Supporting information

An Expanded Genetic Toolbox to Accelerate Creation of *Acholeplasma laidlawii* Driven by Synthetic Genomes

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Supplementary Figures

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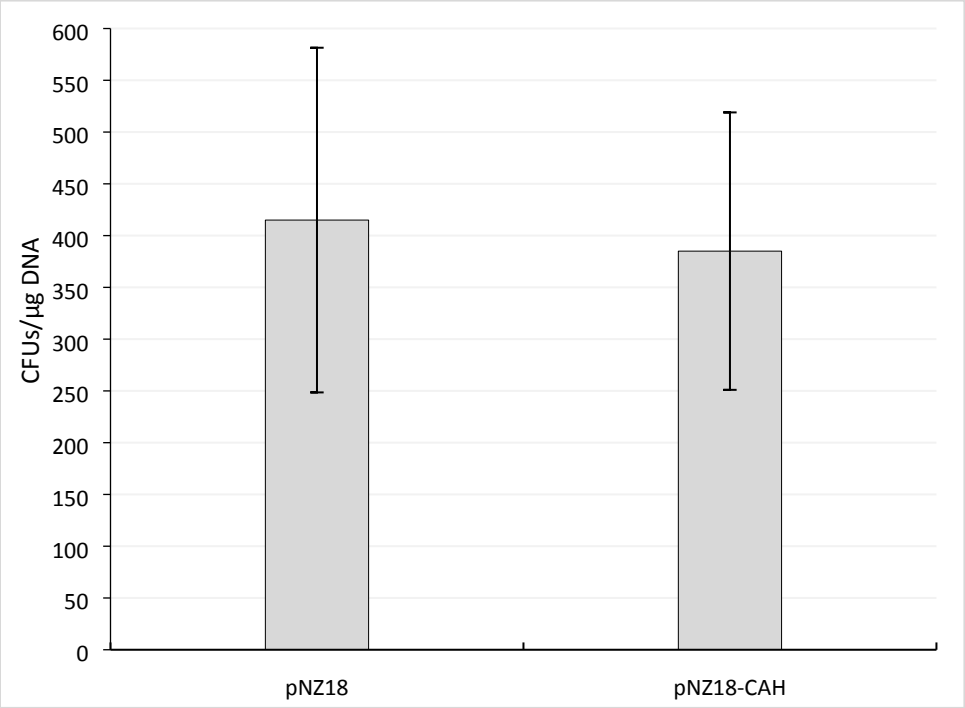
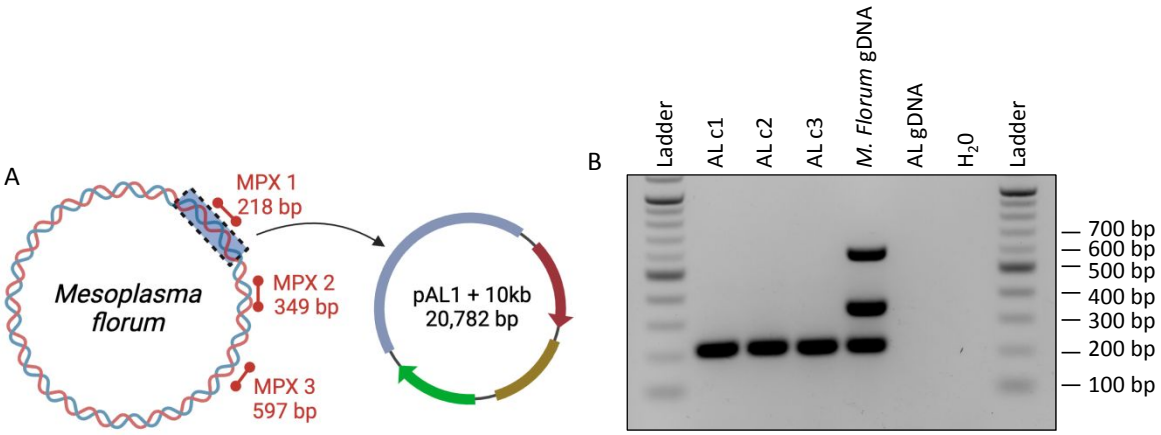
attaataaaaggggtaaaat

Supplementary Figure 1. Sequence of the *A. laidlawii* 8195 origin of replication. The CDS of the DnaA gene is shown in grey, with the start codon highlighted in green and the stop codon highlighted in red. Highlighted in yellow are DnaA boxes predicted by Ori-Finder 2022.



Supplementary Figure 2. Comparison between pNZ18 and pNZ18-CAH. Bars represent the average colony counts from 4 independent electroporation experiments to an evolved *A. laidlawii* 8195 strain using 1 microgram of pNZ18 or pNZ18-CAH, with error bars showing standard error of the mean. The difference in mean colony counts between the two plasmids is not statistically significant, as calculated by a two-tailed student's t-test.



Supplementary Figure 3. Cloning a large fragment in *A. laidlawii*. A) Schematic showing the cloning of a 10 kb fragment (highlighted in blue) from the genome of *Mesoplasma florum* in pAL1, resulting in the plasmid pAL1 + 10 kb. MPX 1 screens for the 10 kb fragment, while MPX 2 and MPX 3 are controls that amplify elsewhere in the *M. florum* genome. Created with BioRender.com B) Gel electrophoresis of a multiplex PCR analysis for MPX 1 – 3. Three *A. laidlawii* colonies (AL c1 – c3) were analyzed following transformation with pAL1 + 10 kb. Positive control: *M. florum* genomic DNA. Negative controls: genomic DNA from untransformed *A. laidlawii* (AL gDNA) and sterile water.

Supplementary Tables

Supplementary Table 1. Optimization of *A. laidlawii* transformation protocol. Each section represents an independent transformation of pAL1 to strain 8195. All experiments were done with cultures of OD_{600} = 0.17 - 0.25, with a recovery time of at least 2 hours at 34°C following transformation. All colonies were counted after 6 days at 34°C.

Condition Tested	Colony Count
Volume of Culture Used (mL)	
1	44
1.5	8
2	7
3	6
Number of Washes Before Transformation	
1	194
2	10
3	11
Amount of DNA Transformed (µg) and use of 10 µg yeast tRNA (+ or -)	
2 -	11
2 +	2
3 -	20
3 +	30
5 -	34
5 +	36
10 -	45
10 +	90
Cell Pretreatment and Incubation (1)	
Pretreatment- 5 µg/mL chloramphenicol for 1 hour	5
Pretreatment- 5 µg/mL tetracycline for 1 hour	4
Pretreatment- 0.25% trypsin for 10 mins	0
Pretreatment- ~1 mM EDTA for 10 mins	92
Pretreatment- ~2 mM EDTA for 10 mins	111
Pretreatment- ~5 mM EDTA for 10 mins	107
Pretreatment- 30 mins on ice	360
Electroporation- 400 Ω instead of 200 Ω	70
After electroporation- 10 mins on ice	266
After electroporation- 10 mins at room temperature	62
Cell Pretreatment and Incubation (2)	
After electroporation- 10 mins at room temperature	220
After electroporation- 10 mins on ice	329
Pretreatment- ~5 mM EDTA for 10 mins	306
Pretreatment: ~5mM EDTA for 10 mins + After transformation: 10 mins on ice	432

Supplementary Table 2. Stability of pAL1 and pNZ18 in *A. laidlawii.* Plasmid stability was analyzed after 50 hours of growth at 37°C.

pNZ18					
Grown with antibiotic			Grown without antibiotic		
No selection	Neo 200	Ratio	No selection	Neo 200	Ratio
158	180	114	613	60	10
pAL1					
Grown with antibiotic			Grown without antibiotic		
No selection	Tet 1	Ratio	No selection	Tet 1	Ratio
184	132	72	725	70	10

Supplementary Table 3. Comparing wild type and evolved *A. laidlawii* strains. Single-nucleotide substitutions within genes that are common to both evolved strains are shown alongside the resulting amino acid change. Bases always refer to the positive strand, and base positions are in reference to a new version of the wild type genome following re-sequencing. K numbers are shown for genes that could be identified by the KEGG Assign KO tool. Genes lacking annotation data are labelled CDS.

Base Position	Gene Name	K Number(s)	WT Base	Evolved Base	WT A.A.	Evolved A.A.
44670	ugpB	K10240	G	T	A	S
79243	mmsB	K00020	G	T	G	C
133815	nusG	K02601	G	T	R	I
208623	aroF	K03856	G	T	A	S
291891	ugpE	K02026	G	T	A	S
296722	murQ	K07106	C	A	A	E
405216	CDS		G	T	E	*
515634	ackA	K00925	G	T	R	I
571912	CDS		G	T	R	I
600951	CDS	K08153	C	A	A	S
631308	CDS		C	A	I	I
667347	amyA	K01182	C	A	R	S
731159	lolD	K02003, K02004	C	A	D	Y
834394	truB	K03177	G	T	P	H
853889	rnjA	K12574	C	A	D	Y

935397	tcyP	K06956	C	A	V	L
1005041	atpA	K02111	G	T	L	L
1013669	mdlB	K18889	C	A	E	*
1034829	CDS	K13280	G	T	L	I
1091422	CDS		G	T	A	E
1107090	CDS		C	A	G	V
1234106	acrR		C	A	A	D
1321675	pdhA	K00161	C	A	G	C
1401987	CDS		C	A	A	S

Supplementary Table 4. Summary of Tn5 transformation experiments to *A. laidlawii*.

Experiment #	Strain	OD_{600}	Recovery time (hours)	Antibiotic Selection (µg/mL)	# of Colonies
1	8195, WT	0.2699	4	Tet1	220
2	8195, WT	0.2000	2	Tet1	11
3	8195, WT	0.0900	2	Tet1	0
4	8195, WT	0.2297	2	Tet1	0
5	8195, WT	0.2077	4	Tet1	0
6	8195, WT	0.2216	4	Tet0.75	17
7	PG-8A	0.2200	2.67	Tet1	1
8	8195, Evolved	~0.25	2	Tet1	23

Supplementary Table 5. Summary of PEG transformation experiments to *A. laidlawii*. Shown for each experiment is the strain used, growth of cultures as determined by pH, the volume of cells plated after the transformation, the plasmid used for each transformation, and the colony counts for each technical replicate. Along with each group of experiments is the list of parameters changed from the standard protocol described in the methods section. Strains: WT = wild type 8195, E = strain evolved for improved electroporation, PEG = a strain transformed and cured from an earlier PEG experiment. SP4- : SP-4 lacking serum; SP4+: SP-4 supplemented with 17% horse serum. N/D – no data

#	Date	Strain	Culture pH	Volumed Plated (µL)	Plasmid	Rep #1	Rep #2	Rep #3	Protocol Notes
1	2023-04-26	PEG	7.27	500	pAL1	0	N/D	N/D	• 3-hour PEG incubation • 2-hour recovery
2	2023-04-26	PEG	7.37	500	pAL1	0	N/D	N/D	
3	2023-04-26	PEG	7.32	500	pAL1	0	N/D	N/D	
4	2023-04-26	E	7.19	500	pAL1	0	N/D	N/D	
5	2023-04-26	E	7.26	500	pAL1	0	N/D	N/D	
6	2023-04-26	E	7.38	500	pAL1	0	N/D	N/D	
7	2023-05-04	PEG	7.20	500	pAL1	6	N/D	N/D	• Cultures were diluted from SP4+ to SP4+ • 1.5-hour PEG incubation • 2-hour incubation
8	2023-05-04	PEG	7.35	500	pAL1	10	N/D	N/D	
9	2023-05-04	PEG	7.43	500	pAL1	20	N/D	N/D	
10	2023-05-04	OC2	7.12	500	pAL1	1	N/D	N/D	
11	2023-05-04	E	7.32	500	pAL1	0	N/D	N/D	
12	2023-05-04	E	7.40	500	pAL1	0	N/D	N/D	
13	2023-05-12	PEG	7.53	500	pAL1	20	N/D	N/D	• Cultures were diluted from SP4+ to SP4+ • 1.5-hour PEG incubation • 2-hour recovery
14	2023-05-12	PEG	7.65	500	pAL1	125	N/D	N/D	
15	2023-05-12	PEG	7.69	500	pAL1	35	N/D	N/D	
16	2023-05-12	PEG	7.44	500	pAL1	36	N/D	N/D	• Cultures were diluted from SP4+ to SP4+

17	2023-05-12	PEG	7.61	500	pAL1	5	N/D	N/D	• 1.5-hour PEG incubation • 2-hour recovery • Used a pool of cured PEG-transformed strains
18	2023-05-12	PEG	7.57	500	pAL1	0	N/D	N/D	
19	2023-05-19	WT	6.89	500	pAL1	0	N/D	N/D	• Cultures were diluted from SP4- to SP4+ • 2-hour PEG incubation • 1.5-hour recovery
20	2023-05-19	WT	7.14	500	pAL1	2	N/D	N/D	
21	2023-05-19	WT	7.29	500	pAL1	0	N/D	N/D	
22	2023-05-19	WT	7.41	500	pAL1	0	N/D	N/D	
23	2023-05-19	WT	7.53	500	pAL1	0	N/D	N/D	
24	2023-05-19	E	6.69	500	pAL1	2	N/D	N/D	
25	2023-05-19	E	7.05	500	pAL1	14	N/D	N/D	
26	2023-05-19	E	7.27	500	pAL1	0	N/D	N/D	
27	2023-05-19	E	7.43	500	pAL1	2	N/D	N/D	
28	2023-05-19	E	7.54	500	pAL1	0	N/D	N/D	
29	2023-05-19	WT	6.89	500	pAL1	0	N/D	N/D	• Cultures were diluted from SP4- to SP4+ • 1.5-hour PEG incubation • 2-hour recovery
30	2023-05-19	WT	7.14	500	pAL1	0	N/D	N/D	
31	2023-05-19	WT	7.29	500	pAL1	0	N/D	N/D	
32	2023-05-19	WT	7.41	500	pAL1	0	N/D	N/D	
33	2023-05-19	WT	7.53	500	pAL1	0	N/D	N/D	
34	2023-05-19	WT	6.89	500	pNZ18	0	N/D	N/D	
35	2023-05-19	WT	7.14	500	pNZ18	0	N/D	N/D	
36	2023-05-19	WT	7.29	500	pNZ18	0	N/D	N/D	

37	2023-05-19	WT	7.41	500	pNZ18	0	N/D	N/D	
38	2023-05-19	WT	7.53	500	pNZ18	0	N/D	N/D	
39	2023-05-19	E	6.69	500	pAL1	0	N/D	N/D	
40	2023-05-19	E	7.05	500	pAL1	1	N/D	N/D	
41	2023-05-19	E	7.27	500	pAL1	1	N/D	N/D	
42	2023-05-19	E	7.43	500	pAL1	1	N/D	N/D	
43	2023-05-19	E	7.54	500	pAL1	0	N/D	N/D	
44	2023-05-19	E	6.69	500	pNZ18	1	N/D	N/D	
45	2023-05-19	E	7.05	500	pNZ18	0	N/D	N/D	
46	2023-05-19	E	7.27	500	pNZ18	0	N/D	N/D	
47	2023-05-19	E	7.43	500	pNZ18	0	N/D	N/D	
48	2023-05-19	E	7.54	500	pNZ18	0	N/D	N/D	
49	2023-06-01	WT	7.29	200	pAL1	0	0	N/D	• Cultures were diluted from SP4+ to SP4+ • 3-hour PEG incubation • 1-hour recovery
50	2023-06-01	WT	7.38	200	pAL1	0	0	N/D	
51	2023-06-01	WT	7.49	200	pAL1	0	0	N/D	
52	2023-06-01	WT	7.64	200	pAL1	0	0	N/D	
53	2023-06-01	E	7.23	200	pAL1	0	0	N/D	
54	2023-06-01	E	7.32	200	pAL1	0	0	N/D	
55	2023-06-01	E	7.40	200	pAL1	0	0	N/D	
56	2023-06-01	E	7.54	200	pAL1	0	0	N/D	
57	2023-06-01	PEG	7.43	200	pAL1	0	0	N/D	

58	2023-06-01	PEG	7.48	200	pAL1	0	0	N/D	
59	2023-06-01	PEG	7.62	200	pAL1	0	0	N/D	
60	2023-06-01	PEG	7.74	200	pAL1	0	0	N/D	
61	2023-06-06	PEG	7.42	200	pAL1	0	N/D	N/D	• Culture was diluted from SP- to SP+ • 2x Fusion Buffer contained 250 mM NaCl • 2-hour PEG incubation • 1.5-hour recovery
62	2023-06-06	PEG	7.42	200	pAL1	1	N/D	N/D	• Culture was diluted from SP- to SP+ • 2x Fusion Buffer contained 250 mM NaCl • 2-hour total PEG incubation: 1-hour incubation without DNA, 1-hour incubation after DNA was added • 1.5-hour recovery
63	2023-06-06	PEG	7.42	200	pAL1	0	N/D	N/D	• Culture was diluted from SP- to SP+ • 2-hour PEG incubation • 1.5-hour recovery
64	2023-06-06	PEG	7.42	200	pAL1	8	N/D	N/D	• Culture was diluted from SP- to SP+ • 2-hour total PEG incubation: 1-hour incubation without DNA, 1-hour incubation after DNA was added • 1.5-hour recovery
65	2023-06-07	WT	7.13	200	pAL1	0	0	0	• Cultures were diluted from SP- to SP+ • 2-hour PEG incubation • 1.5-hour recovery
66	2023-06-07	WT	7.27	200	pAL1	0	0	0	
67	2023-06-07	WT	7.47	200	pAL1	0	0	0	
68	2023-06-07	E	7.31	200	pAL1	0	0	0	

69	2023-06-07	E	7.50	200	pAL1	0	0	0	
70	2023-06-07	E	7.65	200	pAL1	0	0	0	
71	2023-06-07	PEG	7.10	200	pAL1	0	0	0	
72	2023-06-07	PEG	7.29	200	pAL1	0	0	0	
73	2023-06-07	PEG	7.46	200	pAL1	0	0	0	
74	2023-06-20	PEG	6.90	200	pAL1	0	0	0	• Cultures were diluted from SP- to SP+ • 3-hour total PEG incubation: 1-hour incubation without DNA, 2-hour incubation after DNA was added • 1-hour recovery
75	2023-06-20	PEG	7.18	200	pAL1	0	0	0	
76	2023-06-20	PEG	7.35	200	pAL1	0	0	0	
77	2023-06-20	PEG	7.47	200	pAL1	1	2	1	
78	2023-06-20	PEG	7.58	200	pAL1	0	0	0	
79	2023-06-21	PEG	6.93	200	pAL1	0	0	0	• Cultures were diluted from SP- to SP+ • 2-hour total PEG incubation: 1-hour incubation without DNA, 1-hour incubation after DNA was added • 1.5-hour recovery
80	2023-06-21	PEG	7.15	200	pAL1	0	0	0	
81	2023-06-21	PEG	7.34	200	pAL1	1	0	0	
82	2023-06-21	PEG	7.46	200	pAL1	1	1	0	
83	2023-06-21	PEG	7.55	200	pAL1	5	1	1	
84	2023-06-23	PEG	7.01	200	pAL1	0	0	0	• Cultures were diluted from SP- to SP+ •2-hour total PEG incubation: 1-hour incubation without DNA, 1-hour incubation after DNA was added • 1.5-hour recovery
85	2023-06-23	PEG	7.19	200	pAL1	1	0	0	
86	2023-06-23	PEG	7.36	200	pAL1	1	0	0	
87	2023-06-23	PEG	7.44	200	pAL1	2	1	1	
88	2023-06-23	PEG	7.52	200	pAL1	2	0	0	

Supplementary Table 6. List of primers used for the creation and screening of the Tn5 TetM cassette. Underlined sequences show 19-bp mosaic ends.

Primer Name	**Sequence (5' - 3')**		**Description**
BK2348_F	ctgtctcttatacacatctgcggccgctccctttagtgagggttaatgtcgt		Primer for creating Tn5 cassette
BK2348_R	ctgtctcttatacacatctgctactagtattattatgtgattttgttgaa		Primer for creating Tn5 cassette
Primer Name	**Sequence (5' - 3')**	**Size (bp)**	**Description**
BK2580_F	tcacgcattacgtaaaatgg	300 bp	Primer for screening TetM gene
BK2580_R	aaagaacagttttggaaacgaa	300 bp	Primer for screening TetM gene

Supplementary Table 7. List of primers used in the construction of pAL1. Underlined sequences show parts of the primer that bind to the template, where the remaining sequence is an overhang that adds an overlapping region of homology.

Name	Sequence (5' - 3')	Description
BK1970_F	acgtatcagtaaagatttaaaaagattaggatttaaatttgggcccgatcgccaacaaat	Amplifies ARSH4
BK1970_R	caattaatgtgagttagctcactcattaggcaccccaggcggatcgcttgcctgtaactt	Amplifies ARSH4
BK1971_F	aaaagatacgaggcgcgtgtaagttacaggcaagcgatccgcctggggtgcctaatgagt	Amplifies pMB1 replicon, AmpR
BK1971_R	taaatacaactttaccggttagcttaaccttatctccatcgggcccgcggaacccctatt	Amplifies pBM1 replicon, AmpR
BK1972_F	tgtatttagaaaaataaacaaatagggttccgcgggcccgatggagataaggttaagct	Amplifies Homology 1
BK1972_R	caagctcttactatattaccctgttatccctagcgtaacttcttaaaagaaagatactact	Amplifies Homology 1
BK1973_F	ttcttttaagagttacgctagggataacagggtaatatagtaagagcttggtgagcgcta	Amplifies HIS3, CEN6
BK1978_R	cgctataatgaccccgaagcagggttatgcagcggaagattttacatcttcggaaaacaa	Amplifies HIS3, CEN6
BK1981_F	ataacaaagaagaaataataattaataaaaggggtaaaattccctttagtgagggttaat	Amplifies TetM
BK1974_R	tcgacctgcaggcatgcaagcttggcgtaatcatggtcatgctactagtattattatgtg	Amplifies TetM
BK1975_F	cgttatatgttcaacaaaatcacataataatactagtagcatgaccatgattacgccaag	Amplifies LacZα
BK1975_R	tatatattttacacttatttaatacttaattcaaaaaatactatgcggcatcagagcaga	Amplifies LacZα
BK1976_F	atggtgcactctcagtacaatctgctctgatgccgcatagtatttttgaattaagtatt	Amplifies Puro
BK1976_R	ctagttaaagtagacatctacatactccggaaaatctatatttaataataaaaatcggg	Amplifies Puro
BK1977_F	taacaaaaaaatcgggaaatcccgatttttattattaaatatagattttccggagtatg	Amplifies Homology 2

BK1977_R	agagcaaggtaaaaggtagtatttgttggcgatcgggcccaaatttaaatc ctaatcttt	Amplifies Homology 2
BK1979_F	aattaaagaaaaaatagtttttgttttccgaagatgtaaaatcttccgctgc ataaccct	Amplifies OriT
BK1979_R	aagaaaagcaaggtttcataagaaaggtggtttttgattgatcgtcttgcc ttgctcgt	Amplifies OriT
BK1980_F	gagctgtaagtacatcaccgacgagcaaggcaagacgatcaatcaaaaa accacctttct	Amplifies OriC
BK1980_R	agggttttcccagtcacgacattaaccctcactaaagggaattttacccctt ttattaat	Amplifies OriC

Supplementary Table 8. List of primers used to construct and screen pAL1 with a 10 kb fragment cloned from *Mesoplasma florum*. Underlined sequences in assembly primers that amplify the 10 kb fragment show parts that bind to the template, where the remaining sequence is an overhang that adds an overlapping region of homology.

Name	**Sequence (5' - 3')**		**Description**
BK3056_F	GGGCCCGATCGCCAACAAATACTACCTTTTACCTTGCTCT		Linearizes pAL1
BK3056_R	aaatttaaatcctaatctttttaaatctttactgatacgt		Linearizes pAL1
BK3057_F	gatatatctaaacgtatcagtaaagatttaaaaagattaggatttaaatttat gaaaaaaatgcttttaagcttaactgc		Amplifies 10 kb fragment from *M. florum* genome
BK3958_R	GAGAGCAGGAAGAGCAAGGTAAAAGGTAGTATTTGTTGG CGATCGGGCCCatcttctcttgtttcaatgtaagcaaataa		Amplifies 10 kb fragment from *M. florum* genome
Primer Name	**Sequence (5' - 3')**	**Product Size (bp)**	**Description**
BK3063_F	tgaacaatttgctagagccaga	218	Primer for screening 10 kb insert
BK3063_R	ctgcatttgaagaaggcgta	218	Primer for screening 10 kb insert
BK3064_F	tccaatagaaatgtctccacaaga	349	Control primer for screening *M. florum* genome
BK3064_R	ccaacgtttggtctaccaacta	349	Control primer for screening *M. florum* genome
BK3065_F	agaagcaaaatgactggtgaaa	597	Control primer for screening *M. florum* genome
BK3065_R	cctcgaaagcctgtttacctt	597	Control primer for screening *M. florum* genome

Supplementary Methods

Whole-Genome Sequencing.

Cultures of *A. laidlawii* PG-8A and 8195 were grown to a high density, and genomic DNA was extracted using the Monarch High Molecular Weight DNA Extraction Kit (New England Biolabs). Genomic DNA was sequenced using a 9.4.1 Flongle flow cell with the RBK004 rapid barcoding kit following library preparation as instructed by the manufacturer (Oxford Nanopore Technologies). The Flongle flow cell was refueled during the sequencing run to improve output. Runs were basecalled using Guppy v4.5.2 in high-accuracy mode, and the resulting output was assembled using Flye v2.8.1. Genome maps were generated using a custom R script following annotation with Prokka and alignment with minimap2, samtools, and mosdepth[1–5]. Putative DnaA boxes were identified with Ori-Finder 2022[6]. For identifying restriction genes, draft genomes were annotated with RAST[7]. To compare wild type and evolved strains, genomic DNA was isolated the same way, and all strains were sequenced at Plasmidsaurus (plasmidsaurus.com).

Finding Mutations in Evolved Strains.

Following whole-genome sequencing of wild type and two evolved *A. laidlawii* strains, genomes were aligned using GSAlign[8]. A custom Python script using BioPython[9] was used to predict amino acid changes from the resulting GSAlign output. A list of common mutations between the two sequenced evolved strains was created. Genes were assigned K numbers using the KEGG Assign KO tool, and corresponding K numbers were submitted to the Reconstruct tool to identify gene pathways and networks[10].

Screening for the Tetracycline Marker and *M. florum* fragments.

Wild type and Tn5-TetM *A. laidlawii* were screened using Multiplex PCR (Qiagen) with primers listed in Supplementary Table 4. 1 µL of *A. laidlawii* genomic DNA or 1 ng of the Tn5 cassette was used as template. The thermocycler was programmed for 95°C for 5 minutes, 20 cycles of 94°C for 30 seconds, 57°C for 90 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 10 minutes. *A. laidlawii* colonies transformed with pAL1 + 10 kb plasmids were screened the same way using primers listed in Supplementary Table 6, but the number of PCR cycles was increased from 20 to 25.

Plasmid Construction.

The plasmid pAL1 was constructed through homologous recombination of overlapping fragments in yeast. All plasmid parts were first PCR-amplified using PrimeSTAR GXL DNA Polymerase (Takara Bio) with primers that added 40 bp of overlapping homology to each end: assembly primers are listed in Supplementary Table 5. The fragments were then combined in roughly equimolar amounts and co-transformed into *S. cerevisiae* spheroplasts; preparation of spheroplasts has been described previously[11]. Following spheroplast recovery, plasmid DNA was isolated and transformed to *E. coli* Epi300. Individual *E. coli* colonies were screened for the correctly assembled plasmid. The high copy pMB1 replicon, ampicillin resistance gene, and lacZα sequences were amplified from pUC19. The CEN6 and HIS3 sequences were amplified from pDMI2.0 (Karas lab), and the ARSH4 sequence was amplified from pAGE1.0[12]. The tetracycline marker, TetM, was isolated from genomic DNA of *Mesoplasma florum* L1-T-ΔRE[13]. *A. laidlawii* sequences (homology 1 and 2, origin of replication) were amplified from *A. laidlawii* 8195 genomic DNA. The origin of transfer was amplified from Pmod-yeast oriT (Karas lab). The puromycin marker was amplified from a previous *A. laidlawii* plasmid version, which was originally amplified from Pmod-yeast (Karas lab).

A version of pAL1 carrying a 10 kb insert was creating by first using PCR to linearize pAL1 between the homology 2 site and the pUC19 backbone. A 10 kb fragment was then PCR amplified from *Mesoplasma florum* L1-T-ΔRE genomic DNA with primers that add 50 - 51 bp of overlapping homology to the plasmid. The fragments were assembled in *S. cerevisiae* as described above.

A. laidlawii Plasmid Recovery and Digest.

Following transformation experiments, 20 *A. laidlawii* transformants from each plasmid were passed on solid media with the appropriate antibiotic. 10 colonies were then inoculated to 10 mL of SP-4 media. Once grown, cells were pelleted, and DNA was isolated using alkaline lysis with Buffers P1, P2, and P3 (Qiagen) followed by alcohol precipitation. Approximately 1 µL of isolated DNA (~20 – 200 ng) was used to transform *E. coli* as described previously: pAL1 was transformed to strain Epi300, and pNZ18 was transformed to strain MC1061. For each transformation, plasmid DNA was miniprepped from two *E. coli* colonies using EZ-10 Spin Columns (BioBasic). pAL1 and pNZ18 were then digested in a 20-µL reaction with 6 units of EcoRV-HF and 3 units of PshAI (New England Biolabs), respectively, at 37°C for approximately 1 hour.

Plasmid Stability Assay.

A single colony of *A. laidlawii* 8195 harboring either pAL1 or pNZ18 was grown in SP-4 supplemented with tetracycline and neomycin, respectively. Cultures were grown to a high density before being diluted to OD_{600} = 0.01 in i) SP-4 supplemented with the appropriate selective antibiotic and ii) SP-4 without selective antibiotic. After 50 hours of growth at 37°C, cultures were serially diluted and plated on solid SP-4 with and without selective antibiotics. All colonies were counted after 6 days at 37°C, and the ratio of colonies grown on non-selective and selective media was calculated.

Transformation to *E. coli*.

Electrocompetent *E. coli* were prepared by washing cells from 50 mL cultures (OD_{600} = 0.5 - 0.8) three times with ice-cold ddH_2O and concentrating cells in 10% glycerol. Typically, 30 µL of electrocompetent cells was combined with plasmid DNA in a 1.5 mL tube before transferring the cell/DNA mix to a pre-chilled 2 mm electrocuvette. The cuvette was pulsed using the same conditions described for *A. laidlawii*. Cells were recovered in 750 µL - 1 mL SOC media (5 mL 2M $MgCl_2$, 10 mL 250 mM KCl, 20 mL 1 M glucose, 0.5 g/L NaCl, 5 g/L yeast extract, 20 g/L Tryptone) with shaking at 225 rpm for 1 hour at 37°C before plating. For some transformations, electrocompetent *E. coli* Transformax Epi300 cells (Lucigen) were used.

PEG Transformation to *A. laidlawii*.

A culture of *A. laidlawii* 8195 was diluted in SP-4 supplemented with 17% horse serum and grown at 32°C for 16 – 24 hours. 1 mL of culture was centrifuged at 15,596 x g for 5 - 10 minutes at room temperature. Cells were washed with 1 mL of S/T Buffer (250 mM NaCl, 10 mM Tris-HCl [pH = 6.5]) and centrifuged as before. Supernatant was discarded; the cells were resuspended in 200 µL 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. Next, the cells were added to a 50 mL conical tube containing 400 µL of SP-4 minus serum, 1 µL of yeast tRNA (10 µg/µL), 1 µg of plasmid DNA, and an equal volume of a modified 2x fusion buffer (500 mM NaCl, 20 mM $MgCl_2$, 20% PEG 8000, 20 mM Tris-HCl [pH = 6.5]: pH adjusted to 6.5). The cells were pipetted gently to mix and incubated at 32°C for 1 - 3 hours. 5 mL of prewarmed SP-4 plus serum was added, and the cells were left to recover at 32°C for 1 - 2 hours. Finally, the cells were centrifuged at 3,000 x g and 4°C for 20 minutes. Supernatant was discarded, the pellets were resuspended in 1 mL of SP-4 minus serum, and 200 - 500 µL was plated on SP-4 plates supplemented with appropriate antibiotics. Typically, colonies were counted after 6 days at 32°C.

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