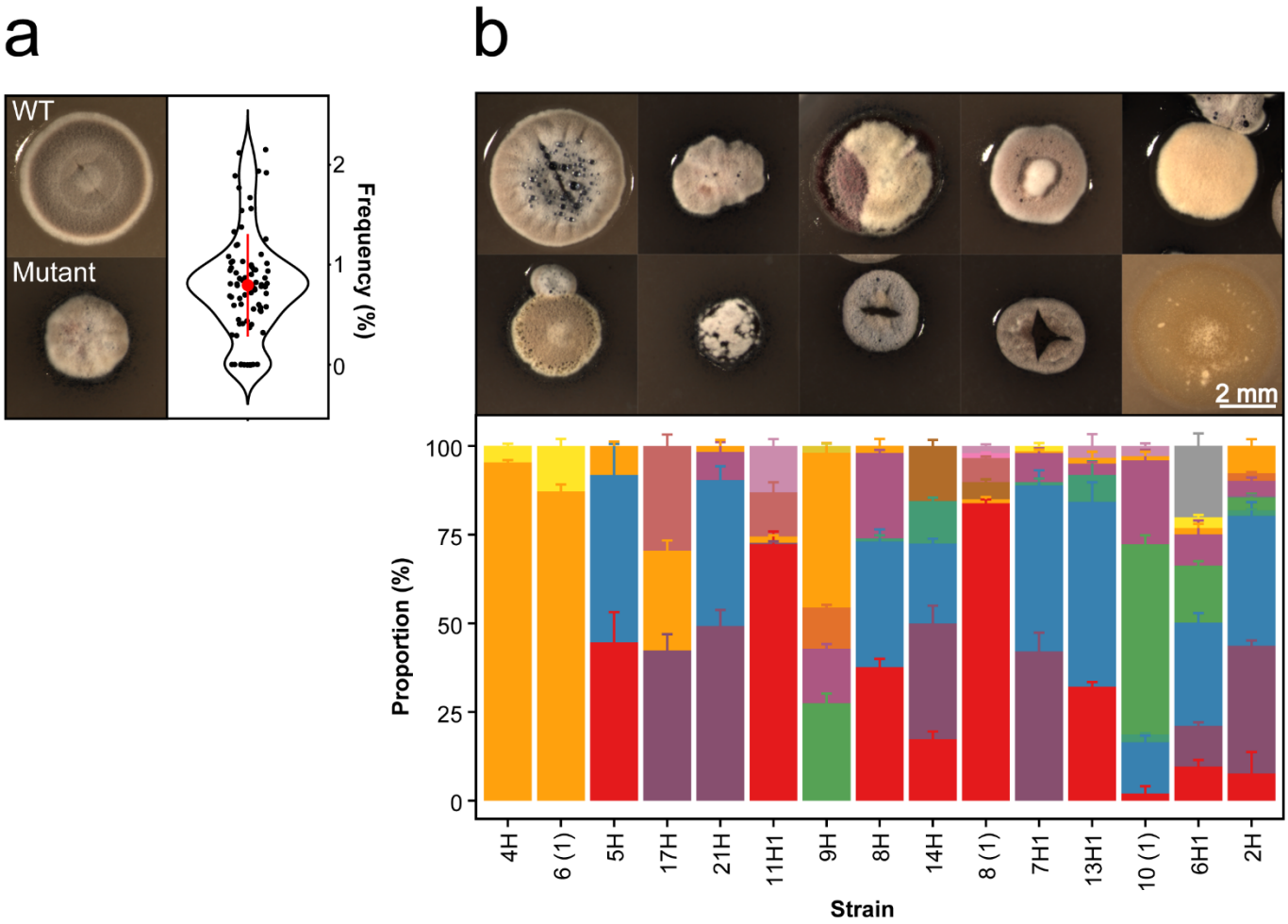

Figure 1: The emergence of phenotypic heterogeneity in colonies of *Streptomyces coelicolor*. (a) Wild-type (top) and mutant (bottom) colonies, and the frequency that mutants emerge from WT colonies (right). (B) Phenotypically diverse progeny (top) emerge after re-streaking mutant colonies that vary in size, shape and pigmentation. Representative colonies are shown. The bottom graph depicts the range of distinct morphologies that emerge after re-streaking 15 random colonies. Each color represents a distinct colony phenotype.

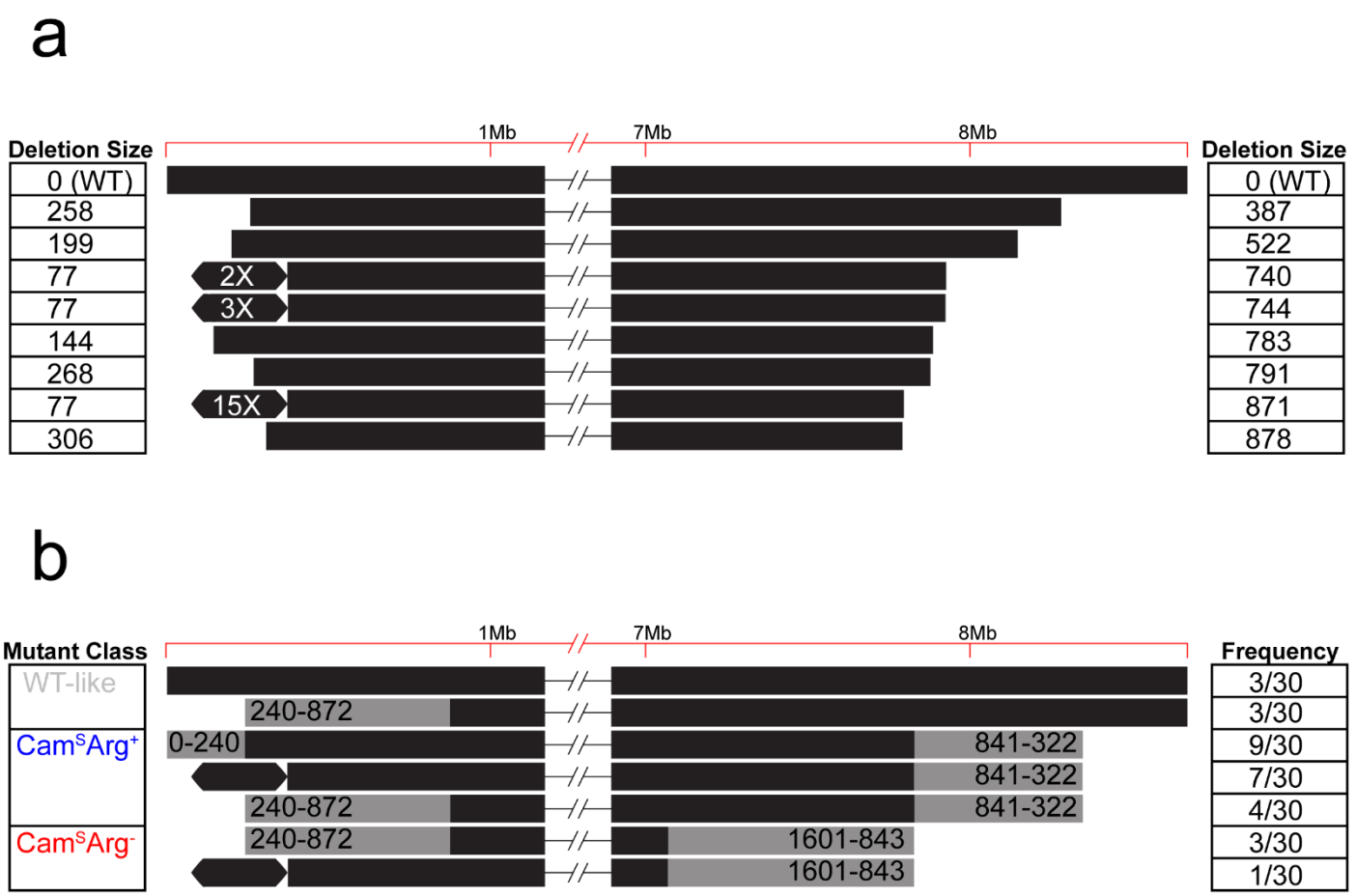

Figure 2: Genome diversity of mutant colonies determined from (a) whole-genome sequencing and (b) Pulsed-field gel electrophoresis (PFGE). Values in (a) correspond to the size (in kb) of genome deletions while the hexagons represent an ~297 kb genome amplification. Each line in (b) depicts the range of deletion sizes (grey) in each mutant class, together with their respective frequencies from 30 sampled mutant strains.

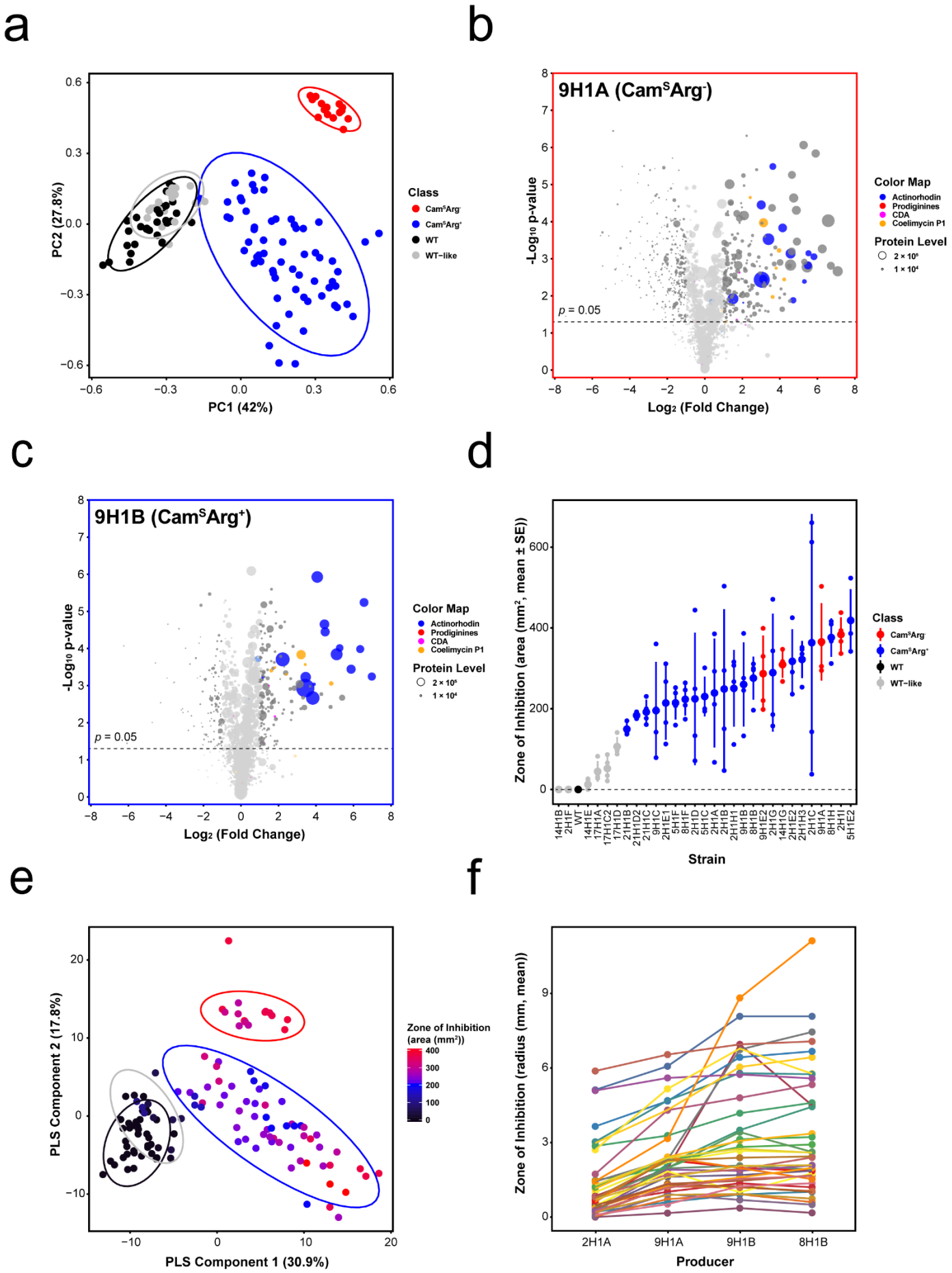
a
PC2 (27.8%)
PC1 (42%)
Class
CamSArg-
CamSArg+
WT
WT−like
b
9H1A (CamSArg-)
-Log10 p-value
Log2 (Fold Change)
p = 0.05
Color Map
Actinorhodin
Prodiginines
CDA
Coelimycin P1
Protein Level
2 × 10⁵
1 × 10⁴
c
9H1B (CamSArg+)
-Log10 p-value
Log2 (Fold Change)
p = 0.05
Color Map
Actinorhodin
Prodiginines
CDA
Coelimycin P1
Protein Level
2 × 10⁵
1 × 10⁴
d
Zone of Inhibition (area (mm², mean ± SE))
Strain
Class
CamSArg-
CamSArg+
WT
WT−like
e
PLS Component 2 (17.8%)
PLS Component 1 (30.9%)
Zone of Inhibition (area (mm²))
f
Zone of Inhibition (radius (mm, mean))
Producer
2H1A
9H1A
9H1B
8H1B

Figure 3: Secondary metabolite production in mutant strains determined by ^{1}H NMR (a and e), quantitative proteomics (b and c) or zones of inhibition on *B. subtilis* (d) or 40 different natural streptomycete isolates (f). (a) PCA plot of ^{1}H NMR data. Each cluster enclosed in a colored ellipse (with 95% CI) corresponds to a mutant class with a different phenotype and degree of genomic instability: WT-like strains (in grey), $Cam^{S}Arg^{+}$ strains (in blue) and $Cam^{S}Arg^{-}$ strains (in red). (b,c) Volcano plots of MS-based quantitative proteomics of two representative strains 9H1A ($Cam^{S}Arg^{-}$) (b) and 9H1B ($Cam^{S}Arg^{+}$) (c). Protein level is indicated by the size of the dot and genes with $\leqq$ 2-fold change and/or $p > 0.05$ are greyed out. (d) Zones of inhibition of each strain when grown with a *B. subtilis* soft-agar overlay. Colors represent the same mutant classes as in (a). The large dot represents the mean of four replicates while error bars represent the standard error. (e) PLS plot of ^{1}H NMR data partitioned by the same clusters as in (a). The heat map indicates the size of the zone of inhibition on *B. subtilis.* (f) Zones of inhibition of 4 representative mutant strains with an overlay of 40 different natural streptomycetes, each represented by a different line. Statistics are given in the main text.

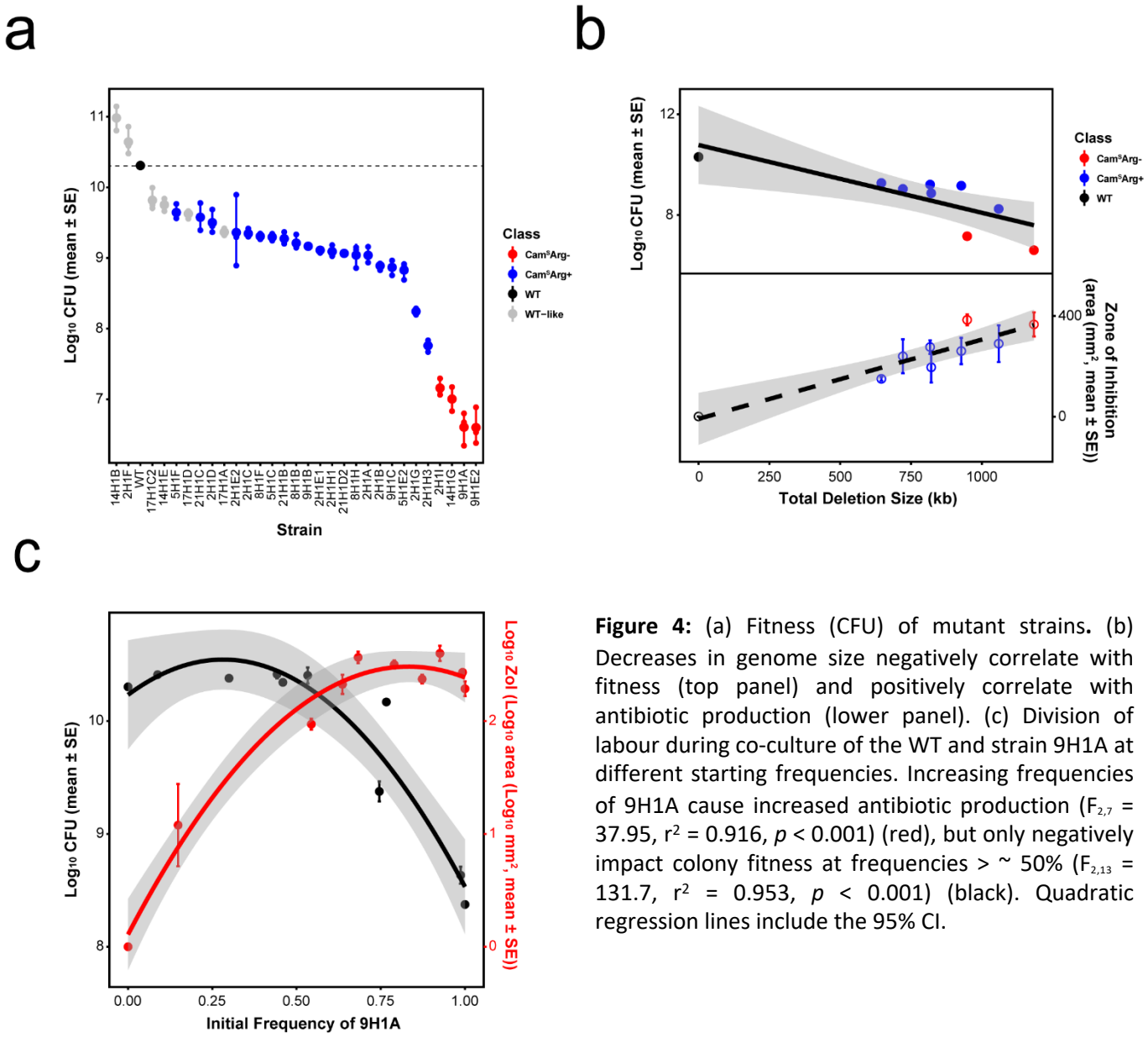

Figure 4: (a) Fitness (CFU) of mutant strains**.** (b) Decreases in genome size negatively correlate with fitness (top panel) and positively correlate with antibiotic production (lower panel). (c) Division of labour during co-culture of the WT and strain 9H1A at different starting frequencies. Increasing frequencies of 9H1A cause increased antibiotic production ($F_{2,7}$ = 37.95, r^2 = 0.916, p < 0.001) (red), but only negatively impact colony fitness at frequencies > ~ 50% ($F_{2,13}$ = 131.7, r^2 = 0.953, p < 0.001) (black). Quadratic regression lines include the 95% CI.

Supplemental Figures: Figure S1- S7

Antibiotic production in *Streptomyces* is organized by a division of labour through terminal genomic differentiation

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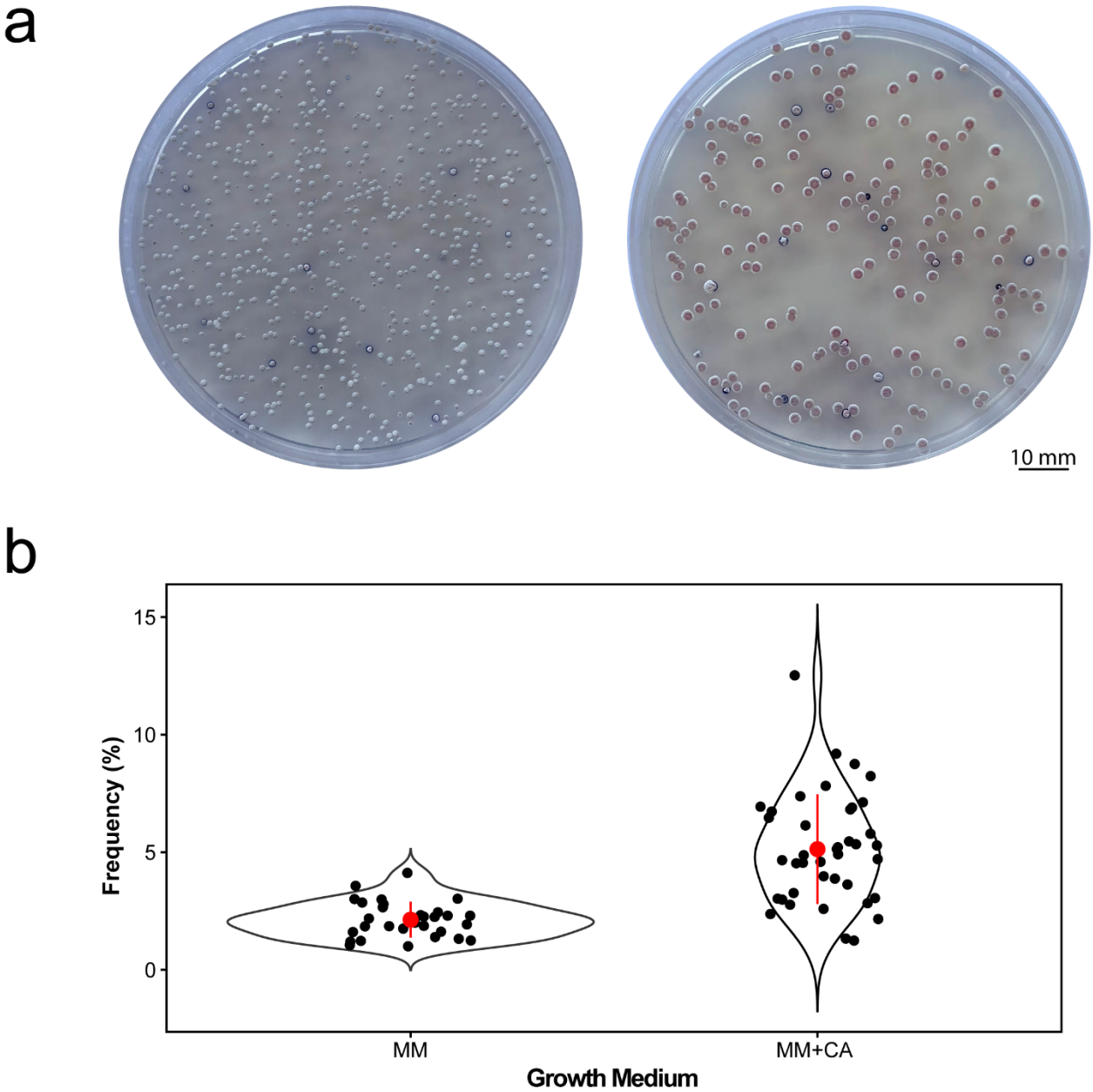
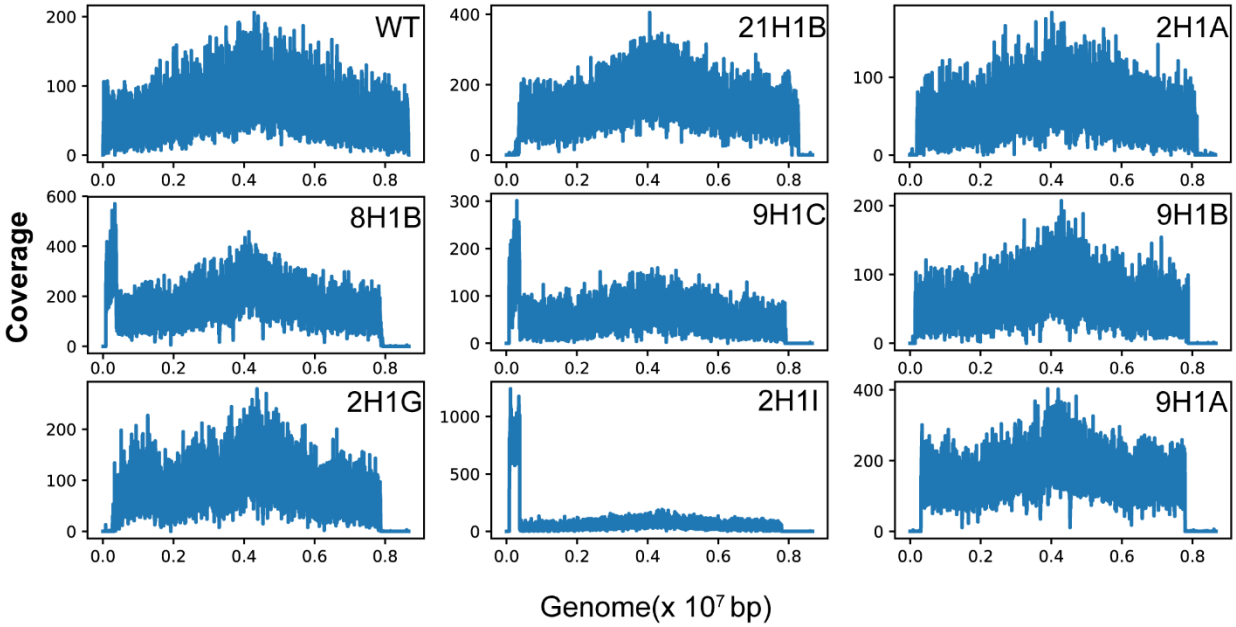


Figure S1: (a) Examples of mutant and wild-type colonies growing on minimal media (MM) (Left) or minimal media supplemented with casamino acids (MM+CA) (Right). (b) The frequency of mutants emerging from WT colonies on both media types.

a



b

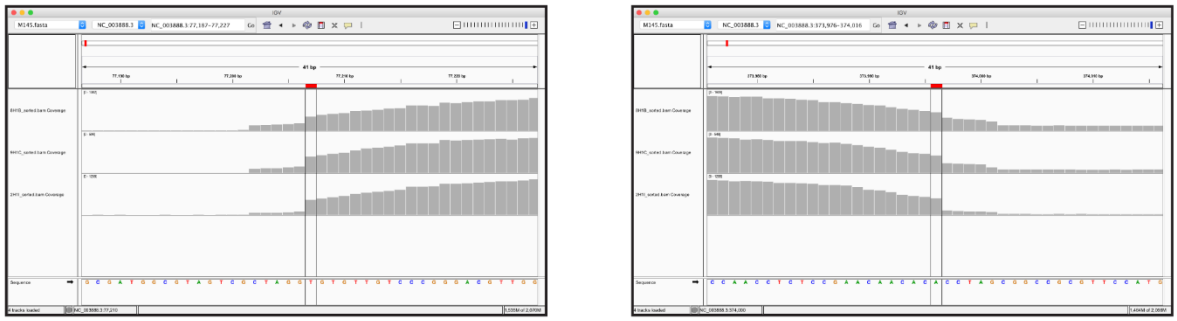


Figure S2: (a) PacBio reads mapped to the *S. coelicolor* M145 reference genome indicating coverage and highlighting breakpoints for genome deletions, where coverage declines to 0, as well as the size of the conserved amplified regions on the left chromosomal arms of strains 8H1B, 9H1C and 2H1I. (b) IGV snapshots of the left and right borders of amplified regions of strains 8H1B, 9H1C and 2H1I.

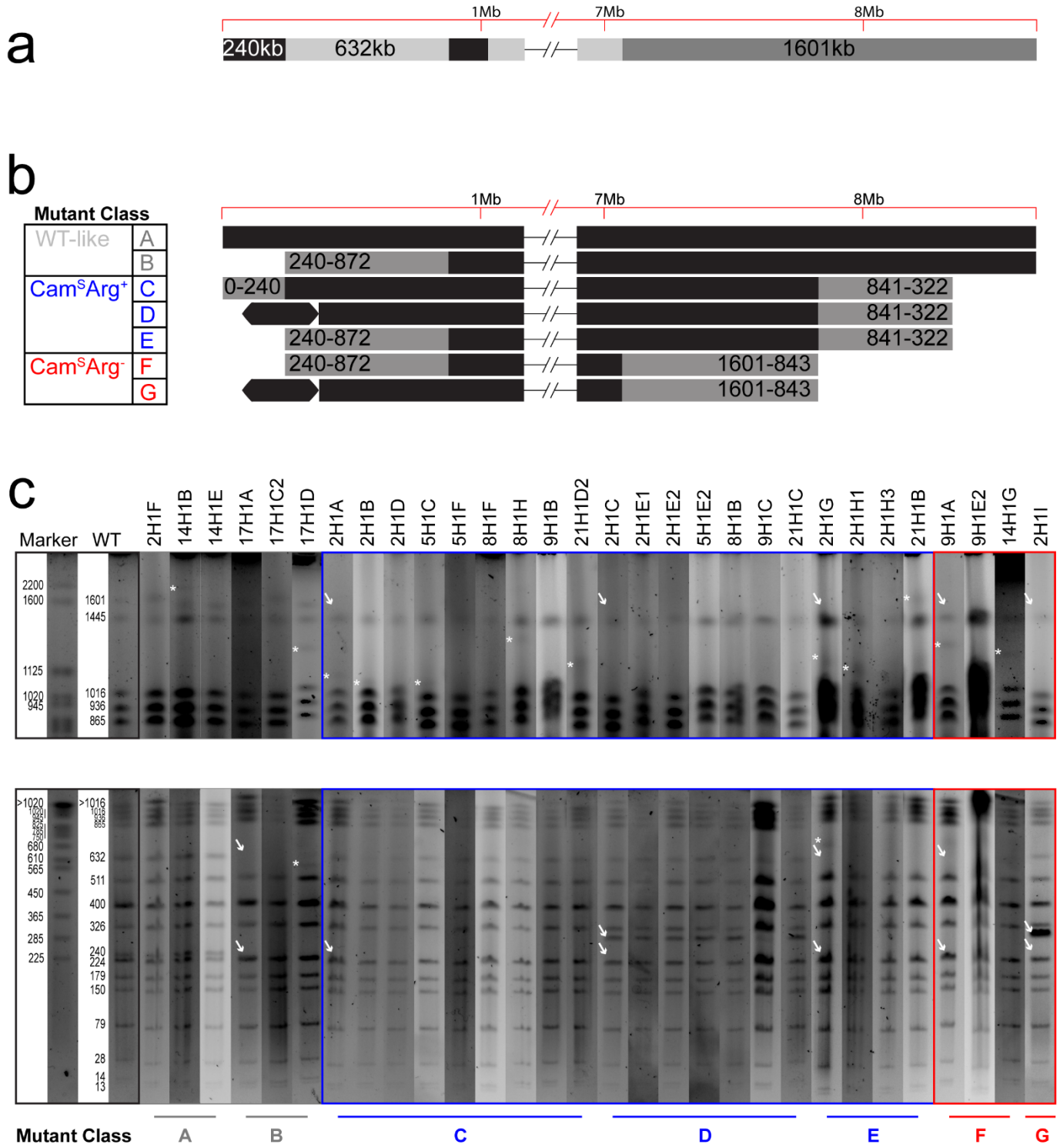


Figure S3: Pulse field gel electrophoresis (PFGE) results of all sampled strains. (a) The schematic of changes to *S. coelicolor* fragments in mutant isolates after AseI digestion. Two bands (240 kb and 632 kb) and one 1601 kb band can be affected on the left and right arms, respectively. (b) Schematic adjusted from Figure 2b with more detailed mutant classes, designated A-G. (c) PFGE results of 30 sampled strains. Two running conditions are used to visualize larger (top panel) or smaller (bottom panel) fragments. (Detailed running conditions are given in the Materials and Methods). White arrows indicate missing or newly appearing of the bands for different mutant class. Asterisks indicate the new bands that were used to evaluate precise genome length.

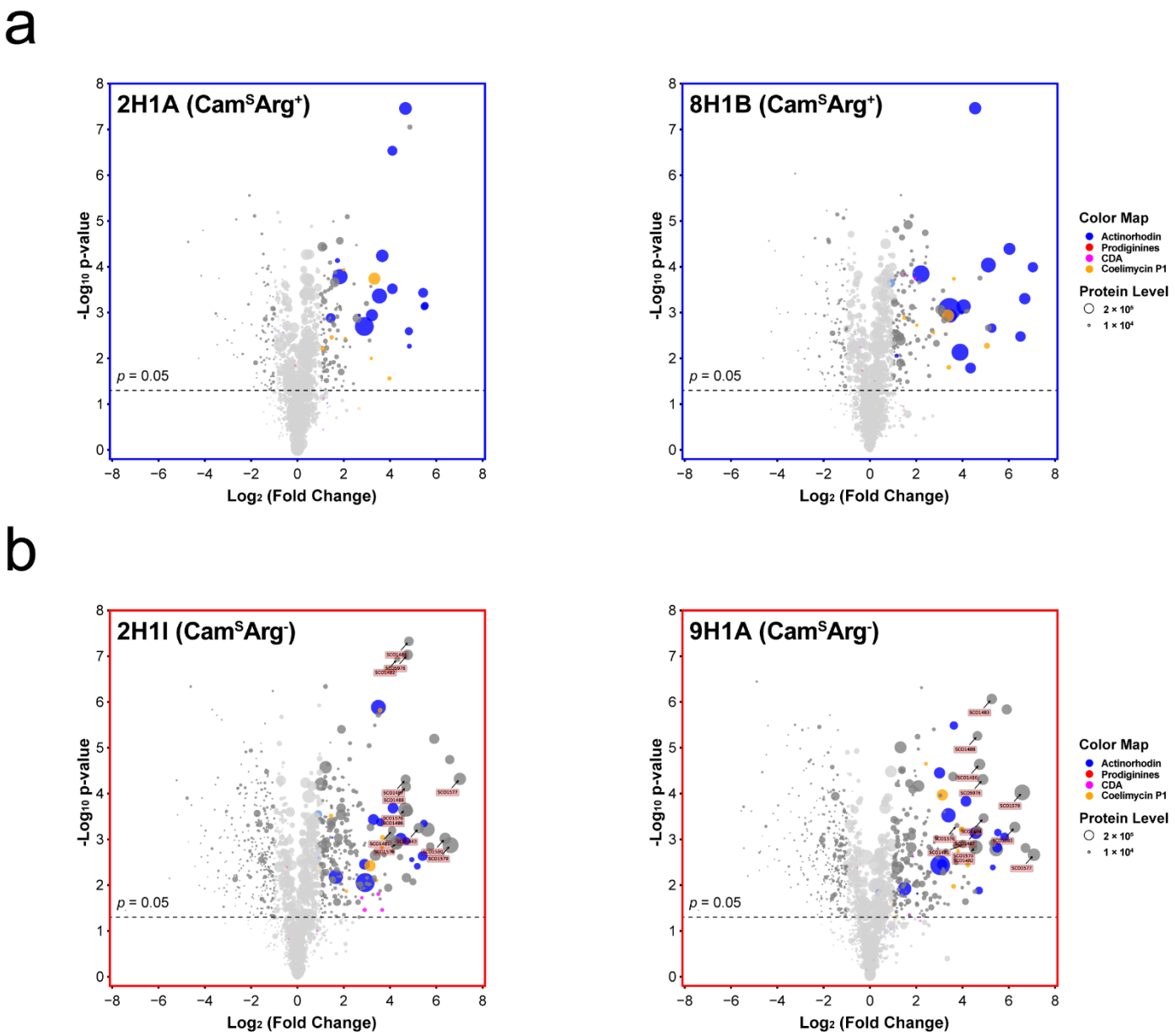


Figure S4: Volcano plots of proteomics from two Cam^{S}Arg$^+$ strains (a) and two Cam^{S}Arg$^-$ strains with annotated genes from arginine and pyrimidine biosynthesis pathways (b).

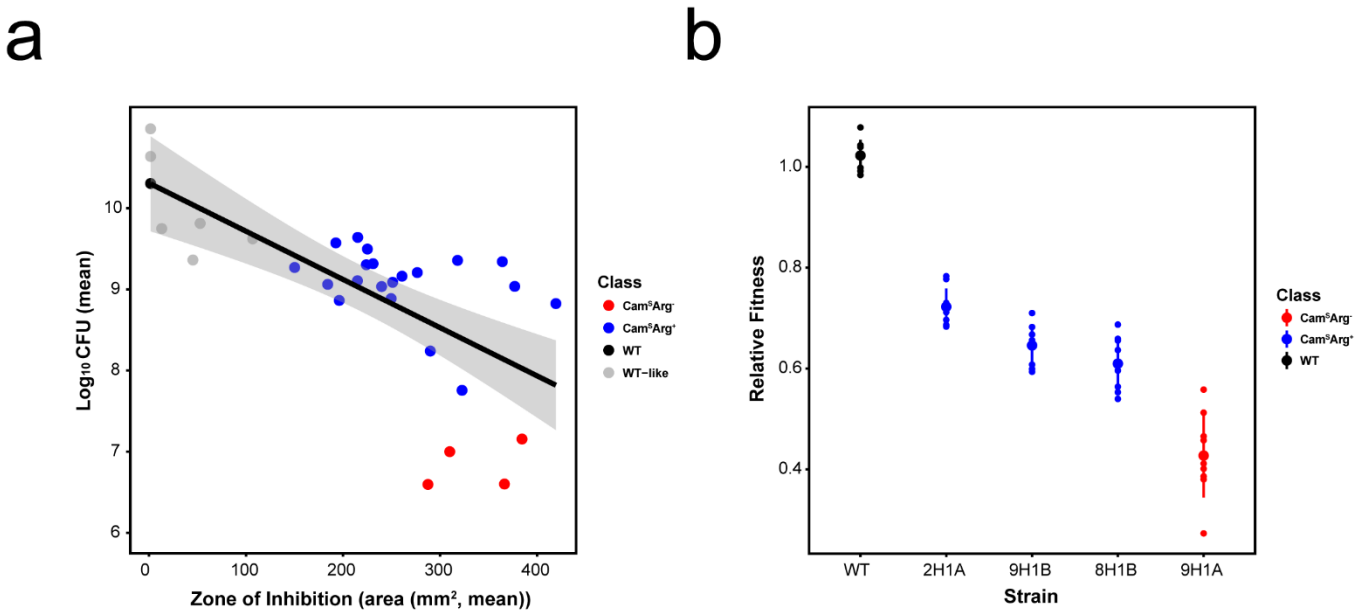


Figure S5: (a) The trade-off between antibiotic production and reproductive capacity, partitioned by different mutant classes. (b) Relative fitness of four selected strains. Detailed methods are given in the Methods and Materials.

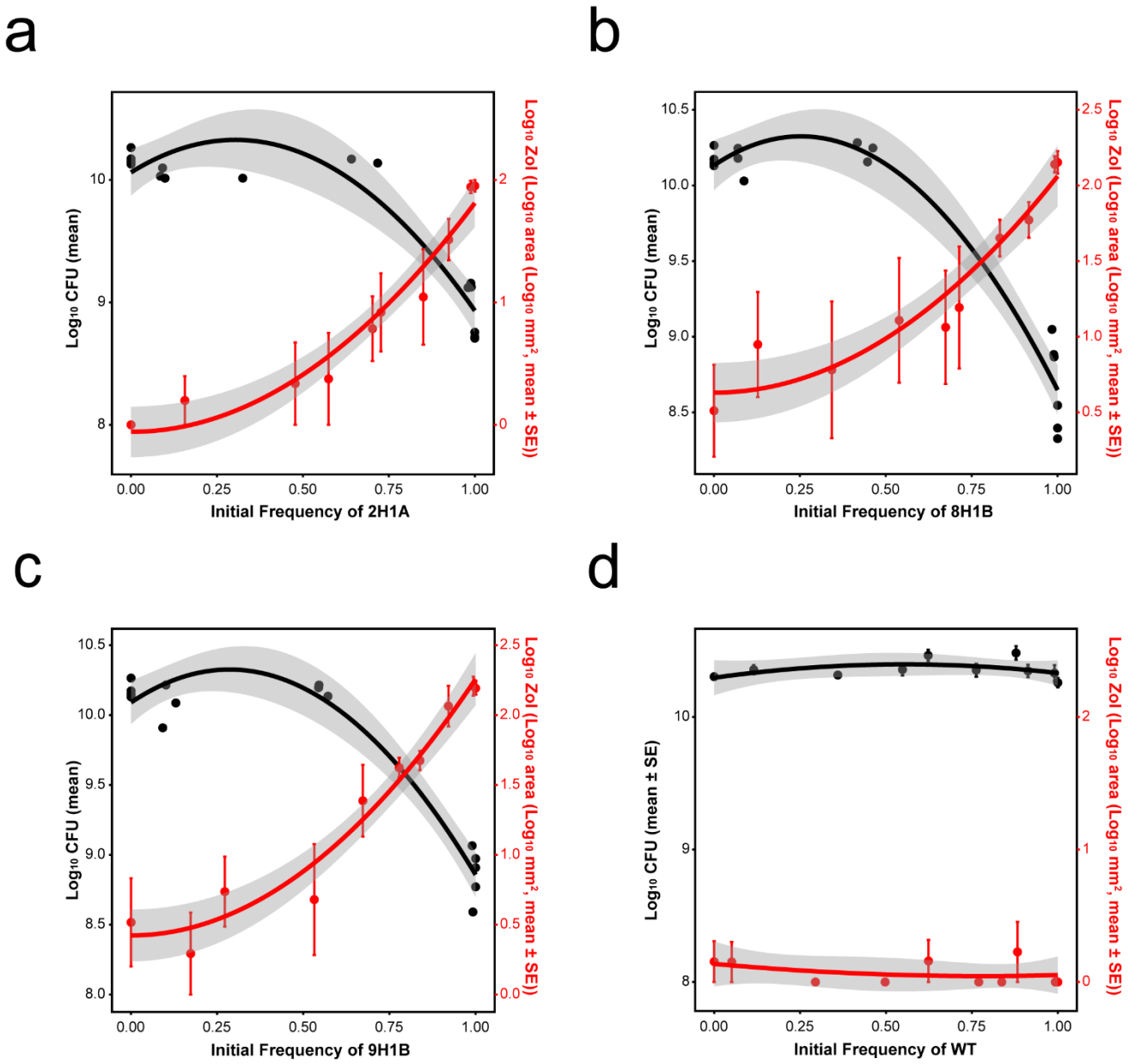


Figure S6: Extended evidence for division of labour during co-culture of the WT and three mutant strains at different starting frequencies. Increasing frequencies of mutants cause increased antibiotic production (red) for (a) 2H1A ($F_{1,8}$ = 170.3, r^2 = 0.955, p < 0.001), (b) 8H1B ($F_{1,8}$ = 105.3, r^2 = 0.929, p < 0.001) and (c) 9H1B ($F_{1,8}$ = 201.1, r^2 = 0.962, p < 0.001) but not (d) WT ($F_{2,7}$ = 0.576, r^2 = 0.141, p = 0.587). Increasing frequencies of mutants only negatively impact colony fitness at frequencies > ~ 50% (black) for (a) 2H1A ($F_{2,13}$ = 59.44, r^2 = 0.901, p < 0.001), (b) 8H1B ($F_{2,13}$ = 131.7, r^2 = 0.953, p < 0.001) and (c) 9H1B ($F_{2,12}$ = 201.1, r^2 = 0.944, p < 0.001) but not (d) WT ($F_{2,7}$ = 1.076, r^2 = 0.235, p = 0.391). Quadratic regression lines include the 95% CI.

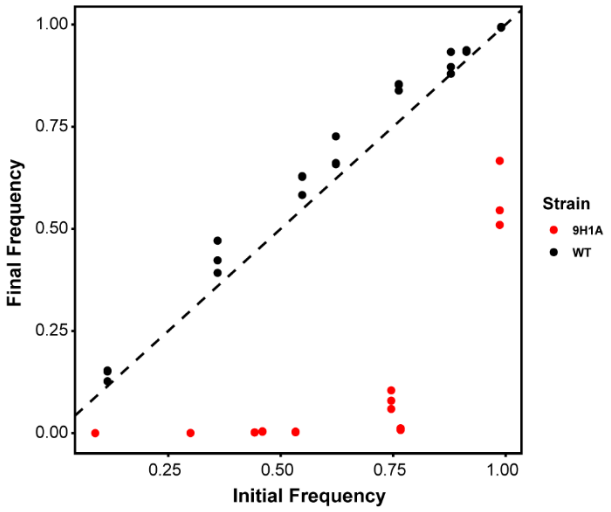


Figure S7: Initial and final frequencies of strains during pairwise competition assays between 9H1A (red) and the wild-type or between two differentially marked wild-type strains (black). The dashed line indicates that initial and final frequencies are equal, while values below the line indicate that the competing strain has declined during the competition assay. While the differentially marked wild-type strains have equal fitness, the mutant strain has dramatically reduced fitness at every starting frequency.