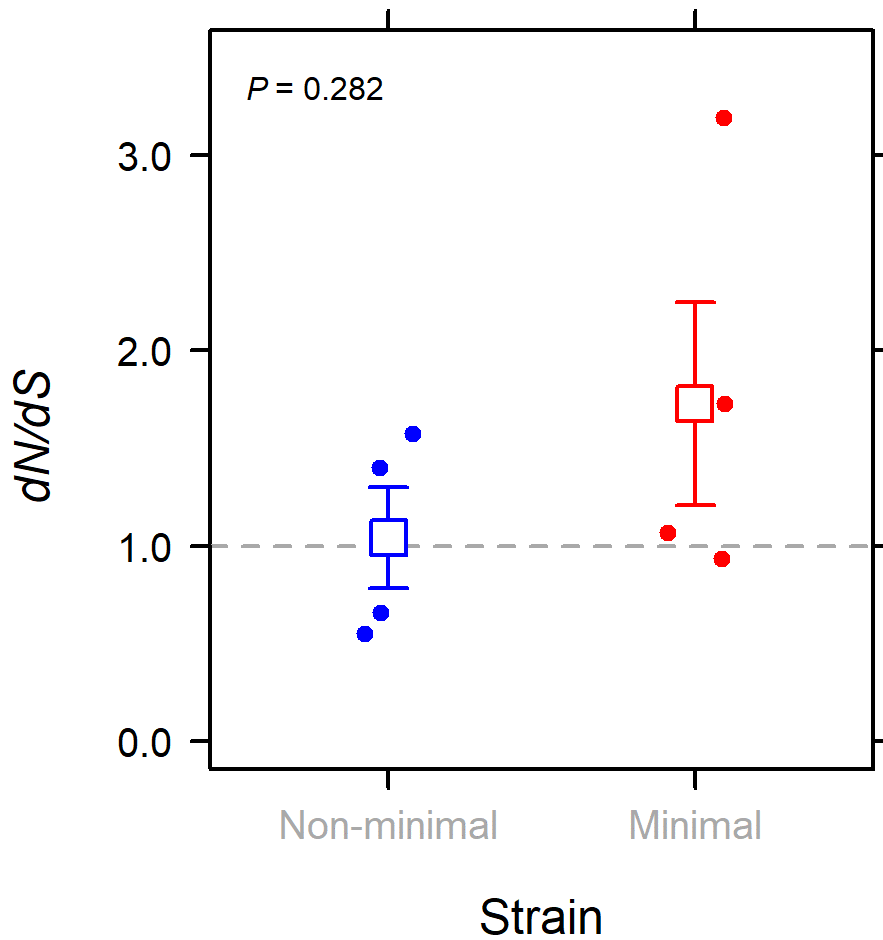


## Supplementary Materials:

2

### Evolution of a minimal cell

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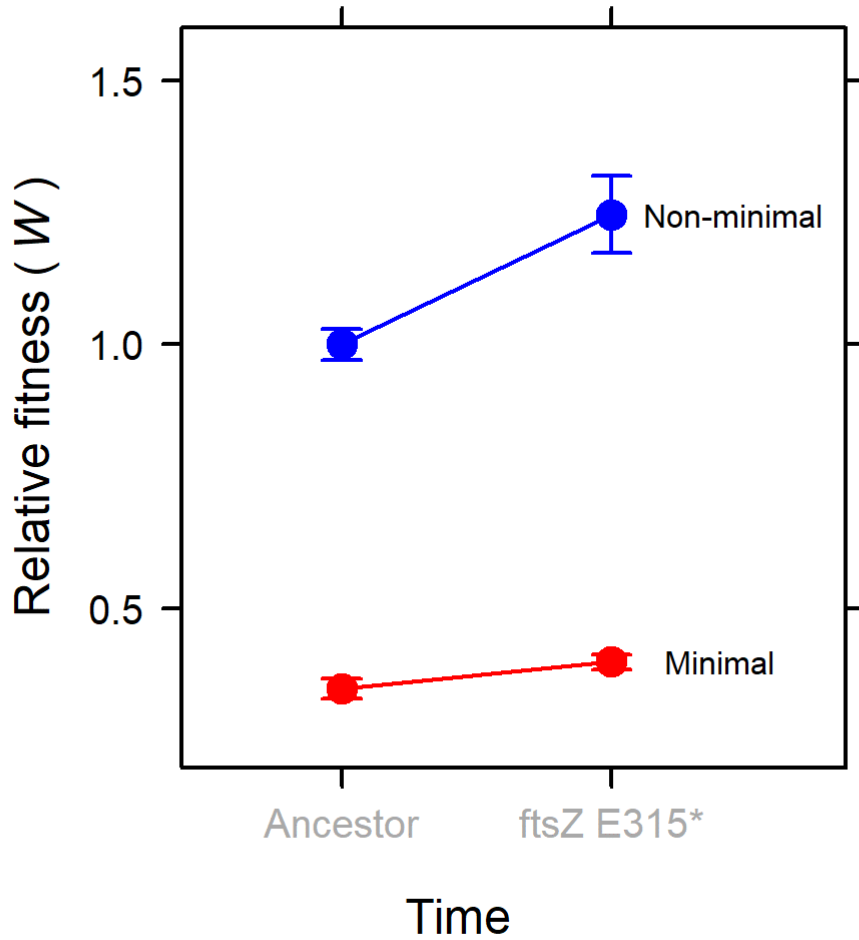
4

**Figure S1.** The ratio of nonsynonymous to synonymous substitutions in populations of *M. mycoides* after 2000 generations of evolution. The numbers of nonsynonymous and synonymous single nucleotide mutations (SNMs) were counted within each of the evolved replicate populations. The normalized ratio of nonsynonymous to synonymous mutations ( $d_N/d_S$ ) can indicate the relative action of positive or negative selection. Values of  $d_N/d_S > 1$  indicate positive selection, while  $d_N/d_S < 1$  can indicate the dominance of negative selection and constraint on adaptation. In contrast to expectations,  $d_N/d_S$  was not lower for the minimal cell, suggesting that it did not experience more constraint during adaptation. Open boxes represent the mean, error bars represent  $\pm$  standard error of the mean (SEM), solid symbols represent values for each replicate population.  $P$ -value corresponds to output from a two-sample  $t$ -test.

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**Table S1.** Mutations observed in *ftsZ* during adaptive evolution across all replicates.

<b>Strain</b>	<b>Replicate ID</b>	<b>Mutation</b>	<b>Mutation effect</b>	<b>Mutant allele frequency</b>
Non-minimal	MM_1	Q349* (CAA→TAA)	Nonsense	1
Non-minimal	MM_3	Q346* (CAA→TAA)	Nonsense	0.079
Non-minimal	MM_4	Δ1 bp (597/1158 nt)	Frameshift	1
Non-minimal	MM_6	G17C (GGT→TGT)	Missense	1
Minimal	MM_11	+CT (1062/1158 nt)	Frameshift	1
Minimal	MM_11	V190I (GTT→ATT)	Missense	1
Minimal	MM_13	E315* (GAA→TAA)	Nonsense	1



18 **Figure S2.** Fitness effects of an *ftsZ* mutation on populations of *Mycoplasma mycoides*. We reengineered the nonsense mutation *ftsZ* E315\* and quantified its effect on fitness in both the non-minimal and minimal cells. The mutation increased fitness by 25% and 14%, respectively. Data represent means  $\pm$  SEM.

22 **Table S2.** Diameter and volume measurements of *M. mycoides* ancestor and evolved cells observed *via* phase contrast microscopy. Values displayed as mean  $\pm$  SEM.

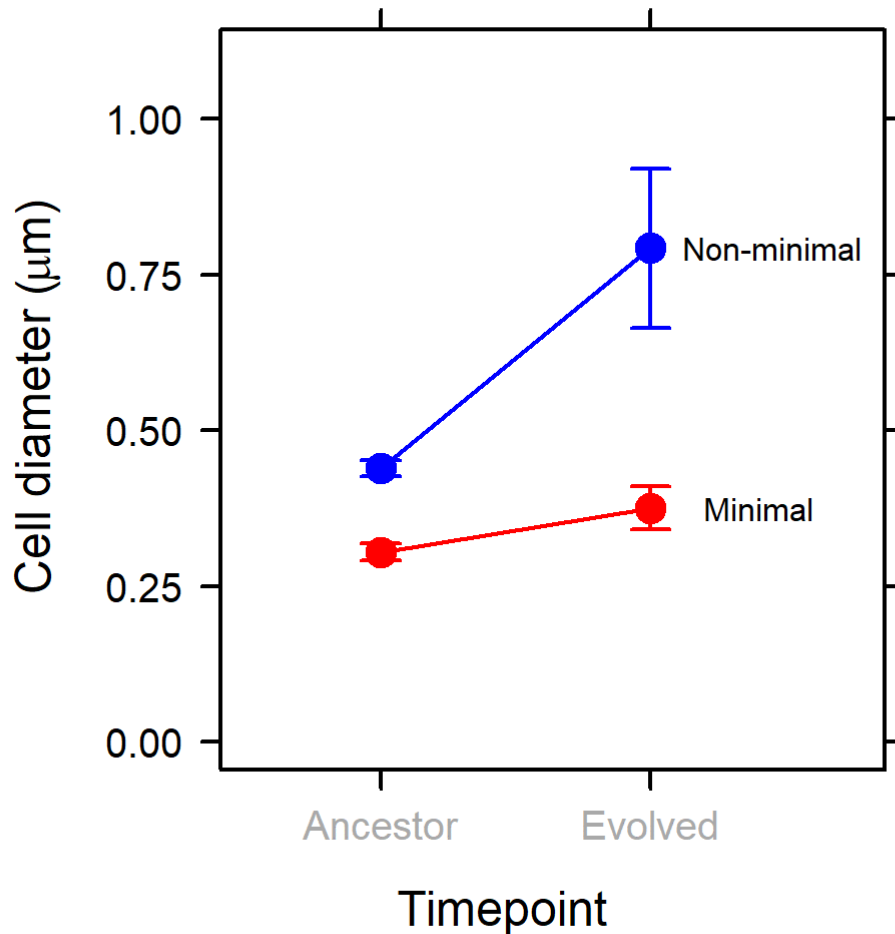
	Non-minimal Cell		Minimal Cell	
	Diameter (nm)	Volume ( $\mu\text{m}^3$ )	Diameter (nm)	Volume ( $\mu\text{m}^3$ )
Ancestor	$575 \pm 28$	$0.123 \pm 0.020$	$422 \pm 23$	$0.058 \pm 0.010$
Evolved	$993 \pm 53$	$0.577 \pm 0.102$	$463 \pm 48$	$0.082 \pm 0.024$

24

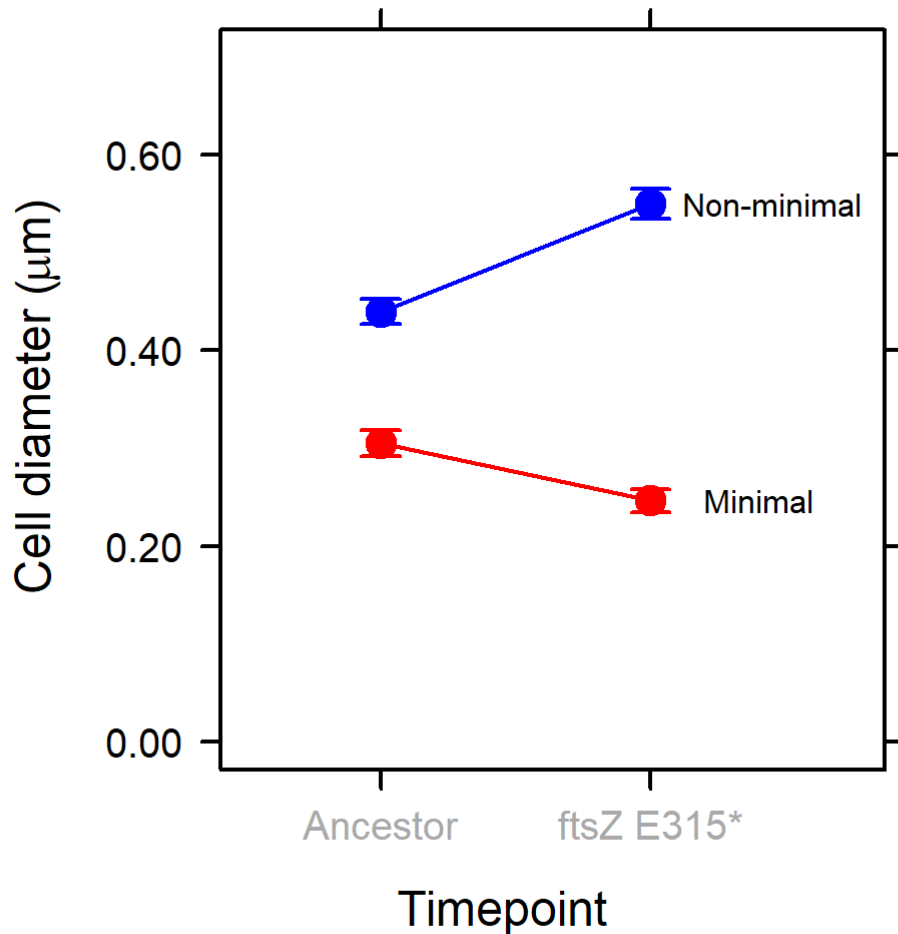
26 **Table S3.** Diameter and volume measurements of *M. mycoides* ancestor, evolved, and *ftsZ* E315\*  
 28 cells observed *via* scanning electron microscopy. Values displayed as mean  $\pm$  SEM.

	Non-minimal Cell		Minimal Cell	
	Diameter (nm)	Volume ( $\mu\text{m}^3$ )	Diameter (nm)	Volume ( $\mu\text{m}^3$ )
Ancestor	439 $\pm$ 13	0.051 $\pm$ 0.004	305 $\pm$ 28	0.021 $\pm$ 0.002
Evolved	792 $\pm$ 128	0.479 $\pm$ 0.260	375 $\pm$ 35	0.044 $\pm$ 0.012
<i>ftsZ</i> E315*	550 $\pm$ 15	0.099 $\pm$ 0.008	246 $\pm$ 12	0.011 $\pm$ 0.001

30



32 **Figure S3.** Cell size of ancestor and evolved cells from the non-minimal and minimal strains ob-  
 34 served using scanning electron microscopy (see Methods). Similar findings are reported in the  
 main text (Fig. 4) using phase microscopy. The evolved phenotypes of the strains diverged: The  
 size of the non-minimal cells increased marginally during evolution ( $t_3 = 2.76$ ,  $P = 0.070$ ), while  
 36 the size of the minimal cell did not change ( $t_3 = 1.01$ ,  $P = 0.459$ ). Data represent means  $\pm$  SEM.  
 Error bars for the ancestor represent experimental replicates, while error bars for evolved lines  
 38 represent evolutionary replicates.



40 **Figure S4.** Cell size of *ftsZ* E315\* mutants compared to the nonmutant for each strain, measured by scanning electron microscopy. The *ftsZ* nonsense mutation caused an increase in wildtype cell size ( $P = 8.71 \times$   
 42  $10^{-8}$ ) but did not increase the minimal cell's size. In fact, the minimal cell *ftsZ* E315\* mutant was smaller than the nonmutant ( $P = 0.0013$ ). These observations are in accord with the observation of increased cell  
 44 size during evolution for the wildtype but not the minimal cell. The data suggest that *ftsZ* mutations could contribute to the mechanism of the divergent cell size trajectory during evolution. Error bars represent  
 46  $\pm 2$  SEM.

**Table S4.** SP4 medium reagents.

<b>Ingredient</b>	<b>Manufacturer</b>	<b>Quantity/L</b>	<b>Unit</b>
Mycoplasma broth base	Hardy Diagnostics	3.5	g
Bacto tryptone	Fisher BioReagents	10	g
Bacto peptone	Fisher BioReagents	5.3	g
Glucose	Sigma-Aldrich	5	g
Yeastolate	Life Technologies	2	g
Epure filtered water		725	mL
CMRL 1066 (10X)	Corning	50	mL
7.5% sodium bicarbonate	Fisher Chemical	14.6	mL
200 mM L-glutamine	Fisher Chemical	5	mL
Yeast extract solution 15%	Gibco	35	mL
Knockout serum replacement	Gibco	170	mL
Penicillin G	Sigma-Aldrich	100	$\mu$ L
Phenol red	Sigma-Aldrich	0.011105	g

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**Table S5.** Genes added to *Mycoplasma mycoides* JCVI-syn3B.

<b>Locus Tag</b>	<b>Annotation</b>
MMSYN1_0520	Conserved hypothetical protein
MMSYN1_0521	Cell division protein sepF
MMSYN1_0522	Cell division protein ftsZ
MMSYN1_0527	Putative metal binding or nucleic acid binding
MMSYN1_0538	Conserved hypothetical protein
MMSYN1_0546	Pseudogene
MMSYN1_0548	tRNA (cytidine(34)-2'-O)-methyltransferase
MMSYN1_0549	Non-canonical purine NTP pyrophosphatase
MMSYN1_0592	Uncharacterized protein
MMSYN1_0593	Uncharacterized protein
MMSYN1_0601	Putative CAAX amino terminal protease, transmembrane
MMSYN1_0602	Pseudogene
MMSYN1_0604	LemA family protein
MMSYN1_0605	Putative phosphate starvation inducible protein (psiF),
MMSYN1_0610	DNA-formamidopyrimidine glycosylase
MMSYN1_0622	Uncharacterized lipoprotein
MMSYN1_0623	Putative adenine glycosylase, methyltransferase, kinase
MMSYN1_0930	50S ribosomal protein L33 rpmG
MMSYN1_0931	Adenylyl-sulfate kinase met14p

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