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Supplementary Materials for

A limit on the evolutionary rescue of an Antarctic bacterium from rising temperatures

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplementary Text

Genetic changes implicated in temperature adaptation

Genetic variants that accumulate at different temperatures affect genes classified into different COG (Clusters of Orthologous Groups (*64*)) functional categories. In control populations evolving at 15°C, and in clones adapted to temperatures ranging between 22°C and 26°C such variants preferentially accumulate in genes involved in motility and signal transduction. In contrast, adaptation to high temperatures, 28°C and 30°C, is preferentially associated with variants of genes involved in protein turnover and chaperone functions, in cell wall/membrane biogenesis, and in energy production and conversion (Fisher test, one-tailed FDR corrected p-value<=0.05) (Fig. S6, Table S4).

In general, the proteomes of psychrophiles harbor fewer aliphatic amino acids (*40*) and their protein core is less hydrophobic to reduce its compactness (*73*). Because the amino acid composition of proteins varies with temperature (*40*), it can help us understand the selective pressures acting during experimental evolution. We observed that at 30°C the amino acids that changed during experimental evolution most frequently into other amino acids are glycine, alanine and serine. The most commonly changing amino acids at other temperatures are as follows. 28°C: serine, arginine, alanine and asparagine; 26°C: glycine, alanine and asparagine; 22°C: alanine; 15°C: arginine and methionine (Table S5). Previous studies suggest that the proteomes of psychrophiles harbor more small amino acids (such as glycine, alanine and serine) than those of mesophiles, possibly to increase protein flexibility (*40*). It is therefore remarkable that glycine, alanine and serine are also the most commonly mutated amino acids at 30°C.

Conversely, at high temperatures (30°C and 28°C) amino acids encoded by genes in the ancestor most frequently turn into leucine and isoleucine (Table S6). These are both aliphatic amino acids that help to maintain the stability of the protein core by increasing internal hydrophobic interactions (*40*). Among the least frequently gained amino acids is alanine, which as mentioned before, is a small amino acid that increases protein flexibility.

Highly parallel mutations are concentrated in few genes and genomic regions

The two most common mutations in our experiment, mutations in *lon* and mutations that reduce the number of copies of chromosome 2, increase their frequency with temperature (Fig. 2B, Fig. S9). Mutations in *lon* appear for the first time at the sublethal 28°C, where only 17% of clones have a mutation affecting this gene. By the time populations are exposed to 30°C, 90% of clones harbor a *lon* mutation. Similarly, mutations that decrease the number of chromosome 2 copies also increase with temperature. While only one clone adapted to 26°C carries this mutation, 46.7% of the clones adapted to 28°C, and 87.5% of clones adapted to 30°C carry it.

Mutations in cell wall biosynthesis are the third most common mutations (85%) in populations adapted to 30°C. The most commonly mutated membrane related genes at high temperatures are *mipA*, *lpxC* and *lapB*. *mipA* encodes for the MltA-interacting protein, an integral outer membrane scaffold protein that forms a complex with two other proteins, MrcB and MltB. This complex is involved in the growth of the murein sacculus (or peptidoglycan exoskeleton), a bag-shaped structure around the cytoplasmic membrane that maintains cell shape by protecting cells from stress, and by helping to avoid cell lysis caused by osmotic pressure (*74*, *75*). Osmotic pressure and the resulting danger of cell lysis increase with temperature, a problem that might be

overcome by mutations that modulate peptidoglycan synthesis. The genes *lpxC* and *lapB* encode UDP-3-O-acyl-N-acetylclucosamine deacetylase and lipopolysaccharide assembly protein B, respectively, both of which are important for the synthesis of lipid A, which anchors the lipopolysaccharides in the plasma membrane (*76*). The gene *lapB* regulates *lpxC,* and is also involved in the synthesis of lipopolysaccharides in the plasma membrane (*76*). Possibly, mutations in *lpxC* and *lapB* might affect outer membrane fluidity, which increases with temperature. Previous studies reported that the structure of lipid A is affected when *P. haloplanktis* is grown at 25°C (*77*). Other commonly mutated genes are involved in phospholipid metabolism (*pgsA*), in peptidoglycan biosynthesis and cell wall elongation (*mrdB*), as well as in the insertion of proteins in the outer membrane (*bamA*). Mutations involved in peptidoglycan synthesis also occur in *E. coli* adapting to high temperatures (*22*).

Mutations in cell wall biosynthesis genes appear for the first time at 26°C, where they are already present in 50% of the clones. At 28°C 97% of clones carry such mutations, whereas at 30°C the percentage of such clones falls slightly to 85%. This implies that not all mutations affecting cell wall biosynthesis identified at 28°C were fixed at that time.

More than half (61.25%) of the populations adapted to 30°C have a mutation involved in energy conversion, specifically in one of the subunits of the F-ATPase. F-type ATPases have two domains. The first is F_0, which is a proton channel integral to the membrane and has 3 subunits (a_1, b_2, c), and the second is F_1 which is the catalytic core and has 5 subunits (α_3,β_3,γ_1,δ_1,ε_1) (*78*). Remarkably, mutations in the F-ATPase only appear at 30°C and most of them are in the α and β subunits of the F_1 domain. We speculate that these mutations help cells cope with the high energetic demands of Lon protease, which requires ATP, and with the energetic demands caused by an increased metabolic rate at high temperature.

Other common genetic variants affect signal transduction (Fig. 2B, Fig. S9, Table S8), but in contrast to the previously mentioned mutations, their incidence at 30°C is no higher than at other temperatures.

<u>Chromosome 2 duplication and its role for adaptation to high temperature</u>
87.5% of the clones adapted to 30°C have mutations that reduce the number of chromosome 2 copies from two – acquired during preadaptation – back to one. Chromosome 2 is thought to have its origin in a plasmid, because it still carries plasmid related genes such as genes involved in plasmid replication (a *repA*-like gene), plasmid partition (*parA* and *parB*) and plasmid maintenance (*kisB*, *kidB*) (*34*).

Mutations to reduce the number of copies of chromosome 2 back to one can follow two alternative genetic routes (Fig. S7), which we confirmed using qPCR experiments (Fig. S8, Table S7). Some clones harbor non-synonymous point mutations in the *repA* gene, a gene involved in plasmid replication located on chromosome 2. Mutations in this gene lead to a reduction in the number of copies of chromosome 2 back to one (Fig. S8, Table S7). Other clones harbor a deletion affecting almost the entire chromosome 2, which results in two chromosomes 2, one of normal size and a small one containing only few genes, including the ones needed for plasmid replication (*repA*) and maintenance (among others, *parA* and *parB* genes which are essential for plasmid partition) (Fig. S8, Table S7). The genes involved in the

metabolism of D-gluconic acid fall in the deleted region. While the copy number of *repA* and *parA* genes in these clones is two-fold larger than in the wild-type clone (Tukey post-hoc test, p-value < 0.05), this is not the case for the gluconate transporter gene, which has the same copy number as the wild-type clone (Tukey post-hoc test, p-value = 0.952) (Fig. S8, Table S7). This observation shows that the second mutational route causes the partial deletion of one of the two copies of chromosome 2. This deletion is associated with increased growth compared to clones with mutations in *repA* (r_{del}=0.484 vs r_{repA}=0.422, Wilcoxon rank sum test, one-tailed p-value=0.035), and compared to clones with no mutations in chromosome 2 or *repA* (r_{del}=0.484 vs r_{nomut}=0.372, Wilcoxon rank sum test, one-tailed p-value=0.010).

Both genetic routes reduce the expression of 546 (13.5%) of the organism's genes. The reduction thus lessens the energetic burden of protein biosynthesis, which consumes up to 50% of the total energy budget of growing bacteria (*79*). More importantly, the reduction also eases the energetic burden of protein folding or re-folding (five ATP molecules per protein (*80*)), and of the even more costly degradation of misfolded proteins (two ATP molecules per peptide bond cleavage for Lon protease (*81*)). Additionally, it also lessens the fitness cost of accumulating misfolded proteins inside the cell (*43*).

Protein misfolding limits adaptation to high temperature

The Lon protease is composed of three domains, the N-terminal domain, the AAA+ domain and the protease domain (*41*). Structural information reveals that the protease is a homohexamer that forms a ring with a central cavity. Protomers (i.e. monomers) with bound ATP alternate with protomers in a nucleotide-free state (*82*). ATP binding and hydrolysis causes large conformational changes that induce the movement of two groups of conserved residues located in the AAA+ domain, which are called pore loop residues. It has been hypothesized that the pore loops bind the protein substrate, and that these movements unfold and translocate the substrate through the central cavity for its degradation (*82*).

The three amino acid insertion we observed during experimental evolution occurs at the end of the N-terminal domain, in a region named the three-helix bundle. It occurs between helix two and three, and increases the separation between the two helices (Fig. 3A). The three-helix bundle region rotates when ATP binds (*82*). Before ATP binding, the three-helix bundle and the pore loops are oriented away from the central axis. After ATP binding the three-helix bundle and the pore loops rotate to face the center of the ring (*82*). Because rotation of pore loops has been suggested to bind and translocate the protein substrate (*82*), it is tempting to speculate that the three amino acid insertion creates an additional loop to interact with the substrates and facilitate their translocation and degradation. The affected region is not highly conserved at the amino acid level in Bacteria and Achaea (Fig. S15), and the three amino acid insertion is not present in any other known organism. Aside from the three amino acid insertion, all other mutations in the Lon protease except one fall in the N-terminal domain (Fig. S15, Table S9).

Adaptation to high temperature does not entail fitness costs at 20°C

Evolution experiments in *E. coli* have shown that adaptation to temperature can be costly (*83*, *84*), for example by decreasing growth at lower temperatures (*84*), but such costs are not universal (*18*, *28*, *85*).

In our experiment clones adapted to 30°C accumulated mutations that affect very important cellular functions, such as bacterial cell wall biosynthesis. To find out whether these mutations can compromise growth at 20°C, the temperature we used to start the experiment, we grew each of the clones adapted to 30°C at 20°C for 24 hours. Surprisingly, all clones adapted to 30°C were able to grow at 20°C, and in some cases, they even grew better than the ancestral clone at 20°C (Fig. S4). A likely explanation is that most clones carry mutations that are general adaptations to the experimental conditions because we find them both in control populations and in populations adapted to different temperatures (Fig. S6, Fig. S9). Examples of these mutations are mutations affecting the two-component system response regulator UvrY (PSHA_RS11640) and the bifunctional diguanylate cyclasephospodiestrease (PSHA_RS16945). In sum, adaptation to high temperatures (30°C) does not compromise growth at lower temperatures (20°C).

Supplementary Methods

Quantitative PCR (qPCR) experiments to confirm the different mutational routes to reduce the number of copies of chromosome 2

We used a set of six genes to study the different mutational routes followed by clones to reduce the number of copies of chromosome 2 from 2 back to 1. Specifically, we used three housekeeping genes located on chromosome 1 (*gyrA*, *rpoB*, *recA*) and three genes located on chromosome 2 (*repA*, *parA*, gluconate transporter) (Table S13). We studied the copy number of these genes in the following 12 clones. The first clone was the *P. haloplanktis* TAC125 wild-type clone, in which we expect only one copy of the three genes located on chromosome 2 to exist. The second clone was the *P. haloplanktis* preadapted clone used to start the evolution experiment (contains the duplication of chromosome 2), in which we expect two copies of the three genes located on chromosome two. Third, we selected a group of three clones that carry the chromosomal duplication and were isolated at different temperatures (22°C-19, 26°C-9, 28°C-23). Fourth, we selected four clones that all carry mutations in the *repA* gene (28°C-10, 30°C-9.2, 30°C-15.1, 30°C-30.1), and in which we expect one copy of the three genes located on chromosome 2. Lastly, we selected three clones with one of the two copies of chromosome 2 partially deleted (28°C-11, 30°C-8.3, 30°C-26.2). The partial deletion affects almost the entire chromosome 2 except few genes (among them *repA* and *parA*). We thus expect two copies of *repA* and *parA*, and one copy of the gluconate transporter to exist in these clones.

We designed primer pairs for qPCR with Primer3Plus (*86*), and used a conventional PCR with Thermopol Taq polymerase (Thermo Fisher Scientific) to confirm the amplification of a single band of the desired size. We determined the optimal primer and genomic DNA concentration, as well as the efficiency of the primer pair by qPCR using a pool of the gDNAs to be tested. We carried out the qPCR reactions in triplicates in 96-well plates (Applied Biosystems), using the Fast SYBR Green Mastermix (Thermo Fisher Scientific), and quantified amplification in a StepOnePlus instrument (Applied Biosystems). Each plate contained 4 replicates of NTC (non-template control). The qPCR conditions were as follows: an initial denaturation step (95°C, 20 sec) followed by 40 cycles of denaturation (95°C, 3 sec) and annealing and extension (60°C, 30 sec). After each run, we recorded a melt curve from 60°C to 95°C, increasing the temperature by 0.5°C every 15 sec. We performed relative quantification qPCR to compare amplification of a gene in different evolved populations of *P. haloplanktis* using the *P. haloplanktis* TAC125 wild-type strain as a control. We used qBASE+ 3.2 (*87*) to analyze the results and GeNorm (*88*) (implemented in qBASE+ 3.2) to select the most stable reference genes, which turned out to be *gyrA* and *rpoB*.

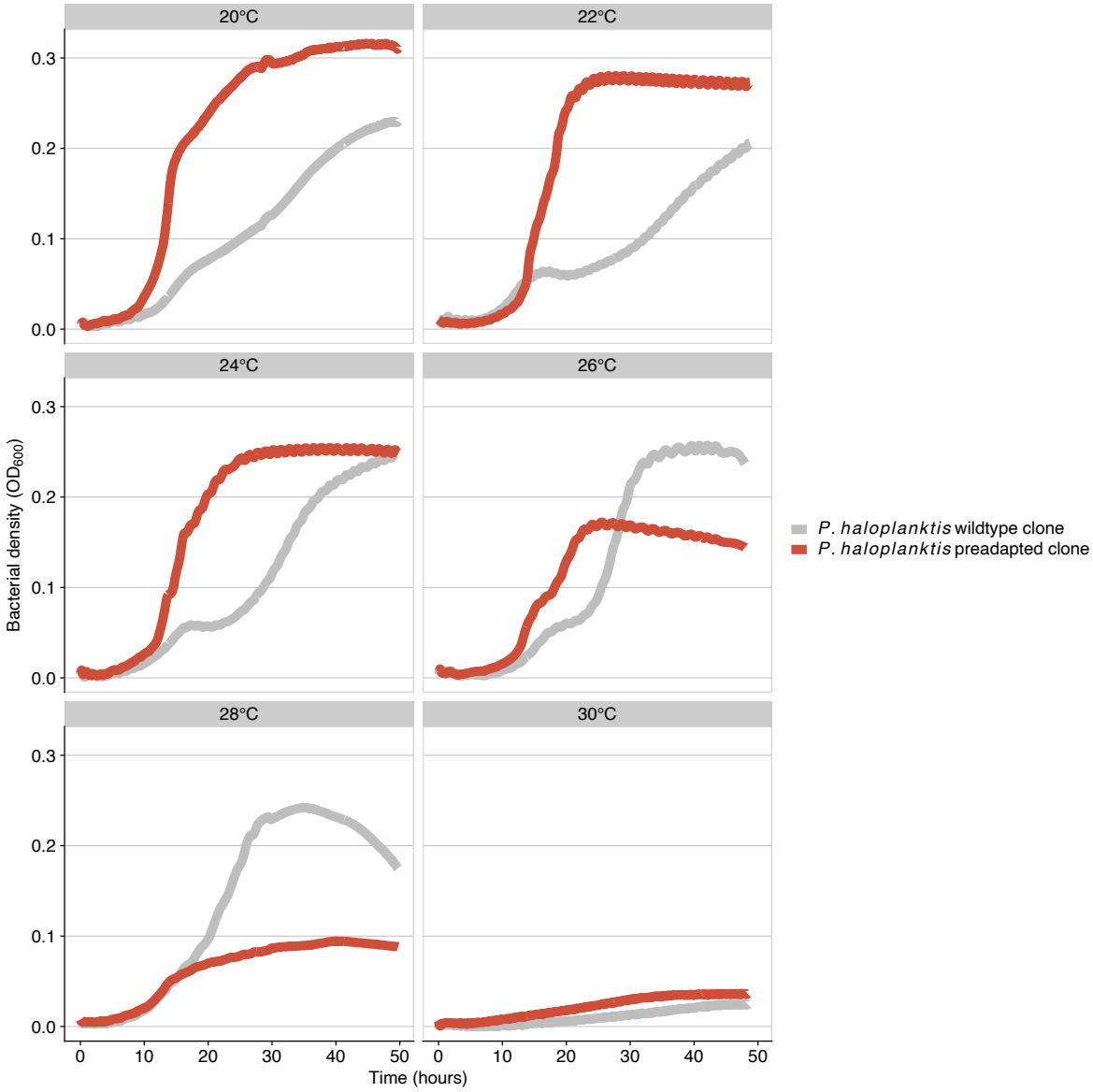


Fig. S1.
The preadapted *P. haloplanktis* TAC125 clone grows better than wild-type *P. haloplanktis* in minimal medium. We measured growth during 48 hours at temperatures ranging between 20°C and 30°C, using a wild-type clone and the preadapted clone. The preadapted clone had been adapted to minimal medium (minimal marine sea water supplemented with 0.1% D-gluconic acid) during 145 generations. Each growth curve is an average of 12 biological replicate populations started from one of the clones.

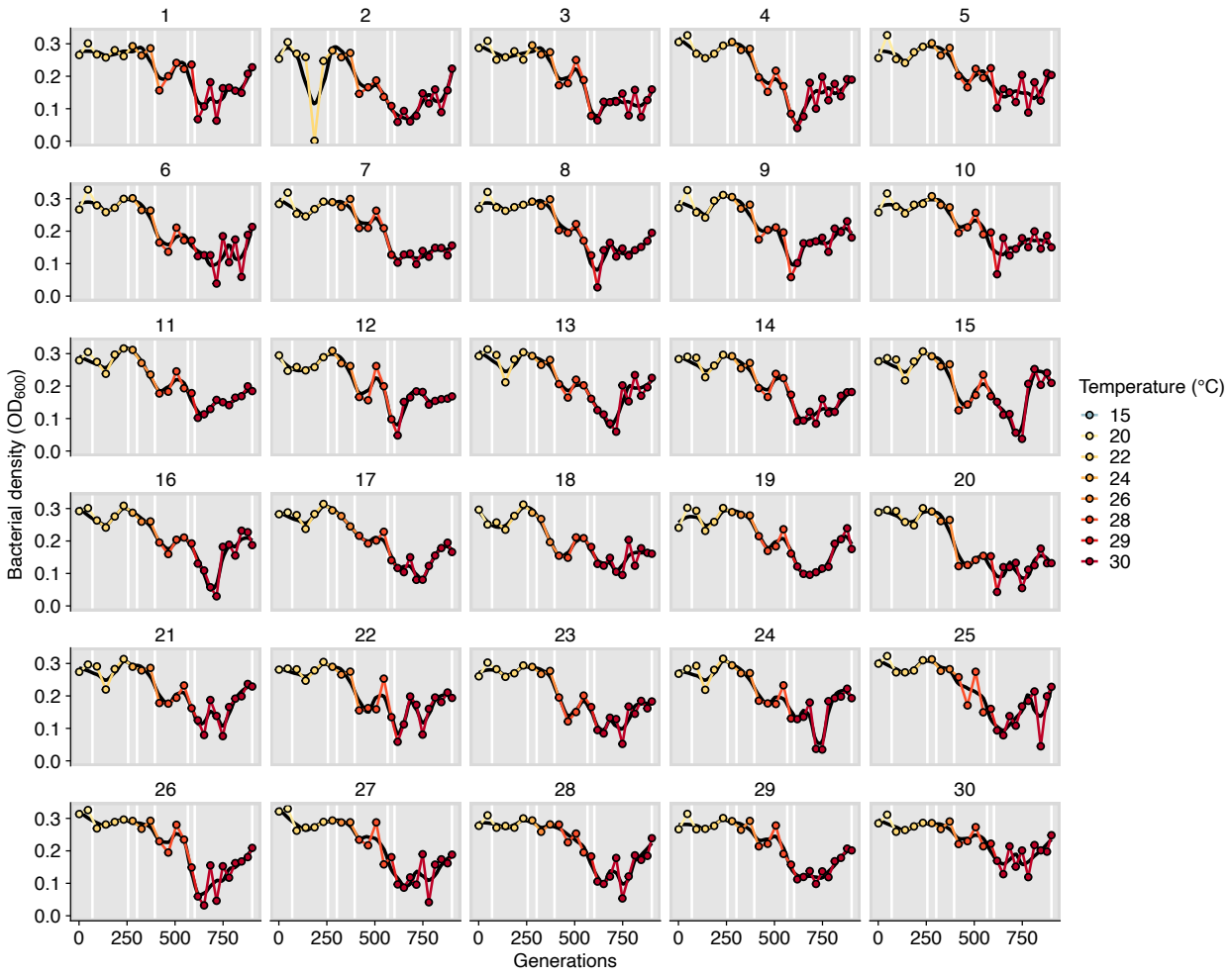


Fig. S2.

Evolutionary rescue upon exposure to 30°C. Bacterial density plotted against number of generations of experimental evolution for each of the 30 evolving populations. The color of the dots indicates the temperature at which the populations were evolving when we measured bacterial density (OD_{600}). We measured bacterial density once per week just before daily transfer to a batch of fresh medium. Vertical white lines show the approximate generation at which the temperature was increased.

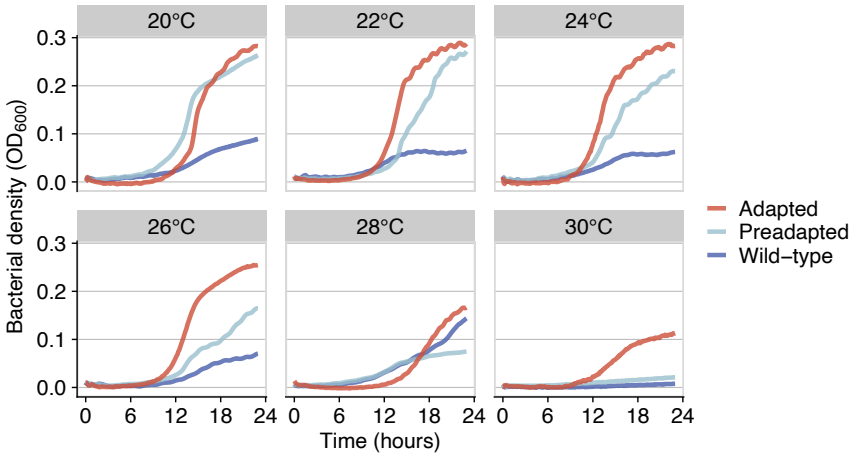
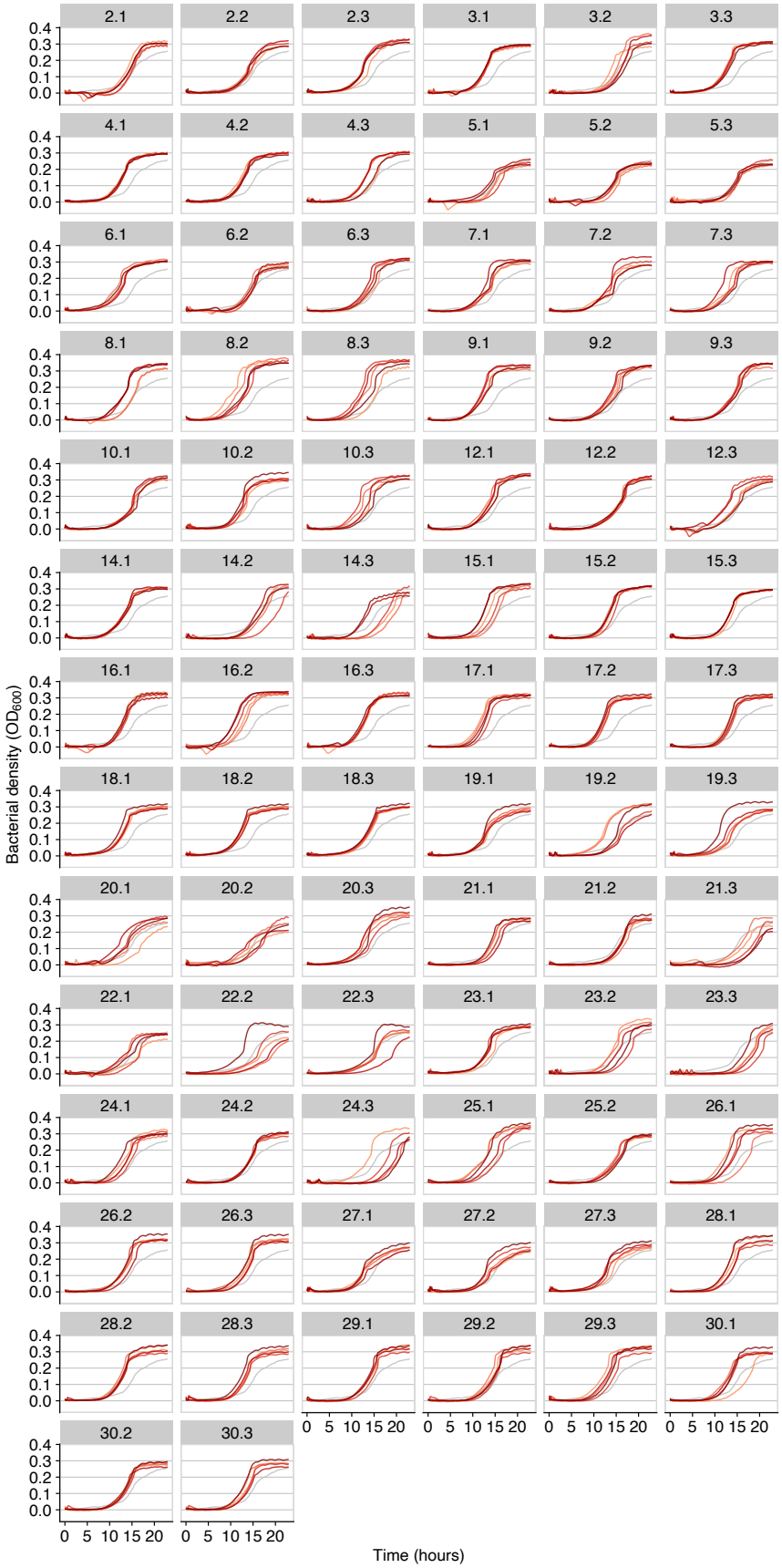


Fig. S3.
Fitness gains increase with temperature. Growth curves measured during 23 hours at different temperatures (see panel labels) using the initial preadapted clone (light blue line), wild-type clone (dark blue line) or populations adapted to each temperature (red line). The growth curve for the preadapted and wild-type clones is the average of 12 biological replicates, and the growth curve for the evolved populations is the average of 90 biological replicates (3 replicate population samples for each of 30 evolving populations).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0.3
0.4
0 5 10 15 20
Bacterial density (OD600)
Time (hours)

Fig. S4.

Adaptation to 30°C does not involve fitness tradeoffs at 20°C. We grew each of the clones adapted to 30°C at 20°C for 23 hours in minimal medium, and measured OD_{600} every 10 minutes. The horizontal axis shows time in hours, and the vertical axis shows bacterial density (OD_{600}). Red indicates growth curves for clones adapted to 30°C, grey indicated the growth curve of the preadapted clone. We measured growth in five biological replicates for each of the evolved clones (colored in different shades of red), and for the preadapted clone (grey line). For the preadapted clone we plot the growth rate averaged across the 5 replicates. Above each panel we indicated the clone number. For each population adapted to 30°C we isolated 3 clones, which we named 'population_number.1', 'population_number.2', 'population_number.3' (e.g. clone 5.1 is the first clone isolated from population 5). The red curves do not fall below the grey curve for most of the clones, indicating that adaptation to 30°C does not involve, in general, fitness tradeoffs at 20°C.

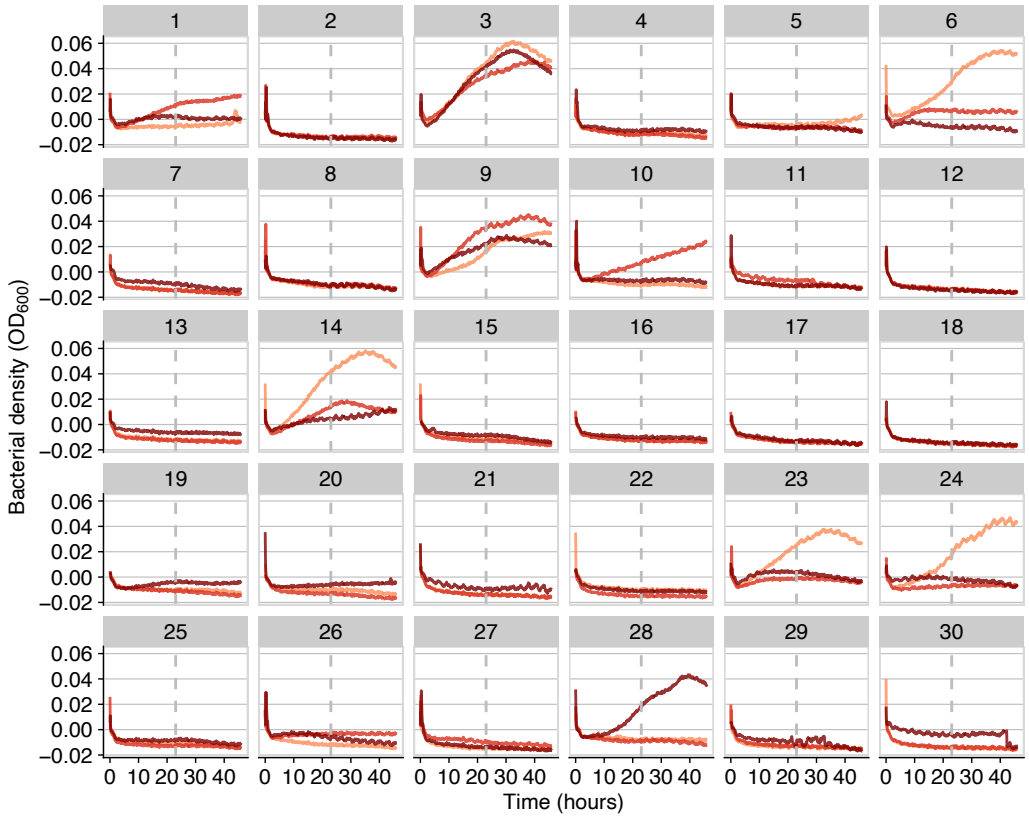


Fig. S5.

Populations adapted to 30°C cannot grow at 32°C. We grew populations adapted to 30°C at 32°C during 48 hours, and measured bacterial density (OD_{600}) every 10 minutes using a plate reader. The x-axis indicates time and the y-axis bacterial density (OD_{600}). Data are based on 3 biological replicates per population.

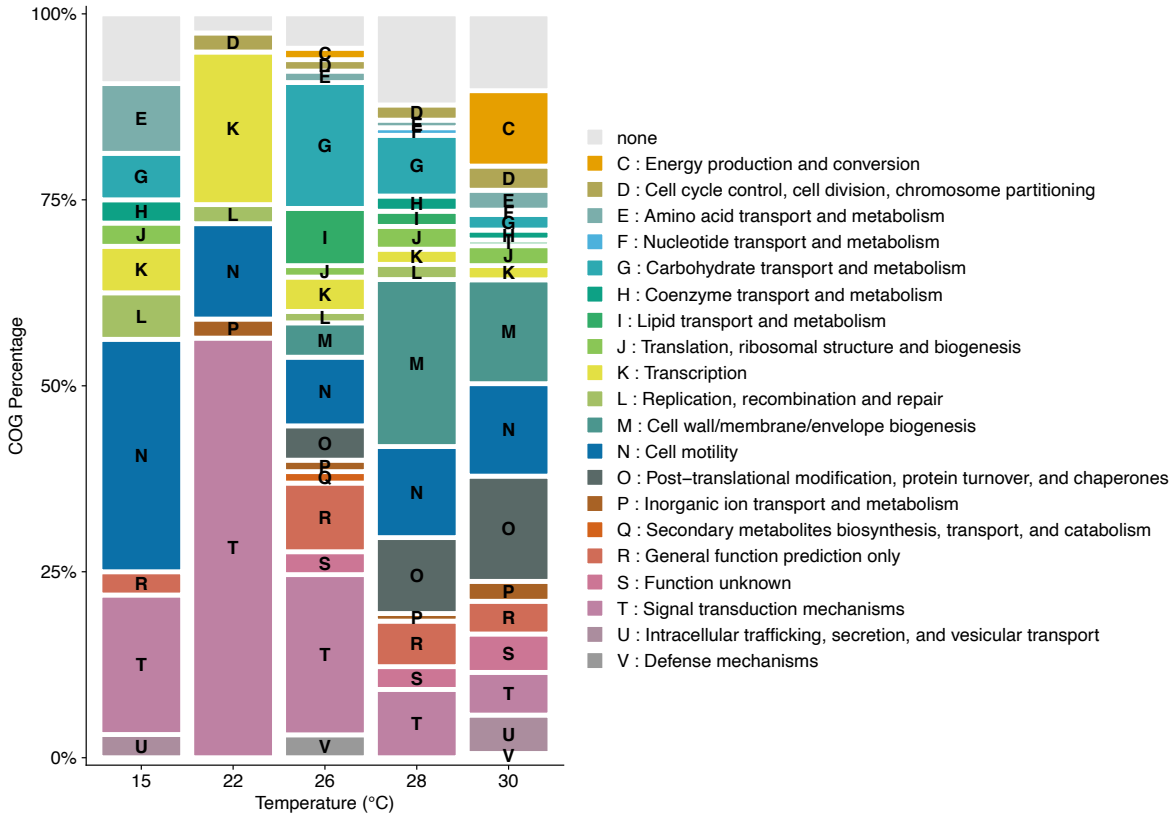


Fig. S6.

The distribution of genetic variants across 20 functional gene categories for populations adapted to different temperatures (horizontal axis). We plotted the percentage of genetic variants (point mutations, indels < 30 nt and deletions affecting only one gene) falling inside each COG (Clusters of Orthologous Groups) category at each temperature (horizontal axis). In grey we indicate the percentage of mutations in genes that do not have a COG category assigned.

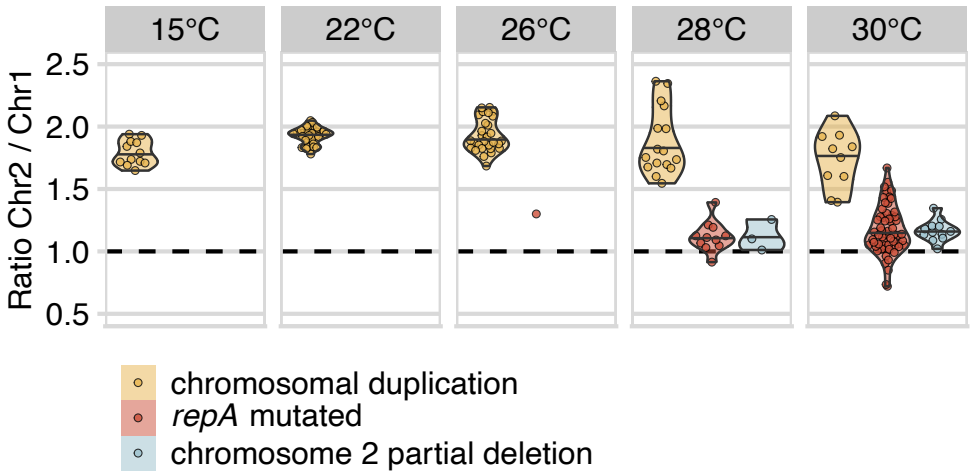


Fig. S7.

Two mutational routes to reduce the number of copies of chromosome 2. Number of copies of chromosome 2 for clones adapted to various temperatures. The number of copies of chromosome 2 is estimated by the ratio between the number of genome sequencing reads that overlap chromosome 2 and chromosome 1. Clones carrying the duplication of chromosome 2 are colored in yellow, clones carrying a mutation in the *repA* gene (a gene involved in replication located on chromosome 2), which reduces the number of copies of chromosome 2 from two to one, are colored in red, and clones with a partial deletion of one of the two copies of chromosome 2 are colored in blue.

The panel for 30°C includes clones isolated from the same populations. Below we detail the clones and populations represented in each category.

Chromosome 2 partial deletion: population 8 (clones 1, 2, 3), populations 26 (clones 1, 2, 3), population 28 (clones 1, 2, 3), population 29 (clones 1, 2, 3).

Chromosomal duplication: population 2 (clone 3), population 12 (clones 1, 2, 3), population 21 (clones 1, 2, 3), population 22 (clones 1, 2, 3).

repA mutated: population 2 (clones 1, 2), population 3 (clones 1, 2, 3), population 4 (clones 1, 2, 3), population 5 (clones 1, 2, 3), population 6 (clones 1, 2, 3), population 7 (clones 1, 2, 3), population 10 (clones 1, 2, 3), population 14 (clones 1, 2, 3), population 15 (clones 1, 2, 3), population 16 (clones 1, 2, 3), population 9 (clones 1, 2, 3), population 17 (clones 1, 2, 3), population 18 (clones 1, 2, 3), population 19 (clones 1, 2, 3), population 20 (clones 1, 2, 3), population 23 (clones 1, 2, 3), population 24 (clones 1, 2, 3), population 25 (clones 1, 2, 3), population 27 (clones 1, 2, 3), population 30 (clones 1, 2, 3).

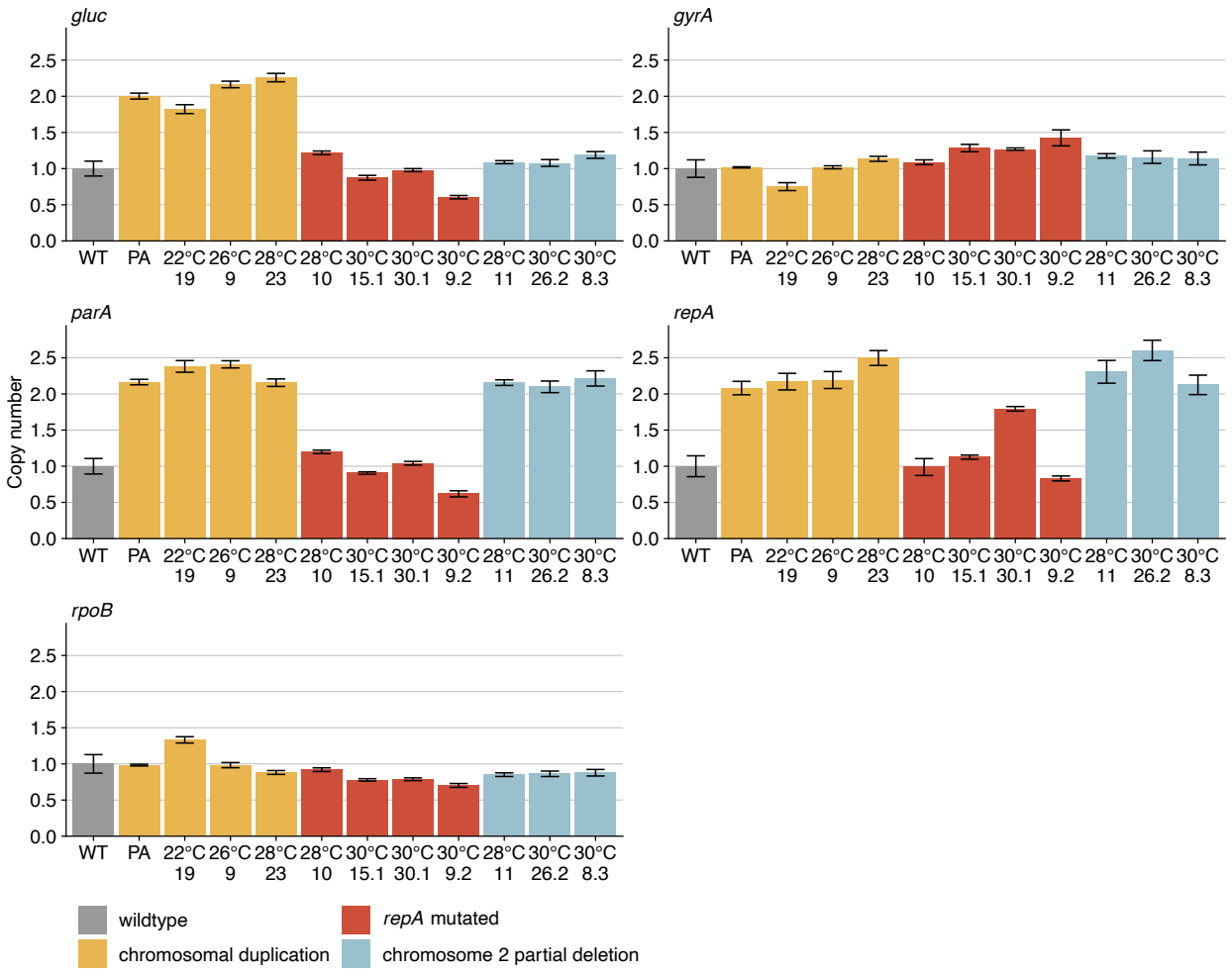


Fig. S8.

Mutational routes to reduce the number of copies of chromosome 2 from two to one. qPCR results confirm the existence of two routes to reduce the number of copies of chromosome 2 from two back to one: mutations in *repA* and the partial deletion of chromosome 2. Copy number for the *gyrA* gene, the *rpoB* gene, the gluconic acid transporter gene (*gluc*), the *parA* gene and for the *repA* gene for clones carrying one or two copies of chromosome 2. Housekeeping genes *gyrA* and *rpoB* show similar values in the wild-type isolate, and in clones with and without the chromosomal duplication adapted to different temperatures. The gluconic acid transporter gene (*gluc*), *parA* and *repA* are located on chromosome 2 but are expected to have different copy number depending on whether the mutation that reverts the chromosome 2 duplication is a mutation in *repA* or a partial deletion of chromosome 2. Clones carrying the duplication of chromosome 2 show higher copy number of the *repA*, *parA* and *gluc* genes compared to the wild-type and to clones compensating the chromosomal duplication through *repA* mutations. Clones compensating the duplication through partial chromosomal 2 deletion also show higher copy number of *repA* and *parA* than the wild-type clone, because both genes are located in the chromosome 2 region that is not partially deleted. The *gluc* gene is located in the chromosome 2 region that is partially deleted and hence, its copy number is the same in clones carrying *repA*

mutations, partial chromosome 2 deletion and the wild-type clone. The copy number of *gluc* is only increased in clones carrying the chromosome 2 duplication. Copy number values are relative to the wild-type *P. haloplanktis* clone. Copy number is quantified on the vertical axes as the CNRQ (Calibrated Normalized Relative Quantity). Error bars represent the standard error of the mean for three technical replicates per sample.

WT: wild-type strain, PA: Preadapted clone, 22°C 19: clone isolated from population 19 adapted to 22°C, 26°C 9: clone isolated from population 9 adapted to 26°C, 28°C 23: clone isolated from population 23 adapted to 28°C, 28°C 10: clone isolated from population 10 adapted to 28°C, 30°C 15.1: clone 1 isolated from population 15 adapted to 30°C, 30°C 30.1: clone 1 isolated from population 30 adapted to 30°C, 30°C 9.2: clone 2 isolated from population 9 adapted to 30°C, 28°C 11: clone isolated from population 11 adapted to 28°C, 30°C 26.2: clone 2 isolated from population 26 adapted to 30°C, 30°C 8.3: clone 3 isolated from population 8 adapted to 30°C.

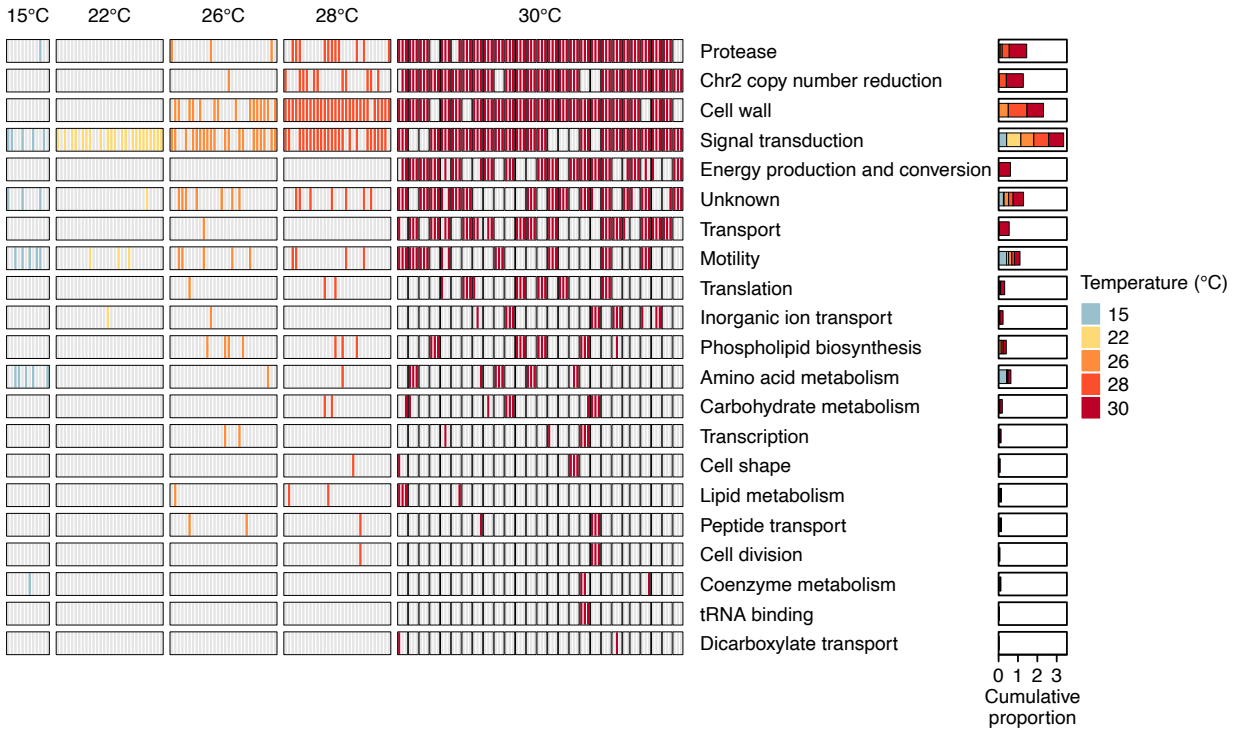


Fig. S9.

As temperature increases variation accumulates preferentially in some classes of genes. We clustered mutated genes into functional categories (Data S3), and we indicate for each sequenced clone at each temperature in which gene category mutations occur. The temperature is displayed on the top horizontal axis, and the functional gene categories on the right vertical axis. Small colored vertical bars denote each of the sequenced clones. Clones adapted to 30°C are grouped by population. Next to each functional category, a bar plot indicates the proportion of clones that harbor a mutation falling in that category at a given temperature. The functional category 'Protease' includes mutations in Lon protease and other proteases.

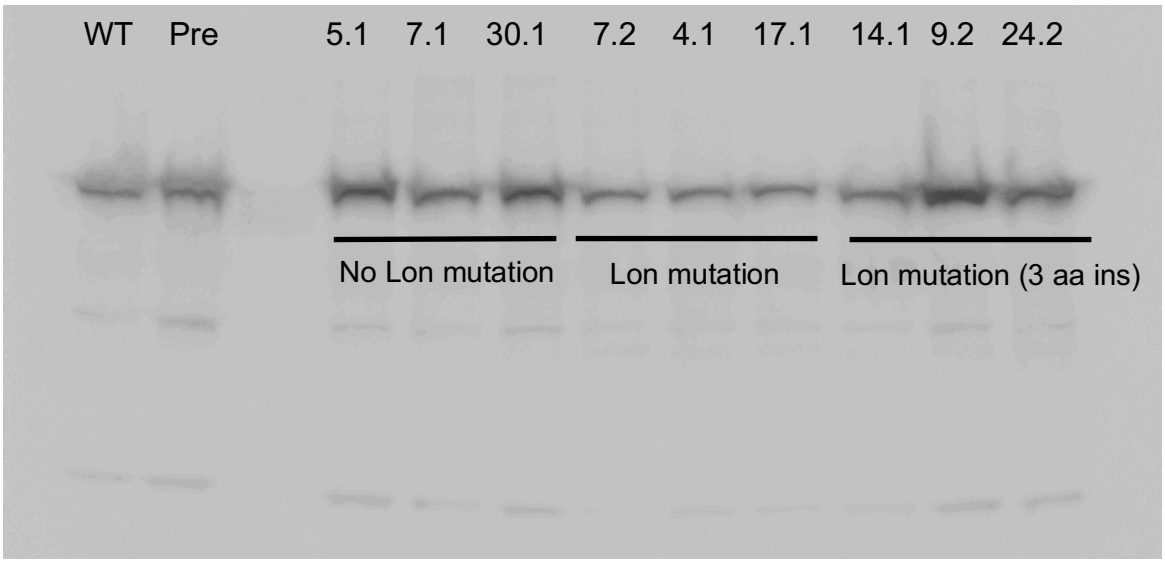


Fig. S10.

Mutations in Lon protease do not affect Lon protease expression. Western blot of wild-type Lon protease and Lon protease from clones adapted to 30°C carrying different mutations in Lon protease. WT: *P. haloplanktis* wild-type clone, Pre: *P. haloplanktis* preadapted clone. The remaining clones are isolated from populations adapted to 30°C. For each population adapted to 30°C we isolated 3 clones, which we named 'population_number.1', 'population_number.2', 'population_number.3' (e.g. clone 5.1 was isolated from population 5). Lon mutation includes 3 types of mutations: His505Tyr (clone 7.2), Leu278_Lys279del (clone 4.1) and Arg292Cys (clone 17.1). Lon mutation (3aa ins) refers to the mutation Met280_Met281insMetSerPro, i.e., a Methionine-Serine-Proline insertion between Met280 and Met281. To prepare this western blot, we grew wild-type clones at 20°C and clones adapted to 30°C at 30°C. We used an antibody against *E. coli* Lon protease (Biorbyt). Because it is a polyclonal antibody, it is highly unlikely that the mutations in Lon protease affect the binding of the antibody. The Lon protease band is the upper-most band. All other bands are unspecific bands, which are typical of polyclonal antibodies.

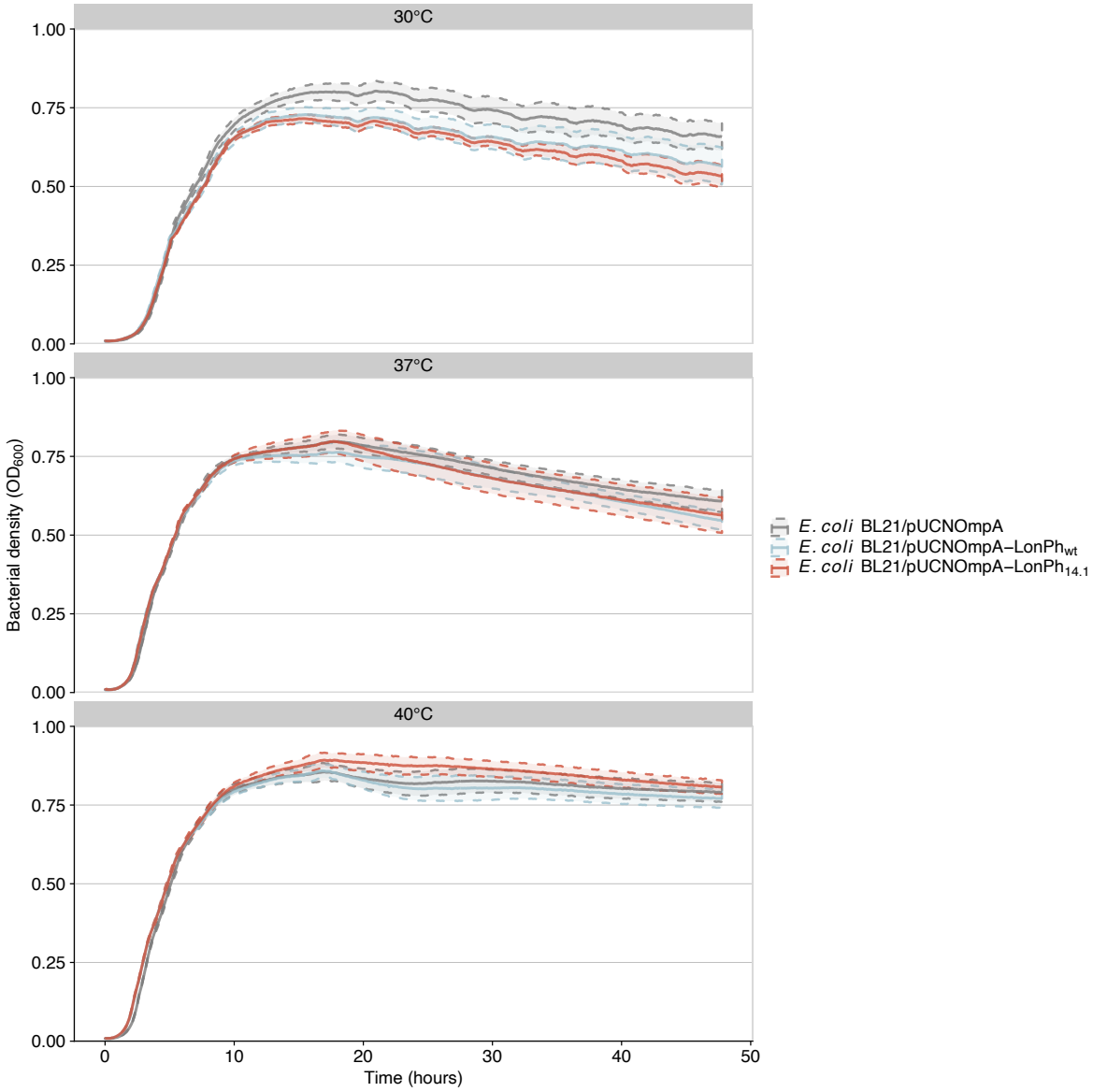


Fig. S11.

***E. coli* cells expressing mutated Lon protease from *P. haloplanktis* grow better at higher temperatures.** 48 hour growth curves measured at 30°C, 37°C and 40°C for *E. coli* BL21 cells carrying an empty plasmid (grey, *E. coli* BL21/pUCNOmpA), a plasmid expressing the wild-type Lon protease from *P. haloplanktis* (blue, *E. coli* BL21/ pUCNOmpA-LonPh$_{wt}$), or a plasmid expressing the mutated (Met-Ser-Pro insertion) Lon protease from *P. haloplanktis* (red, *E. coli* BL21/ pUCNOmpA-LonPh$_{14.1}$). Shaded areas indicate 95% confidence interval of the mean. We measured each growth curve in 12 replicate populations derived from the same Lon protease-expressing clone.

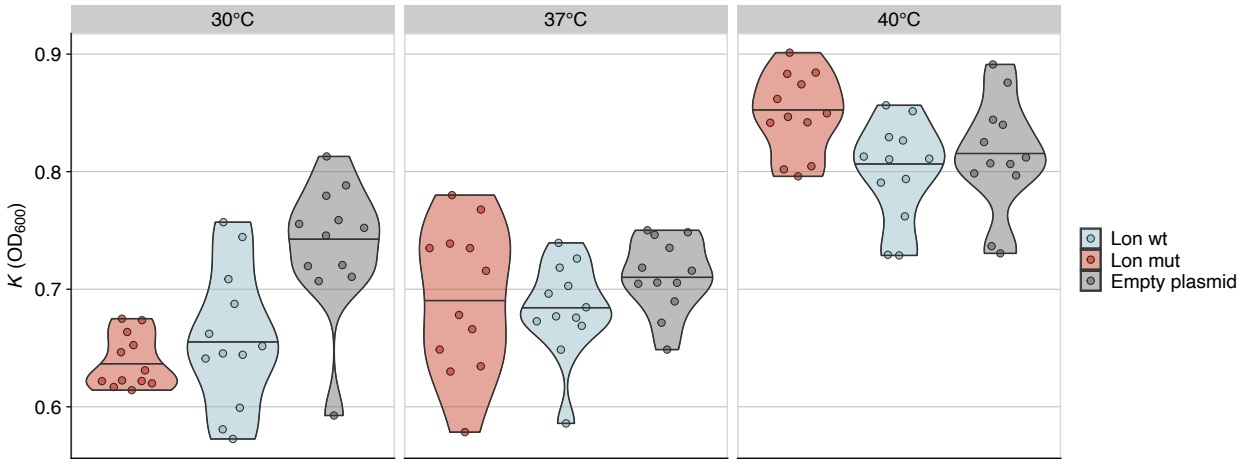
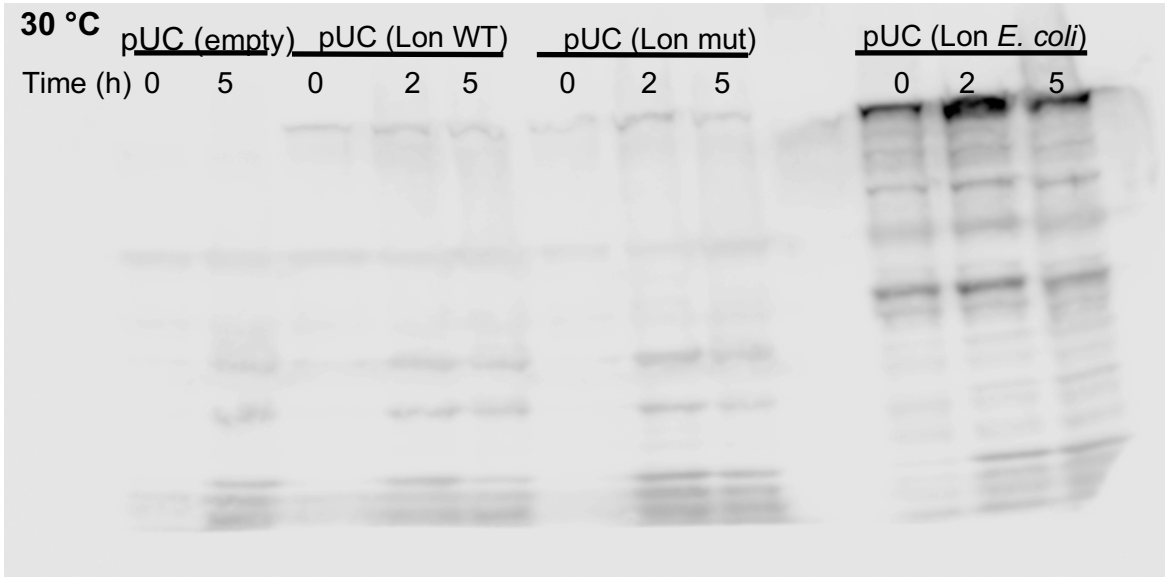


Fig. S12.

Mutations in Lon protease are key for adaptation to high temperature. Maximum population density (*K*) in *E. coli* BL21 strain carrying an empty plasmid (grey), a plasmid expressing the wild-type Lon protease from *P. haloplanktis* (blue) or a plasmid expressing the mutated (Met-Ser-Pro insertion) Lon protease from *P. haloplanktis* (red). We measured growth curves at 30°C, 37°C and 40°C. We performed 12 biological replicates for each *E. coli* and plasmid combination, and for each temperature.


30 °C
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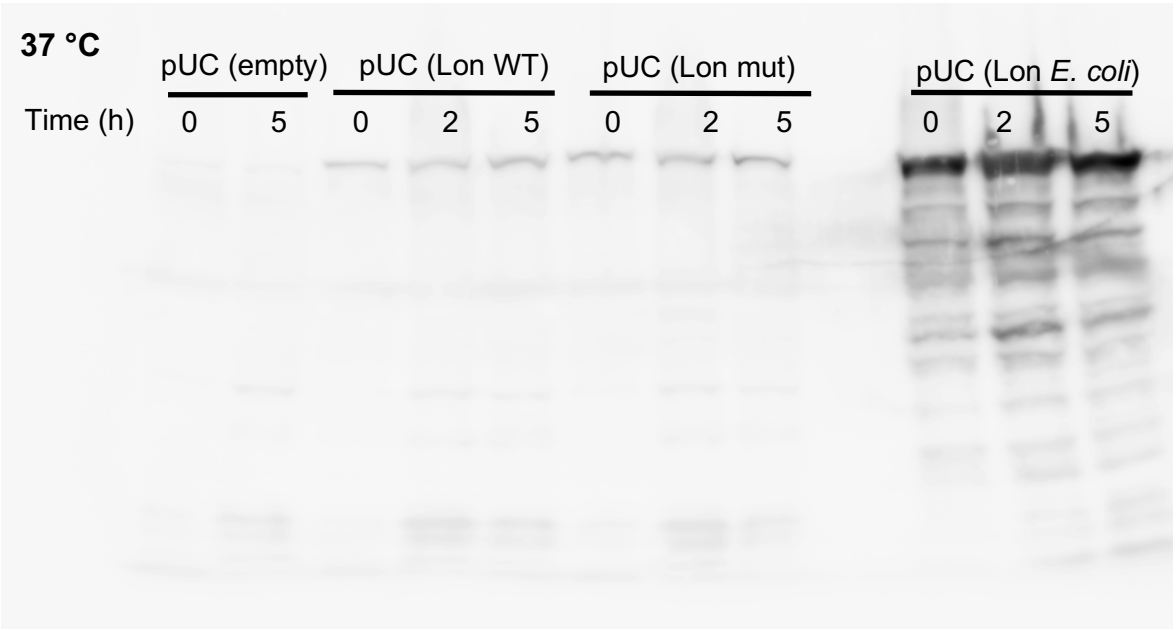
pUC (Lon WT)
pUC (Lon mut)
pUC (Lon E. coli)
Time (h) 0 5 0 2 5 0 2 5 0 2 5


37 °C
pUC (empty)
pUC (Lon WT)
pUC (Lon mut)
pUC (Lon E. coli)
Time (h) 0 5 0 2 5 0 2 5 0 2 5

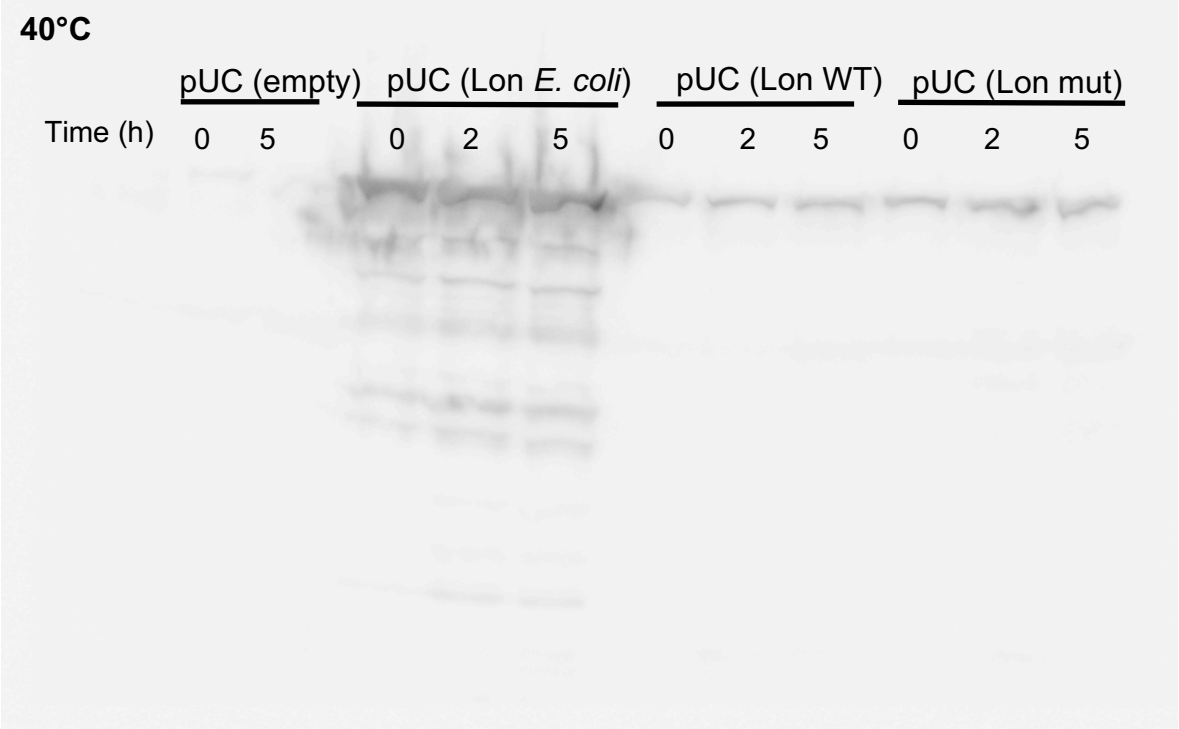


Fig. S13.
Western blot for wild-type Lon protease and mutated Lon protease (3aa insertion) expressed in *E. coli* BL21 cells at 30°C, 37°C and 40°C. We performed western blots to confirm the expression of Lon protease during the growth curves measured to investigate the effect of Lon protease on the growth of *E. coli* at different temperatures. We used an antibody against *E. coli* Lon protease (Biorbyt). Because it is a polyclonal antibody, it is highly unlikely that the mutations in Lon protease affect the binding of the antibody. The Lon protease band is the upper-most band. All other bands are unspecific bands, which are typical of polyclonal antibodies.

> pUCNOmpA-EYFP-1

CTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGCC
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TTATCAGAATCGCAGATCCGGCTTCAGGTTTGCCGGCTGAAAGCGCTATTTCTTCCAGAA
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TACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCC
TTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACC
TCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCG
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GCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTT
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GCTGGCCTTTTGCTCACATCCTAGTGCTTGGATTCTCACCAATAAAAAACGCCCGGCGGC
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AGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA
AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAATGCTTCG
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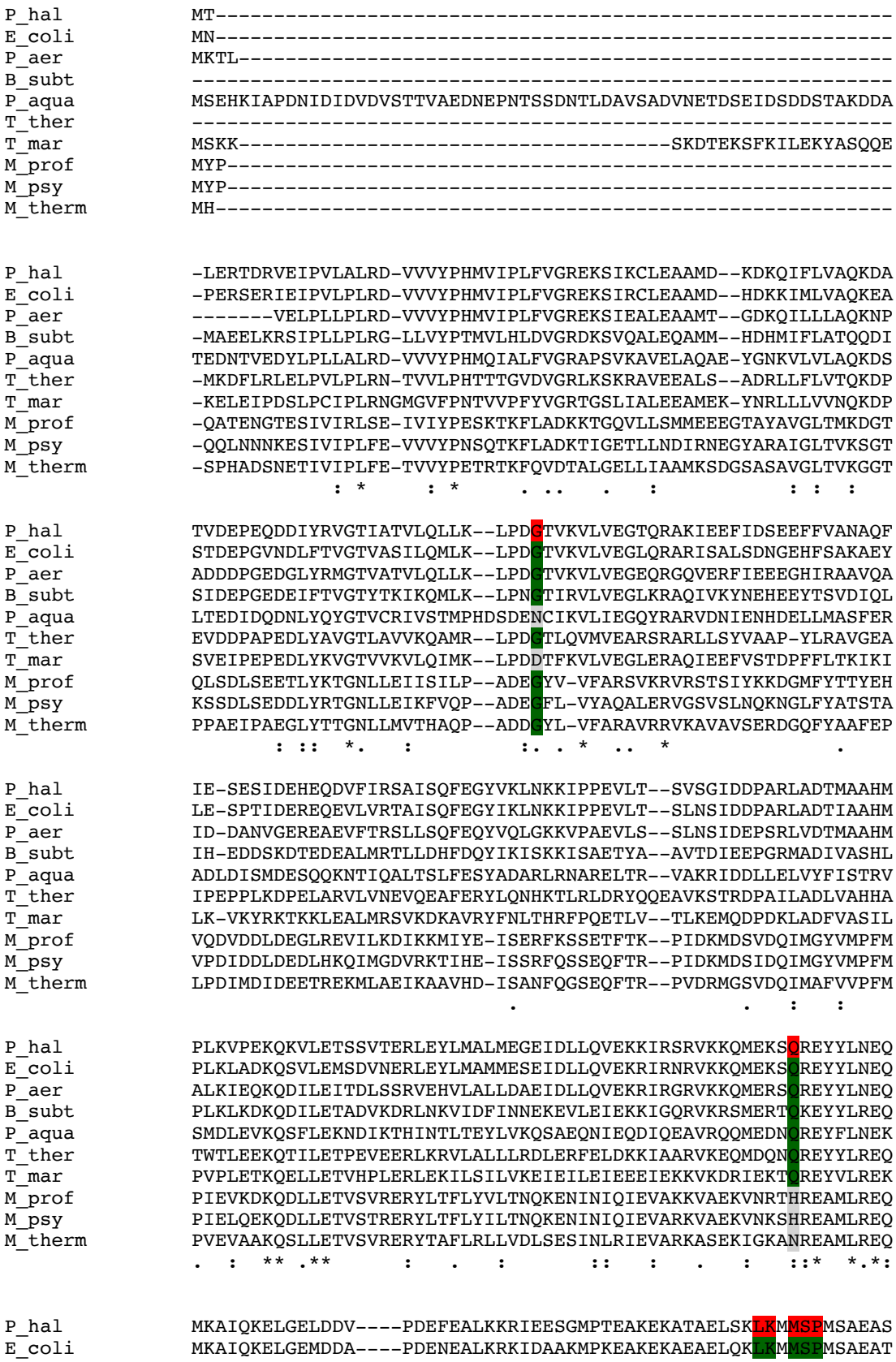
ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCG
GCATGGACGAGCTGTACAAGTGATCTAGAGGCATCAAATAAAACGAAAGGCTCAGTCGAA
AGACTGGGC

Fig. S14.

DNA sequence of the plasmid pUCNOmpA-EYFP-1. Bold indicates the pUC origin of replication, orange shows the ompA promoter and yellow indicates the EYFP gene.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E_coli MN
P_aer MKTL
B_subt
P_aqua MSEHKIAPDNIDIDVDVSTTVAEDNEPNTSSDNTLDAVSADVNETDSEIDSDDSTAKDDA
T_ther
T_mar MSKK SKDTEKSFKILEKYASQQE
M_prof MYP
M_psy MYP
M_therm MH
P_hal -LERTDRVEIPVLALRD-VVVYPHMVIPLFVGREKSIKCLEAAMD--KDKQIFLVAQKDA
E_coli -PERSERIEIPVLPLRD-VVVYPHMVIPLFVGREKSIRCLEAAMD--HDKKIMLVAQKEA
P_aer -------VELPLLPLRD-VVVYPHMVIPLFVGREKSIEALEAAMT--GDKQILLLAQKNP
B_subt -MAEELKRSIPLLPLRG-LLVYPTMVLHLDVGRDKSVQALEQAMM--HDHMIFLATQQDI
P_aqua TEDNTVEDYLPLLALRD-VVVYPHMQIALFVGRAPSVKAVELAQAE-YGNKVLVLAQKDS
T_ther -MKDFLRLELPVLPLRN-TVVLPHTTTGVDVGRLKSKRAVEEALS--ADRLLFLVTQKDP
T_mar -KELEIPDSLPCIPLRNGMGVFPNTVVPFYVGRTGSLIALEEAMEK-YNRLLLVVNQKDP
M_prof -QATENGTESIVIRLSE-IVIYPESKTKFLADKKTGQVLLSMMEEEGTAYAVGLTMKDGT
M_psy -QQLNNNKESIVIPLFE-VVVYPNSQTKFLADKTIGETLLNDIRNEGYARAIGLTVKSGT
M_therm -SPHADSNETIVIPLFE-TVVYPETRTKFQVDTALGELLIAAMKSDGSASAVGLTVKGGT
P_hal TVDEPEQDDIYRVGTIATVLQLLK--LPDGTVKVLVEGTQRAKIEEFIDSEEFFVANAQF
E_coli STDEPGVNDLFTVGTVASILQMLK--LPDGTVKVLVEGLQRARISALSDNGEHFSAKAEY
P_aer ADDDPGEDGLYRMGTVATVLQLLK--LPDGTVKVLVEGEQRGQVERFIEEEGHIRAAVQA
B_subt SIDEPGEDEIFTVGTYTKIKQMLK--LPNGTIRVLVEGLKRAQIVKYNEHEEYTSVDIQL
P_aqua LTEDIDQDNLYQYGTVCRIVSTMPHDSDENCIKVLIEGQYRARVDNIENHDELLMASFER
T_ther EVDDPAPEDLYAVGTLAVVKQAMR--LPDGTLQVMVEARSRARLLSYVAAP-YLRAVGEA
T_mar SVEIPEPEDLYKVGTVVKVLQIMK--LPDDTFKVLVEGLERAQIEEFVSTDPFFLTKIKI
M_prof QLSDLSEETLYKTGNLLEIISILP--ADEGYV-VFARSVKRVRSTSIYKKDGMFYTTYEH
M_psy KSSDLSEDDLYRTGNLLEIKFVQP--ADEGFL-VYAQALERVGSVSLNQKNGLFYATSTA
M_therm PPAEIPAEGLYTTGNLLMVTHAQP--ADDGYL-VFARAVRRVKAVAVSERDGQFYAAFEP
P_hal IE-SESIDEHEQDVFIRSAISQFEGYVKLNKKIPPEVLT--SVSGIDDPARLADTMAAHM
E_coli LE-SPTIDEREQEVLVRTAISQFEGYIKLNKKIPPEVLT--SLNSIDDPARLADTIAAHM
P_aer ID-DANVGEREAEVFTRSLLSQFEQYVQLGKKVPAEVLS--SLNSIDEPSRLVDTMAAHM
B_subt IH-EDDSKDTEDEALMRTLLDHFDQYIKISKKISAETYA--AVTDIEEPGRMADIVASHL
P_aqua ADLDISMDESQQKNTIQALTSLFESYADARLRNARELTR--VAKRIDDLLELVYFISTRV
T_ther IPEPPLKDPELARVLVNEVQEAFERYLQNHKTLRLDRYQQEAVKSTRDPAILADLVAHHA
T_mar LK-VKYRKTKKLEALMRSVKDKAVRYFNLTHRFPQETLV--TLKEMQDPDKLADFVASIL
M_prof VQDVDDLDEGLREVILKDIKKMIYE-ISERFKSSETFTK--PIDKMDSVDQIMGYVMPFM
M_psy VPDIDDLDEDLHKQIMGDVRKTIHE-ISSRFQSSEQFTR--PIDKMDSIDQIMGYVMPFM
M_therm LPDIMDIDEETREKMLAEIKAAVHD-ISANFQGSEQFTR--PVDRMGSVDQIMAFVVPFM
P_hal PLKVPEKQKVLETSSVTERLEYLMALMEGEIDLLQVEKKIRSRVKKQMEKSQREYYLNEQ
E_coli PLKLADKQSVLEMSDVNERLEYLMAMMESEIDLLQVEKRIRNRVKKQMEKSQREYYLNEQ
P_aer ALKIEQKQDILEITDLSSRVEHVLALLDAEIDLLQVEKRIRGRVKKQMERSQREYYLNEQ
B_subt PLKLKDKQDILETADVKDRLNKVIDFINNEKEVLEIEKKIGQRVKRSMERTQKEYYLREQ
P_aqua SMDLEVKQSFLEKNDIKTHINTLTEYLVKQSAEQNIEQDIQEAVRQQMEDNQREYFLNEK
T_ther TWTLEEKQTILETPEVEERLKRVLALLLRDLERFELDKKIAARVKEQMDQNQREYYLREQ
T_mar PVPLETKQELLETVHPLERLEKILSILVKEIEILEIEEEIEKKVKDRIEKTQREYVLREK
M_prof PIEVKDKQDLLETVSVRERYLTFLYVLTNQKENINIQIEVAKKVAEKVNRTHREAMLREQ
M_psy PIELQEKQDLLETVSTRERYLTFLYILTNQKENINIQIEVARKVAEKVNKSHREAMLREQ
M_therm PVEVAAKQSLLETVSVRERYTAFLRLLVDLSESINLRIEVARKASEKIGKANREAMLREQ
P_hal MKAIQKELGELDDV----PDEFEALKKRIEESGMPTEAKEKATAELSKLKMMSPMSAEAS
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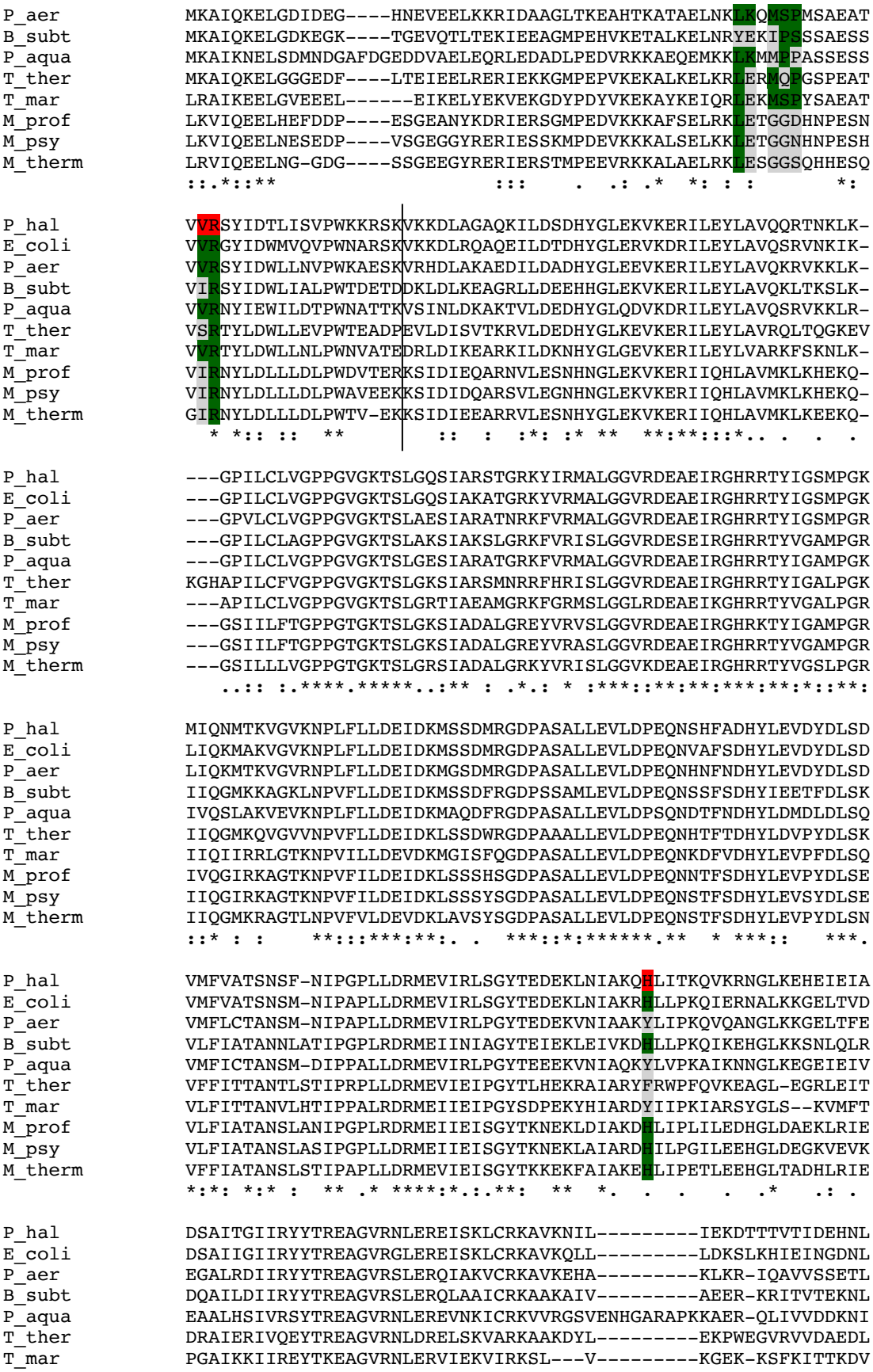
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P_aqua MKAIKNELSDMNDGAFDGEDDVAELEQRLEDADLPEDVRKKAEQEMKKLKMMPPASSESS
T_ther MKAIQKELGGGEDF----LTEIEELRERIEKKGMPEPVKEKALKELKRLERMQPGSPEAT
T_mar LRAIKEELGVEEEL------EIKELYEKVEKGDYPDYVKEKAYKEIQRLEKMSPYSAEAT
M_prof LKVIQEELHEFDDP----ESGEANYKDRIERSGMPEDVKKKAFSELRKLETGGDHNPESN
M_psy LKVIQEELNESEDP----VSGEGGYRERIESSKMPDEVKKKALSELKKLETGGNHNPESH
M_therm LRVIQEELNG-GDG----SSGEEGYRERIERSTMPEEVRKKALAELRKLESGGSQHHESQ
P_hal VVRSYIDTLISVPWKKRSKVKKDLAGAQKILDSDHYGLEKVKERILEYLAVQQRTNKLK-
E_coli VVRGYIDWMVQVPWNARSKVKKDLRQAQEILDTDHYGLERVKDRILEYLAVQSRVNKIK-
P_aer VVRSYIDWLLNVPWKAESKVRHDLAKAEDILDADHYGLEEVKERILEYLAVQKRVKKLK-
B_subt VIRSYIDWLIALPWTDETDDKLDLKEAGRLLDEEHHGLEKVKERILEYLAVQKLTKSLK-
P_aqua VVRNYIEWILDTPWNATTKVSINLDKAKTVLDEDHYGLQDVKDRILEYLAVQSRVKKLR-
T_ther VSRTYLDWLLEVPWTEADPEVLDISVTKRVLDEDHYGLKEVKERILEYLAVRQLTQGKEV
T_mar VVRTYLDWLLNLPWNVATEDRLDIKEARKILDKNHYGLGEVKERILEYLVARKFSKNLK-
M_prof VIRNYLDLLLDLPWDVTERKSIDIEQARNVLESNHNGLEKVKERIIQHLAVMKLKHEKQ-
M_psy VIRNYLDLLLDLPWAVEEKKSIDIDQARSVLEGNHNGLEKVKERIIQHLAVMKLKHEKQ-
M_therm GIRNYLDLLLDLPWTV-EKKSIDIEEARRVLESNHYGLEKVKERIIQHLAVMKLKEEKQ-
P_hal ---GPILCLVGPPGVGKTSLGQSIARSTGRKYIRMALGGVRDEAEIRGHRRTYIGSMPGK
E_coli ---GPILCLVGPPGVGKTSLGQSIAKATGRKYVRMALGGVRDEAEIRGHRRTYIGSMPGK
P_aer ---GPVLCLVGPPGVGKTSLAESIARATNRKFVRMALGGVRDEAEIRGHRRTYIGSMPGR
B_subt ---GPILCLAGPPGVGKTSLAKSIAKSLGRKFVRISLGGVRDESEIRGHRRTYVGAMPGR
P_aqua ---GPILCLVGPPGVGKTSLGESIARATGRKFVRMALGGVRDEAEIRGHRRTYIGAMPGK
T_ther KGHAPILCFVGPPGVGKTSLGKSIARSMNRRFHRISLGGVRDEAEIRGHRRTYIGALPGK
T_mar ---APILCLVGPPGVGKTSLGRTIAEAMGRKFGRMSLGGLRDEAEIKGHRRTYVGALPGR
M_prof ---GSIILFTGPPGTGKTSLGKSIADALGREYVRVSLGGVRDEAEIRGHRKTYIGAMPGR
M_psy ---GSIILFTGPPGTGKTSLGKSIADALGREYVRASLGGVRDEAEIRGHRRTYVGAMPGR
M_therm ---GSILLLVGPPGTGKTSLGRSIADALGRKYVRISLGGVKDEAEIRGHRRTYVGSLPGR
P_hal MIQNMTKVGVKNPLFLLDEIDKMSSDMRGDPASALLEVLDPEQNSHFADHYLEVDYDLSD
E_coli LIQKMAKVGVKNPLFLLDEIDKMSSDMRGDPASALLEVLDPEQNVAFSDHYLEVDYDLSD
P_aer LIQKMTKVGVRNPLFLLDEIDKMGSDMRGDPASALLEVLDPEQNHNFNDHYLEVDYDLSD
B_subt IIQGMKKAGKLNPVFLLDEIDKMSSDFRGDPSSAMLEVLDPEQNSSFSDHYIEETFDLSK
P_aqua IVQSLAKVEVKNPLFLLDEIDKMAQDFRGDPASALLEVLDPSQNDTFNDHYLDMDLDLSQ
T_ther IIQGMKQVGVVNPVFLLDEIDKLSSDWRGDPAAALLEVLDPEQNHTFTDHYLDVPYDLSK
T_mar IIQIIRRLGTKNPVILLDEVDKMGISFQGDPASALLEVLDPEQNKDFVDHYLEVPFDLSQ
M_prof IVQGIRKAGTKNPVFILDEIDKLSSSHSGDPASALLEVLDPEQNNTFSDHYLEVPYDLSE
M_psy IIQGIRKAGTKNPVFILDEIDKLSSSYSGDPASALLEVLDPEQNSTFSDHYLEVSYDLSE
M_therm IIQGMKRAGTLNPVFVLDEVDKLAVSYSGDPASALLEVLDPEQNSTFSDHYLEVPYDLSN
P_hal VMFVATSNSF-NIPGPLLDRMEVIRLSGYTEDEKLNIAKQHLITKQVKRNGLKEHEIEIA
E_coli VMFVATSNSM-NIPAPLLDRMEVIRLSGYTEDEKLNIAKRHLLPKQIERNALKKGELTVD
P_aer VMFLCTANSM-NIPAPLLDRMEVIRLPGYTEDEKVNIAAKYLIPKQVQANGLKKGELTFE
B_subt VLFIATANNLATIPGPLRDRMEIINIAGYTEIEKLEIVKDHLLPKQIKEHGLKKSNLQLR
P_aqua VMFICTANSM-DIPPALLDRMEVIRLPGYTEEEKVNIAQKYLVPKAIKNNGLKEGEIEIV
T_ther VFFITTANTLSTIPRPLLDRMEVIEIPGYTLHEKRAIARYFRWPFQVKEAGL-EGRLEIT
T_mar VLFITTANVLHTIPPALRDRMEIIEIPGYSDPEKYHIARDYIIPKIARSYGLS--KVMFT
M_prof VLFIATANSLANIPGPLRDRMEIIEISGYTKNEKLDIAKDYLIPLILEDHGLDAEKLRIE
M_psy VLFIATANSLASIPGPLLDRMEIIEISGYTKNEKLAIARDHILPGILEEHGLDEGKVEVK
M_therm VFFIATANSLSTIPAPLLDRMEVIEISGYTKKEKFAIAKEHLIPETLEEHGLTADHLRIE
P_hal DSAITGIIRYYTREAGVRNLEREISKLCRKAVKNIL----------IEKDTTTVTIDEHNL
E_coli DSAIIGIIRYYTREAGVRGLEREISKLCRKAVKQLL----------LDKSLKHIEINGDNL
P_aer EGALRDIIRYYTREAGVRSLERQIAKVCRKAVKEHA----------KLKR-IQAVVSSETL
B_subt DQAILDIIRYYTREAGVRSLERQLAAICRKAAKAIV----------AEER-KRITVTEKNL
P_aqua EAALHSIVRSYTREAGVRNLEREVNKICRKVVRGSVENHGARAPKKAER-QLIVVDDKNI
T_ther DRAIERIVQEYTREAGVRNLDRELSKVARKAAKDYL----------EKPWEGVRVVDAEDL
T_mar PGAIKKIIREYTKEAGVRNLERVIEKVIRKSL---V----------KGEK-KSFKITTKDV

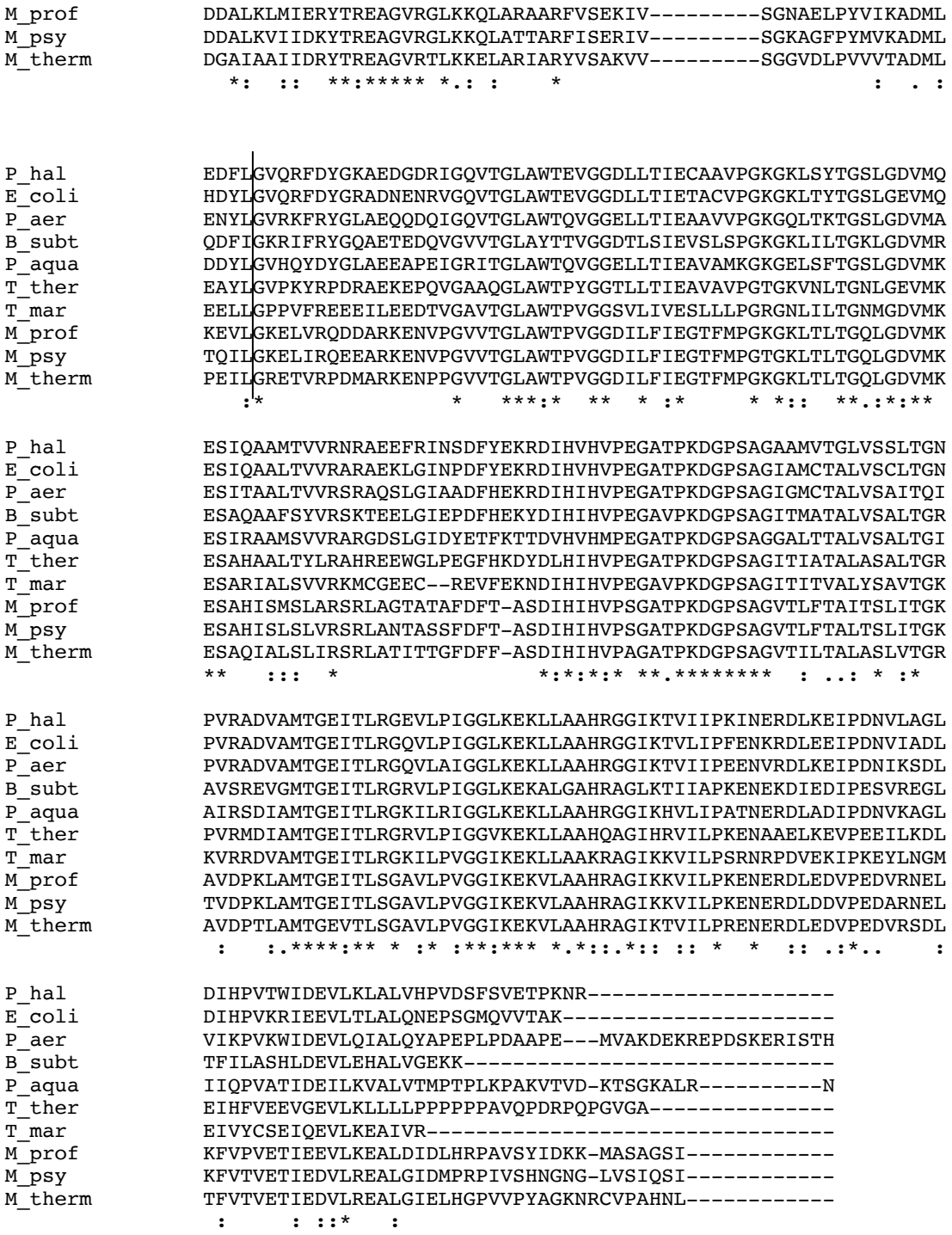

Fig. S15.

Lon protease conservation across species. In the multiple sequence alignment (produced using MAFFT) red indicates the amino acids that are mutated in *P. haloplanktis* adapted to 30°C, green shows amino acid positions that are conserved and grey indicates those amino acids that are not conserved. Three vertical lines separate the three Lon protease domains, i.e., the N-terminal domain, the AAA+ domain, and the protease domain. P_hal: *P. haloplanktis TAC125*, E_coli:

Escherichia coli str. K-12 substr. MG1655 (gram-negative, mesophile), P_aer: *Pseudomonas aeruginosa PAO1* (gram-negative, mesophile), B_subt: *Bacillus subtilis* (gram-positive, mesophile), P_aqua: *Psychrobacter aquaticus* (gram-negative, marine psychrophilic bacterium), T_ther: *Thermus thermophilus* (gram-negative, thermophilic bacterium), T_mar: *Thermotoga maritima* (gram-negative, marine hyperthermophilic bacterium), M_prof: *Methanolobus profundi* (archaea, mesophilic), M_psy: *Methanolobus psychrophilus* (archaea, psychrophilic), M_therm: *Methanoculleus thermophiles* (archaea, thermophilic). We obtained sequences homologous to *P. haloplanktis* Lon protease through a BlastP search against the *nr* database in NCBI. All amino acid sequences shown cover at least 96% of the *P. haloplanktis* Lon protease sequence, are at least 40% identical with *P. haloplanktis* Lon protease, and showed an E-value of 0.0 (BlastP rounds off to 0.0 very small E-values).

Table S1. **List of mutations identified in the *P. haloplanktis* preadapted clone.**

Chromosome	Position	Mutation	Gene	Description
NC_007481	2,737,161	$(AGCTCA)_{2\rightarrow3}$	PSHA_RS12760	Outer membrane channel protein TolC
NC_007482	NA	Chromosomal duplication		Duplication of whole chromosome 2
NC_007482	46,491	Δ6,608 bp	PSHA_RS15050–PSHA_RS15090	9 genes deleted (DEAD/DEAH box helicase, uracil-DNA glycosylase family protein, VOC family protein, hypothetical protein, PepSY domain-containing protein, DUF3325 domain-containing protein, hypothetical protein, YaiI/YqxD family protein, hypothetical protein)

We compared the genome of the preadapted clone of *P. haloplanktis* to the *P. haloplanktis* wild-type isolate to identify the mutations gained during the preadaptation experiment. NA: not applicable.

Table S2. Types of mutations identified in clones adapted to different temperatures.

Type of mutation	30°C	28°C	26°C	22°C	Control	Total
Point mutations	423 (117)	79 (46)	51 (45)	21 (21)	11 (11)	585 (240)
Indels < 30 nt						
Deletion	124 (35)	32 (27)	11 (13)	1 (1)	4 (4)	172 (80)
Insertion	108 (46)	7 (6)	3 (2)	5 (5)	9 (9)	132 (68)
Deletions > 30 nt	34 (11)	3 (3)	3 (0)	2 (2)	0 (0)	42 (16)
Tandem duplications	6 (2)	0 (0)	0 (0)	0 (0)	0 (0)	6 (2)
IS	0 (0)	0 (0)	0 (0)	0 (0)	3 (3)	3 (3)
Total	695 (211)	121 (82)	68 (60)	29 (29)	27 (27)	940 (409)

Control: control populations evolved at a constant temperature of 15°C
IS: insertional sequence (IS) element integration

In parenthesis we indicate mutations with an independent origin. To identify mutations that originated independently from each other, we counted a mutation only at the lowest temperature where it was first observed, and only once if it occurred in more than one of the three clones isolated from the population adapted to 30°.

Table S3. Genotypic consequences of mutations identified in clones adapted to different temperatures.

Type of mutation	Effect	30°C	28°C	26°C	22°C	Control	Total
Point mutations	Initiator codon variant	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
	Intergenic region	23 (8)	7 (2)	5 (4)	3 (3)	4 (4)	42 (21)
	Missense variant	364 (97)	68 (41)	42 (37)	17 (17)	7 (7)	498 (199)
	Start codon lost	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
	Stop codon gained	19 (6)	2 (2)	1 (1)	1 (1)	0 (0)	23 (10)
	Synonymous variant	15 (4)	2 (1)	3 (3)	0 (0)	0 (0)	20 (8)
Indels < 30 nt							
Deletion	Conservative in-frame deletion	51 (6)	19 (18)	2 (2)	1 (1)	0 (0)	73 (27)
	Disruptive in-frame deletion	9 (4)	0 (0)	1 (1)	0 (0)	1 (1)	11 (6)
	Frameshifting variant	35 (17)	3 (2)	3 (3)	0 (0)	2 (2)	43 (24)
	Intergenic region	29 (8)	8 (7)	5 (5)	0 (0)	1 (1)	43 (21)
Insertion	Conservative in-frame insertion	62 (21)	5 (4)	3 (2)	3 (3)	4 (4)	77 (34)
	Disruptive in-frame insertion	17 (9)	0 (0)	0 (0)	0 (0)	0 (0)	17 (9)
	Frameshifting variant	18 (8)	1 (1)	0 (0)	1 (1)	1 (1)	21 (11)
	Intergenic region	11 (8)	1 (1)	0 (0)	1 (1)	4 (4)	17 (14)
Deletion > 30 nt	Frameshifting variant	4 (1)	1 (0)	2 (2)	1 (1)	0 (0)	8 (4)
	Gene fusion	4 (2)	0 (0)	0 (0)	0 (0)	0 (0)	4 (2)
	Intergenic region	11 (4)	1 (0)	1 (0)	1 (1)	0 (0)	14 (5)
	Large deletion	15 (4)	3 (3)	0 (0)	0 (0)	0 (0)	18 (7)
Tandem duplication	Tandem duplication	6 (2)	0 (0)	0 (0)	0 (0)	0 (0)	6 (2)
IS	Insertion sequence	0 (0)	0 (0)	0 (0)	0 (0)	3 (3)	3 (3)
Total		695 (211)	121 (82)	68 (60)	29 (29)	27 (27)	940 (409)

Control: control populations evolved at a constant temperature of 15°C
Conservative in-frame insertion or deletion: one or more codons are inserted or deleted

Disruptive in-frame insertion or deletion: one codon is changed and one or more codons are inserted or deleted
IS: insertional sequence (IS) element integration
In parenthesis we indicate mutations with an independent origin. To identify mutations that originated independently from each other, we counted a mutation only at the lowest temperature where it was first observed, and only once if it occurred in more than one of the three clones isolated from the population adapted to 30°.

Table S4. COG categories significantly enriched at each temperature.

T(°C)	COG	obs	exp	odds ratio	p-value	FDR
15	N: Cell motility	10	3.9	3.38	0.004	0.049
15	L: Replication, recombination and repair	2	0.18	11.93	0.023	0.213
15	E: Amino acid transport and metabolism	3	0.71	4.59	0.042	0.3
22	T: Signal transduction mechanisms	22	3.47	13.57	1.32E-12	1.32E-10
22	K: Transcription	8	1	9.79	2.33E-05	7.76E-04
26	G: Carbohydrate transport and metabolism	11	2.09	6.14	4.03E-05	0.001
26	I: Lipid transport and metabolism	5	0.6	8.95	0.002	0.023
26	T: Signal transduction mechanisms	14	6.68	2.41	0.009	0.089
28	M: Cell wall/membrane/envelope biogenesis	22	10.93	2.36	0.002	0.028
28	G: Carbohydrate transport and metabolism	8	3.45	2.45	0.037	0.284
30	C: Energy production and conversion	53	2.18	27.2	1.47E-08	7.33E-07
30	O: Post-translational modification, protein turnover, and chaperones	74	28.36	2.91	1.57E-04	0.003
30	U: Intracellular trafficking, secretion, and vesicular transport	27	2.18	13.05	2.90E-04	0.005
30	S: Function unknown	27	10.91	2.56	0.033	0.271

For each temperature and COG category, we compared the proportion of mutations assigned to a given COG category at the selected temperature versus at all other temperatures. The table contains only COG categories with a p-value not exceeding 0.05 (one-tailed Fisher test). FDR indicates the false discovery rate according to the Benjamini-Hochberg correction for multiple testing (*66*).

Table S5. Frequency at which each of the 20 amino acids is lost through mutation at each temperature.

OLD AA	30°C	28°C	26°C	22°C	Control
ALA	12,24	12,2	13,51	11,76	14,29
ARG	8,16	**14,63**	8,11	5,88	0
ASN	3,06	0	0	0	14,29
ASP	3,06	12,2	13,51	11,76	14,29
CYS	1,02	0	0	5,88	0
GLN	4,08	2,44	0	5,88	0
GLU	5,1	2,44	8,11	11,76	0
GLY	**13,27**	7,32	**16,22**	**17,65**	0
HIS	4,08	0	0	0	0
ILE	1,02	4,88	5,41	5,88	0
LEU	9,18	2,44	8,11	0	28,57
LYS	0	0	8,11	0	0
MET	3,06	7,32	5,41	5,88	28,57
PHE	4,08	0	2,7	0	0
PRO	6,12	7,32	2,7	11,76	0
SER	11,22	**14,63**	2,7	5,88	0
THR	3,06	9,76	0	0	0
TRP	3,06	0	0	0	0
TYR	1,02	2,44	2,7	0	0
VAL	4,08	0	2,7	0	0

Control: control populations evolved at a constant temperature of 15°C.

Bold type indicates, for each temperature, the amino acid that gets most frequently lost as a result of mutation across all protein coding genes. Numbers are percentages. For each given temperature, we only counted the mutations that are new at that temperature, in other words we did not count mutations that may have appeared at previous temperatures and that became fixed. For clones adapted to 30°C, if more than one of the sequenced clones belonging to the same population had the same mutation, we only counted the mutation once.

Table S6. Frequency at which each of the 20 amino acids is gained through mutation at each temperature.

NEW AA	30°C	28°C	26°C	22°C	Control
ALA	0,00	2,44	2,70	5,88	0,00
ARG	1,02	2,44	0,00	0,00	14,29
ASN	3,06	7,32	**18,92**	11,76	0,00
ASP	5,10	2,44	5,41	11,76	0,00
CYS	6,12	2,44	2,70	0,00	0,00
GLN	1,02	0,00	0,00	0,00	0,00
GLU	2,04	0,00	0,00	0,00	0,00
GLY	1,02	4,88	2,70	0,00	0,00
HIS	6,12	7,32	5,41	5,88	0,00
ILE	7,14	**21,95**	8,11	5,88	**28,57**
LEU	**15,31**	19,51	10,81	11,76	0,00
LYS	7,14	0,00	5,41	5,88	14,29
MET	0,00	2,44	0,00	0,00	0,00
PHE	7,14	0,00	2,70	5,88	0,00
PRO	1,02	7,32	2,70	0,00	0,00
SER	11,22	7,32	8,11	11,76	0,00
THR	9,18	2,44	8,11	**17,65**	0,00
TRP	1,02	0,00	0,00	0,00	0,00
TYR	6,12	7,32	2,70	5,88	14,29
VAL	9,18	2,44	13,51	0,00	**28,57**

Control: control populations evolved at a constant temperature of 15°C.

Bold type indicates, for each temperature, the amino acid that is most frequently gained as a result of mutation across all protein coding genes. Numbers indicate percentage. For each given temperature, we only counted the mutations that are new at that temperature, in other words we did not count mutations that may have appeared at previous temperatures and that became fixed. For clones adapted to 30°C, if more than one of the sequenced clones belonging to the same population had the same mutation, we only counted the mutation once.

Table S7. Copy number differences between clones carrying different mutations affecting the number of copies of chromosome 2.

Gene	ANOVA p-value	Comparison	Fold-Change	95% CI	Tukey p-value
parA	8.018E-004				
		Partial chr2 deletion/Chr 2 duplication	0.948	0.612 - 1.467	8.018E-01
		Partial chr2 deletion/RepA mutation	2.358	1.524 - 3.65	2.200E-05
		Partial chr2 deletion/WT	2.156	1.114 - 4.173	1.965E-03
		Chr 2 duplication/RepA mutation	2.488	1.66 - 3.728	7.890E-05
		Chr 2 duplication/WT	2.274	1.2 - 4.311	8.208E-04
		RepA mutation/WT	0.914	0.482 - 1.733	9.896E-01
gluc	2.193E-003				
		Partial chr2 deletion/Chr 2 duplication	0.544	0.341 - 0.866	1.093E-03
		Partial chr2 deletion/RepA mutation	1.254	0.788 - 1.997	5.735E-01
		Partial chr2 deletion/WT	1.117	0.553 - 2.257	9.520E-01
		Chr 2 duplication/RepA mutation	2.307	1.5 - 3.549	1.690E-04
		Chr 2 duplication/WT	2.055	1.04 - 4.06	5.803E-03
		RepA mutation/WT	0.891	0.451 - 1.76	9.816E-01
repA	3.111E-003				
		Partial chr2 deletion/Chr 2 duplication	1.048	0.622 - 1.766	9.642E-01
		Partial chr2 deletion/RepA mutation	2.058	1.221 - 3.469	4.911E-03
		Partial chr2 deletion/WT	2.336	1.061 - 5.144	2.191E-02
		Chr 2 duplication/RepA mutation	1.964	1.212 - 3.185	5.611E-03
		Chr 2 duplication/WT	2.230	1.038 - 4.788	2.862E-02
		RepA mutation/WT	1.135	0.529 - 2.437	9.468E-01

For each gene, *repA*, *parA* and gluconate transporter (all three located on chromosome 2) we performed one-way ANOVA test to assess differential copy number among 4 groups of samples: wild-type, chromosomal duplication, partial chromosome 2 deletion and *repA* mutation. The null hypothesis is that samples do not differ in their means. The p-value of the one-way ANOVA test is indicated in the ANOVA p-value column. Then we did a Tukey post-hoc test for each pair-wise comparison to test if there are differences in copy number between two given groups tested. Fold-Change column indicates the fold change in copy number between nominator and denominator for each comparison. 95% confidence interval of the ratio is indicated. For each comparison we provide the p-value that resulted from the Tukey post-hoc test.

Wild-type includes the copy number results from the wild-type clone. Chromosome 2 duplication includes the copy number results from 4 clones (*P. haloplanktis* preadapted clone, 22°C-19, 26°C-9, 28°C-23), partial chr2 deletion include the copy number results from 3 clones (28°C-11, 30°C-26.2, 30°C-8.3), and *repA* mutations include the copy number results from 4 clones (28°C-10, 30°C-9.2, 30°C-15.1, 30°C-30.1).

Table S8. List of most commonly mutated genes at each temperature.

Gene ID	Gene description	Function	Type mutations	N mutations	New mutations
Controls (15°C)					
PSHA_RS11640	Flagellar motor component	Motility	SNP, indel	5	5
PSHA_RS17145	Gluconate transporter	Amino acid metabolism	SNP	3	3
PSHA_RS03600	Two-component sensor histidine kinase BarA	Signal transduction	SNP	2	2
PSHA_RS09465	UvrY/SirA/GacA family response regulator	Signal transduction	indel	2	2
22°C					
PSHA_RS09465	UvrY/SirA/GacA family response regulator	Signal transduction	SNP, indel	8	8
PSHA_RS16945	Putative GGDEF domain membrane associated protein	Signal transduction	SNP	6	6
PSHA_RS03600	Two-component sensor histidine kinase BarA	Signal transduction	SNP, indel	5	5
PSHA_RS11640	Flagellar motor component	Motility	SNP, indel	3	3
26°C					
PSHA_RS16945	Putative GGDEF domain membrane associated protein	Signal transduction	SNP, indel	12	8
PSHA_RS07030	Lipopolysaccharide assembly protein LapB	Cell wall	SNP, indel	10	10
PSHA_RS08920	DUF3413 domain-containing protein	Unknown	SNP	6	6
PSHA_RS11640	Flagellar motor component	Motility	SNP, indel	5	3
PSHA_RS03600	Two-component sensor histidine kinase BarA	Signal transduction	SNP	4	3
PSHA_RS09455	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Phospholipid biosynthesis	SNP, indel	3	3
28°C					
PSHA_RS04215	MipA/OmpV family protein	Cell wall	SNP, indel	20	19
PSHA_RS16945	Putative GGDEF domain membrane associated protein	Signal transduction	SNP, indel	16	5
PSHA_RS07030	Lipopolysaccharide assembly protein LapB	Cell wall	SNP	13	6
PSHA_RS17540	Putative replication protein (repA-like)	Replication	SNP	11	10
PSHA_RS08920	DUF3413 domain-containing protein	Unknown	SNP	6	2
PSHA_RS10175	Lon protease	Protease	SNP, indel	5	5
PSHA_RS11640	Flagellar motor component	Motility	SNP, indel	4	0
PSHA_RS12300	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	Cell wall	SNP	4	3
PSHA_RS12695	ATP-dependent zinc protease	Protease	indel	4	4
PSHA_RS09455	CDP-diacylglycerol--glycerol-3-phosphate 3-	Phospholipid biosynthesis	SNP, indel	3	1

	phosphatidyltransferase				
30°C					
PSHA_RS10175	Lon protease	Protease	SNP, indel	72 (25)	60 (21)
PSHA_RS17540	Putative replication protein (repA-like)	Replication	SNP	58 (21)	38 (14)
PSHA_RS04215	MipA/OmpV family protein	Cell wall	SNP, indel	48 (16)	9 (3)
PSHA_RS16945	Putative GGDEF domain membrane associated protein	Signal transduction	SNP, indel	41 (14)	6 (2)
PSHA_RS07030	Lipopolysaccharide assembly protein LapB	Cell wall	SNP	27 (10)	2 (1)
PSHA_RS08920	DUF3413 domain-containing protein	Unknown	SNP	23 (8)	9 (3)
PSHA_RS05885	TonB2 protein	Transpot	indel	17 (9)	17 (9)
PSHA_RS14760	Membrane-bound ATP synthase, F1 sector, beta-subunit	Energy production and conversion	SNP	16 (8)	16 (8)
PSHA_RS12300	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	Cell wall	SNP	15 (5)	9 (3)
PSHA_RS12695	ATP-dependent zinc protease	Protease	indel, deletion	15 (5)	9 (3)
PSHA_RS12760	Outer membrane protein tolC precursor	Transport	SNP	13 (5)	13 (5)
	Chromosome 2 deletion		large deletion	12 (4)	6 (2)
PSHA_RS04170	Sensory histidine kinase in two-component regulatory system with RstA	Signal transduction	SNP, indel	12 (5)	12 (5)
PSHA_RS09455	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Phospholipid biosynthesis	SNP, indel	12 (4)	3(1)
PSHA_RS10020	Outer membrane protein assembly factor BamA	Cell wall	SNP	12 (4)	12 (4)
PSHA_RS11640	Flagellar motor component	Motility	SNP, indel	12 (4)	12 (4)
PSHA_RS05005	Peptidoglycan glycosyltransferase MrdB	Cell wall	SNP	11 (5)	11 (5)
PSHA_RS07245	DUF1852 domain-containing protein	Unknown	SNP	11 (6)	11 (6)
PSHA_RS14770	Membrane-bound ATP synthase, F1 sector, alpha-subunit	Energy production and conversion	SNP	11 (5)	11 (5)
PSHA_RS01875	Type IV-A pilus assembly ATPase PilB	Motility	indel	8 (3)	8 (3)
PSHA_RS12850	DUF885 domain-containing protein	Unknown	indel	7 (5)	7 (5)
PSHA_RS13195	SUMF1/EgtB/PvdOfamily nonheme iron enzyme	Unknown	SNP, indel, deletion	8 (3)	8 (3)
PSHA_RS16885	Conserved protein of unknown function	Unknown	SNP	9 (3)	9 (3)

The number of mutations identified at 30°C corresponds to mutations found in all clones, irrespective of whether they were found in more than one of the three clones we had sequenced from the same population at 30°C. In parentheses we indicated the number of independent mutations at 30°C, that is, if more than one of the three clones had the same mutation, we only counted the mutation once. New mutations refer to mutations that appear for the first time at that temperature.

Table S9. List of Lon protease mutations in clones adapted to 30°C.

Position	Mutation Type	Effect	Clone
2177758	SNP	His505Tyr	7.2
2178397	SNP	Arg292Cys	17.1, 17.2, 17.3
2178399	SNP	Val291Glu	2.3
2178425	9 nt insertion	Met280_Met281insMetSerPro	2.1, 2.2, 3.1, 3.2, 3.3, 8.1, 8.2, 8.3, 9.1, 9.2, 9.3, 10.1, 10.2, 10.2b, 12.1, 12.2, 12.3, 14.1, 14.2, 14.3, 15.1, 15.2, 15.3, 16.1, 16.2, 16.3, 18.1, 18.2, 18.3, 19.1, 19.2, 19.3, 21.1, 21.2, 21.3, 22.1, 22.2, 22.3, 23.1, 23.2, 23.3, 24.1, 24.2, 24.3, 25.1, 25.2, 26.1, 26.2, 26.3, 27.1, 27.2, 27.3, 28.1, 28.2, 28.3, 29.1, 29.2, 29.3
2178433	6 nt deletion	Leu278_Lys279del	4.1, 4.2, 4.3
2178597	SNP	Gln225Leu	6.1, 6.2, 6.3
2179014	SNP	Gly86Val	20.1, 20.2, 20.3
2179220	intergenic	NA	6.2

Table S10. *E. coli* BL21 cells expressing mutated Lon protease grow to higher population density at 40°C.

Temperature	Group 1	Group 2	W	p-value
30°C	Lon mut	Empty plasmid	12	<0.001
	Lon mut	Lon wt	58	0.795
	Empty plasmid	Lon wt	22	0.001
37°C	Lon mut	Empty plasmid	59	0.779
	Lon mut	Lon wt	78	0.378
	Empty plasmid	Lon wt	40	0.970
40°C	Lon mut	Empty plasmid	102	0.044
	Lon mut	Lon wt	115	0.006
	Empty plasmid	Lon wt	61	0.743

We cloned Lon protease wild-type and mutated Lon protease (with the 3aa insertion) into a plasmid and expressed the protease in *E. coli* BL21 cells. To assess the effect of the Lon protease mutation at different temperatures we measured growth curves of *E. coli* BL21 cells carrying the different plasmid variants. We measured each growth curve in 12 replicate populations derived from the same Lon protease-expressing clone. We performed pairwise comparisons of the maximum population density between *E. coli* BL21 cells carrying different plasmid variants. To assess statistical significance we performed a one-sided Wilcoxon rank-sum test.

Table S11. List of oligonucleotide primers used in this study.

Oligo name	Sequence	Purpose
1597378_fw	5'-AGCTGTGGCAGTTTTTGGGG-3'	Primer specific to *P. haloplanktis* TAC125 PSHA_RS07527 gene. Control for contamination during experimental evolution
1597378_rv	5'-CATTGCTATTGCTGTTAAGCGG-3'	Primer specific to *P. haloplanktis* TAC125 PSHA_RS07527 gene. Control for contamination during experimental evolution
398078_fw	5'-CATTTACTTACAGGGGCTCTGG-3'	Primer specific to *P. haloplanktis* TAC125 PSHA_RS01895 gene. Control for contamination during experimental evolution
398078_rv	5'-GCTCCGTGATTTCATCTCGC-3'	Primer specific to *P. haloplanktis* TAC125 PSHA_RS01895 gene. Control for contamination during experimental evolution
16s_fw	5'-GATCATGGCTCAGATTGAACGC-3'	Primer to amplify 16S from *E. coli* and *P. haloplanktis* TAC125. Control for contamination during experimental evolution
16s_rv	5'-AGGCACCAAACCATCTCTGG-3'	Primer to amplify 16S from *E. coli* and *P. haloplanktis* TAC125. Control for contamination during experimental evolution
EG11498_fw	5'-AACAGGATGTGAACCGACCC-3'	Primer specific to *E. coli* EG11498 gene. Control for contamination during experimental evolution
EG11498_rv	5'-GGCTGTGGAGGATTTTTGCG-3'	Primer specific to *E. coli* EG11498 gene. Control for contamination during experimental evolution
EG11973_fw	5'-GCGGACATTGTCATTTCGGG-3'	Primer specific to *E. coli* EG11973 gene. Control for contamination during experimental evolution
EG11973_rv	5'-AACCACTTCAGGGCTAACGG-3'	Primer specific to *E. coli* EG11973 gene. Control for contamination during experimental evolution
Lon Ph fw1	5'-GATATACATCTCGAGATGACGCTTGAGAGAACC GATCG-3'	Primer to amplify $LonPh_{wt}$ and $LonPh_{14.1}$ from Ph gDNA
Lon Ph rv1	5'-CTGATGCCTCTAGATTATCGGTTTTTCGGCGTT TCG-3'	Primer to amplify $LonPh_{wt}$ and $LonPh_{14.1}$ from Ph gDNA
Seq 1523 Phrv	5'-GCCATTACGCTTAACTTGCTTGG-3'	Primer to check for the 3 aa insertion on Lon Ph

fwpUCN empty	5'-GAGATATACATCTCGAGTCTAGAGGCATCAAAT AAAACGAAAGG-3'	Primer to remove EYFP from pUCNOmpA and obtain an empty vector
rvpUCN empty	5'-GATGCCTCTAGACTCGAGATGTATATCTCCTTC-3'	Primer to remove EYFP from pUCNOmpA and obtain an empty vector
A PhLonpUC	5'-GATTTAAGAAGGAGATATACATCTCGAGATGACGC TTGAGAGAACCGATCGAG-3'	Primer to amplify $LonPh_{wt}$ or $LonPh_{14.1}$ for Gibson assembly in pUCNOmpA plasmid series
B PhLonpUC	5'-CTCGATCGGTTCTCTCAAGCGTCATCTCGAGATGT ATATCTCCTTCTTAAATC-3'	Primer to amplify pUCNOmpA plasmid for Gibson assembly with $LonPh_{wt}$ and $LonPh_{14.1}$
C PhLonpUC	5'-GTCGAAACGCCGAAAAACCGATAATCTAGAGGCAT C AAATAAAACGAAAGGC-3'	Primer to amplify pUCNOmpA plasmid for Gibson assembly with $LonPh_{wt}$ and $LonPh_{14.1}$
D PhLonpUC	5'-GCCTTTCGTTTTATTTGATGCCTCTAGATTATCGGT TTTTCGGCGTTTCGAC-3'	Primer to amplify $LonPh_{wt}$ or $LonPh_{14.1}$ for Gibson assembly in pUCNOmpA plasmid series
seqR2	5'-ACAAACTAGCAACACCAGAAC-3'	Primer to sequence pUCNOmpA plasmids
seqF2	5'-ATTTTGGATGATAACGAGGCG-3'	Primer to sequence pUCNOmpA plasmids

(Ph: *P. haloplanktis*; gDNA: genomic DNA)

Table S12. List of plasmids used in this study.

Plasmid	Resistance marker	Reference
pUCNOmpA-EYFP-1	Kan	Fig. S14
pUCNOmpA	Kan	This study
pUCNOmpA-LonPh$_{wt}$	Kan	This study
pUCNOmpA-LonPh$_{14.1}$	Kan	This study

Table S13. List of oligonucleotide primers and genes used for qPCR.

Gene	Forward primer	Reverse Primer	Fragment size amplified	Primer efficiency	Primer concentration (nM)
rpoB (PSHA_RS01095)	GTGCGTGTAGAA CGTGCTGT	AACTGAGACGAG CCGAAGAA	128 bp	102%	200
gyrA (PSHA_RS06995)	AAAATGCCTGAA GGACAACG	CGACTCTTCGCTG GGTAGTC	125 bp	107%	200
gluconate transporter (PSHA_RS17145)	GCGAGAAAAACG CTAACTGG	ACACTACCACCGC CTTTACG	130 bp	82%	200
repA (PSHA_RS17540)	ACTTTAACAGCGC CCAAGAA	CTGCATAACGCCA AAGATGA	81 bp	99%	300
recA (PSHA_RS03395)	CAGATACTGGCG AACAAGCA	TCGCCTTCAATTT CAGCTTT	118 bp	109%	400
parA (PSHA_RS14855)	GCTGAAGTTGCCG AAAAGTC	TCGTGGCGTCTTT TTAAGGT	149 bp	102%	400

(Primer efficiency larger than 100% indicates that there might be inhibitors of the polymerase enzyme)

Data S1. (separate file)
List of mutations identified in all sequenced clones across temperatures.

Data S2. (separate file)
List of types of mutations identified in all sequenced clones across temperatures.

Data S3. (separate file)
List of genes mutated in this study and their corresponding classification into functions.

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