1 Supporting information

1.1 Primer design and gene synthesis

The following primers were used in this study:

Table S.1: Primers summary.

Primer	Sequence	Usage
oFS39	atcttaaggaggagttttcgtcgacAGGGTTGTGGGTTGTACGGAgttttagagctagaaatagcaagtt	Anneal with oFS79 to assemble BM4 dsDNA oligomer.
oFS40	atcttaaggaggagttttcgtcgacATTTCTGATATTACTGTCACgttttagagctagaaatagcaagtt	Anneal with oFS80 to assemble BM5 dsDNA oligomer.
oFS41	atcttaaggaggagttttcgtcgacACCGATACCGTTTACGAAATgttttagagctagaaatagcaagtt	Anneal with oFS81 to assemble BM6 dsDNA oligomer
oFS42	atcttaaggaggagttttcgtcgacTGAAGATCAGGCTATCACTGgttttagagctagaaatagcaagtt	Anneal with oFS82 to assemble BM7 dsDNA oligomer.
oFS43	atcttaaggaggagttttcgtcgacTCCGGAGCTCCGATAAAAAAgttttagagctagaaatagcaagtt	Anneal with oFS83 to assemble BM8 dsDNA oligomer.
oFS44	atcttaaggaggagttttcgtcgacTATTGATTCTCTTCAAGTAGgttttagagctagaaatagcaagtt	Anneal with oFS84 to assemble BM9 dsDNA oligomer.
oFS45	atcttaaggaggagttttcgtcgacCCATTGTACTATCATGCTAGgttttagagctagaaatagcaagtt	Anneal with oFS85 to assemble BM10 dsDNA oligomer.
oFS46	atcttaaggaggagttttcgtcgacATGCAGTCGGCTGTAGAAAGgttttagagctagaaatagcaagtt	Anneal with oFS86 to assemble BM11 dsDNA oligomer.
oFS47	atcttaaggaggagttttcgtcgacCGACTGCATTTTATTATGTAgttttagagctagaaatagcaagtt	Anneal with oFS87 to assemble BM12 dsDNA oligomer.
oFS66	atacataaatgcgatcgcGTACCGCTGCTATCTGCC	Amplify *pyrE* homology-directed recombination cassette with oFS67.
oFS67	atacataaatgcgatcgcGTACCGCTGCTATCTGCC	Amplify *pyrE* homology-directed recombination cassette with oFS66.
oFS73	CTAGATTTATATTTAGTCCCTTGCCTTGC	Sequencing of sgRNA cassette.
oFS79	aacttgctatttctagctctaaaacTACATAATAAAATGCAGTCGGTCGACgaaaactcctcctta agat	Anneal with oFS39 to assemble BM4 dsDNA oligomer.
oFS80	aacttgctatttctagctctaaaacCTTTCTACAGCCGACTGCATGTCGACgaaaactcctcctta agat	Anneal with oFS40 to assemble BM5 dsDNA oligomer.
oFS81	aacttgctatttctagctctaaaacCTAGCATGATAGTACAATGGGTCGACgaaaactcctcctta agat	Anneal with oFS41 to assemble BM6 dsDNA oligomer.
oFS82	aacttgctatttctagctctaaaacCTACTTGAAGAGAATCAATAGTCGACgaaaactcctcctta agat	Anneal with oFS42 to assemble BM7 dsDNA oligomer.
oFS83	aacttgctatttctagctctaaaacTTTTTTATCGGAGCTCCGGAGTCGACgaaaactcctcctta agat	Anneal with oFS43 to assemble BM8 dsDNA oligomer.

oFS84	aacttgctatttctagctctaaaacCAGTGATAGCCTGATCTTCAGTCGACgaaaactcctcctaagat	Anneal with oFS44 to assemble BM9 dsDNA oligomer.
oFS85	aacttgctatttctagctctaaaacATTTCGTAAACGGTATCGGTGTCGACgaaaactcctcctaagat	Anneal with oFS45 to assemble BM10 dsDNA oligomer.
oFS86	aacttgctatttctagctctaaaacGTGACAGTAATATCAGAAATGTCGACgaaaactcctcctaagat	Anneal with oFS46 to assemble BM11 dsDNA oligomer.
oFS87	aacttgctatttctagctctaaaacTCCGTACAACCCACAACCCTGTCGACgaaaactcctcctaagat	Anneal with oFS47 to assemble BM12 dsDNA oligomer.
oFS105	gagcttatgcaattcaagtaggtactgcaaac	Screening and sequencing of *pyrE* genomic locus
oFS106	catcaaagctatactattttccgtatttacattgggg	Screening and sequencing of *pyrE* genomic locus
oFS109	caattgttcaaaaaaataatggcggcgcgccCCTGTAATCGGAGCATCTGG	Amplify *pyrE* LHA with oFS119 to assemble the *pyrE* knock-out vector.
oFS112	catttgcaggcttcttatttttatgcgatcgcGTACCGCTGCTATCTGCC	Amplify *pyrE* RHA with oFS120 to assemble the *pyrE* knock-out vector.
oFS119	ATCCATAACTGTCCTCCTAAATTATTCCTC	Amplify *pyrE* LHA with oFS109 to assemble the *pyrE* knock-out vector.
oFS120	AAATAAGTCGAAAAAATCAATGCACGATGC	Amplify *pyrE* RHA with oFS112 to assemble the *pyrE* knock-out vector.
oFS57	GAAACTTAATCATATGCGCTAAGG	Sequencing of Cas9.
oFS58	ATGGATAAGAAATACTCAATAGGCTTAG	Sequencing of Cas9.
oFS59	GCTTTGTCATTGGGTTTGAC	Sequencing of Cas9.
oFS60	GTCGATAAAGGTGCTTCAGC	Sequencing of Cas9.
oFS61	GAACATATTGCAAATTTAGCTGG	Sequencing of Cas9.
oFS62	CTGACTTCCGAAAAGATTTCC	Sequencing of Cas9.
oFS63	GAGTTAGAAAACGGTCGTAAACG	Sequencing of Cas9.
oFS68	GCAAAATACATTCGTTGATG	Sequencing of pMTL vector series/confirm plasmid loss.
oFS69	GTCAAGTATGAAATCATAAATAAAG	Sequencing of pMTL vector series.
oFS70	GATAAATAGTTAACTTCAGGTTTGTC	Sequencing of pMTL vector series.
oFS71	CTGTGGATAACCGTATTACC	Sequencing of pMTL vector series.
oFS72	CAAGAAGAGCGACTTCGC	Sequencing of pMTL vector series.
oFS73	CTAGATTTATATTTAGTCCCTTGCCTTGC	Sequencing of pMTL vector series.
oFS74	CTGTTATGCCTTTTGACTATC	Sequencing of pMTL vector series.
oFS75	GTCAAAATACTCTTTTCTGTTCC	Sequencing of pMTL vector series/confirm plasmid loss.
oFS77	CATTGAAAGAAGTAGGAGCAC	Sequencing of *pyrE* complementation homologous region.
oFS88	GGTCATAGCTGTTTCCTG	Sequencing/cPCR of pMTL vector series.

oFS105	gagcttatgcaattcaagtaggtactgcaaac	Screen *pyrE* for bookmark complementation.
oFS106	catcaaagctatactattttccgtatttacatttggg	Screen *pyrE* for bookmark complementation.
oFS208	TGCATAGTAGACAGAAGAGC	Sequencing of *pyrE* complementation homologous region.
oFS215	AATCAATGCACGATGCAG	Sequencing of *pyrE* complementation homologous region.

The following dsDNA was ordered from IDT DNA technology:

Table S.2: DNA synthesis summary

Name	Sequence	Usage
BMa	GCATCGTGCATTGATTTTTTCGACTTATTTAGGGTTGTGGGTTGTACGGAAGGATTTCTGATATTACTGTCACAGGACCGATACCGTTTACGAAATAGGTGAAGATCAGGCTATCACTGAGGTCCGGAGCTCCGATAAAAAATGGTATTGATTCTCTTCAAGTAGAGGCCATTGTACTATCATGCTAGAGGATGCAGTCGGCTGTAGAAAGAGGCGACTGCATTTTATTATGTAAGGATCCATAACTGTCCTCCTAAATTATTCCTC	HiFi with amplicon of oFS109-oFS119 and oFS112-oFS120, and with pMTL431511-CLAU-pyrE digested with AscI and AsisI to make *pyrE* knock-out vector.

1.2 sgRNA design

All the seed sequences used to design sgRNAs to target protospacers in this study were either picked from literature or designed using Benchling sgRNA design tool (https://benchling.com, 2017).

Table S.3: Seed sequences used in sgRNA. For each seed sequence, the original publication and the vector in which it was assembled are given.

Seed	Sequence	Publication	Construct
BM4	AGGGTTGTGGGTTGTACGGA	Jiang et al., 2013	pMTL431511_BM4
BM5	ATTTCTGATATTACTGTCAC	Jiang et al., 2013	pMTL431511_BM5
BM6	ACCGATACCGTTTACGAAAT	Jiang et al., 2013	pMTL431511_BM6
BM7	TGAAGATCAGGCTATCACTG	Altenbuchner, 2016	pMTL431511_BM7
BM8	TCCGGAGCTCCGATAAAAAA	Altenbuchner, 2016	pMTL431511_BM8
BM9	TATTGATTCTCTTCAAGTAG	Altenbuchner, 2016	pMTL431511_BM9
BM10	CCATTGTACTATCATGCTAG	Oh & Van Pijkeren, 2014	pMTL431511_BM10
BM11	ATGCAGTCGGCTGTAGAAAG	Oh & Van Pijkeren, 2014	pMTL431511_BM11
BM12	CGACTGCATTTTATTATGTA	Oh & Van Pijkeren, 2014	pMTL431511_BM12
pyrE	CTATGAACTTGCAAGGCAAA	Ingle et al., 2019	pMTL431511_BMa

1.3 Complementation efficiency calculation

Table S.4: Raw data from the calculation of the complementation efficiency of each bookmark protospacer, across three independent conjugations.

Bookmark protospacer	Replicate	Colony count (CFU)	Complemented	Screened	Efficiency
BM4	I	1132	6	8	75%
BM4	II	750	8	8	100%
BM4	III	1154	10	10	100%
BM5	I	1616	8	9	89%
BM5	II	1475	7	7	100%
BM5	III	536	11	11	100%
BM6	I	414	12	12	100%
BM6	II	946	6	7	86%
BM6	III	113	7	7	100%
BM7	I	849	3	5	60%
BM7	II	615	3	3	100%
BM7	III	971	8	9	89%
BM8	I	1178	3	3	100%
BM8	II	308	8	9	89%
BM8	III	433	4	4	100%
BM9	I	412	7	7	100%
BM9	II	690	7	7	100%
BM9	III	481	5	5	100%
BM10	I	410	5	6	83%
BM10	II	308	2	2	100%
BM10	III	436	6	7	86%
BM11	I	183	5	5	100%
BM11	II	156	7	8	88%
BM11	III	259	9	11	82%
BM12	I	235	2	6	33%
BM12	II	280	4	4	100%
BM12	III	568	4	4	100%
WT	I	0			
WT	II	0			
WT	III	0			

1.4 Uncropped gel

Figure S.1: Uncropped electrophoresis gel of 6 C. autoethanogenum colonies obtained after conjugation of pMTL431511_BMa. A 20kb band present in all lanes was dismissed from the analysis of the gel, and removed from the main text to improve clarity. Indeed, this band appears not only in the negative control (-) where no DNA template was present, it also appeared in the empty lanes of the gel, where no PCR reaction had been loaded. We assume this band must have been the result of some kind of DNA contamination of the TAE buffer.

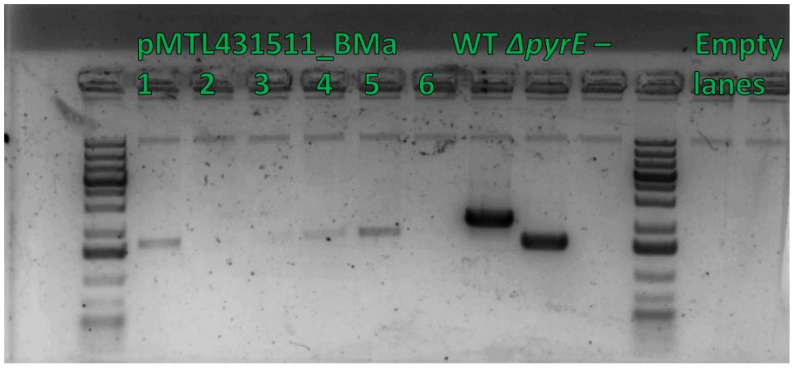

Figure S.2: Subsequent PCRs of the ΔpyrE::BMa strain screened in Figure S1. *In accordance with the hypothesis that the 20 kb band observed in Figure S.1 could be safely dismissed as the product of a contamination of the gel, subsequent PCRs of the strains used as template in Figure S.1 did not exhibit any 20kb band.*