Longevity of growth-arrested bacteria requires protein synthesis and a stringent response

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Abstract

Gram-negative bacteria in infections, biofilms and industrial settings often stop growing due to nutrient depletion, immune responses or environmental stresses. Bacteria in this state tend to be tolerant to antibiotics and are often referred to as dormant. Here we describe the physiology of *Rhodopseudomonas palustris*, a phototrophic alpha-proteobacterium, in its growth arrested state. *R. palustris* can maintain almost full viability for over four months and we found that ATP synthesis, proteins synthesis and a stringent response were required for this remarkable longevity. *R. palustris* is an extreme example of a bacterial species that will stay alive for long periods of time as a relatively homogeneous populations of cells. There is evidence that other gram-negative species also continue to synthesis proteins during growth arrest and that a stringent response is required for their longevity as well. Our observations challenge that notion that growth-arrested cells are dormant and expands our mechanistic understanding of a crucial but understudied phase of the bacterial life cycle.

Introduction

Bacteria in nature often exist in viable but non-growing states without forming differentiated structures like spores (*1, 2*). Yet this crucial phase of the bacterial life cycle is underexplored. Factors that limit cell replication include nutrient limitation, competition within microbial communities and stresses like oxygen-depletion and extreme temperatures (*3-5*). Non-growing pathogenic bacteria present in human infections present challenges for treatment because antibiotics tend to target processes such as cell-wall biosynthesis or DNA replication that are active in growing, but not non-growing cells. One example of a bacterium that is recalcitrant to antibiotic treatment *in vivo* is *Mycobacterium tuberculosis*, which forms latent infections that persist over many years in the absence of overt signs of growth (*6*). Then there are bacterial

56 antibiotic persisters, a small subpopulation of non-growing or slowly growing cells that develop
57 in many bacterial infections and are tolerant to antibiotic treatment (*7-9*).
58
59 Studying non-growing bacteria is also important in environmental, industrial and other non-
60 medical contexts. For example, non-growing bacteria are excellent biocatalysts because they
61 can convert substrates that might be used for growth to value-added products. In past work we
62 have studied production of hydrogen gas, a biofuel, by the phototrophic α-proteobacterium
63 *Rhodopseudomonas palustris.* When illuminated to allow ATP synthesis by
64 photophosphorylation, non-growing cells diverted cell resources to hydrogen gas production and
65 generated 300% more hydrogen gas than growing cells (*10*). We also found that non-growing *R.*
66 *palustris* cells embedded in thin strips of latex film remained viable and produced hydrogen gas
67 for over four months (*11*).
68
69 These results prompted us to develop *R. palustris* as a model for studies of bacterial longevity,
70 and to ask the question: are there universal features of long-lived bacteria that we can uncover
71 from studies of this proteobacterium? Several attributes make *R. palustris* a good model for
72 addressing this question. First, it generates ATP and protonmotive force from light, even when it
73 is not growing (Figure 1). Also, *R. palustris* carries out photophosphorylation under anaerobic
74 conditions and does not generate oxygen, thus avoiding complications of oxidative stress during
75 growth-arrest. A Tn-seq study of growth-arrested *R. palustris* identified 116 genes, which we
76 call longevity genes, that are essential for long-term viability of non-growing cells, but are not
77 required for growth (*12*). Among these are genes that are conserved in bacteria and associated
78 with basic cellular processes including protein synthesis. Here, we carried out a physiological
79 characterization of *R. palustris* in its growth-arrested, but viable state. We found that active
80 translation and optimized ribosomes were critical for the longevity of *R. palustris,* as was
81 (p)ppGpp. This goes against the conventional description of non-growth as a dormant and

inactive state. We suggest that protein synthesis and other cellular activities are likely important for many bacteria to maintain viability when they are not growing.

Results

Growth-arrested *R. palustris* cells do not appear to undergo cycles of death and regrowth. A major barrier to studies of bacterial longevity in the laboratory is that most heterotrophic bacteria, the best studied of which is *E. coli*, undergo cycles of death and growth after entry into stationary phase (*1, 13*). Typically a fraction of cells survive long-term stationary phase by feeding on nutrients released from dead cells. This leads to physiological and genetic diversity in the population and there is strong selection pressure for mutations that enable cannibalism (*14, 15*). In previous work we reported that populations of growth-arrested *R. palustris* cells were nearly 100% viable and morphologically homogenous (*12*). To firmly establish that cells were truly growth-arrested, rather than undergoing cycles of growth and death, we introduced an unstable plasmid expressing a gentamycin (Gm) resistance gene into wild-type *R. palustris*. When Gm is absent, the percentage of growing cells that retain this plasmid would be expected to decrease (*16*). On the other hand, a non-growing culture would be expected to retain the plasmid in Gm-free medium. When this strain (WT::pBBR^{Gm}) was inoculated into Gm-free medium, about 60% of the cells had lost the plasmid by the time they reached stationary phase. When these cells were transferred into fresh Gm-free medium and successively transferred several times over a period of 20 days after reaching stationary phase, the plasmid continued to be lost. In contrast, growth-arrested cells retained the plasmid over the same period of time (Figure 2a), suggesting that most or all of the cells were indeed in a non-growing state.

In further support of the conclusion that growth-arrested *R. palustris* does not undergo cycles of growth and death, we found that non-growing cells were tolerant to the cell wall synthesis

inhibitor ampicillin and the DNA synthesis inhibitor nalidixic acid at concentrations that prevented growth (Figure 2b). However, we also found that the viabilities of non-growing cells were compromised by protein synthesis inhibitors (Figure 2b and SI Figure 1). In all these cases and throughout this study, growth arrest was imposed by providing cells a growth-limiting amount of the carbon source acetate.

Long-lived, growth-arrested cells continue to synthesize proteins. Growth-arrested *R. palustris* was sensitive to Gm, an aminoglycoside antibiotic that affects protein synthesis by causing mistranslation, which results in accumulation of damaged proteins. We found that over 99% of growth-arrested cells lost viability over a few days when treated with Gm at a final concentration of 50 μg/ml (Figure 2b). Although cells died relatively rapidly, they were not as sensitive to Gm as were growing cells, which died within a few hours after exposure to this antibiotic. Chloramphenicol (Cm) belongs to another class of antibiotic that targets ribosomes. Although typically bacteriostatic, Cm was effective in killing growth-arrested *R. palustris* over a prolonged period of time (Figure 2b and SI Figure 1). These results suggest that active translation is important for *R. palustris* longevity. In support of the conclusion that growth-arrested cells continue to synthesize proteins, proteomics experiments revealed that after 6 days and 20 days of growth-arrest, 289 and 525 proteins, respectively, were present at greater than two-fold higher levels than in growing cells (Figure 3a and SI Table 1). Among these were enzymes involved in the degradation of compounds, including benzoate and fatty acids (SI Table 1 and 2), that are preferred carbon sources for *R. palustris*, suggesting that cells respond to growth-arrest by increasing the translation of proteins that may aid in scavenging carbon. In the period between 6 and 20 days post growth-arrest, 51 proteins of unknown function increased in abundance. Some proteins (163 and 310 proteins at days 6 and 20 post growth-arrest) were diminished in abundance by two-fold or more following growth-arrest (Figure 3a and SI Table 1). This set included 53 out of 55 ribosomal proteins that were identified in our

proteomics dataset. On average, ribosome proteins were at 25-50% of their original levels after 6 days and 20 days of growth-arrest (Figure 3b). However, not all translation related proteins were decreased in their abundances. For example, translation elongation factors made up ~5% of the total protein from growing cells as well as growth-arrested cells. Similar patterns were also observed for proteins involved in aminoacyl-tRNA biosynthesis (tRNA charging) and for two previously identified longevity proteins YbeY and Era (Figure 3c).

Characterization of protein synthesis apparatus in long-lived cells. We isolated *R. palustris* ribosomes by sucrose gradient centrifugation and found that the ribosome profile of growing *R. palustris* cells included 30S, 50S and 70S populations, as is typical for growing bacteria (Figure 4a). There was also a small peak corresponding to a 100S population, which is likely a dormant form of ribosomes, as has been seen in other bacteria (*17*). The ribosome profile of growth-arrested *R. palustris* cells resembled that of growing cells (Figure 4a and 4b), but the quantity of the 70S population was lower by day 25 post growth-arrest. Ribosome degradation is often accompanied by the non-specific degradation of rRNA (*18-21*). In growing *R. palustris*, rRNA made up about 70% of the total RNA (Figure 4c). After growth-arrest, rRNA accounted for about 50% of the total RNA, and it stayed at about this level for 120 d (Figure 4c). The ratio between 23S and 16S rRNA remained stable during this time (SI Figure 2), and little degradation was observed (Figure 4c). The relative levels of aminoacylated (or charged) and free forms of tRNAs are important for promoting and inhibiting translation (*22*). We used Northern blot analysis to estimate the percentage of tryptophan-charged $tRNA_{trp}$ (Trp-$tRNA_{trp}$) relative to $tRNA_{trp}$. In growing cells, ~60% of $tRNA_{trp}$ was in the charged Trp-$tRNA_{trp}$ form (Figure 4d and 4e). This level is comparable to that reported in other bacteria (*23*). The percentage of Trp-$tRNA_{trp}$ increased to ~85% upon growth-arrest and was maintained at this level for ~20 days (Figure 4d and 4e).

Two proteins identified as essential for *R. palustris* longevity are critical for ribosome integrity in growth-arrested cells. Among the longevity genes that we have identified in *R. palustris* (*12*), *ybeY* and *era* are predicted to encode proteins that interact with each other and the S11 ribosome protein during ribosome maturation (*24-27*). *ybeY* or *era* deletion mutants have compromised longevity but grow normally (*12*). Here we found that the ribosome profiles of growing cells were similar between the wild-type and these mutants (Figure 5a). All had distinctive 30S, 50S and 70S populations, although the 50S subunit was slightly more abundant in the mutants. The ribosome profiles of growth-arrested *ΔybeY* and *Δera* mutant cells were noticeably different from that of the wild-type, however. A prominent 50S subunit peak was seen but the amounts of 30S subunits and 70S ribosomes were greatly diminished (Figure 5a).

As predicted based on studies of their *E. coli* homologs (*24-27*), *ΔybeY* and *Δera* strains were defective in 16S rRNA maturation. An extra band immediately above the 16S rRNA was present in gels of RNA from both mutants (SI Figure 2). Northern blot experiments confirmed that the extra band was 17S rRNA, a precursor of 16S rRNA (Figure 5b). In growth-arrested cells, both 16S rRNA and 17S rRNA species were severely depleted in the mutated strains (Figure 5b, SI Figure 2). This likely led to the defective ribosome production shown in Figure 5a, and the compromised longevity of *R. palustris*. *ybeY* or *era* deletion mutants had wild-type levels of charged tRNA-Trp (SI Figure 3), suggesting that tRNA aminoacylation is not directly coordinated with the process of ribosome maturation.

As shown in Figure 3b, there was no significant change in the abundance of YbeY and Era proteins in growth-arrested cells. Since the core ribosomal proteins were decreased 2 to 4-fold in abundance (Figure 3b), the relative abundance of YbeY and Era was increased on a per ribosome basis following growth-arrest. To test whether we could change the effects of YbeY on longevity by modulating its relative abundance, we constructed and expressed a YbeY variant

predicted based on work in *E. coli*, to interact weakly with ribosomes. In *E. coli*, the interaction between YbeY and ribosomes is partially disrupted by replacement of a conserved aspartate with an arginine (*28*). When we made the corresponding $YbeY_{D118R}$ change in *R. palustris*, the strain was compromised in its longevity to a similar degree as the *ΔybeY* mutant (Figure 6a). When we overexpressed $YbeY_{D118R}$ *in trans* (*ΔybeY*::pBBP-*ybeY*$_{D118R}$) however, the longevity defect was complemented (Figure 6a). The quantity of 16S rRNA was similar to that of the wild-type, and no 17S rRNA was observed during growth-arrest (Figure 6b). Also, the ribosome profile of the growth-arrested *ΔybeY*::pBBP-*ybeY*$_{D118R}$ complemented strain was comparable to that of growth-arrested wild-type cells (Figure 6c).

The stringent response is important for longevity. Our results suggest that translation is reduced but still maintained in growth-arrested cells. A well-known mechanism that contributes to this is the stringent response mediated by guanosine polyphosphate [(p)ppGpp] (*29, 30*). In many bacteria, the level of (p)ppGpp increases upon growth-arrest and this inhibits the transcription of ribosomal genes, although the mechanism by which this is achieved varies among bacteria (*29-31*). As with other bacteria, we detected little to no (p)ppGpp in exponentially growing *R. palustris*. However, the level of (p)ppGpp increased sharply as growing cells reached stationary phase and remained at relatively high levels after growth-arrest (Figure 7a).

Unlike *E. coli*, but like most other bacteria outside the γ- and β-proteobacteria, *R. palustris* has a single bifunctional (p)ppGpp synthesis/hydrolysis *rsh* gene, here called rsh_{rp}, that has multiple domains including a hydrolase and RelA_SpoT domain (Figure 7b). Our Tn-seq results indicated that this gene (*rpa2693*) was essential for *R. palustris* longevity, but not for growth (*12, 32, 33*). However, we were unable to delete the full-length rsh_{rp} gene. A subsequent inspection of the locations of the Tn-insertions in this gene in our bank of Tn mutagenized cells revealed

that there were relatively few insertions in its RelA_SpoT domain (Figure 7b). When we made domain specific deletions in Rsh_{rp}, we obtained a hydrolase domain deletion mutant ($\Delta Rsh_{rp64\text{-}192}$, Figure 7b) that produced very little (p)ppGpp during growth or growth-arrest. (Figure 7c). This mutant grew with a generation time of 9.4 ± 0.6 h, compared to a wild-type 6.4 ± 0.2 h generation time, a growth rate difference of about 30%. In contrast to this relatively modest effect on the growth rate the $\Delta Rsh_{rp60\text{-}189}$ mutation had a severe effect on longevity (Figure 7d).

Discussion

We found that illuminated *R. palustris* responds to growth-arrest by reducing transcription of ribosomal genes and making fewer ribosomes as is typical of most bacteria when they slow their growth rate. Growth-arrested cells continued to synthesize proteins, some at elevated levels relative to growing cells, over the period of 20 days that we followed protein synthesis by proteomics (SI Table 1). We saw no evidence of significant rRNA degradation even 120 d post-growth arrest (Figure 4c). The levels of proteins involved in tRNA charging and translation elongation and were at similar levels in growing and growth-arrested cells (Figure 3c), which is consistent with the notion from a previous report that elongation rates are maintained as bacteria slow down their growth-rates even towards zero growth (*34*). In addition, a tRNA that we followed was almost fully charged 20 d into growth-arrest (Figure 4d and 4e). Not only does protein synthesis continue after growth-arrest, but it is essential for viability of non-growing cells as indicated by their sensitivity to Gm and Cm (Figure 2b, SI Figure 1).

There are reports of continued protein synthesis in other species of bacteria in growth-arrest, the more convincing of which come from studies in which production of fluorescent proteins in single cells was used as a proxy for protein synthesis. Antibiotic persisters in early stages of formation, as well as non-growing *E. coli* and *M. tuberculosis* have all been inferred to carry out protein synthesis (*35-37*). These results suggest that maintenance of some level of protein

synthesis may be required for viability of many non-growing bacteria. From this it follows that it would make sense to prioritize development of antibiotics that target protein synthesis in growth-arrested cells. This will likely require understanding in detail what happens to bacterial ribosomes following growth-arrest. Bacterial ribosomes are known to be differentially post-translationally modified in stationary phase cells (*38*). Two longevity genes, *ybeY* and *era*, were essential for normal ribosomes to form in non-growing *R. palustris* (Figure 3b). Era is a well-studied bacterial GTPase (*27*), and genes for it and YbeY, are present and conserved in most bacteria (*25*). Our data are consistent with studies of the corresponding *E coli* proteins showing that YbeY and Era are involved in rRNA processing in *R. palustris*. Drawing on results from *E. coli* (*28*) it is likely that the *R. palustris* proteins interact with each other as well as with ribosome protein S11. When we looked at rRNAs formed by Δ*ybeY* and Δ*era* mutants that were actively growing, it was apparent that growing cells and growth-arrested cells had the same defect in 17S rRNA processing (Figure 5b), but only the growth-arrested cells had a phenotype. This highlights a feature of at least some *R. palustris* longevity proteins, which is that they are functional in growing cells as well as in non-growing cells, but it is only when the additional demands of growth-arrest on cellular functionality are imposed that essentiality becomes apparent. Our work on Era and YbeY has led us to conclude that optimized ribosomes are a feature of long-lived cells.

In early stationary phase and when in growth arrest, *R. palustris* synthesizes increasing amounts of (p)ppGpp, indicating that cells mount a stringent response. The gene *rpa2693*, which we call rsh_{rp}, is among the genes identified by Tn-seq to be essential for longevity (*12*). A $\Delta rsh_{rp60\text{-}189}$ mutant did not produce detectable (p)ppGpp and the viability of this mutant decreased rapidly after growth-arrest (Figure 7c). We do not know why this deletion, which is in the hydrolase domain of Rsh_{rp}, affected (p)ppGpp synthesis, but one idea is that it destabilized or altered the structure of the protein so that its ability to synthesize (p)ppGpp was

compromised. In *R. palustris* the signal for (p)ppGpp synthesis appears to be nutrient limitation or slowing growth rate. A future area of investigation is to understand why (p)ppGpp is essential for *R. palustris* longevity and to identify genes and proteins whose activities may be modulated by (p)ppGpp. (p)ppGpp has recently been shown to bind to Era from *Staphylococcus aureus* and *E. coli* to inhibit its GTPase activity (*39-41*), and this may influence ribosome biogenesis and integrity. There is good evidence that (p)ppGpp is important for development of antibiotic persisters (*42*) and for long term survival of latent and growth-arrested *M. tuberculosis* (*43, 44*).

R. palustris is an extreme example of a bacterial species for which an entire population of non-growing cells maintains viability for very long periods of time. It is likely that this is primarily due to its ability to generate ATP by photophosphorylation when it is in growth arrest, as growth-arrested cells incubated in dark do not survive well*(12)*. This feature of *R. palustris* makes it a good model for understanding molecular mechanisms of longevity that can be extended to other bacteria that remain alive in growth arrest, but only as heterogeneous populations. One hypothesis is that varying ATP levels per cell underlie this heterogeneity. In addition to a universal requirement for ATP to support viability of growth-arrested bacteria, we can say that protein synthesis, optimized ribosomes and (p)ppGpp likely lie at the nexus of diverse strategies used by non-growing bacteria to survive and even thrive.

Methods

Bacterial strains, growth and incubation conditions. *R. palustris* strain CGA009 was used as the wild-type for this study. Anaerobic cultures were grown under illumination at 30℃, using a defined media PM with 20 mM sodium acetate or 10mM sodium succinate as the carbon source (*45*). The longevity of *R. palustris* cultures was determined by counting the colony-forming units as described previously (*12*). In-frame deletions of *R. palustris* genes were created by using suicide vector pJQ200SK as described before (*46*). For *in trans* expression in *R.*

palustris, the target genes were cloned into plasmid pBBPgdh and the plasmids were mobilized into *R. palustris* by conjugation using *E. coli* strain S17-1 (*47*). Media were supplemented with 100 µg/ml of gentamicin for *in trans* complementation experiments.

Ribosome purification. Ribosomes from *R. palustris* cells were isolated following a method adapted from a previous study (*48*). About 35 ml of culture was grown to the desired state and poured over a 10-ml ice sheet (fast-cool). The ice sheet melted within 10 min and the cells were collected by centrifugation at 4000 rpm for 15 min. The cell pellet was resuspended in 2 ml of Buffer A (20mM HEPES-KOH, pH 7.5, 30mM NaCl, 8mM $MgCl_2$) with 1 µl/ml RNasin (Promega). Three hundred microliter of 0.1 mm Zirconia/Silica beads (Biospec Products) were added to every ml of sample. The samples were lysed with a Mini-Beadbeater-24 (Biospec Products) at 3500 rpm for 1 min at 4℃, kept on ice for 1 min and the cycle was repeated 4 more times. The micro-beads and cell debris were removed by centrifugation at 15000 rpm for 10 min. The lysates were further clarified by spinning at 30,000 g in Beckman TL-1000 ultracentrifuge for 30 min, followed by pelleting ribosomes at 100,000 g for 4 hours. The pellet was resuspended with Buffer A overnight on ice.

Sucrose gradient analysis. About five A_{260} units of ribosomes (less than 200 µl in volume) were applied on top of 11 ml of a sucrose gradient (7-47%). The samples were centrifuged through the gradient at 39,000 rpm for 4.5 h with a SW41 rotor, and then were separated with a Brandel gradient pump. The sucrose gradient was made with Buffer A.

Ribosomal RNA analysis. Total RNA was purified from 3-5 ml of *R. palustris* culture using an miRNeasy Mini Kit. The rRNAs were then analyzed with an RNA ScreenTape system (Agilent). To probe 16S rRNA by Northern blot, 1 µg of total RNA was heated at 70℃ for 10 min and immediately chilled on ice. The samples were then separated on a 1% agarose gel with 1X TBE

buffer. After electrophoresis, RNA was transferred to an Amersham Hybond-XL membrane (GE Lifesciences) with a downward nucleic acid transfer system overnight using 20X SSC buffer. A probe (5'-TCGTGCCTCAGCGTCAGTAATGGCCCAGTGA) labeled with DIG was used to detect 16S rRNA following the manufacturer's instruction (DIG, Roche).

Charging analysis of $tRNA_{Trp}$. Total RNA was isolated with QIAzol lysis reagent according to the manufacturer's instructions (Qiagen). The dried RNA pellet was dissolved and stored in 10 mM sodium acetate, pH 4.5. A deacylated control sample was prepared by incubating 10 µg of total RNA with 1 mM EDTA and 100 mM Tris-HCl pH 9.0 at 42℃ for an hour (*49*). Northern blot and acid-urea gel electrophoresis were performed essentially as described previously (*23, 49*). Loading dye (0.01% xylene cyanol, 0.01% bromophenol blue, 8 M Urea, 1 mM EDTA and 10 mM sodium acetate, pH 5.0) was mixed with sample at 1:1 ratio. Samples were loaded on a 12% acid-urea gel of 20 cm and then were run at 15 W for 12 h at 4℃. Transfer of RNA to an Amersham Hybond-XL membrane (GE Lifesciences) was performed in 0.5* TBE buffer at 650 mA for 30 min 4℃. The aminoacylated and the deacylated $tRNA_{Trp}$ were detected using a DIG-labeled probe (5'-GGTTTTGGAGACCGGTGCTCTACCAATTGAG) following the manufacturer's instruction (DIG, Roche).

Determination of cellular (p)ppGpp levels. *In vivo* (p)ppGpp labeling and detection were performed as described with some modifications (*50*). Anaerobic *R. palustris* cultures were grown in 1.5 ml PM medium with 100 µCi/ml of $Na_2H^{32}PO_4$ (PerkinElmer-NEX011001MC) added at the time of inoculation. At the desired time points, 200-300 µl of culture was collected and extracted with 8 M formic acid. Supernatants of the extracts were spotted on PEI-cellulose TLC plates (Sigma-Aldrich) and separated with 1.5 M KH_2PO_4 (pH 3.4). The plates were then dried and visualized by phosphor imaging. The level of (p)ppGpp was calculated by the ratio of (ppGpp + ppGpp) / (GTP + ppGpp + pppGpp).

Proteomic analysis. Cells were lysed, digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry as described in (*51*). Collected data were analyzed using MaxQuant software (v 1.5.5.1) (*32, 52*) for peptide/protein identification and quantification. Identification of peptides was done by searching against the *R. palustris* ATCC BAA-98 (strain CGA009) sequences from the Uniprot Knowledgebase (downloaded on August 30, 2016). The search was performed first with a larger parent mass tolerance (20 ppm) to identify peptides and re-calibrate peptide masses. After re-calibration, the parent mass tolerance was set to 4.5 ppm for a second round of search. During the searches oxidation of methionine as a variable modification, and trypsin digestion was required in both peptide termini. Identifications were filtered with 1% false-discovery rate at peptide-spectrum match and protein levels. The function “matching between runs” was checked to detect peptide peaks in LC-MS/MS runs that they were not fragmented. Label-free quantification (LFQ) values were used to quantitative analysis. Proteins were considered differentially abundant with a p-value ≤0.05 by T.test considering two tails and equal variance.

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Abbreviations

Tn: transposon.

Author contribution

L.Y. and C.S.H. designed the research. L.Y. and H.M. performed the antibiotics assays with *R. palustris*. L.Y. made the *R. palustris* mutants. L.Y. grew the *R. palustris* cells. S.H.P. and E.S.N. performed proteomic analysis. L.Y. and D.R.M. carried out the *in vitro* analysis of ribosome. L.Y. carried out the analysis of rRNA and tRNA. L.Y. performed the analysis of (p)ppGpp. All authors analyzed the data. L.Y., E.S.N. and C.S.H. wrote the paper. All authors contributed to the revision of the manuscript.

Competing interests

The authors declare no competing financial interests.

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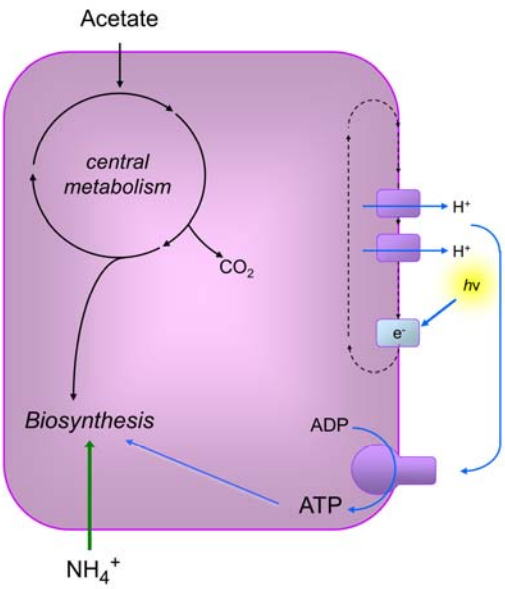


Figure 1. Metabolism of *R. palustris* during anaerobic growth in light. Acetate was used as the carbon source and ammonium as the nitrogen source for growth in this study. *R. palustris* generates ATP and protommotive force from light energy by cyclic photophosphorylation. Energy generation and carbon use are independent from each other. In this study growth-arrest occurred when *R. palustris* had used all available acetate. In this circumstance cells can continue to generate ATP and protonmotive force from light.

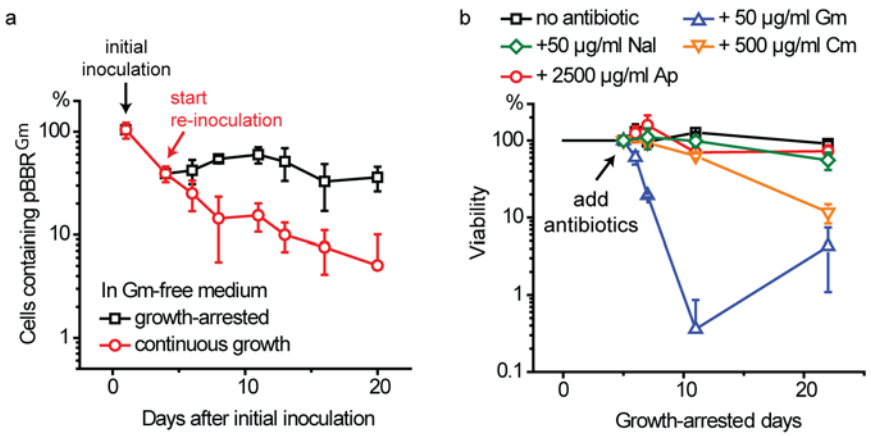


Figure 2. Growth-arrested *R. palustris* cells do not appear to undergo cycles of death and regrowth. a: Percentage of *R. palustris* cells that continue to maintain plasmid pBBR^{Gm} during continuous growth (re-inoculated in medium with fresh carbon source repeatedly) or during growth-arrest (no treatment after the initial carbon source was depleted). Gm-free medium was used for this experiment. b: Growth-arrested *R. palustris* is sensitive to antibiotics targeting ribosomes. Antibiotics are added five days after growth-arrest to independent cultures. Gm: gentamycin. Cm: chloramphenicol. Nal: nalidixic acid. Ap: ampicillin. Error bars indicate the standard deviation (n>3).

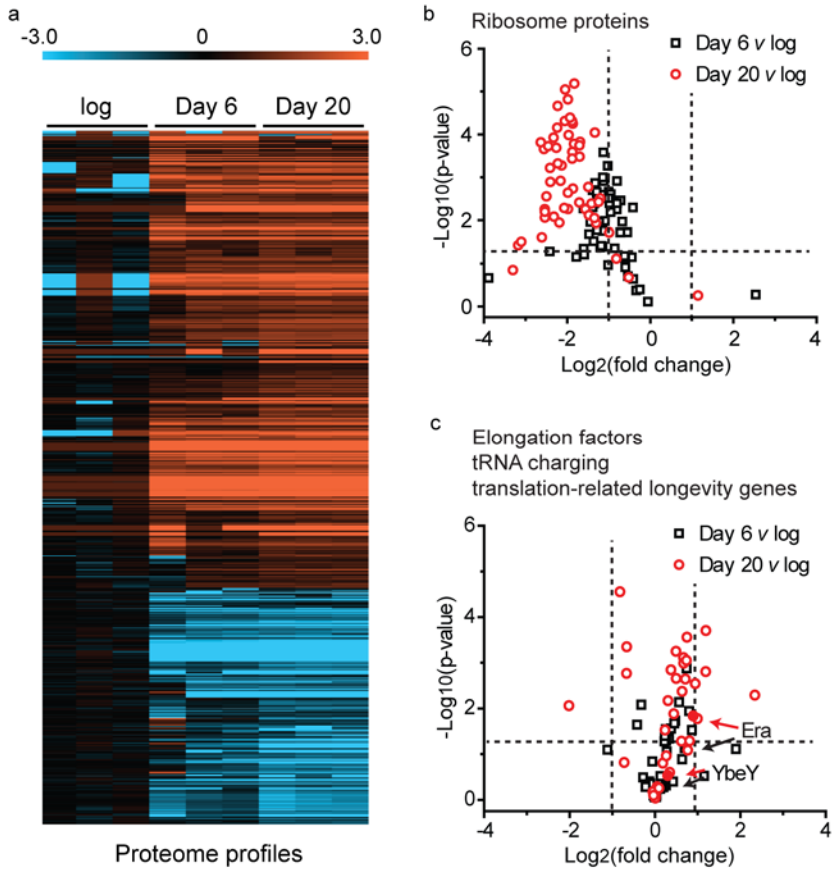


Figure 3. *R. palustris* protein abundances. Growing cells and growth-arrested cells are compared. a: Log_2-fold changes in abundances of all identified *R. palustris* proteins in growing cells (log), 6 days after growth-arrest (Day 6) and 20 days after growth arrest (Day 20) (n = 3, SI Table 1). b: The average relative quantities of ribosome proteins at day 6 (black, Day 6) and day 20 (red, Day 20) growth-arrest relative to the growing cells (log). The horizontal dashed line indicates the position of p = 0.05 (Student's *t*-test), and the vertical dashed lines indicate the position of two-fold abundance changes. Ribosome proteins decreased to ~50% and ~25% after 6 days and 20 days of growth-arrest, respectively. c: The average relative quantities of proteins involved in tRNA charging (KEGG map00970, SI Table 2), translation elongation factors (SI Table 2) and two previously identified longevity proteins [YbeY and Era, (*12*)]. The horizontal dashed line indicates the position of p = 0.05 (Student's *t*-test), and the vertical dashed lines indicate the position of two-fold abundance changes.

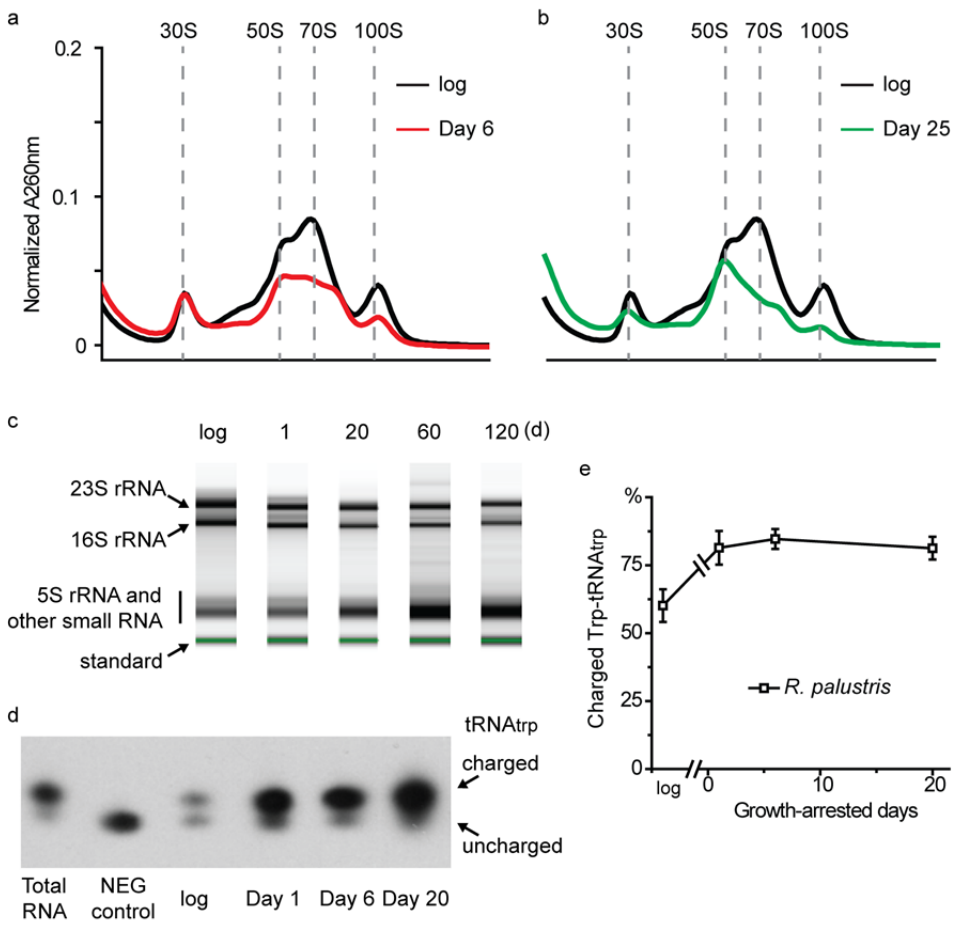


Figure 4. Translation is intact and active in growth-arrested *R. palustris*. a: The ribosome profile of cells at 6 days (Day 6) post growth-arrest is shown and compared to ribosomes from growing *R. palustris* (log). b: The ribosome profile of cells at 25 days (Day 25) post growth-arrest is shown and compared to ribosomes from growing *R. palustris* (log). c: Total RNA was purified and analyzed from growing and growth arrested *R. palustris* cells. The quantification of replicated experiments is shown in SI Figure 2. The standard is the internal standard of the RNA ScreenTape system used (Agilent). d: The charged and uncharged species of $tRNA_{trp}$ are shown here with a representative Northern blot. The aminoacylated (charged) and deacylated (uncharged) species of $tRNA_{trp}$ were separated with an acid urea gel and probed with a $tRNA_{trp}$ probe. e: Quantification of the charged and uncharged species of $tRNA_{trp}$ as analyzed in Figure 4d. Error bars represent the standard deviation (n>3).

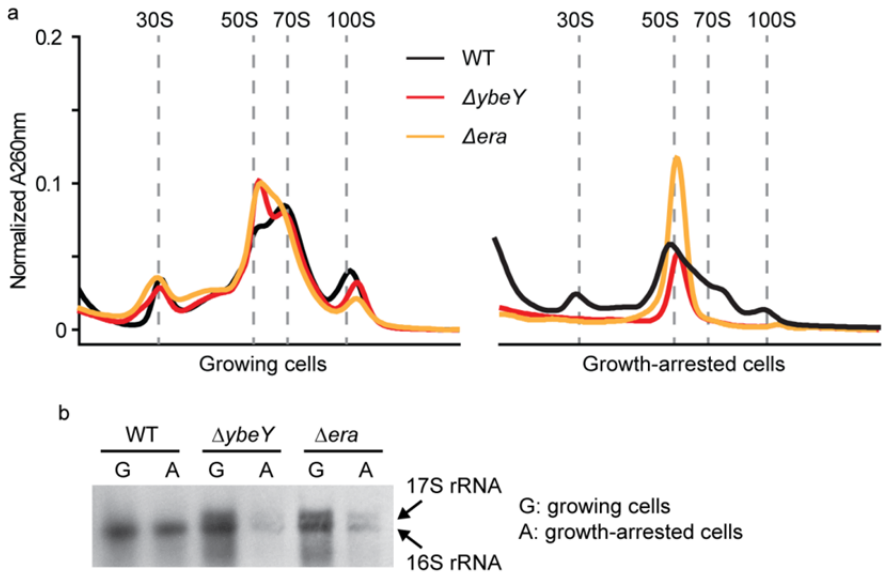


Figure 5. YbeY and Era are important for ribosome maintenance during growth-arrest. a: Deletion of either *ybeY* or *era* had little effect on ribosomes from growing *R. palustris*, while the ribosomes from growth-arrested cultures of *ybeY* and *era* deletion mutants lacked 30S subunits. b: *R. palustris* total RNA was analyzed by Northern blot with a probe targeting 16S rRNA. In growing cells, both Δ*ybeY* and Δ*era* mutants accumulated 17S rRNA but maintained substantial levels of 16S rRNA. In growth-arrested cells, both 17S rRNA and 16S rRNA species were depleted.

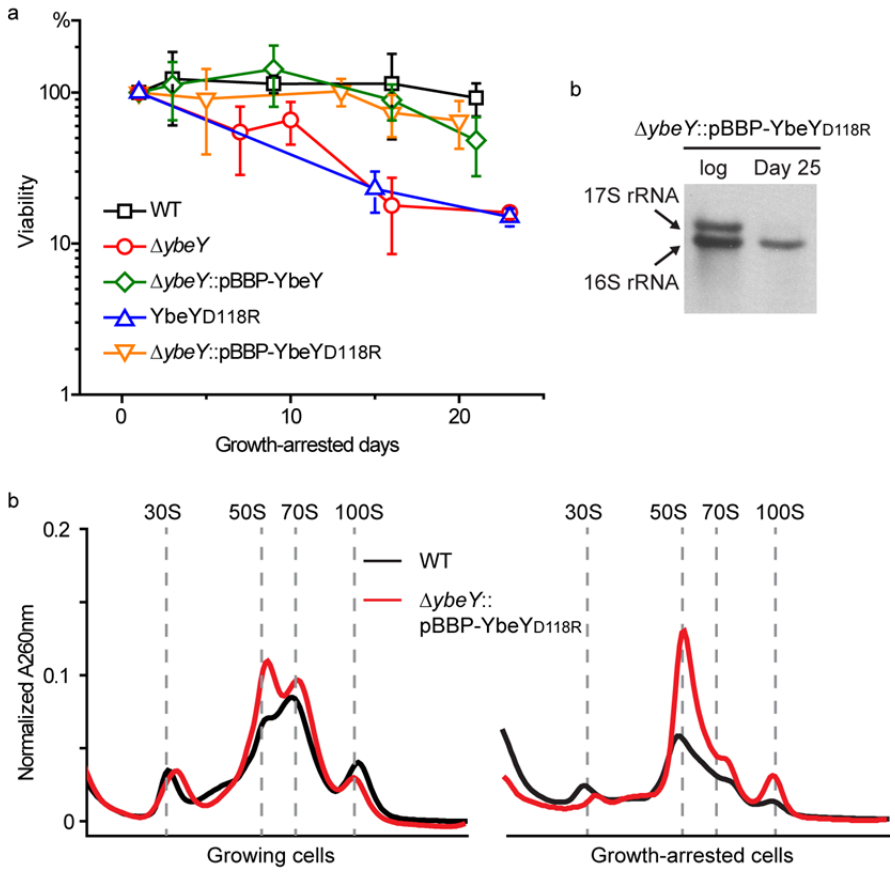


Figure 6. Overexpression of $YbeY_{D118R}$ complemented the longevity defect of a Δ*ybeY* mutant. a: Longevity of different *R. palustris* strains. Deletion of the *ybeY* gene compromised the longevity of growth-arrested cells (Δ*ybeY*), and this was complemented by expressing this gene *in trans* (Δ*ybeY*::pBBP-YbeY) (*12*). A $ybeY_{D118R}$ mutant had a longevity defect similar to that of the Δ*ybeY* deletion mutant. Overexpression of $ybeY_{D118R}$ *in trans*, restored the longevity of a Δ*ybeY* mutant (Δ*ybeY*::pBBP-$YbeY_{D118R}$). Error bars indicate the standard deviation (n>3). b: *R. palustris* total RNA was analyzed by Northern blot with a probe targeting 16S rRNA. In the growth-arrested cells of Δ*ybeY*::pBBP-$ybeY_{D118R}$, only 16S rRNA appeared and no 17S rRNA was detected. c: The ribosomes from Δ*ybeY*::pBBP-$YbeY_{D118R}$ were analyzed on a sucrose gradient. In the growth-arrested cells, 30S, 50S and 70S ribosomes were clearly seen, though the abundance of the 50S subunit is higher than that of WT.

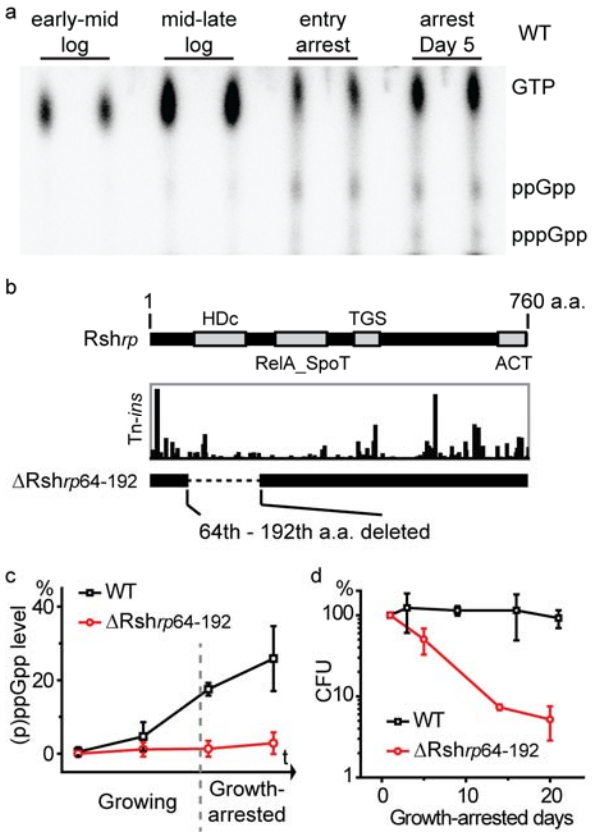
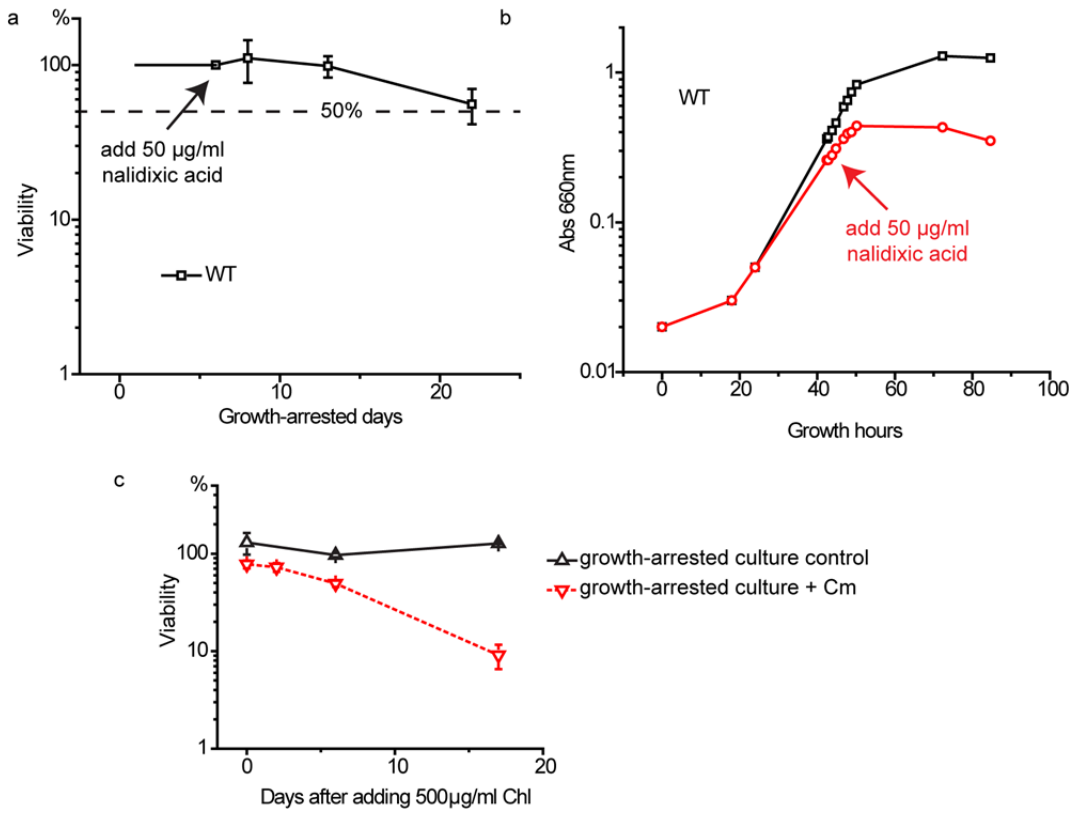
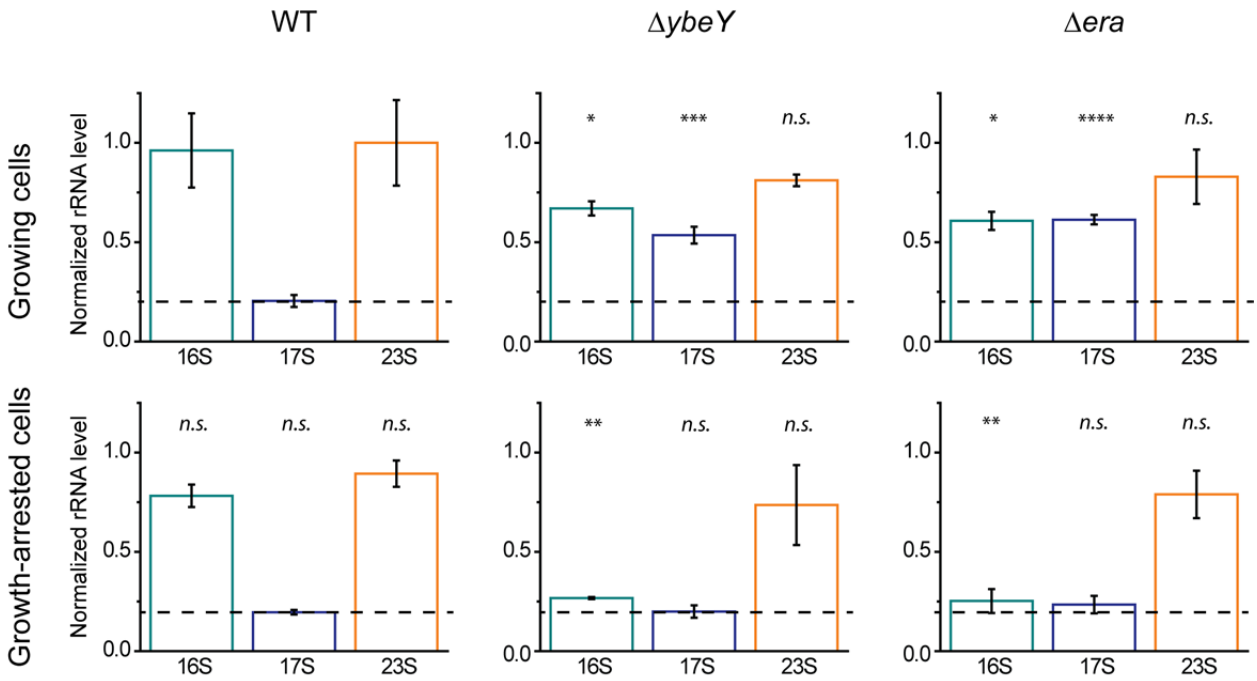


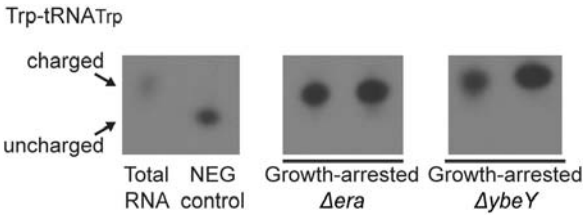
Figure 7. Growth-arrested cells with very low levels of (p)ppGpp have compromised longevity. a: The level of (p)ppGpp was low in exponentially growing *R. palustris* and increased during growth-arrest. b: The domain structure of Rsh_{rp} (top). Locations and relative quantities of Tn insertions in Rsh_{rp} (*12*). A $\Delta Rsh_{rp64\text{-}192}$ mutant was made by deleting the sequence encoding the 64th-192th amino acids of Rsh_{rp} from *R. palustris.* c: (p)ppGpp synthesis was severely compromised in the $\Delta Rsh_{rp64\text{-}192}$ strain. The time points shown correspond to the time points shown in Figure 7a, representing early-mid log phase, mid-late log phase, entry into growth-arrest and day 5 following growth-arrest. d: The longevity of $\Delta rsh_{rp64\text{-}192}$ was severely compromised. Less than 10% of cells remained viable after 2 weeks of growth-arrest. Error bars indicate the standard deviation (n>3).



SI Figure 1. The responses of *R. palustris* to various antibiotics at different growth stages. Antibiotics are added to growing cultures or five days after growth-arrest. Cm: chloramphenicol. Nal: nalidixic acid. Error bars indicate the standard deviation (n>3). For growing cells treated with Nal, the number of viable cells was determined by A_{660} and a representative result is shown (n > 3).



SI Figure 2. rRNA levels in different *R. palustris* strains at different growth-stages. The quantities of 16S, 17S and 23S rRNA are determined using an RNA ScreenTape system (Agilent) and normalized against the levels of 23S rRNA in growing cells of wild-type *R. palustris*. An unpaired *t* test was performed with at least three replicates (n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ****: $p < 0.001$, as determined by Student's *t*-test).



SI Figure 3. Trp-tRNATrp remained highly charged in the growth-arrested cells of the strains of Δ*ybeY* and Δ*era*.