

CRP Binding Site      -35 Box      -10 Box

```

aggaattccc tgaatgtgac gcaaatcaact tcaaaagagtg agcaacagtc agtatcatca
tccttaaggg acttacactg cgtttagtga agtttctcac tcgttgtcag tcatagtagt

```

Stem-Loop Structure

```

tgtgagttaa accctcgccg cctgacgggtg agggttttct tttgggtatg tttatctgcg
acactcaatt tgggagcggc ggactgccac tcccaaaaga aaaccatac aaatagacgc

```

*rnk* Ribosome Binding Site      *rnk* Start

```

acactcgcag caccgacgac atggagtaaa atgtccaga ccaactatca tcattaacga
tgtgagcgtc gtggctgctg tacctcattt tacagggtct ggttgatagt agtaattgct

```

```

cctggatgcc gaacgcacg atattctgct ggagcaacc gcctatgctg gtttgccaat
ggacctacgg cttgcgtagc tataagacga cctcgttggg cggatacgcg caaacggtta

```

Amplification Junction → Truncated *citG*

```

cgccgacgcg ttaaacgcag agttggatcg cgccaaaatg ttattgcaa aagggggcat
cgggctgcgc aatttgcgtc tcaacctagc gcgggtttac aataacgtt ttccccgta

```

```

tcgaacccc gcgatctcg attatctccg gcagttcgac agggagtgta tcgaacgaaa
agcttggggg cggctagagc taatagaggc cgtaagctg tccctcacat agcttgcttt

```

```

tctcagtcca ggcggcagtg ctgacctact gatccttacc tggtttttag cacagattta
agagtcaggt ccgcccgtcac gactggatga ctaggaatgg accaaaaatc gtgtctaaat

```

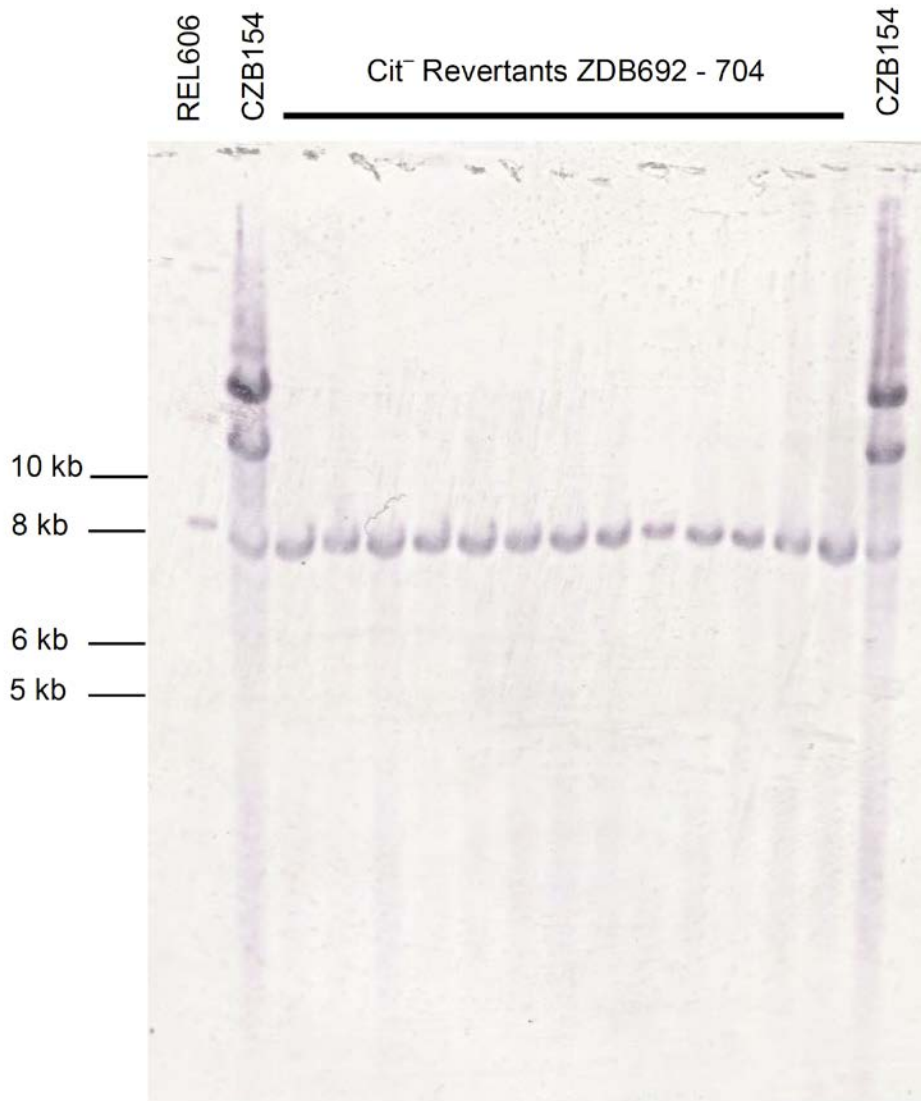
*citT* Ribosome Binding Site      *citT* Start

```

attattttaag cacttgataa atttggaat attaatthtc ggagaaccgc atgtcttta
taataaatcc gtgaactatt taaaccttta taattaaaag cctcttgggc atacagaaat

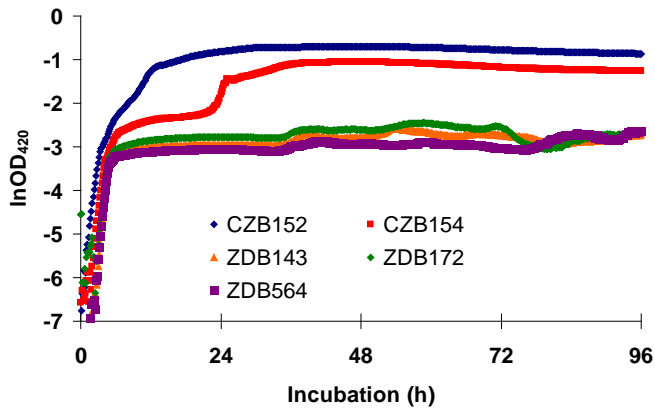
```

Supplementary Figure 1 | Annotation of sequence adjacent to the *cit* amplification boundary found in *Cit*<sup>+</sup> genomes.



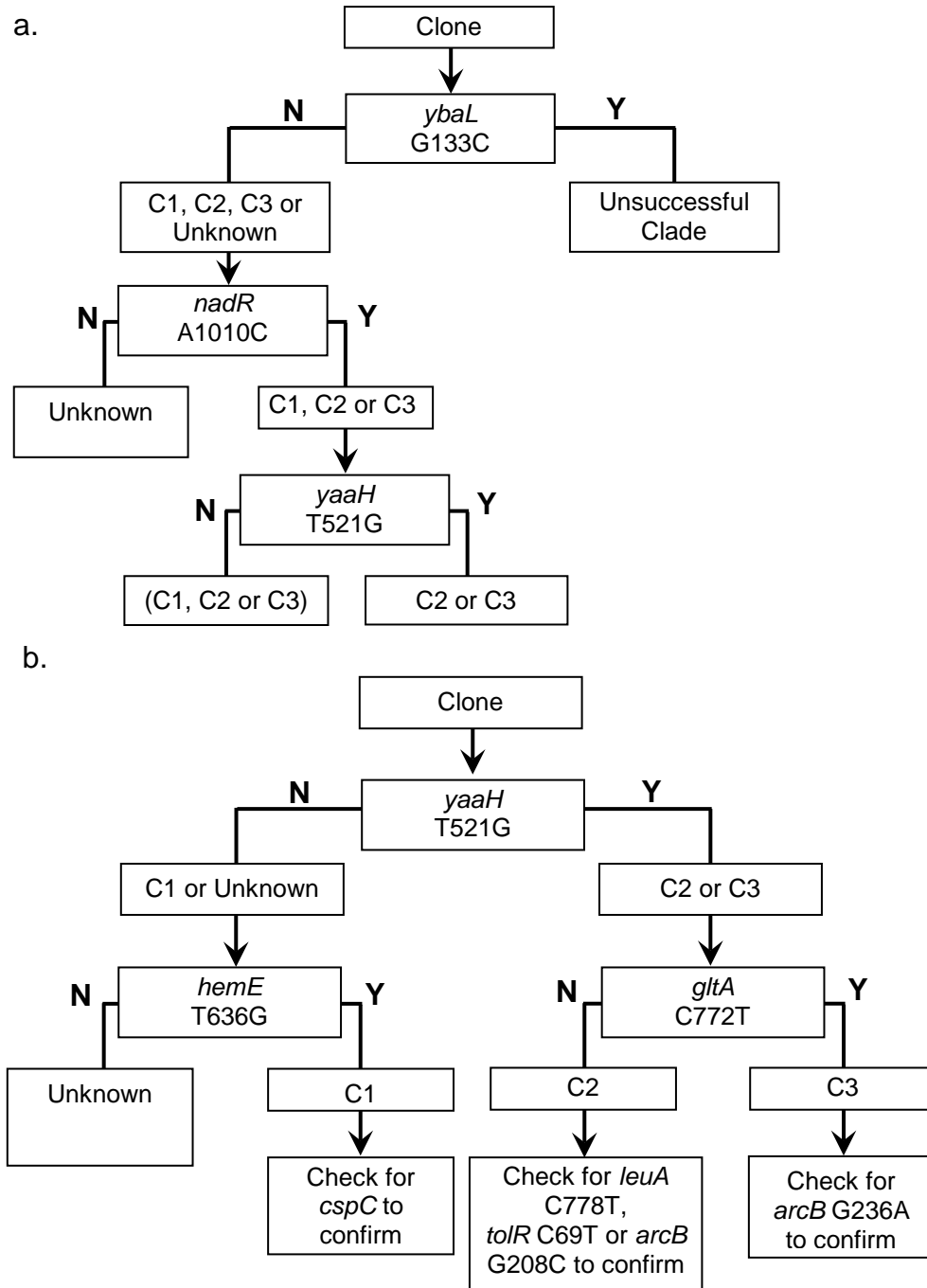
**Supplementary Figure 2 | Reversion to Cit<sup>-</sup> phenotype is associated with loss of *cit* amplification.**

The 33,000-generation Cit<sup>+</sup> clone, CZB154, has an increased *citT* band size relative to the ancestral strain, REL606, as a consequence of the *cit* amplification. By contrast, all 13 independent Cit<sup>-</sup> revertants of CZB154 have reduced *citT* band sizes, consistent with the loss of the *cit* amplification.

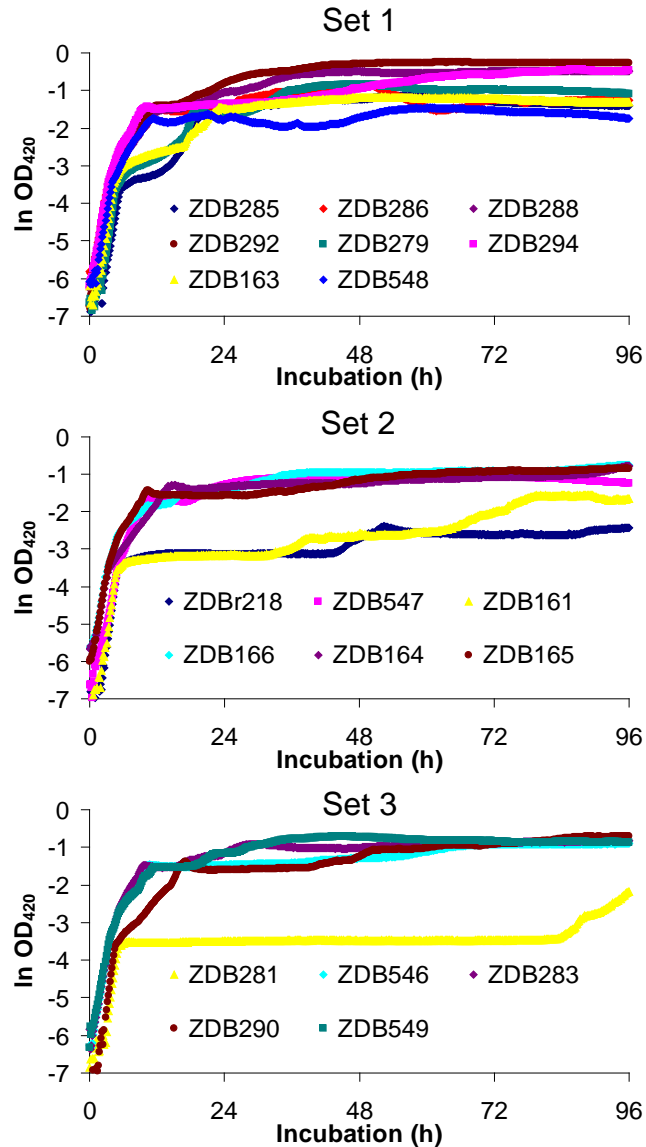


### Supplementary Figure 3 | Growth of early Cit<sup>+</sup> clones in DM25.

Early Cit<sup>+</sup> clones from population Ara-3 improved greatly in their capacity to grow on the citrate in DM25 over time. Cit<sup>+</sup> clones from generations 31,500 (ZDB564), 32,000 (ZDB172), and 32,500 (ZDB143) showed little growth on citrate even after 96 h, while two clones from generation 33,000 (CZB152 and CZB154) achieved substantial growth on citrate within 24 h. This improvement in citrate utilization allowed Cit<sup>+</sup> clones to rise from a minority of the population to numerical dominance.



**Supplementary Figure 4 | Keys for placement of clones used in replay experiments.** The phylogenetic placement of each clone used in the replay experiments was determined by scoring the presence or absence of informative mutations according to the keys above. Clones sampled before generation 20,000 were placed using according to **a**, while those from generation 20,000 and later were placed using **b**. The lower resolution for earlier clones reflects the lesser divergence in earlier generations.



**Supplementary Figure 5 | Growth of  $Cit^+$  mutants derived in replay experiments.**

The 19 spontaneous  $Cit^+$  mutants isolated during the course of replay experiments vary in how quickly they transition from the glucose to the citrate and how well they grow on the citrate in DM25. Here mutants are grouped according to the mutations responsible for the  $Cit^+$  phenotype. The eight mutants in Set 1 have tandem *citT* duplications similar to that which evolved in the original population. The six mutants in Set 2 mutants have IS3 insertions in *citG*. The five mutants in Set 3 have a variety of mutations, as described in the main text and Supplementary Table 12.

<b>Supplementary Table 1   Historical Ara-3 Clones Subjected to Whole Genome Sequencing</b>		
<b>Generation</b>	<b>Clones</b>	<b>Clade</b>
0	REL606	N/A
2,000	REL1166A	?
5,000	ZDB409	?
10,000	ZDB429	UC
15,000	ZDB446	UC
20,000	ZDB458	(C1,C2)
	ZDB464*	(C1,C2)
	ZDB467	(C1,C2)
25,000	ZDB477	C1
	ZDB483	C3
30,000	ZDB16	C1
	ZDB357	C2
31,500	ZDB199*	C1
	ZDB200	C2
	ZDB564	Cit <sup>+</sup>
32,000	ZDB30*	C3
	ZDB172	Cit <sup>+</sup>
32,500	ZDB158	C2
	ZDB143	Cit <sup>+</sup>
33,000	CZB199	C1
	CZB152	Cit <sup>+</sup>
	CZB154	Cit <sup>+</sup>
34,000	ZDB83	Cit <sup>+</sup>
	ZDB87	C2
36,000	ZDB96	Cit <sup>+</sup>
	ZDB99	C1
38,000	ZDB107	Cit <sup>+</sup>
	ZDB111	C2
40,000	REL10979	Cit <sup>+</sup>
	REL10988	C2

\*Known potentiated clone

## **Supplementary Table 2 | Mutations in Population Ara-3 Clones Subjected to Whole Genome Sequencing**

This table shows all of the mutations found in the 29 genomes from population Ara-3 that were sequenced for this study. Owing to its large size, this table is provided in a separate zip file. When opened, this zip file generates a summary table showing all of the mutations in html format; separate tables that show the mutations for each individual genome in html format; and machine-readable data files for each individual genome.

**Supplementary Table 3 | Results of PCR screens on whole-population samples for *cit* amplification**

<b>Generation</b>	<b>Replicate</b>	<b><i>cit</i> Amplification Detected?</b>
25,000	A	–
	B	–
	C	–
30,000	A	–
	B	–
	C	–
31,500	A	–
	B	–
	C	–
32,000	A	–
	B	–
	C	+
32,500	A	–
	B	–
	C	–
33,000	A	+
	B	+
	C	+
33,500	A	+
	B	+
	C	+
34,000	A	+
	B	+
	C	+
36,000	A	+
	B	+
	C	+



**Supplementary Table 4 | PCR screens for *cit* amplification in Ara-3 clones**

Generation	Clone	Cit Phenotype	Amplification Detected?	Generation	Clone	Cit Phenotype	Amplification Detected?
31,500	ZDB25	-	No	35,000	ZDB89	+	Yes
	ZDB26	-	No		ZDB90	+	Yes
	ZDB27	-	No		ZDB91	+	Yes
	ZDB564	+	Yes		ZDB92	-	No
	ZDB565	+	Yes		ZDB93	+	Yes
	ZDB566	+	Yes		ZDB94	+	Yes
32,000	ZDB28	-	No	36,000	ZDB95	+	Yes
	ZDB29	-	No		ZDB96	+	Yes
	ZDB30	-	No		ZDB97	+	Yes
	ZDB172	+	Yes		ZDB98	+	Yes
	ZDB173	+	Yes		ZDB99	-	No
	ZDB179	+	Yes		ZDB100	-	No
32,500	ZDB31	-	No	37,000	ZDB101	+	Yes
	ZDB32	-	No		ZDB102	+	Yes
	ZDB33	-	No		ZDB103	-	No
	ZDB143	+	Yes		ZDB104	-	No
	ZDB144	+	Yes		ZDB105	-	No
	ZDB145	+	Yes		ZDB106	-	No
33,000	CZB199	-	No	38,000	ZDB107	+	Yes
	CZB204	-	No		ZDB108	+	Yes
	CZB205	-	No		ZDB109	+	Yes
	CZB151	+	Yes		ZDB110	-	No
	CZB152	+	Yes		ZDB111	-	No
	CZB154	+	Yes		ZDB112	+	Yes
34,000	ZDB83	-	No	40,000	REL10979	+	Yes
	ZDB84	-	No		REL10980	+	Yes
	ZDB85	-	No		REL10981	+	Yes
	ZDB86	+	Yes		REL10988	-	No
	ZDB87	+	Yes		REL10989	-	No
	ZDB88	+	Yes		REL10990	-	No

Supplementary Table 5   Point mutations in early Cit <sup>+</sup> genomes											
Genome Position	Nucleotide Change	Type	Amino-acid Change	Gene	Product	Clone (generation)	ZDB564 (31,500)	ZDB172 (32,000)	ZDB143 (32,500)	CZB152 (33,000)	CZB154 (33,000)
447290	T → C	NC	N/A	<i>tesB/ybaY</i>	Acyl-CoA thioesterase II / predicted outer membrane lipoprotein		Gray	Gray	Gray	Gray	Red/Crosshatched
1399744	T → C	NS	I228V	<i>abgB</i>	Predicted peptidase / aminobenoyl-glutamate utilization protein		Gray	Gray	Red	Gray	Gray
2241625	C → A	NS	A259S	<i>ccmH</i>	Heme lyase subunit		Gray	Gray	Red	Gray	Gray
2443160	C → A	S	N/A	<i>glk</i>	Glucokinase		Gray	Gray	Red	Gray	Gray
3612959	C → T	NC	N/A	<i>dctA/yhjK</i>	C4-dicarboxylate transporter / Predicted diguanylate cyclase		Gray	Gray	Gray	Gray	Red/Crosshatched

Mutations shown are those that were not uniformly found in all five Cit<sup>+</sup> clones under study. Red fill indicates presence of mutation. Crosshatching further indicates mutation was also present in Cit<sup>+</sup> clones from generations 34,000, 36,000, and 38,000. Gray fill indicates absence of mutation.

Supplementary Table 6   IS-element insertions in early Cit <sup>+</sup> genomes									
Genome Position	Element	Gene	Product	Clone (generation)	ZDB564 (31,500)	ZDB172 (32,000)	ZDB143 (32,500)	CZB152 (33,000)	CZB154 (33,000)
595335	IS 150	<i>fepA/fes</i>	Outer membrane transporter/Ferric enterobactin esterase		Gray	Gray	Gray	Red	Gray
620126	IS 150	<i>dsbG</i>	Periplasmic disulfide isomerase/Thiol-disulphide oxidase		Gray	Gray	Gray	Red	Gray
1028311	IS 150	<i>uup</i>	Fused predicted transporter subunits of ABC superfamily: ATP-binding components		Gray	Red	Gray	Gray	Gray
2322345	IS 186	<i>menC</i>	O-succinylbenzoate		Gray	Gray	Gray	Gray	Crosshatched
2877315	IS 150	<i>kduD</i>	2-deoxy-D-fluconate/3-dehydrogenase		Gray	Gray	Red	Gray	Gray
4252526	IS 150	<i>uvrA</i>	Excinuclease ABC subunit A		Gray	Gray	Red	Gray	Gray

Mutations shown are those that were not uniformly found in all five Cit<sup>+</sup> clones under study. Red fill indicates presence of mutation. Crosshatching further indicates mutation was also present in Cit<sup>+</sup> clones from generations 34,000, 36,000, and 38,000. Gray fill indicates absence of mutation.

Supplementary Table 7   Deletions in early Cit <sup>+</sup> genomes									
Genome Start	Genome End	Size (bp)	Genes Deleted	Clone (generation)	ZDB564 (31,500)	ZDB172 (32,000)	ZDB143 (32,500)	CZB152 (33,000)	CZB154 (33,000)
590472	599560	9088	<i>hokE, insL-3, entD, fepA, fes, ybdZ</i>						
590472	595335	4863	<i>hokE, insL-3, entD, fepA</i>						
1345210	1345211	1	<i>yciR/mb</i>						
3786737	3786738	1	Noncoding						

Mutations shown are those that were not uniformly found in all five Cit<sup>+</sup> clones under study. Red fill indicates presence of mutation. Crosshatching further indicates mutation was also present in Cit<sup>+</sup> clones from generations 34,000, 36,000, and 38,000. Gray fill indicates absence of mutation.

**Supplementary Table 8 | Amplifications in early Cit<sup>+</sup> genomes**

Genome Start	Genome End	Size (bp)	Genes Duplicated	Clone (Generation)	ZDB564 (31,500)	ZDB172 (32,000)	ZDB143 (32,500)	CZB152 (33,000)	CZB154 (33,000)
12452	12455	3 (x2)	dnaK (internal fragment)						
599561	666130	66569 (x2)	<i>[entF], fepE, fepC, fepG, fepD, ybdA, fepB, entC, entE, entB, entA, ybdB, cstA, cstA, ybdD, ybdH, ybdL, ybdM, ybdN, insB-8, insA-8, dsbG, ahpC, ahpF, ybdQ,ybdR, rnk, rna, citT, citG, citX, citF, citE,citD, citC, insB-9, insA-9, citA, citB, dcuC, crcA, cspE, ccrB, ybeM, tatE, lipA,ybeF, lipB, ybeD, dacA, rlpA, mrdB, mrdA, ybeA, ybeB, phpB, nadD, holA, rlpB, leuS, ybeL, ybeQ, ybeQ, ybeR, ybeV, hscC, rihA, gltL, insJ-2, insK-2, [gltL]</i>						
619885	634746	14861 (x3)	<i>[dsbG], ahpC, ahpF, ybdQ, ybdR, rnk, rna, citT, citG, citX, citF, citE, citD, citC, insB-9, insA-9</i>						
1729052	1995783	266731 (x3)	<i>[ydhV], ydhY, ydhZ, pykF, lpp, ynhG, sufE, sufS, sufD, sufC, sufB, sufA, ydiH, ydil, ydiJ, ydiK, ydiL, ydiM, ydiM, ydiN, ydiB, aroD, ydiF, ydiO, ydiP, ydiQ, ydiR, ydiS, ydiT, ydiD, ppsA, ydiA, aroH, ydiE, ydiU, ydiV, nlpC, btuD, btuE, btuC, himA, pheT, pheS, pheM, rplT, rpml, infC, thrS, arpB, arpB, ECB_01690, ydiY, pfkB, ydiZ ,yniA, yniB, yniC, ydjM, ydjN, ydjO, ceda, katE, ydjC, celF, celD, celC, celB, celA, osmE, nadE, ydjQ, ydjR, spy, astE, astB, astD, astA, astC, xthA, ydjX, ydjY, ydjY, ydjZ, ynjA, ynjB, ynjC, ynjD, ynjE, ynjF, nudG, ynjH, gdhA, ynjI, topB, selD, ydjA, sppA, ansA, pncA, ydjE, ydjF, ydjG, ydjH ,ydjI, ydjJ, ydjK, ydjL, yeaC, yeaA, gapA, yeaD, yeaE, mipA, yeaG, yeaH, yeaI, yeaJ, yeaK, ECB_01757, yeaL, yeaM, yeaN, yeaO, yoaF, yeaP, yeaQ, yoaG, yeaR, insL-4, yeaS, yeaT, yeaU, yeaV, yeaW, yeaX, md, fadD, yeaY, yeaZ, yoaA, yoaB, yoaC, yoaH, pabB, yeaB,sdaA, yoaD, yoaE, manX, manY, manZ,yobD, yebN, rrmA, cspC, yobF, yebO,yobG, ECB_01797, kdgR, yebQ, htpX,prc, proQ, yebR, yebS, yebT, yebU, yebV, yebW, pphA, yebY, yebZ, yobA, holE, yobB, exoX, ptrB, yebE, yebF,</i>						

**Supplementary Table 8 | Amplifications in early Cit<sup>+</sup> genomes**

Genome Start	Genome End	Size (bp)	Genes Duplicated	Clone (Generation)	ZDB564 (31,500)	ZDB172 (32,000)	ZDB143 (32,500)	CZB152 (33,000)	CZB154 (33,000)
			<i>yebG, purT, eda, edd, zwf, yebK, pykA, msbB, yebA, znuA, znuC, znuB, ruvB, ruvA, yebB, ruvC, yebC, ntpA, aspS, yecD, yecE, yecN, yecO, yecP, torZ, torY, cutC, yecM, argS, yecT, flhE, flhA, flhB, cheZ, cheY, cheB, cheR, tap, tar, cheW, cheA, motB, motA, flhC, flhD, yecG, otsA, otsB, araH, araG, araF, yecl, yecJ, yecR, ftn, yecH, tyrP, yecA, leuZ, cystT, glyW, pgsA, uvrC, yvrY, insA-13, insB-13, yedU, yedV, yedW, yedX, yedY, yedZ, yodA, yodB, serU, yeel, asnT, yeeJ, yeel, yeeL, shiA, amn, yeeN</i>						
3268052	2086894	1181158 (x2)	<i>rpmA, rplU, ispB, nlp, murA, yrbA, yrbB, yrbC, yrbD, yrbE, yrbF, yrbG, yrbH, yrbI, yrbK, yhbN, yhbG, rpoN, yhbH, ptsN, yhbJ, ptsO, yrbL, mtgA, yhbL, arcB, yhcC, gltB, gltD, yhcG, ECB_03080, yhcH, nanK, nanE, nanT, nanA, nanR, dcuD, sspB, sspA, rpsI, rplM, yhcM, yhcB, degQ, degS, mdh, argR, yhcN, yhcO, yhcP, yhcQ, yhcR, yhcS, tldD, yhdP, rng, maf, mreD, mreC, mreB, yhdA, yhdH, accB, accC, yhdT, panF, prmA, yhdG, fis, yhdJ, yhdU, envR, acrE, acrF, yhdV, yhdW, yhdX, yhdY, yhdZ, rrfF, thrV, rrfD, rrlD, alaU, ileU, rrsD, yrdA, yrdB, aroE, yrdC, yrdD, smg, smf, def, fmt, rrmB, trkA, mscL, yhdL, zntR, yhdN, rplQ, rpoA, rpsD, rpsK, rpsM, rpmJ, prlA, rplO, rpmD, rpsE, rplR, rplF, rpsH, rpsN, rplE, rplX, rplN, rpsQ, rpmC, rplP, rpsC, rplV, rpsS, rplB, rplW, rplD, rplC, rpsJ, pioO, gspA, gspC, gspD, gspE, gspF, gspG, gspH, gspI, gspJ, gspK, gspL, gspM, gspO, bfr, bfd, chiA, tuf, fusa, rpsG, rpsL, yheL, yheM, yheN, yheO, fkpA, slyX, slyD, kefB, yheR, yheS, yheT, yheU, prkB, yhfA, crp, yhfK, argD, pabA, fic, yhfG, ppiA, yhfC, nirB, nirD, nirC, cysG, yhfL, yhfM, yhfN, frlC, yhfQ, yhfR, yhfS, insB-23, insA-23, yhfS, yhfT, yhfU, yhfV, yhfW, yhfX, yhfY, yhfZ, trpS, gph, rpe, dam, damX, aroB, aroK, hofQ, yrfA, yrfB, yrfC, yrfD, mrcA, yrfE, yrfF, yrfG, hslR, hslO, yhgE, pckA, envZ, ompR, greB, yhgF, feoA, feoB, yhgG, yhgA, bioH, yhgH, yhgI, gntT, malQ, malP, malT, rtcA, rtcB, rtcR, glpR, glpG, glpE, glpD, yzgL, ECB_03279, yzgL, glgP, glgA, glgC, glgX, glgB, asd, yhgN,</i>						

**Supplementary Table 8 | Amplifications in early Cit<sup>+</sup> genomes**

Genome Start	Genome End	Size (bp)	Genes Duplicated	Clone (Generation)	ZDB564 (31,500)	ZDB172 (32,000)	ZDB143 (32,500)	CZB152 (33,000)	CZB154 (33,000)
			<i>gntU, gntK, gntR, yhhW, yhhX, yhhY, yhhZ, yrhA, yrhB, ggt, yhhA, ugpQ, ugpC, ugpE, ugpA, ugpB, livF, livG, livM, livH, livK, yhhK, livJ, rpoH, ftsX, ftsE, ftsY, yhhF, yhhL, yhhM, yhhN, zntA, sirA, yhhQ, dcrB, yhhS, yhhT, acpT, nikA, nikB, nikC, nikD, nike, nikR, rhsB, yhhH, yrhC, yhhI, yhhJ, yhiH, yhil, yhiJ, yhiKL, yhiM, yhiN, pitA, yhiO, uspA, yhiP, yhiQ, priC, yhiR, gor, arsR, arsB, arsC, yhiS, slp, yhiF, yhiD, hdeB, hdeA, hdeD, yhiE, yhiU, yhiV, yhiW, gadX, gadA, yhjA, treF, yhjB, yhjC, yhjD, yhjE, yhjG, yhjH, kdgK, yhjJ, dctA, yhjK, bcsC, bcsZ, yhjN, yhjO, yhjQ, yhjR, yhjS, yhjT, yhjU, ldrD, ldrD, ldrD, yhjV, dppF, dppD, dppC, dppB, dppA, proK, yhjW, yhjX, yhjY, tag, yiaC, bisC, yiaD, tkrA, yiaF, yiaG, cspA, hokA, insJ-4, insK-4</i>						

Mutations shown are those that were not uniformly found in all five Cit<sup>+</sup> clones under study. Red fill indicates presence of mutation. Crosshatching further indicates mutation was also present in Cit<sup>+</sup> clones from generations 34,000, 36,000, and 38,000. Gray fill indicates absence of mutation.

Supplementary Table 9   Estimated <i>citT</i> copy number										
Gen (k)	Clone	Junction Number				Coverage			Predicted Configuration	Predicted <i>rnk-citT</i> Modules
		Anc <i>rnk</i>	Anc <i>citT</i>	<i>rnk-citT</i>	Relative <i>rnk-citT</i>	<i>citT</i>	Reference Regions	Relative <i>citT</i>		
31.5	ZDB564	52	49	42	0.83	120.8	64.9	1.86	2×	1
32	ZDB172	45	39	82	1.95	139.3	23.8	5.86	2× (3×)	4
32.5	ZDB143	116	76	301	3.14	457.2	112.8	4.05	4×	3
33	CZB152	22	23	192	8.53	267.4	32.7	8.18	9×	8
	CZB154	398	252	345	1.06	797.6	130.0	6.14	3× (2×)	3
34	ZDB83	57	43	125	2.50	214.7	62.9	3.42	4×	3
36	ZDB96	26	22	87	3.63	97.4	28.8	3.39	4×	3
38	ZDB107	51	32	133	3.20	204.7	60.0	3.41	4×	3
40	REL10979	36	32	73	2.15	115.6	32.5	3.56	4×	3

For each *Cit*<sup>+</sup> genome, the number of new junctions per genome was estimated from the relative number of reads supporting the new *rnk-citT* junction produced by the amplification versus the number of reads supporting the ancestral *rnk* and *citT* junctions. The total number of *citT* copies per genome was estimated by comparing read-depth coverage of the amplified *citT* region to coverage of regions that appear to be single copy in all genomes (comprising ~20 kb total including the *ara* operon and *tufB* gene). Together these data can be used to predict the likely configuration of *citT* amplification copies in each genome. Examination of read-depth coverage over a larger area supports the observation that there are nested amplifications in CZB154 and ZDB172. For example, the CZB154 genome contains three copies of a larger region, and each copy of that region contains two tandem copies with the usual *rnk-citT* junction.



**Supplementary Table 10 | Phylogenetically informative mutations**

<b>Gene</b>	<b>Gene Product</b>	<b>Genome Position</b>	<b>Gene Position</b>	<b>Ancestral Nucleotide</b>	<b>Evolved Nucleotide</b>	<b>Associated Clades</b>
<i>ybaL</i>	Predicted transporter with NAD(P)-binding Rossmann-fold domain	475173	133	G	C	UC
<i>nadR</i>	Nicotinamide-nucleotide adenyltransferase	4616538	1010	A	C	C1,C2,C3
<i>hemE</i>	Uroporphyrinogen decarboxylase	4177963	636	T	G	C1
<i>cspC</i>	Stress protein, member of the CspA-family	1886011	4	C	A	C1
<i>yaaH</i>	Conserved inner membrane protein associated with acetate transport	9972	521	T	G	C2,C3
<i>leuA</i>	2-isopropylmalate synthase	85556	778	C	T	C2
<i>tolR</i>	Membrane spanning protein in TolA-TolQ-TolR complex	756799	69	C	T	C2
<i>arcB</i>	Hybrid sensory histidine kinase in two-component regulatory system with ArcA. Aerobic respiration control sensor	3288053	208	G	C	C2
		3288026	236	T	A	C3
<i>gltA</i>	Citrate synthase	734488	772	G	A	C3



















**Supplementary Table 11 | Phylogenetic placement of Cit<sup>-</sup> replay clones**

Generation	Clone	Phylogenetically Informative Mutations											Clade
		Gene	<i>ybaL</i>	<i>nadR</i>	<i>hemE</i>	<i>cspC</i>	<i>yaaH</i>	<i>leuA</i>	<i>tolR</i>	<i>arcB</i>	<i>arcB</i>	<i>gltA</i>	
		Gene Position	133	1010	636	4	521	778	69	208	236	772	
		Ancestral Nucleotide	G	A	T	C	T	C	C	G	T	G	
		Evolved Nucleotide	C	C	G	A	G	T	T	C	A	A	
32,500	ZDB155												C1
32,500	ZDB156												C1
32,500	ZDB157												C3
32,500	ZDB158*												C2
32,500	ZDB159												C2
32,500	ZDB160												C1
32,500	ZDB398												C1
32,500	ZDB399												C2

\*Genome has been sequenced.

Red fill indicates presence of mutation has been established by sequencing. Gray fill indicates absence of mutation has been established by sequencing. No fill indicates that the presence or absence of mutation was not examined.

Clade refers to UC, C1, C2, or C3 as shown in Fig. 1 of the main text. When two or more clades are grouped by parentheses, either the clone belongs to the basal group or the clone's placement could not be resolved further based on the available data. n.d. indicates that the clone belongs to some other early clade or its placement could not be resolved based on the available data.

**Supplementary Table 12 | Mutations affecting *cit* region in Cit<sup>+</sup> replay mutants**

Generation	Cit <sup>-</sup> Parent	Clade	Cit <sup>+</sup> Mutant	Mutation affecting <i>cit</i> Region
20,000	ZDB464*	C3	ZDB285*	2978-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.
			ZDB286	2656-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.
27,000	ZDB309	C3	ZDB288	2070-bp tandem <i>cit</i> duplication that creates <i>rna-citT</i> regulatory module.
	ZDB310	C3	ZDB290*	~5,000-bp tandem duplication. Basis of <i>citT</i> activation unknown.
30,500	ZDB20	C3	ZDB547	IS3 insertion in <i>citG</i> .
31,000	ZDB390	C1	ZDB292	2706-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.
31,500	ZDB25	C3	ZDBr218	IS3 insertion in <i>citG</i>
	ZDB199	C1	ZDB279	2745-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.
32,000	ZDB28	C3	ZDB161	IS3 insertion in <i>citG</i> .
	ZDB29	C2	ZDB166	IS3 insertion in <i>citG</i> .
	ZDB30*	C3	ZDB164	IS3 insertion in <i>citG</i> .
			ZDB165*	IS3 insertion in <i>citG</i> .
			ZDB283*	~568-kbp inversion that places much of the <i>cit</i> operon under control of the <i>fimB</i> promoter.
	ZDB294*		2663-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.	
ZDB183	C3	ZDB281*	~14.3-kbp duplication. Basis of <i>citT</i> activation unresolved.	
32,500	ZDB31	C3	ZDB163	2990-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.
			ZDB546	Unknown rearrangement or duplication affecting <i>citT</i> .
			ZDB549	422-bp deletion in <i>citG</i> . Basis of <i>citT</i> activation unresolved.
	ZDB32	C2	ZDB548	3144-bp tandem <i>cit</i> duplication that creates <i>rnk-citT</i> regulatory module.

\*Genome has been sequenced.

**Supplementary Table 13 | Annotated differences between genomes of four Cit<sup>+</sup> mutants and their Cit<sup>-</sup> parent clones**

Supplementary Table 13a   Pair 1: ZDB464 (Generation 20,000) and Cit <sup>+</sup> Mutant ZDB285					Cit <sup>-</sup> Parent	Cit <sup>+</sup> Mutant
Position	Mutation	Description	Gene or Genes Involved	Product(s)	ZDB464	ZDB285
626102	2794 bp duplication	Tandem duplication with one junction in <i>citG</i> and the other between <i>rna</i> and <i>rnk</i> ; presumed Cit <sup>+</sup> actualizing mutation	<i>citG</i>	Triphosphoribosul-dephospho-CoA transferase		
			<i>citT</i>	Citrate transporter		
			<i>rna</i>	Ribonuclease I		
848202	IS150 Insertion	Insertion in a coding region	<i>ybiS</i>	Hypothetical protein		
1360374	IS150 Insertion	Insertion in a coding region	<i>ycjC</i>	DNA-binding transcriptional repressor		
3270443	IS150 Insertion	Insertion in a non-coding, intergenic region; promoters not disrupted	Site between <i>nlp/murA</i>	DNA-binding transcriptional activator of maltose metabolism/UDP-N-acetylglucosamine 1-carboxyvinyltransferase		

Supplementary Table 13b   Pair 2: ZDB30 (Generation 32,000) and Cit <sup>+</sup> Mutant ZDB165					Cit <sup>-</sup> Parent	Cit <sup>+</sup> Mutant
Position	Mutation	Description	Gene or Genes Involved	Product(s)	ZDB30	ZDB165
628716	IS3 Insertion	Insertion in a coding region; presumed Cit <sup>+</sup> actualizing mutation	<i>citG</i>	Triphosphoribosul-dephospho-CoA transferase		
1651966	Δ795 bp	Deletion of multiple coding regions	<i>ydgG</i>	Transporter of quorum signal AI-2		
			<i>pntB</i>	Pyridine nucleotide transhydrogenase, β subunit		

Supplementary Table 13c   Pair 3: ZDB30 (Generation 32,000) and Cit <sup>+</sup> ZDB293					Cit <sup>-</sup> Parent	Cit <sup>+</sup> Mutant
Position	Mutation	Description	Gene or Genes Involved	Gene Product(s)	ZDB30	ZDB293
632864	568476 bp inversion	Most of <i>cit</i> operon structural genes placed downstream of <i>fimB</i> promoter; new junctions in <i>citC</i> and between <i>yjhA</i> and <i>fimB</i> ; presumed Cit <sup>+</sup> actualizing mutation	<i>citC</i>	Citrate lyase synthetase		
			<i>fimB</i>	Regulator of <i>fimA</i> pili subunit		
687047	IS150 Insertion	Insertion in a coding region	<i>nagE</i>	Fused N-acetyl glucosamine specific PTS enzyme: IIC, IIB, and IIA components		

Supplementary Table 13d   Pair 4: ZDB30 (Generation 32,000) and Cit <sup>+</sup> Mutant ZDB294					Cit <sup>-</sup> Parent	Cit <sup>+</sup> Mutant
Position	Mutation	Description	Gene or Genes Involved	Gene Product(s)	ZDB30	ZDB294
626107	2661 bp duplication	Tandem duplication with junctions in <i>citG</i> and between <i>rna</i> and <i>rnk</i> ; presumed Cit <sup>+</sup> actualizing mutation	<i>citG</i>	Triphosphoribosul–dephospho–CoA transferase	Gray	Red
			<i>citT</i>	Citrate transporter		
			<i>rna</i>	Ribonuclease I		
1607917	Δ1 bp	Single nucleotide deletion in a coding region	<i>ECB_1510</i>	Putative tail component of prophage	Gray	Red
1729055	Δ2462 bp	IS150–mediated deletion of multiple coding regions	<i>ydhV</i>	Predicted oxidoreductase	Gray	Red
			<i>ydhY</i>	Predicted 4Fe–4S ferredoxin–type protein		
2157881	Δ12781 bp	IS150–mediated deletion of multiple coding regions.	<i>yehM</i>	Conserved protein	Gray	Red
			<i>yehP</i>	Conserved protein		
			<i>yehQ</i>	Possible pseudogene		
			<i>yehR</i>	Conserved protein		
			<i>yehS</i>	Conserved protein		
			<i>yehT</i>	Predicted sensory kinase in two–component system with YehU		
			<i>yehU</i>	Predicted sensory kinase in two–component system with YehT		
			<i>yehV</i>	MerR–like regulator		
			<i>ECB_02057</i>	Unknown function		
			<i>yehW</i>	Membrane component of an ABC transporter involved in osmoprotection		
			<i>yehX</i>	Membrane component of an ABC transporter involved in osmoprotection		
			<i>yehY</i>	Membrane component of an ABC transporter involved in osmoprotection		
<i>yehZ</i>	Periplasmic component of an ABC transporter involved in osmoprotection					

Red fill indicates presence of mutation. Gray fill indicates absence of corresponding mutation.

**Supplementary Table 14 | Primer pairs used in this study**

<b>Gene or Region Amplified</b>	<b>Primer Name</b>	<b>Primer Sequence</b>	<b>PCR Product Size</b>
<i>ybaL</i> mutation	<i>ybaL</i> mut F	5' CATCGCCCTGTTCCATCATTCT 3'	503 bp
	<i>ybaL</i> mut R	5' ACCCCGCTTATCACCACCATTGTT 3'	
<i>nadR</i> mutation	<i>nadR</i> mut F	5' ATGGTCGCGATTATGTCTTTTCAC 3'	459 bp
	<i>nadR</i> mut R	5' CGTTTCATCGCGGTTATCTCTG 3'	
<i>hemE</i> mutation	<i>hemE</i> mut F	5' GTGCCGACGCGATGGGGTTAG 3'	524 bp
	<i>hemE</i> mut R	5' CACTGTCCGCCGCTTTGGTA 3'	
<i>cspC</i> mutation	<i>cspC</i> mut F	5' GGGCAAATATCCGAACG 3'	412 bp
	<i>cspC</i> mut R	5' AGCCTTATATTGGTGCCTCAT 3'	
<i>yaaH</i> mutation	<i>yaaH</i> mut F	5' CTTTCGCGTCAGGTTGGTGTG 3'	1030 bp
	<i>yaaH</i> mut R	5' CCTGCCTGCGCCGGATGGTTAG 3'	
<i>leuA</i> mutation	<i>leuA</i> mut F	5' GAATGCGCCGCTGCCAACA 3'	497 bp
	<i>leuA</i> mut R	5' GCCTCAACCAGCGCGTAAACAAA 3'	
<i>tolR</i> mutation	<i>tolR</i> mut F	5' GCCTCAACCAGCGCGTAAACAAA 3'	400 bp
	<i>tolR</i> mut R	5' ACTTCCGCCACCACCTGCTCTG 3'	
<i>arcB</i> mutation	<i>arcB</i> mut F	5' TGTGCGGACCAAAGCCCATCA 3'	709 bp
	<i>arcB</i> mut R	5' GCCCTCGTCGTTCTTGCCATTGT 3'	
<i>gltA</i> mutation	<i>gltA</i> mut F	5' TGTGTTTAACGGAGCTGATTTCTT 3'	626 bp
	<i>gltA</i> mut R	5' GCTGGCGACCGATTCTAACTACCT 3'	
<i>cit</i> amplification	<i>cit</i> Tout F	5' GTCCTGGGTGATTATTTACGGCT 3'	1807 bp
	<i>cit</i> Tout R	5' CAATAACGCAAATAGTAACCGCAA 3'	
<i>citAmpJ</i> fragment	<i>citAmpJ</i> F	5' TTTTTTGGATCCGGTTTCAATGCCCCCTTTTT 3'	529 bp
	<i>citAmpJ</i> R	5' TTTTTTGTGCGACGGTAACCCTGCGTATTTGACTGAA 3'	
<i>rnk</i> promoter region of <i>rnk-citT</i> module for expression studies	nctForward	5' AAAAAAGGATCCGACACCCATCACCACAGT 3'	707 bp
	nctReverse	5' AAAAACTCGAGACGCCATCAACGCTCCGCTTTCT 3'	
<i>citT-citG</i> fragment for gene-gorging	<i>citT-citG</i> F	5' AACCAGCCAGGCCCATTTTCAGC 3'	648 bp
	<i>citT-citG</i> R	5' AAAAAAGGATCCCACGCCTTGCCGCATTACCTCACT 3'	
<i>citG</i> frag fragment for gene-gorging	<i>citG</i> frag F	5' TTTTTTGGATCCGGGGTTCGAATGCCCCCTTTTT 3'	694 bp
	<i>citG</i> frag R	5' GCACAAAGATATGGCGCTGGAAGA 3'	
<i>rnk</i> promoter and <i>cit</i> amplification junction construct for gene-gorging	<i>citT-citG</i> Gorge F	5' TAGGGATAACAGGGTAATAACCAGCCAGGCCCATTTTCAGC 3'	1889 bp*
	<i>citG</i> frag R	5' GCACAAAGATATGGCGCTGGAAGA 3'	
<i>rnk-citT</i> module for cloning into pUC19	<i>citTAmpX</i> F	5' AAAAAAGGATCCGGGCAGCAACCGATTTAGG 3'	2490 bp
	<i>citTAmpX</i> R	5' AAAAAAGTGCACAACGCTCCGCTTTCTGC 3'	
<i>citT</i> internal fragment for Southern hybridizations	<i>citT</i> probe F	5' AGCCGTAATAATCACCCAGGAC 3'	1173 bp
	<i>citT</i> probe R	5' TTGCGGTTACTATTTGCGTTATTG 3'	
Amplification of genomic region immediately upstream of <i>citT</i>	<i>citT</i> upstrm R	5' CTCTCCCGCCGCGACTATTCA 3'	1264 bp <sup>†</sup>
	<i>citT</i> upstrm F	5' CAATAACGCAAATAGTAACCGCAA 3'	

\*Length for fully assembled construct.

<sup>†</sup>Length without deletions or insertions.

<b>Supplementary Table 15   Clones used in growth-trajectory experiments</b>			
<b>Clone</b>	<b>Generation</b>	<b>Description</b>	<b>Growth Curve Locations</b>
REL606	0	Ancestor	Fig. 6
CZB152	33,000	Cit <sup>+</sup> clone from main population	Fig. 5, Supplementary Fig. 3
CZB154	33,000	Cit <sup>+</sup> clone from main population	Supplementary Fig. 3
ZDB30	32,000	Potentiated Cit <sup>-</sup> clone from Clade 3	Fig. 4, 5, 6
ZDB143	32,500	Cit <sup>+</sup> clone from main population	Supplementary Fig. 3
ZDB161	32,000	Cit <sup>+</sup> mutant of ZDB28	Supplementary Fig. 5
ZDB163	32,500	Cit <sup>+</sup> mutant of ZDB31	Supplementary Fig. 5
ZDB164	32,000	Cit <sup>+</sup> mutant of ZDB30	Supplementary Fig. 5
ZDB165	32,000	Cit <sup>+</sup> mutant of ZDB30	Supplementary Fig. 5
ZDB166	32,000	Cit <sup>+</sup> mutant of ZDB29	Supplementary Fig. 5
ZDB172	32,000	Cit <sup>+</sup> clone from main population	Fig. 4, Supplementary Fig. 3
ZDB199	31,500	Potentiated Cit <sup>-</sup> clone from Clade 1	Fig. 6
ZDB200	31,500	Cit <sup>-</sup> clone from Clade 2	Fig. 6
ZDB279	31,500	Cit <sup>+</sup> mutant of ZDB199	Supplementary Fig. 5
ZDB281	32,000	Cit <sup>+</sup> mutant of ZDB183	Supplementary Fig. 5
ZDB283	32,000	Cit <sup>+</sup> mutant of ZDB30	Supplementary Fig. 5
ZDB285	20,000	Cit <sup>+</sup> mutant of ZDB464	Supplementary Fig. 5
ZDB286	20,000	Cit <sup>+</sup> mutant of ZDB464	Supplementary Fig. 5
ZDB288	27,000	Cit <sup>+</sup> mutant of ZDB309	Supplementary Fig. 5
ZDB290	27,000	Cit <sup>+</sup> mutant of ZDB310	Supplementary Fig. 5
ZDB292	31,000	Cit <sup>+</sup> mutant of ZDB390	Supplementary Fig. 5
ZDB294	32,000	Cit <sup>+</sup> mutant of ZDB30	Supplementary Fig. 5
ZDB546	32,500	Cit <sup>+</sup> mutant of ZDB31	Supplementary Fig. 5
ZDB547	30,500	Cit <sup>+</sup> mutant of ZDB20	Supplementary Fig. 5
ZDB548	32,500	Cit <sup>+</sup> mutant of ZDB32	Supplementary Fig. 5
ZDB549	32,500	Cit <sup>+</sup> mutant of ZDB31	Supplementary Fig. 5
ZDB564	31,500	Cit <sup>+</sup> clone from main population	Fig. 4, 5, Supplementary Fig. 3
ZDB595	32,000	Cit <sup>+</sup> isogenic construct of ZDB30 in which <i>rnk-citT</i> module promoter was inserted into the chromosome	Fig. 4
ZDB611	0	pUC19:: <i>rnk-citT</i> transformant of REL606	Fig. 6
ZDB612	32,000	pUC19:: <i>rnk-citT</i> transformant of ZDB30	Fig. 5, 6
ZDB614	31,500	pUC19:: <i>rnk-citT</i> transformant of ZDB199	Fig. 6
ZDB615	31,500	pUC19:: <i>rnk-citT</i> transformant of ZDB200	Fig. 6
ZDBr218	31,500	Cit <sup>+</sup> mutant of ZDB25	Supplementary Fig. 5