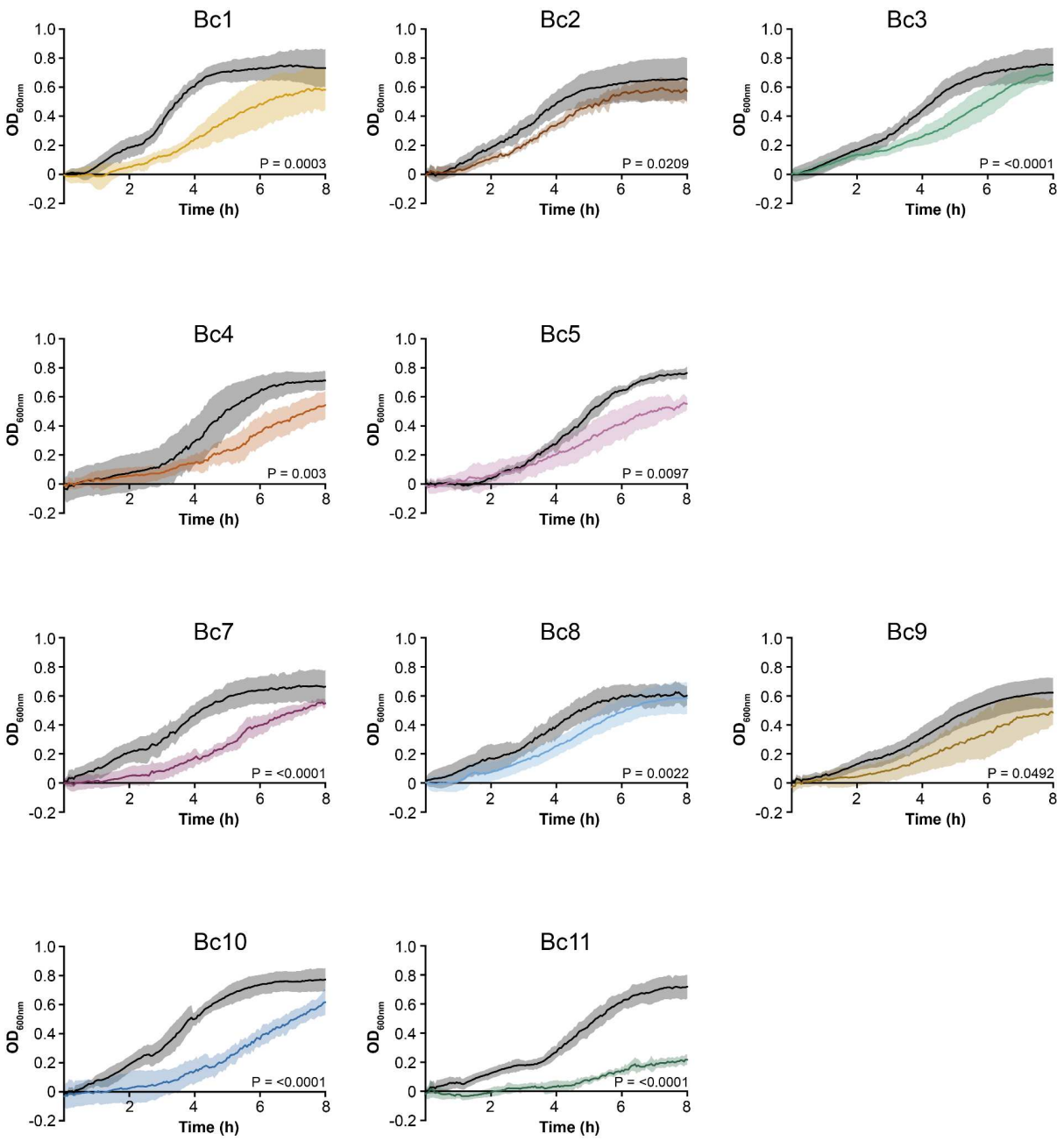
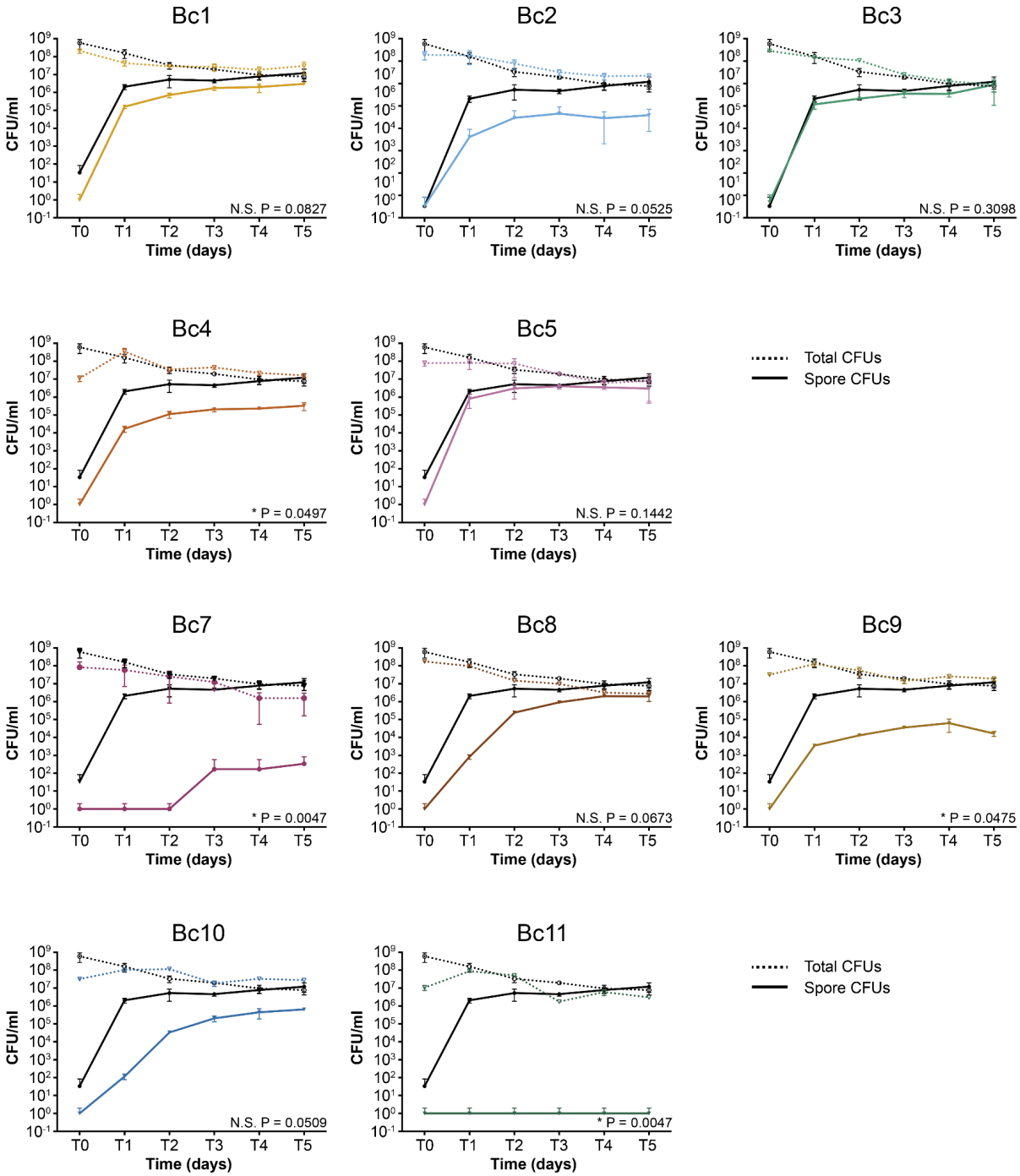
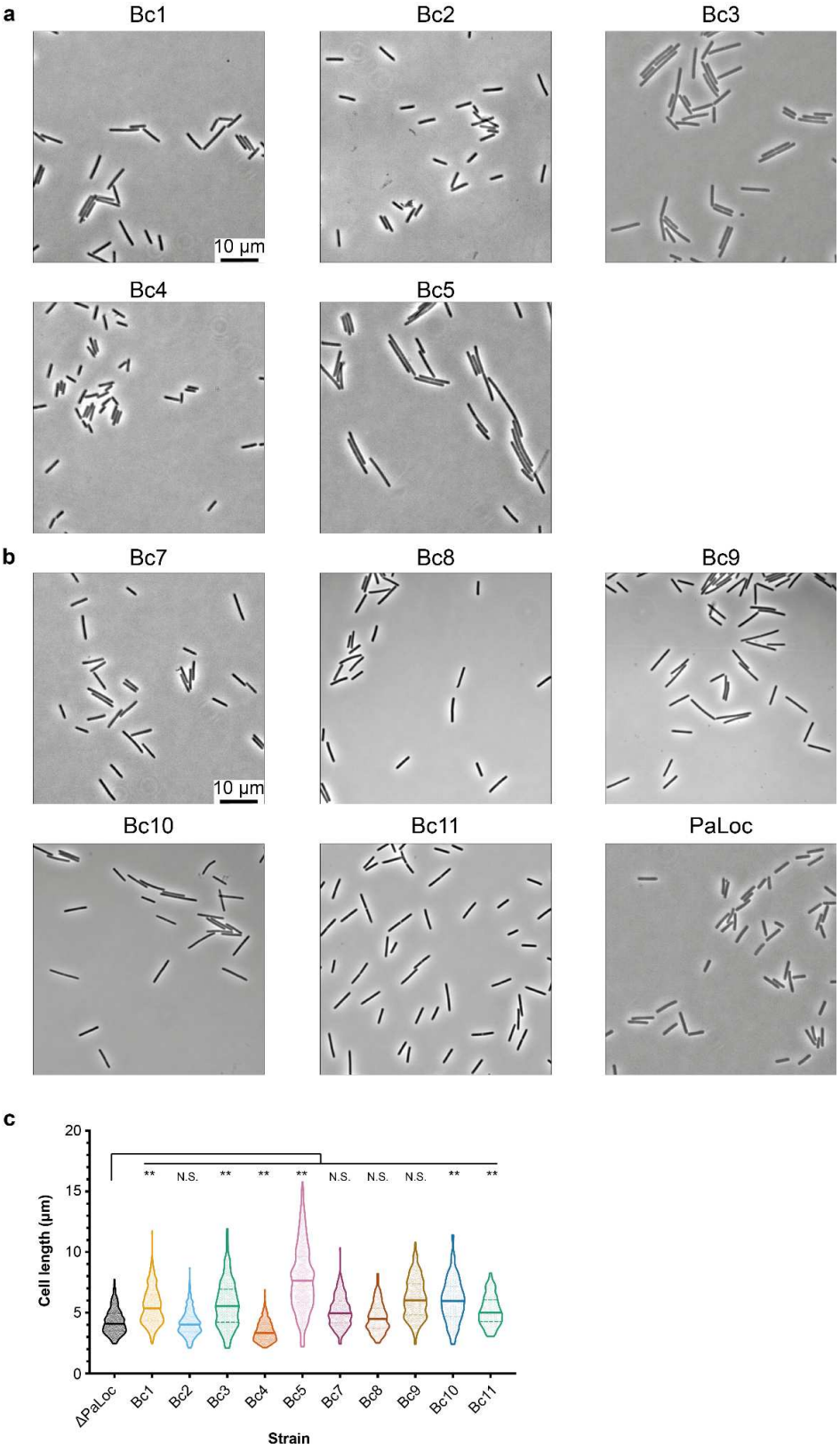


Supplementary Figure 1. Growth of evolved endpoint clones. Growth over time in rich media (TY broth) was measured at 600 nm in a 96 well microplate spectrometer. Growth of each endpoint clone (coloured lines) was compared to its matched control (black lines). Shown are the mean and standard deviation of repeats, assayed at minimum in biological and technical triplicate. For each strain, area under the curve was determined using the GrowthCurver R package and these were compared using Student's t-tests with Welch's correction, with the P value shown on each graph. All pairwise differences are significant.



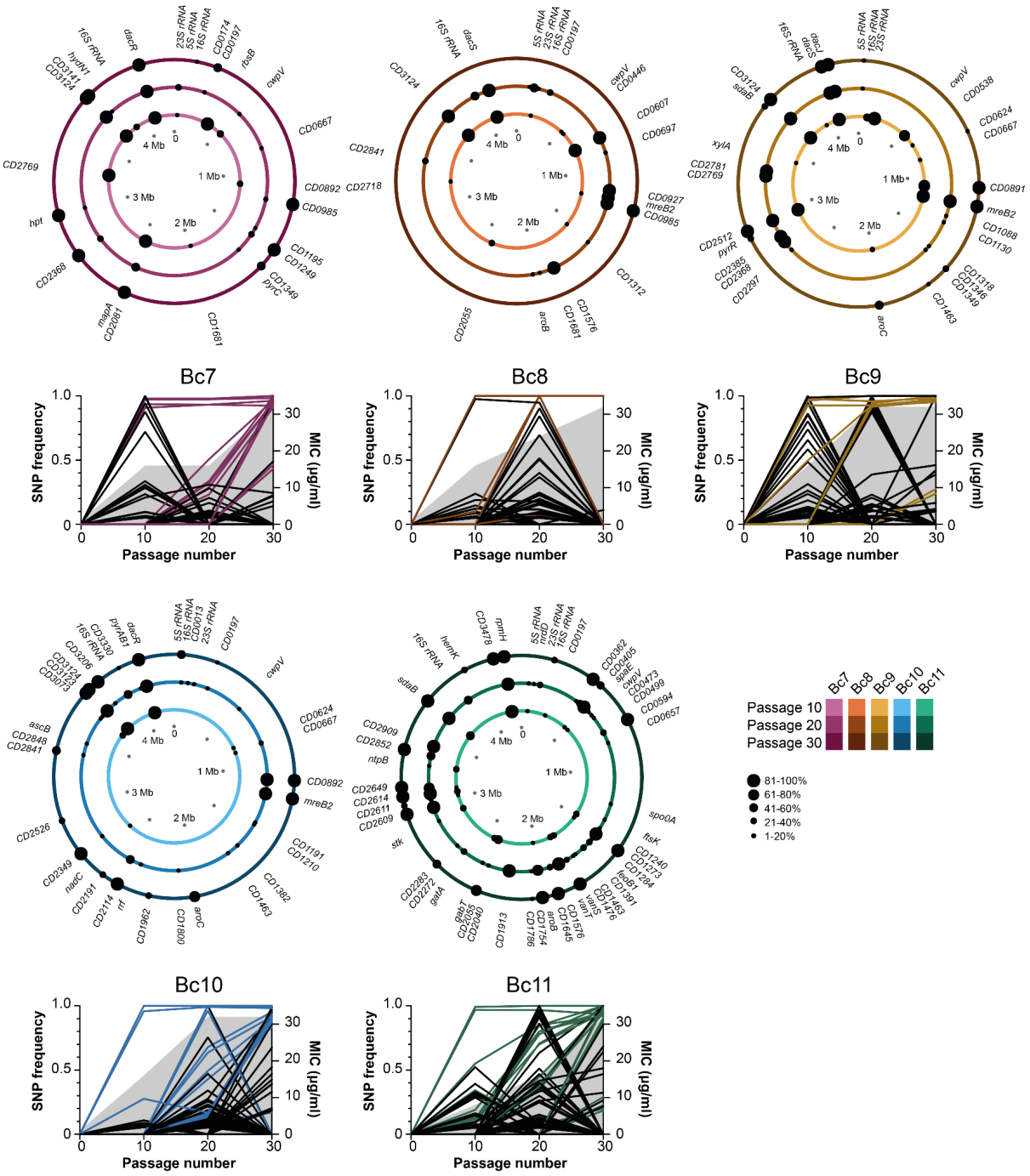
Supplementary Figure 2. Sporulation of evolved endpoint clones. Sporulation efficiencies of each endpoint clone (coloured lines) were compared to the parental R20291Δ*PaLoc* (black lines). Stationary phase cultures were incubated anaerobically for 5 days with samples taken daily to enumerate total colony forming units (CFUs, dotted lines) and spores (solid lines), following incubation at 65°C for 30 min to kill vegetative cells. Shown are the mean and standard deviations of biological duplicates assayed in triplicate. For each strain,

spore CFU area under the curve was determined using Graphpad Prism and these were compared using Dunnett's T3 multiple comparisons test with the adjusted P value shown on each graph. * = significant difference, N.S. = not significant.



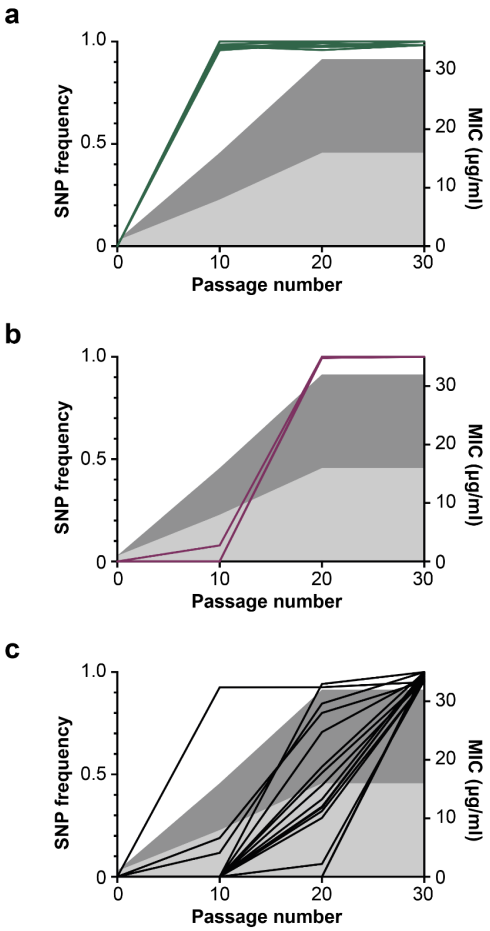
Supplementary Figure 3. Cell morphology of evolved endpoint clones. Phase contrast light microscopy of mid-log cultures of each wild type (**a**) and hypermutating (**b**) endpoint clone, with R20291Δ*PaLoc* for comparison. Shown is a representative field of view for each strain. **c**

Images were analysed using MicrobeJ to determine lengths of at least 185 individual cells for each strain. Shown is an all point violin plot with the median indicated by a solid horizontal line. Statistical significance of evolved isolates against the R20291Δ*PaLoc* control was calculated using a one-way ANOVA with Dunnett's T3 multiple comparisons test, ** = P<0.0001, N.S. = not significant.



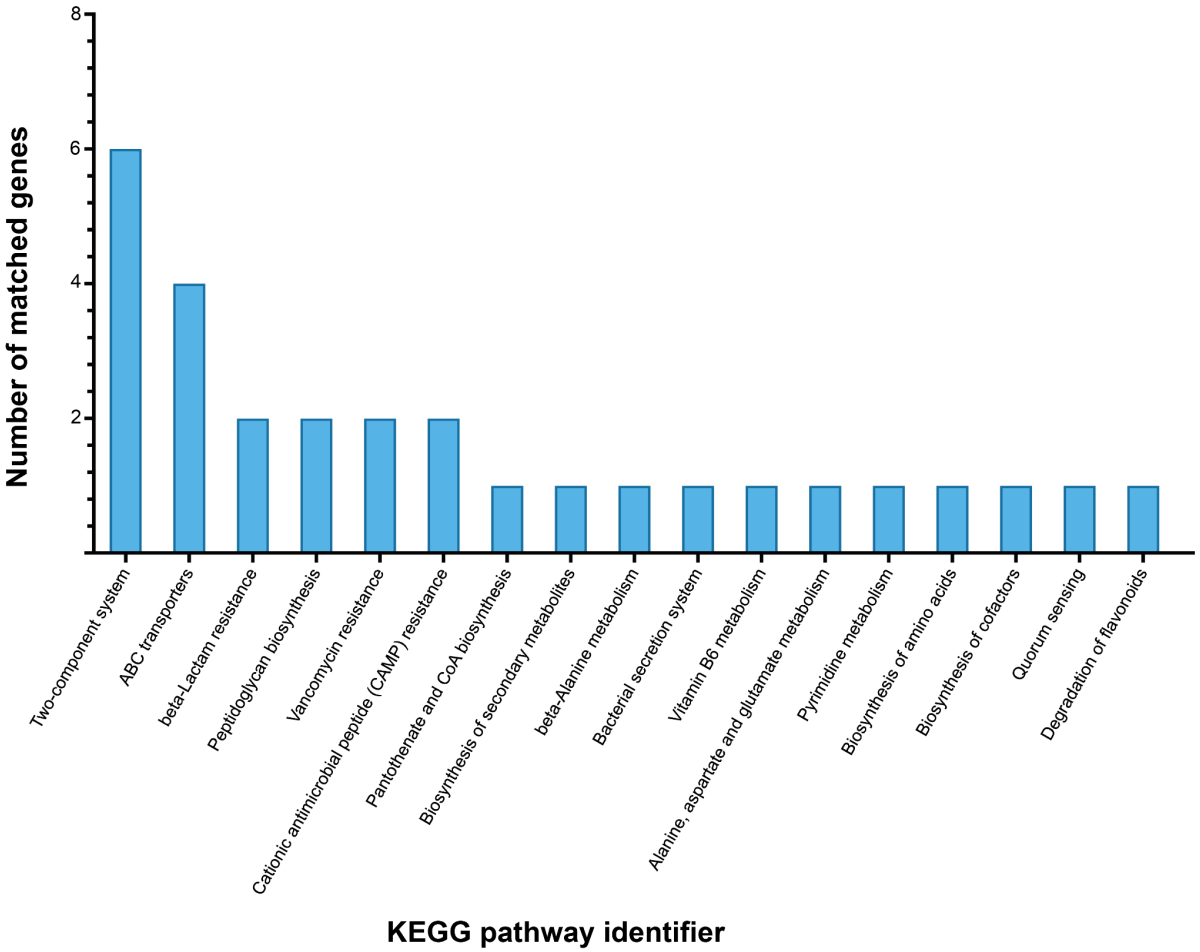
Supplementary Figure 4. Genomic location of gene variants over time. Accumulation of variants in the hyper-mutating *C. difficile* lineages. Each circle plot represents the 4.2 Mb genome of a single evolving population after 10 (inner ring), 20 (middle ring) and 30 passages (outer ring), with the locations of non-synonymous within gene variants indicated with black circles and the penetrance of each mutation in the population indicated by the size of the circle. The line graphs show the frequency of all variants (intergenic, synonymous, non-synonymous and nonsense) in each population. The vancomycin MIC for

each population is also indicated by the shaded region. Mutations also identified in the respective end point clone (Fig. 1c) are highlighted by the coloured lines. A full list of all variants shown here is included in Supplementary Data 3.



Fixed at P10	Fixed at P20	Fixed at P30
CD0982 Bc9	*CD0108* Bc4	*CD0362* Bc11
CD1159 Bc4	*CD0892* Bc10	*CD0801* Bc5
CD3124 Bc2	*CD0892* Bc10	*CD0985* Bc7
CD3124 Bc7	*CD0982* Bc10	*CD1249* Bc7
CD3124 Bc9	*CD0985* Bc8	***vanT* Bc3**
CD3124 Bc10	***vanT* Bc4**	***vanT* Bc11**
CD3193 Bc2	***vanT* Bc5**	*CD1645* Bc11
dacS Bc1		*CD2081* Bc7
***dacS* Bc2**		*CD2512* Bc9
***dacS* Bc9**		*CD2579* Bc7
***dacR* Bc7**		*CD2609* Bc11
***dacR* Bc10**		*CD2614* Bc11
		CD2649 Bc11
		CD2852 Bc11
		CD3141 Bc7
		***dacJ* Bc9**
		CD3478 Bc11

Supplementary Figure 5. Fixation of mutations during evolution. Shown are the individual variants which fix (>95% penetrance) in all 10 parallel populations during vancomycin resistance evolution after 10 (**a**), 20 (**b**) and 30 passages (**c**). The frequency of each variant within their respective population is shown and the genes affected at each timepoint are shown in the table on the right. The genes in the *CD3437-9* cluster are highlighted in yellow and *vanT* (*CD1526*) in green. The range of vancomycin MICs observed across all populations (lowest, light grey; highest, dark grey) is indicated by the shaded areas.



Supplementary Figure 6. KEGG pathway analysis of genes identified in evolved populations. Mutations occurring within genes (except those in the transiently hypermutating Bc1 P20) were coloured according to their barcoded replicate line, and pathways were visualised in KEGG (Kyoto Encyclopedia of Genes and Genomes) colour mapper[1]. Two-component systems and ABC transporters were the best-represented functional classes.

Supplementary Table 1: Strains used in this study

Strain	Characteristics	Source
General Strains – C. difficile		
R20291	*C. difficile* ribotype 027 strain isolated during an outbreak at Stoke Mandeville hospital, UK in 2006.	[2]
R20291Δ*PaLoc*	R20291 with the entire pathogenicity locus (*tcdD, tcdB, tcdE, tcdA, dxtA)*, except the first codon of *dxtA*, deleted.	This study
R20291Δ*PaLoc*Δ*mutSL*	R20291Δ*PaLoc* with the entire *mutSL* locus (*mutS, mutL*), except the first codon of *mutS* and the last 2 codons of *mutL*, deleted.	This study
General Strains – E. coli		
CA434	*E. coli* conjugative donor. HB101 carrying R702.	[3]
NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.*	New England Biolabs
Barcoded Strains		
R20291Δ*PaLoc pyrE::*barcode 1	R20291Δ*PaLoc* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 1 (AAGTCCTCG)	This study
R20291Δ*PaLoc pyrE::*barcode 2	R20291Δ*PaLoc* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 2 (TCTTGACCG)	This study
R20291Δ*PaLoc pyrE::*barcode 3	R20291Δ*PaLoc* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 3 (AACAACACC)	This study
R20291Δ*PaLoc pyrE::*barcode 4	R20291Δ*PaLoc* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 4 (AACAGGTGG)	This study
R20291Δ*PaLoc pyrE::*barcode 5	R20291Δ*PaLoc* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 5 (ACCGATTAG)	This study
R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 7	R20291Δ*PaLoc*Δ*mutSL* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 7 (CCTCCAACT)	This study
R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 8	R20291Δ*PaLoc*Δ*mutSL* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 8 (CGAGGACAT)	This study
R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 9	R20291Δ*PaLoc*Δ*mutSL* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 9 (CTGGTTCTA)	This study
R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 10	R20291Δ*PaLoc*Δ*mutSL* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 10 (GGATGTTGG)	This study

R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 11	R20291Δ*PaLoc*Δ*mutSL* with a 218 bp insertion between *CD0188* (*pyrE*) and *CD0189,* including 9 bp Barcode 11 (GTCACCAGT)	This study
Evolved Strains		
Bc1	R20291Δ*PaLoc pyrE::*barcode 1 isolated after 60 days of vancomycin selection pressure.	This study
Bc2	R20291Δ*PaLoc pyrE::*barcode 2 isolated after 60 days of vancomycin selection pressure.	This study
Bc3	R20291Δ*PaLoc pyrE::*barcode 3 isolated after 60 days of vancomycin selection pressure.	This study
Bc4	R20291Δ*PaLoc pyrE::*barcode 4 isolated after 60 days of vancomycin selection pressure.	This study
Bc5	R20291Δ*PaLoc pyrE::*barcode 5 isolated after 60 days of vancomycin selection pressure.	This study
Bc7	R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 7 isolated after 60 days of vancomycin selection pressure.	This study
Bc8	R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 8 isolated after 60 days of vancomycin selection pressure.	This study
Bc9	R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 9 isolated after 60 days of vancomycin selection pressure.	This study
Bc10	R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 10 isolated after 60 days of vancomycin selection pressure.	This study
Bc11	R20291Δ*PaLoc*Δ*mutSL pyrE::*barcode 11 isolated after 60 days of vancomycin selection pressure.	This study
Recapitulated strains		
R20291Δ*PaLoc dacS*c.548T>C	R20291Δ*PaLoc* with *dacS* 548T>C point mutation identified in Evolved R20291Δ*PaLoc*Δ*mutSL pyrE::*barcodes 8 and 9	This study
R20291Δ*PaLoc dacS*c.714G>T	R20291Δ*PaLoc* with *dacS* 714G>T point mutation identified in Evolved R20291Δ*PaLoc pyrE*::barcode 1	This study

Supplementary Table 2: Primers used in this study

Oligonucleotide	Sequence	Use
Primers for Cloning		
RF920	cgtagaaatacggtgtttttttgttaccctaTGGAATTTAGATATAAAAACCAATTC	Amplification of homology arm upstream of *PaLoc* with RF921
RF921	atttattttggtgtgGACAACATTGGAATTAAATCAG	Amplification of homology arm upstream of *PaLoc* with RF920
RF922	aattccaatgttgtcCACACCAAAATAAATGCC	Amplification of homology arm downstream of *PaLoc* with RF923
RF923	gggattttggtcatgagattatcaaaaaggCCCAACTATGGAAAAACC	Amplification of homology arm downstream of *PaLoc* with RF922
RF2066	AatacggtgtttttttgttaccctagagctcCCACTTATAATTTCTAATGAAACTGTG	Amplification of homology arm upstream of *mutSL* with RF2067
RF2067	ccaaatattttacatCATTATCAAACCTCCTTCTTTTC	Amplification of homology arm upstream of *mutSL* with RF2066
RF2068	GGAGGTTTGATAATGATGTAAAATATTTGGATATTTAAAATATATGGAAAG	Amplification of homology arm downstream of *mutSL* with RF2069
RF2069	TTGGTCATGAGATTATCAAAAAGGGGATCCGCCCTTTAACTTGCACTC	Amplification of homology arm downstream of *mutSL* with RF2068
Primers for Barcoding		
RF1810	GAAAAAGGCTTCTCTCATGAGAAG	To linearise pJAK081 to add barcode fragments
RF1811	GGTACCATAAAAATAAGAAGCCTGC	To linearise pJAK081 to add barcode fragments
RF1902	ACC GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 3
RF1903	GTTGTT AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 3
RF1904	GTGG GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 4
RF1905	CTGTT AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 4
RF1906	GATTAG GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 5
RF1907	GGT AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 5
RF1912	CAACT GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 7
RF1913	GAGG AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 7

RF1914	GACAT GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 8
RF1915	CTCG AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 8
RF1916	GTTCTA GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 9
RF1917	CAG AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 9
RF1918	TTGG GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 10
RF1919	CATCC AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 10
RF1920	CAGT GAAAAAGGCTTCTCTCATGAGAAG	Inverse PCR of pJAK201 to introduce barcode 11
RF1921	GTGAC AAATGGAAGATGGAATAGAAGTAAGC	Inverse PCR of pJAK201 to introduce barcode 11
Primers for qPCR		
RF2504	CATCATTACCAGGTGTAGCAGTG	Amplification of ~200bp *rpoA* fragment for qPCR
RF2505	GGAGGACAGATTATATCTGCACC	Amplification of ~200bp *rpoA* fragment for qPCR
RF2506	CAATCACATCATTAGCAATTTATTCCATG	Amplification of ~200bp *dacS* fragment for qPCR
RF2507	GTTCATCAATATCATCCTTTTCTTTATCC	Amplification of ~200bp *dacS* fragment for qPCR
RF2508	GGATGGGATAGAAGTTTGTAGAAAAG	Amplification of ~200bp *dacR* fragment for qPCR
RF2509	CTCTTCTAATCAGTGATTTCACTCTC	Amplification of ~200bp *dacR* fragment for qPCR
RF2510	CAACATGATTCAGAACAAGATGTTGAG	Amplification of ~200bp *dacJ* fragment for qPCR
RF2511	GCTTGCTTAACTAAATCTTCAACTGC	Amplification of ~200bp *dacJ* fragment for qPCR
RF2512	CTGATTTTAGAAAAGTATATAAACACGG C	Amplification of ~200bp *rnpA* fragment for qPCR
RF2513	CTTGCTATAAATACTATATCATATCCAGG	Amplification of ~200bp *rnpA* fragment for qPCR

Supplementary Table 3: Plasmids used in this study

Plasmid	Characteristics	Source
General Plasmids		
pJAK112	pMTL-SC7215 based vector with added BamHI and SacI restriction sites for cloning.	[4]
pJAK143	*PaLoc* deletion – 1200bp homology arms upstream and downstream of the *PaLoc* for deletion of the entire pathogenicity locus (*tcdD, tcdB, tcdE, tcdA, dxtA)*, except the first codon of *dxtA*.	[5]
pJEB002	*mutSL* deletion – 1200 bp homology arms upstream and downstream of *mutSL* for deletion of the entire *mutSL* locus (*mutS, mutL*), except the first codon of *mutS* and the last 2 codons of *mutL.*	This study
pMTL-SC7215	Allele exchange vector for *codA*-based selection.	[6]
Plasmids for Barcoding		
pJAK081	pMTL-SC7215 based vector with 1,200 bp homology arms for insertion of DNA sequences between *CD0188* (*pyrE*) and *CD0189* in the *C. difficile* R20291 genome.	This study
pJAK201	*pyrE::*barcode 1 – pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 1 (AAGTCCTCG)	This study
pJAK202	*pyrE::*barcode 2 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 2 (TCTTGACCG)	This study
pJAK203	*pyrE::*barcode 3 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode (AACAACACC)	This study
pJAK204	*pyrE::*barcode 4 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 4 (AACAGGTGG)	This study
pJAK205	*pyrE::*barcode 5 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 5 (ACCGATTAG)	This study
pJAK207	*pyrE::*barcode 7 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 7 (CCTCCAACT)	This study
pJAK208	*pyrE::*barcode 8 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 8 (CGAGGACAT)	This study
pJAK209	*pyrE::*barcode 9 pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 9 (CTGGTTCTA)	This study
pJAK210	*pyrE::*barcode 10 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode 10 (GGATGTTGG)	This study
pJAK211	*pyrE::*barcode 11 - pJAK081 based vector, with a 218 bp insertion containing 9 nt Barcode e 11 (GTCACCAGT)	This study
Plasmids for Recapitulating		
pJEB019	*dacS*c.548T>C - pJAK112 based vector containing 1,926 bp homology arms centred on a *dacS* 548T>C point mutation.	This study
pJEB026	*dacS*c.714G>T - pJAK112 based vector containing 1,926 bp homology arms centred on a *dacS* 714G>T point mutation.	This study
qRT-PCR Plasmids		

pJEB029	qPCR 1 - pUC-GW-Kan vector including ~200 bp fragments of *rpoA, dacS, dacR, dacJ* and *rnpA* for qRT-PCR	This study

Supplementary Table 4. DNA Accession numbers

References

1 Kanehisa, M. *et al.* Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res* **42**, D199-205 (2014). https://doi.org:10.1093/nar/gkt1076

2 Stabler, R. A. *et al.* Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biol* **10**, R102 (2009). https://doi.org:10.1186/gb-2009-10-9-r102

3 Purdy, D. *et al.* Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. *Mol Microbiol* **46**, 439-452 (2002). https://doi.org:10.1046/j.1365-2958.2002.03134.x

4 Fuchs, M. *et al.* An RNA-centric global view of *Clostridioides difficile* reveals broad activity of Hfq in a clinically important gram-positive bacterium. *Proc Natl Acad Sci U S A* **118** (2021). https://doi.org:10.1073/pnas.2103579118

5 Ormsby, M. J. *et al.* An intact S-layer is advantageous to *Clostridioides difficile* within the host. *PLoS Pathog* **19**, e1011015 (2023). https://doi.org:10.1371/journal.ppat.1011015

6 Cartman, S. T., Kelly, M. L., Heeg, D., Heap, J. T. & Minton, N. P. Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the *tcdC* genotype and toxin production. *Appl Environ Microbiol* **78**, 4683-4690 (2012). https://doi.org:10.1128/AEM.00249-12