Supplementary Materials

Absence of ppGpp Leads to Increased Mobilization of Intermediately Accumulated Poly(3-hydroxybutyrate) (PHB) in *Ralstonia eutropha* H16

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Suppl. materials, document S1: Optimization of ppGpp extraction from *Ralstonia eutropha* and quantification

First, a nucleotide extraction and quantification protocol was tested using a method similar to the ones described by Theobald et. al. (1). The cells were lysed in 35% perchloric acid with 80 µM EDTA. After 15 min, the pH was neutralized with 1 M K_2HPO_4 and 5 M KOH. HPLC with a KH_2PO_4/K_2HPO_4-methanol gradient was run on a C18 column as described by Hardiman et al. (2) replacing acetonitrile with methanol. ATP, ADP, AMP and GTP could be separated using this method but the intracellular ppGpp content of *R. eutropha* was under the detection limit of the UV detector. Another nucleotide extraction method (3) was tested and samples were analyzed with the HPLC method adapted from (2) but no nucleotides were detected.

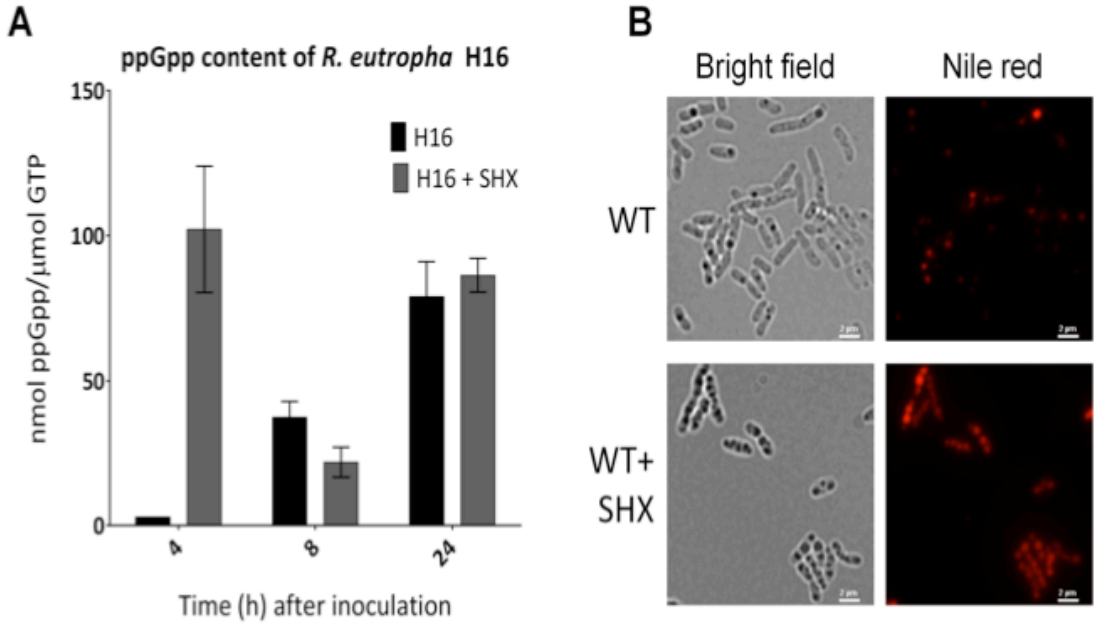
We therefore tried the HPLC-MS detection method of Kästle et al. (3). However, using this method no nucleotides could be detected from samples that had been extracted with perchloric acid. The nucleotide extraction method from Kästle et. al (3) was tested in combination with the HPLC-MS quantification described by the same authors. Peaks corresponding to ppGpp could be indeed detected in samples prepared from *E. coli* cells but application of the same method for *R. eutropha* cells resulted in only very weak signal intensity for ppGpp. Therefore, the number of harvested cells had to be increased. Different culture volumes were tested, and as the ppGpp content of *R. eutropha* turned out to be very low, we decided to harvest 100 ml quantities.

Two different methods for cell harvesting were compared: harvest by (i) fast filtration over a 0.22 µm sterile filter with vacuum or (ii) centrifugation with ice (7 min, 5000 rpm, 4°C). No significant differences were observed, and we decided to use the centrifugation method because the filtration-based method was complicated at higher culture volumes by long filtration times.

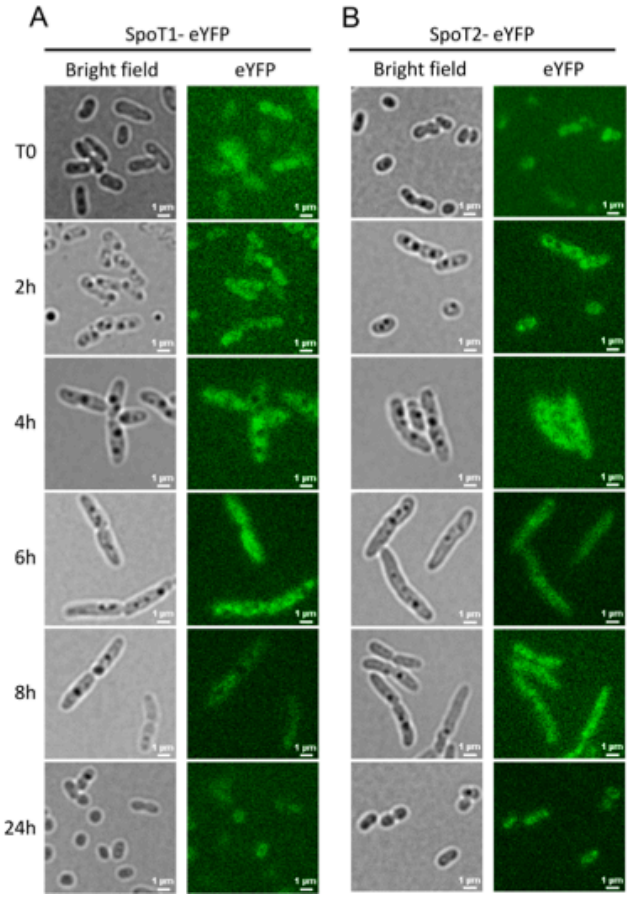
Two nucleotide extraction methods were compared: first, centrifuged cells were resuspended in 60% ethanol and disrupted by vortexing, using a French press, or by shaking at full speed for 2x20 s with glass beads in a microtube homogenizer. Second, the nucleotide extraction method with ethanol was compared with the extraction method described by (4). Both methods gave comparable ppGpp signal intensities; however, the form of the signals obtained by the extraction method described by Ihara et. al (4) indicated a more clean preparation of ppGpp. Furthermore the pre-filter of the MS was much cleaner using the method described by Ihara et. al. We therefore decided to use this method for all *R. eutropha* samples.

A further improvement in the efficiency of ppGpp extraction was achieved by increasing the volume of formic acid used for cell lysis. Furthermore, different sizes of resin bed volumes (30 mg, 60 mg and 100 mg sorbent) for the nucleotide concentration were compared. The cartridges with 60 mg resin material gave best results. We also observed that the nucleotides, including ppGpp, were very stable once they were resuspended in water. The detected peak area for ppGpp even slightly increased after an overnight incubation at room temperature. An additional cleanup of the purified nucleotides on commercial PCR cleanup columns (as described in (4)) did not improve the quality of the peak and the purity of the sample and was therefore omitted in our method. The

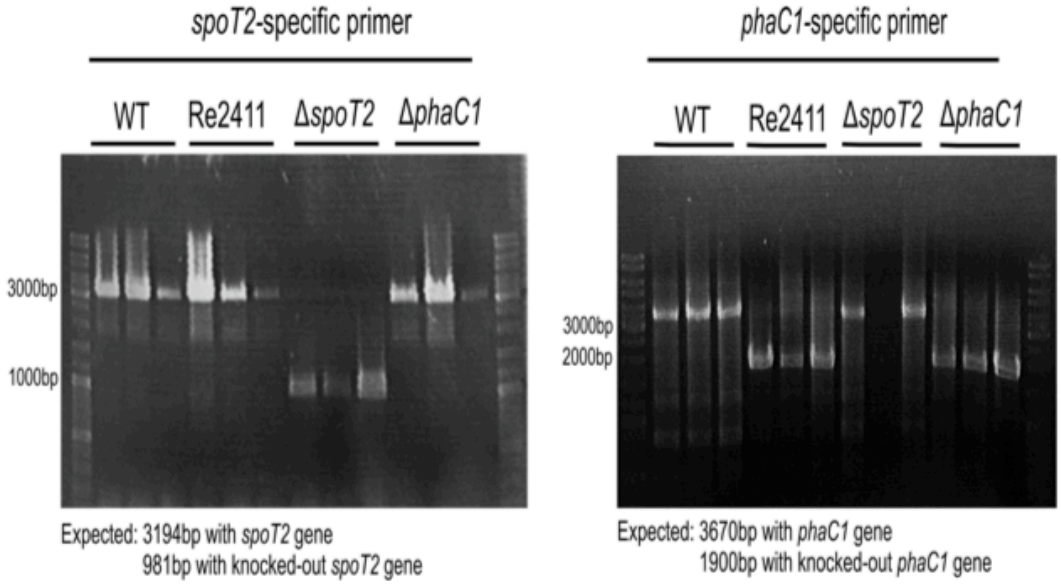
optimized protocol for the extraction of ppGpp and other nucleotides (dNTPs) from *R. eutropha* cells is shown in the Materials and Methods section. We assume that our protocol might also work for efficient ppGpp extraction from other hard-to-disrupt Gram-negative soil bacteria.



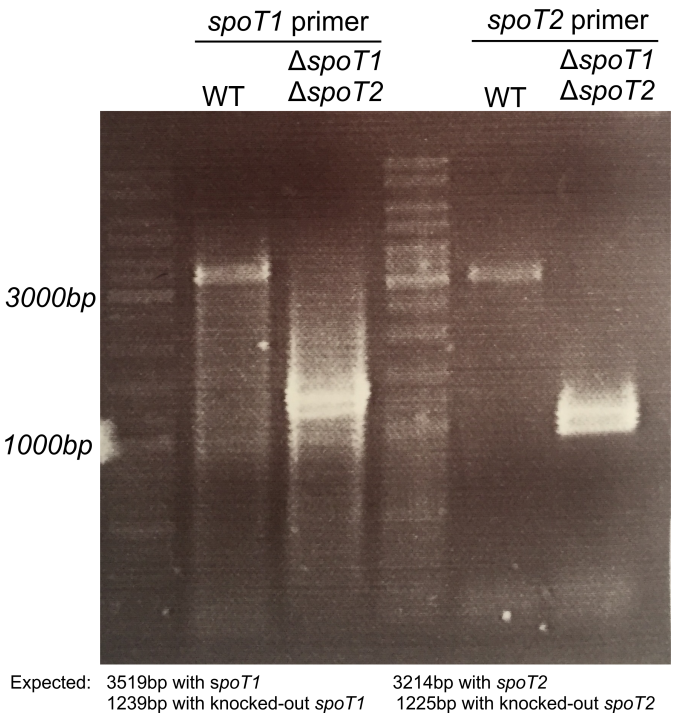
Suppl. materials, Fig. 2: Effect of serine hydroxamate on the accumulation of PHB and on cell morphologies in *R. eutropha* H16.
Bacteria were grown on NB-gluconate medium and 1.5 mM (wt/vol) serine hydroxamate (SHX) was added at t=0h. Samples were taken at indicated time points and ppGpp concentrations (**A**) and PHB granule formation at t=8h (**B**) were determined (bright field, Nile red channel). Note the high numbers of PHB granules in the presence of SHX. The experiment was performed in biological triplicates. The data points/columns and the error bars correspond to the means and standard deviation, respectively.



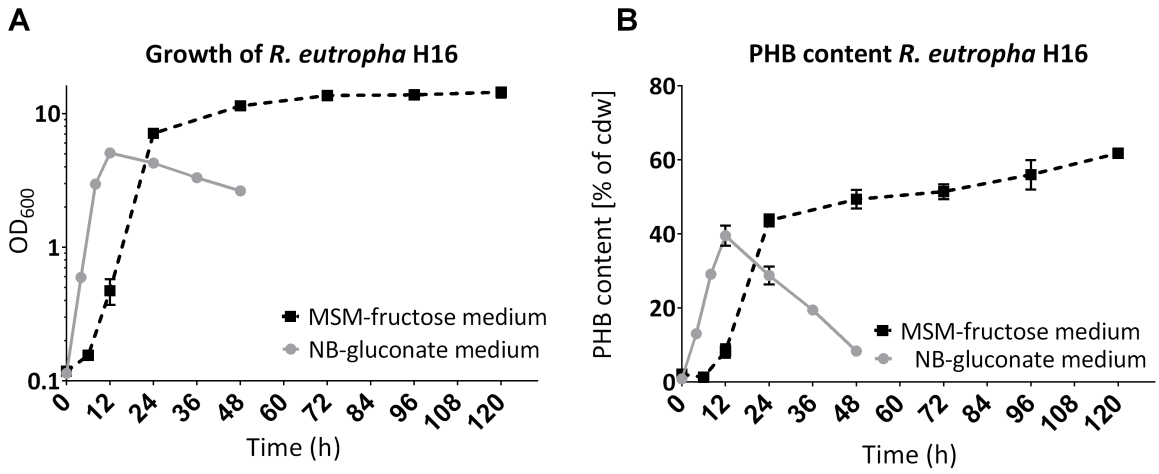
Suppl. materials, Fig. S3: Expression of chromosomally integrated *spoT1-eyfp* and *spoT2-eyfp* fusions in *R. eutropha* H16 during growth on NB medium. The *spoT1* and *spoT2* genes were replaced on the chromosome by *spoT1-eyfp* or *spoT2-eyfp* as described in the Materials and Method section. *R. eutropha* wild type strains (in the absence of eYfp) don´t have any autofluorescence (not shown).



Suppl. materials, Fig. S4: Size determinations of PCR-amplified *phaC1* and *spoT2* loci of *R. eutropha* strains by agarose gel electrophoresis. Standard PCR (30 cycles) with primers specific for the up- and downstream regions (**suppl. materials Table S5)** of the *phaC1* and *spoT2* genes of *R. eutropha* was performed with three single colonies of each strain. The theoretical (expected) values for wild type and for the *phaC1* or *spoT2* gene deletion mutants are indicated below the gel image. *R. eutropha* wild type (WT), the Re2411 strain and the Δ*phaC1* strain have a wild-type *spoT2* locus (≈3.2 kb band); the reduced size of ≈ 1 kbp for the Δ*spoT2* strain is in agreement with the deletion of the *spoT2* gene. *R. eutropha* wild type (WT) and two colonies of the Δ*spoT2* strain have a wild-type *phaC1* locus (the PCR for one colony of the Δ*spoT2* strain was not successful); the reduced sizes of the PCR products for the Re2411 and the Δ*phaC1* strains indicate the deletion of the *phaC1* gene in both strains. For each strain, the PCR product of one colony was DNA-sequenced and confirmed the presence of wild type *phaC1* loci in the wild type and in the Δ*spoT2* strain, the presence of wild-type *spoT2* loci in the wild type, in Re2411 and in the Δ*phaC1* strain and precise deletions of *phaC1* in Re2411 and in the Δ*phaC1* strain and a precise deletion of *spoT2* only in the Δ*spoT2* mutant.



Suppl. materials, Fig. S5: Size determinations of PCR-amplified *spoT1* and *spoT2* loci of a *R. eutropha* mutant by agarose gel electrophoresis. Standard PCR (30 cycles) with primers specific for the up- and downstream regions (**suppl. materials Table S5)** of the *spoT1* and *spoT2* genes of *R. eutropha* was performed. The theoretical (expected) values for wild type and for the *spoT1* or *spoT2* gene deletion mutants are indicated below the gel image. The *R. eutropha* H16 WT strain has a wild-type *spoT1* locus (≈3.5 kbp band) and a wild-type *spoT2* locus (≈3.2 kbp band). The PCR products of the *R. eutropha* Δ*spoT1*Δ+*spoT2* strain have a reduced size of ≈ 1.2 kbp for the *spoT1* locus and ≈ 1kbp for the *spoT2* locus. These data are in agreement with the deletion of the *spoT1* and *spoT2* gene. The PCR products were DNA-sequenced and confirmed the presence of wild type *spoT1* and *spoT2* loci in the wild type and a precise deletion of *spoT1 and spoT2* in the Δ*spoT1*+Δ*spoT2* mutant.



Suppl. materials, Fig. S6: Growth and PHB contents of *R. eutropha* H16 on mineral salts medium (MSM) compared to complex medium. *R. eutropha* wild type (WT) was grown either on complex medium (NB-medium + 0.2% sodium gluconate) or on MSM medium (2% fructose). OD_{600} was determined photospectroscopically (**A**) and the PHB content (in % per cdw) was quantified by gas chromatography (**B**).

Suppl. materials Table S7: Designation of PHB depolymerases (PhaZs) and PHB oligomer hydrolases (PhaYs) of *R. eutropha*

Protein/ (KEGG)	Locus tag/ Organization	Length (aa)	Mw [kDa]
PhaZa1 (PhaZ1)	H16_A1150	419	47.3
PhaZa2 (PhaZ2)	H16_A2862	404	44.8
PhaZa3 (PhaZ5)	H16_B1014	407	45.2
PhaZa4 (PhaZ4)	H16_PHG178	245*)	27.4*)
PhaZa5 (PhaZ3)	H16_B0339	412	45.2
PhaZb (PhaY1)	H16_A2251	718	74.3
PhaZc (PhaY2)	H16_A1335	293	31.6
PhaZd1 (PhaZ6)	H16_B2073	362	39.2
PhaZd2 (PhaZ7)	H16_B2401	365	38.4

*) correct gene start questionable

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