Title: Whole genome microarray and gene deletion studies reveal regulation of the polyhydroxyalkanoate production cycle by the stringent response in *Ralstonia eutropha* H16.

Running Title: Microarray analysis of *Ralstonia eutropha* H16

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Abstract

PHB production and mobilization in *Ralstonia eutropha* is well studied, but in only a small number of instances has PHB production been explored in relation to other cellular processes. We examined global gene expression of wild-type *R. eutropha* throughout the PHB cycle; growth on fructose, PHB production using fructose following ammonium depletion, and PHB utilization in the absence of exogenous carbon after ammonium was resupplied. Our results confirm or lend support to previous results regarding expression of PHB-related genes and enzymes. Additionally, genes for many different cellular processes, such as DNA replication, cell division and translation, are selectively repressed during PHB production. In contrast, expression of genes under control of the alternative sigma factor $\sigma^{54}$ increases sharply during PHB production and is repressed again during PHB utilization. The global gene regulation during PHB production is strongly reminiscent of the gene expression pattern observed during stringent response in other species. Furthermore, a ppGpp synthase deletion mutant did not show accumulation of PHB and chemical induction of the stringent response with DL-norvaline caused increased accumulation of PHB in the presence of ammonium. These results indicate that the stringent response is required for PHB accumulation in *R. eutropha*, helping to elucidate a thus far unknown physiological basis for this process.
Many organisms have been reported to accumulate storage compounds when encountering nutrient stress (10). One of the best studied examples of such a storage compound is poly(3-hydroxybutyrate) (PHB). Accumulation of PHB has been observed in a wide range of organisms and stress conditions (2, 47). PHB accumulation has been intensively studied for both its industrial potential and its role in survival of environmental stress (36, 49, 59). PHB accumulation is regulated, in part, by PhaR, which represses transcription of the phasin gene instrumental in PHB granule formation, phaP1 (42, 73). When PHB starts accumulating in the cell, PhaR binds to the polymer instead of its DNA binding site, thus removing repression of phaP1 expression (32, 71). High expression of phaP1 subsequently results in proper formation of PHB granules, coated predominantly by PhaP1, PhaR, the PHB synthase PhaC1 and the PHB depolymerase PhaZ1 (24, 48). Although this process is well studied, thus far it is unclear what governs the threshold accumulation to set this regulatory network in motion.

One possibility is that initiation of PHB accumulation occurs as a result of the stringent response. This process is a mechanism providing protection against nutrient stress in a wide range of organisms (for recent reviews, see (33, 41)). The stringent response is governed by the nucleotide guanosine-tetraphosphate (ppGpp), which destabilizes the RNA polymerase-σ70 holoenzyme and thus strongly reduces transcription of genes under control of σ70 or its homologs in other organisms (22, 33). This inhibition of σ70 controlled genes results in strong induction of genes under control of alternative σ-factors, such as σ54 (6). Additionally, ppGpp has been shown to directly inhibit translation (28, 60). This process could potentially reduce alternative carbon sinks, thus triggering PHB accumulation.
To determine if the physiological basis for PHB carbon storage could be found in the stringent response, we examine the model organism for polymer accumulation, *Ralstonia eutropha* H16. *R. eutropha* H16 is probably the most widely used model organism for the study of PHB accumulation. A large number of scientific works have been published about the PHB production and utilization of *R. eutropha* (referred to in this work as the “PHB Cycle”), including a number of reviews (47, 48, 58, 59). The genome sequence of *R. eutropha* H16 has, in the past decade, become available (40, 52), further establishing it as the organism of choice for the study of PHB accumulation. However, the stringent response has not been studied in *R. eutropha* previously.

Using the available sequence, genome-wide expression studies could be designed to examine global gene expression under different culture conditions, to elucidate the myriad of changes that occur in the cell during transition from growth to PHB production to PHB utilization. In the case of the *R. eutropha* PHB cycle, such a study can be validated by the many works in the literature that have closely studied the components of the cycle and can potentially shed new light on the physiology of PHB storage. Although there exists an earlier published analysis of genome wide expression focusing on PHB production in *R. eutropha*, the study did not identify a physiological origin for the initial PHB accumulation (37). Furthermore, it only focused on the comparison of growth to PHB accumulation, and neglected the study of gene expression during PHB utilization.

In this study we used custom designed *R. eutropha* microarray chips to compare gene expression under three conditions: cell growth (with ammonium as nitrogen source) in the presence of fructose as the sole carbon source, PHB production (after ammonium has been depleted) in the presence of fructose as the sole carbon source and utilization of PHB as the sole carbon source (after ammonium has been resupplied). The emerging global gene expression pattern was further studied through promoter identification of the regulated genes. The physiological origin of the
observed global expression changes was studied in more detail using a \textit{R. eutropha} strain lacking the \textit{relA} homolog and chemically inducing the stringent response using DL-norvaline. The role of the $\sigma^S$ gene, \textit{rpoS}, in the PHB cycle was also examined.

\textbf{Materials and Methods}

\textbf{Bacterial strains and materials.} Bacterial strains and plasmids used in this study are listed in Table 1. All chemicals and commercial reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). \textit{Pfu} DNA polymerase and other DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA).

\textbf{Design of custom \textit{Ralstonia eutropha} H16 microarray chips.} Probe sets representing 6626 protein-encoding genes and 3 rRNA genes from the \textit{R. eutropha} H16 genome, as annotated per Pohlmann, et al (40), were printed on an 11 $\mu$m array (49-5241 format, Affymetrix, Santa Clara, CA) as described previously (9). Custom \textit{R. eutropha} H16 gene expression microarray chips were constructed according to the quality control guidelines outlined by the manufacturer (www.affymetrix.com), after submission of design parameters.

\textbf{Cell growth and total cellular RNA isolation procedure.} Individual colonies of \textit{R. eutropha} H16 grown on a tryptic soy agar (TSA) plate were inoculated into 5 mL of dextrose-free tryptic soy broth (TSB, Becton Dickinson, Sparks, MD) and grown for 24 h. Cultures for microarray analysis were all performed in triplicate. Aliquots of 0.5 mL of overnight culture were inoculated into 250 mL shake flasks containing 50 mL of minimal medium, modified from (35), containing 0.1 $\%$ NH$_4$Cl and 2 $\%$ (w/v) fructose. These cultures were grown for 24 h. Overnight cultures were inoculated to an initial OD$_{600}$ of 0.1 into 250 mL shake flasks containing 50 mL of minimal
medium containing 0.05 % NH₄Cl and 2 % (w/v) fructose. Cultures were grown for 12 h. Cultures for sampling were inoculated to an initial OD₆₀₀ of 0.05 in 250 mL shake flasks containing 50 mL of minimal medium with 0.05 % NH₄Cl and 2 % (w/v) fructose. All flask cultures were grown at 30 °C with agitation (200 rpm). Unless otherwise mentioned, all growth media in this study contained 10 µg/mL gentamicin. In selecting culture conditions for microarray analysis, we focused on logarithmic growth (when the nitrogen source in the culture was depleted by 50%), PHB production (when nitrogen in the culture was depleted), and PHB utilization (when cells were actively utilizing intracellular PHB stores as a carbon and energy source). The concentration of NH₄⁺ in the growth medium was monitored using an Ammonia Assay Kit (Sigma-Aldrich) following the manufacturer’s instructions. An aliquot of cells (OD₆₀₀ equivalent = 2.5) was harvested at an NH₄⁺ concentration of approximately 0.025 %, and another aliquot of cells (also an OD₆₀₀ equivalent = 2.5) was harvested 2 h after depletion of nitrogen in the media. For PHB utilization samples, cells were grown in PHB production medium for 48 h, washed and transferred to PHB utilization medium, which was free of extracellular carbon and contained 0.1% NH₄Cl, allowing cells to utilize PHB stores as the main carbon source. Intracellular PHB content was monitored (see below) and, after 6 h of incubation, cells were harvested and later used for RNA isolation. All culture aliquots were treated with 2 volumes of RNA Protect reagent (QIAgen, Valencia, CA). Cells were centrifuged at 5000 rpm, growth medium was removed, and cell pellets were stored at -80°C until RNA extraction.

Cellular RNA isolation, labeling and hybridization to Affymetrix array chips were performed as described previously (9).

**Microarray data analysis.** Microarray data analysis was performed as described previously (9), using Affymetrix GCOS v.1.4 for data extraction and robust multichip average (RMA; ArrayStar...
Software, Madison, WI) with quantile normalization for further analysis. Annotation of genes in
the final output was performed based on Pohlmann, et al. (40). Genes of interest with a
statistically significant change in expression (p < 0.01) were selected for further study. Gene
expression values are denoted as log(2) of actual expression values, similar to previous work (9).

Growth of strains and PHB quantitation. A relA homolog (Supplemental Figure S1) (spoT2,
locus tag H16_A1337) was identified in the R. eutropha H16 genome using the genome
information broker (GIB) (19). A deletion mutant strain, denoted Re2411 (Table 1), was
constructed using methods described previously (9) using primer pairs listed in Supplemental
Table 1. Four cultures of H16 and Re2411 were grown in 50 mL minimal medium containing 0.1
% NH4Cl and 2 % fructose for 70 h at 30 °C with agitation (200 rpm). Aliquots of culture (5 mL)
were removed at 0, 12, 20, 44 and 69 h for intracellular PHB quantitation.

An rpoS homolog (H16_A2373) was also identified using GIB (19). A deletion mutant strain,
denoted Re2424 (Table 1), was constructed using methods described previously (9), using primer
pairs listed in Supplemental Table 1. An rpoS complementation plasmid, pASCB1 (Table 1), was
constructed by inserting the rpoS gene into pBBR1MCS-2, as follows. The R. eutropha rpoS
gene was amplified by PCR using primers rpoScompFW and rpoScompFW (Supplemental Table
1). The PCR product was cut using SalI and XbaI restriction endonucleases and ligated into a
Sall/XbaI-cut pBBR1MCS-2. Three cultures of H16/pBBR1MCS-2, Re2424/pBBR1MCS-2, and
Re2424/pASCB1 were grown in 50 mL minimal medium containing 0.1 % NH4Cl and 2 %
fructose for 72 h at 30 °C with agitation (200 rpm). Aliquots of culture (5 mL) were removed at
0, 24, 48 and 72 h for intracellular PHB quantitation. The remaining cultures were washed twice
with sterile 0.85% saline solution and the cells were resuspended in PHB utilization medium

7
containing 0.2 % NH₄Cl for 72 h at 30 °C with agitation (200 rpm). Aliquots of culture (5 mL) were removed at 0, 24, 48 and 72 h for intracellular PHB quantitation.

For quantitation of intracellular PHB, 5 mL aliquots of culture were transferred to pre-weighed borosilicate glass tubes at various time points during the PHB production cycle. Cells were pelleted, washed with 5 mL of cold water and pelleted again and dried in vacuo at 80°C. The PHB content and cell dry weight (CDW) were determined from the dried samples using established methods (7, 26).

Enzyme assays. Cultures of H16 and Re2411 were grown in 50 mL minimal medium containing 0.1 % NH₄Cl and 2 % fructose for 72 h at 30 °C with agitation (200 rpm). Cells were grown for up to 24 h, then pelleted and stored at -80°C. Pellets were thawed on ice and resuspended in 20 mM Tris-HCl buffer (pH 7.5) for PHB synthase and malate dehydrogenase assays, 150 mM EPPS buffer (pH 8.0) for β-ketothiolase assays, 50 mM KH₂PO₄ (pH 6.0) for Acetoacetyl-CoA dehydrogenase assays, and 100 mM Tris-HCl (pH 8.0) for citrate synthase assays. One milliliter of suspended cells was placed, along with ~100 μL 0.1-mm zirconia/silica beads (Biospec products, Bartlesville, OK), in a 2-mL microcentrifuge tube and sealed. Tubes were loaded onto a FastPrep-24 machine (MP Biomedicals, Solon, OH) and treated twice at 6.0 m/s for 30 sec, with a 5-min rest period in between treatments. Following lysis, samples were centrifuged for 5 min at 4°C to pellet cell debris. Clarified cell extracts were removed to a clean tube and enzyme assays were performed.

Discontinuous PHB synthase (PhaC) activity assays were performed as described previously (15), using 3HB-CoA as the substrate. The 3HB-CoA sample was a generous gift from Dr. Mimi Cho and Professor JoAnne Stubbe (Department of Chemistry, MIT). Activities in cell extracts
were monitored for 5 min for each assay. β-ketothiolase (PhaA) activity assays were performed as described previously (55). Acetoacetyl-CoA reductase (PhaB) assays were performed as described previously (12, 23). Citrate synthase assay was performed according to (18, 56), using acetyl-CoA and oxaloacetate as the substrates. Malate dehydrogenase activity was monitored by following reduction of NAD\(^+\) to NADH during production of oxaloacetate from malate. The assay mixture was composed of 600 μL 100 mM Tris-HCl (pH 7.6), 100 μL 8 mM MnSO\(_4\), 100 μL 5 mM NAD\(^+\), 100 μL 8 mM malic acid. The reaction was started upon addition of 100 μL cell extract, and activity was monitored by following\( A_{340} \) over time. In all cases, one unit (U) of enzyme activity is the amount of enzyme needed to convert 1 μmol substrate to product per min at 25°C.

**Reduced pyridine nucleotide cofactor quantitation.** Intracellular levels of NADH and NADPH were quantified in *R. eutropha* H16 and Re2411 cells during growth (6 h culture) and nitrogen limitation (24 h culture) in minimal medium containing 0.1 % NH\(_4\)Cl, 2 % fructose, and 10 μg/mL gentamicin. A volume of cells equivalent to an OD\(_{600}\) of 5.0 were harvested, centrifuged for 5 min at 2800 × \( g \), washed in 5 mL of 0.85% saline, and stored at -80°C. Reduced pyridine nucleotides (NADH and NADPH) were extracted from cells according to (20). Intracellular NADH concentrations were measured using an alcohol dehydrogenase assay coupled to the reduction of phenazine ethosulfate (PES) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), as described previously (20, 66). The substrate was 3% (v/v) ethanol. Intracellular NADPH concentrations were measured by coupling with glucose 6-phosphate dehydrogenase coupled to reduction of PES and MTT, as previously described (20, 74). The substrate was 25 mM glucose 6-phosphate. NADH and NADPH quantitations were performed by comparison with a standard curve of each nucleotide cofactor.
Chemical induction of the stringent response. Six individual cultures of H16 were grown on 50 mL minimal medium containing 0.1 % NH₄Cl and 2 % fructose at 30 °C with agitation (200 rpm). During exponential growth (OD₆₀₀ ≈ 0.7), 5 mL aliquots of culture were sampled for CDW determination and PHB quantitation. After 20 min, another 5 mL sample was taken and DL-norvaline was added to three cultures of H16 to a final concentration of 0.1 % (w/v). At the time of addition, 5 mL of culture was sampled for CDW determination and PHB quantitation. Sampling was repeated every 20 min for 100 min after addition of DL-norvaline. PHB content and CDW were determined as described above.

Promoter analysis. Upstream sequences of strongly regulated genes were retrieved from the GIB and further analyzed using MEGA 5 (61). Potential σ₅⁴ promoters were manually identified based on the consensus sequence published previously (3). Potential σ₇₀ promoters were identified using BPROM (Softberry).

Microarray data accession number. Microarray data discussed in this work have been deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through the GEO series accession number GSE21145.

Results

*R. eutropha* H16 fructose cultures – Gene expression in the presence or absence of nitrogen.

We have examined global gene expression in *R. eutropha* H16 under growth conditions in the presence of nitrogen, under nitrogen depletion, and upon reintroduction of nitrogen. A total of 2263 genes exhibited a greater than 2-fold change in expression (p < 0.01) when nitrogen was depleted in fructose cultures. Of these 2263 genes, roughly half were observed to be upregulated
and half were downregulated after nitrogen depletion. The magnitude of this change suggests that major physiological changes occur in *R. eutropha* upon entrance into PHB production.

Table 2 breaks down these 2263 genes into functional groups, categorized as per Tatusov, *et al.* (62). Transcription of several of these groups of genes was shown to be generally repressed during PHB production. The downregulated functional groups include genes involved in nucleotide metabolism (55.7% down), DNA replication (32.2% down), cell envelope biogenesis (28% down), cell division (40.6% down), and translation (67.6% down) (Table 2). Additionally, cell motility and secretion genes were observed to be generally downregulated (30.6% down) in the absence of nitrogen (Table 2). An earlier study has shown that *R. eutropha* stops producing flagella and that the amount of flagellin decrease in cells during nitrogen starvation (45). The *fliC* gene was also shown to be downregulated in stationary phase in a previous *R. eutropha* gene expression study (37). Our results show that the flagellin gene, *fliC* (H16_B2360), is indeed downregulated, 9.3-fold, during PHB production compared to growth. Furthermore, genes of the TCA cycle were all observed to be downregulated during PHB production (Table 3), although some exhibited more of a decrease in expression than others. These data suggest that there is a change in the flux of acetyl-CoA in the cell when *R. eutropha* enters the PHB production phase, providing a potential additional level of control for PHB production, since the precursor of PHB is produced using two molecules of acetyl-CoA.

Expression of a large group of genes involved in inorganic ion or carbohydrate transport and metabolism was shown to be increased during nitrogen starvation. The upregulated genes involved in inorganic ion transport and metabolism can largely be accounted for by genes involved in nitrogen uptake and metabolism. For example, an operon encoding potential nitrogen scavenging transporters and enzymes (locus tags H16_A1075 through H16_A1087) is
upregulated an average of 491-fold during nitrogen starvation. The upregulated genes involved in carbohydrate metabolism were most notably involved in fructose transport and metabolism and, surprisingly, the two cbb-operons encoding Calvin cycle enzymes for CO₂ fixation.

The pattern of this expression change is similar to those observed during the stringent response in various other species (11, 14, 34, 63) suggesting a central role for the nucleotide guanosine tetraphosphate (ppGpp) in regulation of PHB production. As mentioned above, inhibition of σ⁷⁰ controlled genes by action of ppGpp results in a strong induction of genes under control of alternative σ-factors, such as σ⁵⁴ (6). To further examine this possibility, we have analyzed the upstream sequences of the most strongly regulated genes. σ⁵⁴-family promoter elements were identified in the upstream sequences of 79 out of 96 genes with a greater than 50-fold increase in expression (Supplemental Figure S2). In the upstream sequences of most strongly downregulated genes the ‘housekeeping’ σ⁷⁰-family promoter was identified. In agreement with earlier observations in *E. coli* (21), a guanine is conserved at the -5 position of rRNA promoters and promoters of the most strongly repressed genes under nitrogen starvation (data not shown).

**Involvement of RelA (SpoT2) in PHB biosynthesis.** To ascertain that changes in gene expression mentioned above can be attributed to a stringent response, we created a ppGpp synthase (*spoT2*, locus tag H16_A1337) deletion strain, H16ΔspoT2, denoted Re2411 (Table 1). Under nitrogen starvation, Re2411 accumulated no detectable PHB (Figure 1A), confirming the hypothesis that the stringent response is required for PHB accumulation. To obtain further evidence that the lack of PHB accumulation could be attributed to a lack of stringent response, DL-norvaline was used to chemically induce a stringent response during exponential growth in *R. eutropha* H16. Following addition of DL-norvaline, wild-type *R. eutropha* cultures accumulated more PHB than an untreated control group (Figure 1B).
It has been suggested that expression of PHB production genes (*phaCAB*) is constitutive throughout the PHB cycle (29). This suggestion was confirmed by our gene expression data (see below). Thus, it is likely that the stringent response in *R. eutropha* is controlling PHB production at a level other than transcription, such as enzyme activity. To investigate whether SpoT2 (RelA) affects activities of key PHB biosynthetic enzymes, we examined activities in extracts of the wild-type and *spoT2* mutant strains during nitrogen limitation (Table 4). No significant difference in PhaA or PhaC activities was seen in H16 or Re2411, but the *spoT2* mutant strain did exhibit a small (<2 fold), but significant, decrease in PhaB activity. It has been shown in *R. eutropha* that both PhaB1 and PhaB3 enzymes exhibit acetoacetyl-CoA reductase activity in minimal medium cultures with fructose as the carbon source (12). It is unclear whether one or both PhaB enzymes are responsible for the decrease in activity in strain Re2411. Since TCA cycle genes were shown to be downregulated during PHB production in *R. eutropha* H16, we examined some TCA cycle enzyme activities in strains H16 and Re2411, to determine if the lack of *relA* affected the activity, and thus the flow of carbon, through the TCA cycle. Table 4 shows that malate dehydrogenase activity was not significantly altered in the presence or absence of an intact RelA enzyme, suggesting that these activities were not modulated by the stringent response. Citrate synthase activity, on the other hand, was lower in extracts of strain Re2411 compared to those of the wild type suggesting expression or activity of citrate synthase is occurring in the *relA* mutant. Given the reduction in PhaB activity, we hypothesized that this would have an effect on intracellular concentrations of reduced cofactors, both of which were shown to be able to act as substrates in acetoacetyl-CoA reductase reactions (12). Quantitation of NADH and NADPH levels in strains H16 and Re2411 revealed an increase in intracellular NADH concentration of Re2411 cells during nitrogen starvation in minimal medium (24 h). No
significant differences in NADPH levels were detected in either strain during growth or nitrogen
limitation (Figure 2). The results shown here suggest a link to RelA activity, PhaB activity, and
reduced cofactor levels in the *R. eutropha* cell during PHB production.

It has been shown previously that *R. eutropha* strains incapable of producing PHB will excrete
pyruvate and other metabolites under conditions that would otherwise permit the biosynthesis of
PHB (57). With carbon unable to flow into storage polymer during nutrient limitation, it can be
reasoned that the cell must do something with this carbon, if conventional storage is not an
option. We examined supernatants of wild-type and mutant *R. eutropha* cultures and determined
that Re2411 also secretes pyruvate into the culture media, due to the strain’s inability to produce
PHB (Supplemental Figure S3).

*R. eutropha* H16 gene expression changes of cells in PHB utilization, compared to growth on
fructose. Although PHB utilization in *R. eutropha* has been less well-studied than PHB
production, several important physiological changes occur when the culture resumes growth
(PHB utilization in the presence of nitrogen and absence of exogenous carbon). Consistent with
past works in the literature regarding changes in expression of PHB-related genes like *phaP* and
*phaZ* during transition from PHB production to PHB utilization (29, 42, 44, 72), our results show
that expression patterns of these genes are linked to and generally follow the production of
intracellular polymer (see below). Furthermore, we observed that the global gene expression
pattern returns to that observed during growth on fructose. To study the differences between the
physiology of extracellular carbon source utilization versus intracellular carbon source utilization
we have compared gene expression during growth and PHB utilization.
Table 4 shows a breakdown of genes exhibiting a change in expression during growth with PHB utilization (cells containing intracellular PHB grown in the presence of no extracellular carbon and abundant nitrogen), compared to growth on fructose. Expression of a total of 1166 genes changed greater than 2-fold between growth on fructose and PHB utilization. Of these genes, 745 are repressed during PHB utilization relative to growth on fructose. Table 4 categorizes these genes in functional groups according to Tatusov, et al. (62). A large group of genes repressed (relative to growth on fructose) during PHB utilization were also seen to be repressed during PHB production. These genes mostly classify within the functional groups cell motility (15% down), amino acid biosynthesis (26.7% down), carbohydrate metabolism (34.1% down), and translation (19.5% down).

Furthermore, the differences between expression levels observed when comparing growth on fructose and PHB were generally much smaller than the differences observed between growth and PHB production. The major exception being the seven gene frc-operon (locus tag H16_B1497 – H16_B1503) encoding a regulator, a fructose specific transporter and three proteins in the Entner-Doudoroff catabolic pathway, which was repressed an average 66 fold. Additionally, expression of both copies of RuBisCO (locus tags H16_B1394 & H16_B1395, and PHG426 & PHG427) was reduced an average 14 fold.

The genes that were most strongly induced during PHB utilization encode hypothetical proteins or proteins with a general predicted function only, reflecting the limited knowledge on this process. Interestingly, expression of four (out of six predicted in the genome) genes encoding cold-shock family proteins increases an average 9-fold, suggesting a role for these gene products during PHB utilization.
Finally, a group of genes induced during PHB production remained induced during PHB utilization. Most notably, the periplasmic nitrate reductase (locus tag H16_PHG209 – H16_PHG213) is induced an average 20-fold. Additionally, genes encoding a bos quinol oxidase (locus tag H16_B1025 – H16_B1028) and a bb3 cytochrome c oxidase (locus tag H16_B2058 – H16_B2062) were upregulated an average 15-fold and 10-fold respectively.

Changes in expression of PHB-related genes throughout the PHB cycle. Figure 3 illustrates the changes in expression of all PHB production pathway and related genes present in *R. eutropha* H16. The main PHB production gene cluster in *R. eutropha, phaCAB*, the regulator gene *phaR*, and recently-discovered granule-associated nucleation factor gene *phaM* (39) did not appear to exhibit dramatic changes in expression under PHB production or utilization conditions (Figure 3A, B, C, and E), confirming that these genes are constitutively expressed throughout the cell cycle and PHA production as hypothesized previously (29). One caveat regarding this result is that the *p*-value of each gene expression comparison is high, suggesting high variation among these probe sets. However, this is still an interesting result, because it suggests that the regulation of PHB production in *R. eutropha* may not be achieved through the regulation of *phaCAB* expression alone, given the irregular changes in expression pattern of the genes in that operon.

Also of note is the increase in expression of the *phaZ* genes under nitrogen limitation. The *phaZ1* gene increased in expression slightly when cells produce PHB. This gene was observed to maintain a similar level of expression in PHB utilization compared to PHB production (Figure 3F). This makes sense, as PhaZ1 has been shown to play an integral role in PHB utilization (65, 72). The most dramatic increase in expression is the *phaZ2* gene, encoding an intracellular PHB depolymerase, which increases 256-fold (Figure 3F). These results are similar to quantitative RT-PCR results examining the amount of *phaZ1* and *phaZ2* transcription in the H16 cell,
published previously (29). The *phaZ2* results also agree with a trend seen in a previous transcriptome analysis of *R. eutropha* (37). The *phaZ2* gene also exhibited a dramatic decrease in expression from PHB production to PHB utilization conditions. As PhaZ2 has also been shown to play a role in PHB utilization (72), the gene expression pattern suggests a role for PhaZ2 that is much different than PhaZ1 in the PHB cycle. Three genes in the *R. eutropha* genome are predicted to encode extracellular PhaZ enzymes, albeit no extracellular PHB depolymerase activity has ever been detected in *R. eutropha* cultures. One of these putative depolymerase genes, *phaZ6*, was shown to be upregulated during PHB production (Figure 3G). These results agree with a trend observed previously (37).

The *phaP1* gene exhibited an increase in expression when cells were producing PHB, compared to growth (Figure 3D). This is not surprising, since an increase of production of the PhaP1 protein has been linked to PHB production by way of the PhaR regulatory protein (42, 73). In a previous transcriptome study, *phaP1* expression was shown to increase in stationary growth phase (37). The *phaP1* gene shows a decrease in expression during PHB utilization, compared to PHB production (Figure 3D). Other phasin genes (*phaP2*, *phaP4*, and newly-discovered *phaP5* (38)) were observed to be upregulated during nitrogen limitation (Figure 3D). Although it has been shown that the other *phaP* genes are expressed during PHB production (42, 44), the respective proteins are clearly less abundant on the PHB granule (24, 43).

Besides the genes discussed in Figure 3, there are many other genes whose putative products have been associated with PHB production (48). Aside from *phaA* and *bktB*, there are 13 other β-ketothiolase homologs present in *R. eutropha* (31, 37, 48). According to recent transcriptome analysis, 6 of the 15 total β-ketothiolase homologs (including *phaA* and *bktB*) were actually expressed during growth and PHB production using gluconate as a carbon source (31).
Examination of our array data revealed that 7 β-ketothiolase homologs (including phaA and bktB) were observed to be expressed under growth and PHB production on fructose (Supplemental Table 2). Four of the 5 additional active β-ketothiolase homologs in our study are the same as those that were discovered previously (31), the one exception being H16_B0759, which our data has shown to be expressed during PHB production (Supplemental Table 2).

Several acetoacetyl-CoA reductase genes and homologs are also present in the R. eutropha genome (48). Two acetoacetyl-CoA reductases, PhaB1 and PhaB3, have been shown to be responsible for most of the 3HB-CoA production in the cell, using fructose as the extracellular source of carbon (12). Of the other acetoacetyl-CoA reductase homologs, only 8 are shown to be expressed in the R. eutropha cell at any time during this study (Supplemental Table 3).

A role for RpoS (σS) in the PHB cycle. In E. coli, the rpoS gene is upregulated in response to many stresses, among them carbon starvation (5). Since PHB utilization conditions represent a form of extracellular carbon starvation, we postulated that RpoS in R. eutropha may play a role in intracellular PHB mobilization. To address this possible relationship, we constructed an rpoS deletion mutant strain of R. eutropha and observed the behavior of the resulting strain, Re2424, under different stress conditions. As is common in rpoS mutant strains of other bacterial species (25, 50), R. eutropha Re2424 exhibited decreased resistance to hydrogen peroxide, as compared to wild type (Supplemental Figure S4). Also, no change in PHB production was seen between the wild-type and rpoS mutant strains (data not shown). During PHB utilization, however, Re2424 exhibited an increased rate and an increased overall level of polymer mobilization, as compared to strain H16. Strain Re2424 was complemented with rpoS gene in trans on plasmid pASCB1 (Table 1), and the resulting strain utilized intracellular PHB at a similar rate and to a similar extent as wild type (Figure 4).
In recent years, genome wide expression microarray studies have been conducted to shed new light on the metabolism of *R. eutropha*, solidly establishing the technique in this organism (9, 37). Although a previous microarray study aimed at providing new insights in the PHB cycle in *R. eutropha* has been published (37), the resolution of that study was much lower, showing only 297 genes changing expression between growth and PHB production (as opposed to 2267 genes in this study). Moreover, no explanation for the global changes in gene expression was provided.

In contrast, this study suggests that the bacterial alarmone, guanosine tetraphosphate (ppGpp), plays a significant role in the production of PHB in *R. eutropha* and can account for the global expression pattern observed in both studies. However, the set of genes from the previous work (37) included *accC2* (encoding biotin carboxylase), *fabG* (encoding 3-oxoacyl-[ACP] reductase), *fabZ* (encoding 3-hydroxymyristoyl-[ACP] dehydratase), and H16_A3307 (encoding a putative enoyl-CoA hydratase). Our microarray data has confirmed these trends seen in the previous study, as transcription of all these genes decreased in PHB production compared to growth (data not shown).

Some connections between ppGpp and the PHB cycle have been reported previously, but to date few works have shown evidence of a relationship between PHB synthesis and the stringent response. The PHB negative phenotype, similar to that observed in Re2411 in this study, has previously been observed in a *Rhizobium etli rsh*-negative mutant (13). However, the phenotype received little attention in that study and no follow-up work has been reported. Evidence for stringent response control of PHB production in *R. eutropha* is observed in how strain Re2411 stores its reducing equivalents when it is unable to synthesize PHB. Typically, reducing equivalents are stored in the PHB polymer during times of stress, through the reduction of...
acetoacetyl-CoA by NADPH-dependent action of PhaB enzymes (12, 17). Since NADH levels in the cell are higher than NADPH levels and that the electrons of NADH mainly get transferred to oxygen for respiration (2, 51), reducing power is most likely stored by *R. eutropha* during growth in laboratory and fermentative cultures as NADH. Transhydrogenase gene clusters are present in the *R. eutropha* genome that could potentially convert NADH to NADPH for supplying to PhaB activity during PHB production. In the *relA* mutant strain Re2411, NADH levels remain high during nitrogen limitation conditions (Figure 2), thus implicating RelA and ppGpp in the distribution of reducing potential by affecting PHB production during nitrogen starvation. This increase in cellular NADH levels could potentially result in the decreased citrate synthase activity observed in extracts of strain Re2411 (Table 4). This was also suggested in a previous study where the presence of increasing reduced nucleotide cofactor concentrations were shown to have inhibitory effects on *R. eutropha* citrate synthase activity (30).

Furthermore, Ruiz *et al.* (49) have shown a correlation between ppGpp accumulation and PHB degradation in *Pseudomonas oleovorans*, but ppGpp levels during PHB accumulation were not reported. A more recent study has shown statistically significant correlations of PHA production in acetate-fed, mixed microbial consortia with ppGpp levels in the biomass (1). The findings of the aforementioned works, combined with the results of our study, suggest an integral regulatory role for ppGpp throughout the PHB cycle. The absolute PHB negative phenotype observed in *ΔrelA* strain Re2411 (Figure 1A) suggests that nitrogen shortage causes amino acid deprivation, thus activating RelA-dependent ppGpp synthesis and PHB accumulation (68) (Supplemental Figure S5). In addition to ppGpp synthase activity of RelA, ppGpp synthase activity of SpoT has been reported in response to stimuli different than amino acid deprivation (53, 67, 70). Recently, SpoT has been shown to modulate the stringent response during fatty acid shortage, sensing the...
availability of fatty acids through an interaction with ACP (4) It is tempting to speculate that SpoT, in a similar fashion, interacts with a heretofore uncharacterized sensor of products of PHB utilization, triggering the observed ppGpp spike coinciding with a commencement of PHB degradation (49) (Supplemental Figure S5). SpoT-dependent ppGpp accumulation during PHB utilization could explain, for example, the continued repression of genes involved in translation, amino acid biosynthesis and cell motility during PHB utilization observed in this study. An integral role of ppGpp throughout the PHB cycle is further supported by a recent study showing that different intracellular concentrations of ppGpp result in expression of different (sub)sets of genes in *E. coli* (64). This finding suggests the possibility that, likewise, gene expression during PHB production and utilization is governed by different intracellular ppGpp levels. Although we have not identified specific regulators that are directly influenced by ppGpp, the magnitude and variation in intensity of the expression changes throughout the PHB cycle suggests the action of a multitude of effectors.

It is clear, however, that RpoS is playing a role in PHB utilization. Deletion of *rpoS* results in increased PHB mobilization, suggesting that one of the roles of σS is repression of over-utilization of intracellular polymer. The exact role of the master regulator remains to be elucidated, but given the PHB utilization phenotype of the *rpoS* mutant strain (Figure 4), it is clear that σS is regulating nutrient homeostasis during the PHB cycle. RpoS has been shown to have a role in polyhydroxyalkanoate (PHA) biosynthesis in *Pseudomonas oleovorans* (16, 50). However, in *Pseudomonas putida*, an *rpoS* mutation resulted in an increased PHA degradation rate (46), similar to what was observed in this study. The *rpoS* mutant strain in the aforementioned study also demonstrated reduced survival under hydrogen peroxide stress,
similar to our observations with *R. eutropha*. This suggests that PHB accumulation and survival
during stress conditions are intertwined and regulated, at least in part, by RpoS in *R. eutropha*.

Furthermore, the ubiquitous presence of ppGpp as a signaling molecule among prokaryotes and
plants could indicate that the results obtained in this study also apply to other types of carbon
storage in a wide variety of organisms. In fact, glycogen accumulation is hampered in a *relA*
mutant *E. coli* strain (8). Although the effect is not as absolute as the effect observed in this
study, it lends support to a general role of the stringent response in carbon storage.

Although earlier studies have touched on a connection between PHB and ppGpp, we are the first
to provide evidence of a relationship between ppGpp synthase enzyme, RelA, and the
accumulation of PHB. This finding links *R. eutropha* PHB accumulation to the rapidly growing
body of knowledge on the stringent response, potentially providing a new level of understanding
in the process of PHB accumulation usable to optimize production of PHB or other storage
compounds.

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hybridization and processing of microarray samples. We thank Mr. Aidan Smith for assistance
with plasmid constructions. We thank Mr. John Quimby, Ms. Jingnan Lu, and Dr. Jens
Plassmeier for help with editing and review of this manuscript. C.B. is supported by Advanced
Research Projects Agency – Energy (ARPA-E). D.S. is supported by the following foundations:
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Gravenhage and dr. Hendrik Muller’s Vaderlandsch Fonds, This work was funded by the
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between scientists at MIT, Universiti Sains Malaysia, Universiti Putra Malaysia, and SIRIM Berhad. The authors would like to thank the members of this program for their collegial collaborations.


Table 1: Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
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<tr>
<td>R. eutropha</td>
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<td></td>
</tr>
<tr>
<td>H16</td>
<td>Wild type, Gen^x</td>
<td>(69)</td>
</tr>
<tr>
<td>Re2411</td>
<td>H16 Δ(H16_A1337) (ΔrelA)</td>
<td>This study</td>
</tr>
<tr>
<td>Re2424</td>
<td>H16 Δ(H16_A2373) (ΔrpoS)</td>
<td>This study</td>
</tr>
<tr>
<td>Re2061</td>
<td>H16ΔphaCAB</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>Strain for conjugative transfer of plasmids into R. eutropha</td>
<td>(54)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<tr>
<td>pJV7</td>
<td>phaC deletion plasmid, confers Kanamycin resistance</td>
<td>(12)</td>
</tr>
<tr>
<td>pBBR1MCS-2</td>
<td>Broad host range cloning vector, confers Kanamycin resistance</td>
<td>(27)</td>
</tr>
<tr>
<td>pDS1</td>
<td>spoT2 deletion plasmid, confers Kanamycin resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pCB300</td>
<td>rpoS deletion plasmid, confers Kanamycin resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pCJB-CAB</td>
<td>phaCAB deletion plasmid, confers Kanamycin resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pASCB1</td>
<td>pBBR1MCS-2 with R. eutropha rpoS gene inserted into the multiple cloning site</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2: *R. eutropha* H16 gene expression microarray experiment comparing samples of cells growing in the presence of nitrogen (+ N) to samples in PHB production (- N).

<table>
<thead>
<tr>
<th>Code</th>
<th>Functional group¹</th>
<th>No. (%) of genes</th>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>Translation, ribosomal structure and biogenesis</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td>K</td>
<td>Transcription</td>
<td>42 (5.3)</td>
</tr>
<tr>
<td>L</td>
<td>DNA replication, recombination and repair</td>
<td>5 (3.1)</td>
</tr>
<tr>
<td>D</td>
<td>Cell division and chromosomal partitioning</td>
<td>1 (3.6)</td>
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<tr>
<td>O</td>
<td>Posttranslational modification, protein turnover, chaperones</td>
<td>9 (5.7)</td>
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<td>M</td>
<td>Cell envelope biogenesis, outer membrane</td>
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<td>N</td>
<td>Cell motility and secretion</td>
<td>8 (4.4)</td>
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<tr>
<td>P</td>
<td>Inorganic ion transport and metabolism</td>
<td>12 (11.7)</td>
</tr>
<tr>
<td>T</td>
<td>Signal transduction mechanisms</td>
<td>9 (1.1)</td>
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<tr>
<td>C</td>
<td>Energy production and conversion</td>
<td>35 (7.2)</td>
</tr>
<tr>
<td>G</td>
<td>Carbohydrate metabolism and transport</td>
<td>13 (8.7)</td>
</tr>
<tr>
<td>E</td>
<td>Amino acid metabolism and transport</td>
<td>28 (9.5)</td>
</tr>
<tr>
<td>F</td>
<td>Nucleotide metabolism and transport</td>
<td>2 (2.7)</td>
</tr>
<tr>
<td>H</td>
<td>Coenzyme metabolism</td>
<td>3 (1.9)</td>
</tr>
<tr>
<td>I</td>
<td>Lipid metabolism (includes genes in PHB cycle)</td>
<td>20 (6.0)</td>
</tr>
<tr>
<td>Q</td>
<td>Secondary metabolite biosynthesis, transport, and catabolism</td>
<td>8 (8.2)</td>
</tr>
<tr>
<td>R</td>
<td>General function prediction only</td>
<td>51 (6.9)</td>
</tr>
<tr>
<td>S</td>
<td>Function unknown</td>
<td>121 (6.6)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>374</td>
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</tbody>
</table>

¹Functional group categories are based on those of Tatusov, *et al* (62).
Genes in the “Up” category have undergone an upregulation of expression in PHB production compared to growth using fructose as a carbon source. Genes in the “Down” category have undergone a downregulation of genes in PHB production compared to growth using fructose as a carbon source. The percentage genes affected per functional group was based on the number of genes in each group exhibiting significant changes in expression as a percentage of the total number of *R. eutropha* genes in a given functional group based on (40). For all genes represented in this table, *p* < 0.01.
Table 3: Changes in expression of TCA cycle genes during nitrogen depletion in minimal medium cultures with fructose.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log(2) expression: Fructose growth</th>
<th>Log(2) expression: PHB production</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Citrate synthase</td>
<td>11.8</td>
<td>9.5</td>
</tr>
<tr>
<td>2. Aconitase</td>
<td>10.8</td>
<td>8.7</td>
</tr>
<tr>
<td>3. Isocitrate dehydrogenase</td>
<td>10.4</td>
<td>9.5</td>
</tr>
<tr>
<td>4. α-ketoglutarate dehydrogenase</td>
<td>(odhA = 11.5), (odhB = 10.7), (odhL = 11.9)</td>
<td>(odhA = 11.0), (odhB = 10.3), (odhL = 9.8)</td>
</tr>
<tr>
<td>5. Succinyl-CoA synthetase</td>
<td>(sucC = 12.2), (sucD = 11.9)</td>
<td>(sucC = 8.2), (sucD = 6.6)</td>
</tr>
<tr>
<td>6. Succinate dehydrogenase</td>
<td>(sdhA = 12.1), (sdhB = 12.6), (sdhC = 13.1), (sdhD = 13.8)</td>
<td>(sdhA = 10.0), (sdhB = 10.4), (sdhC = 11.0), (sdhD = 12.0)</td>
</tr>
<tr>
<td>7. Fumarase</td>
<td>11.2</td>
<td>8.8</td>
</tr>
<tr>
<td>8. Malate dehydrogenase</td>
<td>11.2</td>
<td>8.3</td>
</tr>
</tbody>
</table>

*All \(p\) values < 0.01, except \(odhB\) (0.04)
Table 4. Key PHB production and citric acid cycle enzyme activities in extracts of nitrogen-limited<sup>a</sup> *R. eutropha* cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PhaA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PhaB&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PhaC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MDH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CS&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H16</td>
<td>25 ± 5</td>
<td>26 ± 2</td>
<td>68 ± 17</td>
<td>5.2 ± 1.5</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td>Re2411</td>
<td>30 ± 6</td>
<td>15 ± 4</td>
<td>45 ± 25</td>
<td>6.0 ± 2.0</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

All enzyme activities defined in U/mg protein; One unit (U) is equivalent to the amount of enzyme needed to convert 1 μmol substrate to product per min at 25°C, except for MDH activity, which is defined as the amount of enzyme needed to convert 1 μmol substrate to product per h at 25°C. All enzyme assays were performed in replicates of three or more.

<sup>a</sup>Cells were grown in minimal medium containing 0.1 % (w/v) NH₄Cl and 2 % (w/v) fructose as described in Materials and Methods. Nitrogen limitation occurred at 12-14 h of culture time.

<sup>b</sup>Cells were harvested for enzyme activity determinations 6-8 h after the onset of nitrogen limitation.

<sup>b</sup>PhaA = β-ketothiolase; PhaB = acetoacetyl-CoA reductase; PhaC = PHB synthase; MDH = malate dehydrogenase; CS = citrate synthase.
Table 5: *R. eutropha* H16 gene expression during growth on fructose compared to gene expression during growth on intracellular PHB

<table>
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<tr>
<th>Code</th>
<th>Functional group</th>
<th>No. (%) of genes</th>
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<th>&gt; 4 fold</th>
<th>2-4 fold</th>
<th>&gt; 4 fold</th>
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<td></td>
<td></td>
<td></td>
<td>Up</td>
<td>Down</td>
<td>Up</td>
<td>Down</td>
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<td></td>
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<td>2-4 fold</td>
<td>&gt; 4 fold</td>
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<td></td>
</tr>
<tr>
<td>J</td>
<td>Translation, ribosomal structure and biogenesis</td>
<td>7 (3.9) 3 (1.7) 30 (16.7) 5 (2.8)</td>
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<td></td>
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<td></td>
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<tr>
<td>K</td>
<td>Transcription</td>
<td>26 (3.3) 5 (0.6) 34 (4.3) 10 (1.3)</td>
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<tr>
<td>L</td>
<td>DNA replication, recombination and repair</td>
<td>2 (1.2) 1 (0.6) 22 (13.6) 3 (2.7)</td>
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<td></td>
<td><strong>Cellular processes</strong></td>
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<td>Cell division and chromosomal partitioning</td>
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<td>O</td>
<td>Posttranslational modification, protein turnover, chaperones</td>
<td>8 (5.1) 3 (1.9) 11 (7.0) 5 (3.2)</td>
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<tr>
<td>M</td>
<td>Cell envelope biogenesis, outer membrane</td>
<td>3 (1.3) 0 (0.0) 21 (9.0) 19 (8.2)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>N</td>
<td>Cell motility and secretion</td>
<td>3 (1.7) 2 (1.1) 18 (10.0) 9 (5.0)</td>
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<td>P</td>
<td>Inorganic ion transport and metabolism</td>
<td>7 (6.8) 11 (10.7) 19 (18.6) 3 (9.0)</td>
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<tr>
<td>T</td>
<td>Signal transduction mechanisms</td>
<td>5 (0.6) 10 (1.3) 16 (2.0) 4 (0.5)</td>
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<td><strong>Metabolism</strong></td>
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<tr>
<td>C</td>
<td>Energy production and conversion</td>
<td>17 (3.5) 15 (3.1) 24 (4.9) 28 (5.8)</td>
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<td>G</td>
<td>Carbohydrate metabolism and transport</td>
<td>5 (3.3) 5 (3.3) 24 (16.1) 27 (18.0)</td>
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<tr>
<td>E</td>
<td>Amino acid metabolism and transport</td>
<td>10 (3.4) 8 (2.7) 52 (17.6) 27 (9.1)</td>
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<td>F</td>
<td>Nucleotide metabolism and transport</td>
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<td></td>
<td></td>
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<tr>
<td>H</td>
<td>Coenzyme metabolism</td>
<td>6 (3.8) 3 (1.9) 18 (9.1) 5 (3.2)</td>
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<td>I</td>
<td>Lipid metabolism (includes genes in PHB cycle)</td>
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<tr>
<td>Q</td>
<td>Secondary metabolite biosynthesis, transport, and catabolism</td>
<td>1 (1.0) 4 (4.1) 6 (6.1) 4 (4.1)</td>
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<td><strong>Uncharacterized or poorly characterized</strong></td>
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<td><strong>Total</strong></td>
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</table>

1Functional group categories are based on those of Tatusov, *et al.* (62).
2Genes in the “Up” category have undergone an upregulation of expression in PHB utilization compared to growth using fructose as a carbon source. Genes in the “Down” category have
undergone a downregulation of genes in PHB utilization compared to growth using fructose as a carbon source. The percentage genes affected per functional group was based on the number of genes in each group exhibiting significant changes in expression as a percentage of the total number of *R. eutropha* genes in a given functional group based on (40). For all genes represented in this table, $p < 0.01$. 
Figure legends:

**Figure 1.** PHB production in *R. eutropha* H16 in relation to ppGpp accumulation. (A) *R. eutropha* strain H16 and the ppGpp synthase deletion strain (Re2411) were incubated in minimal medium for 69 h. Intracellular PHB content was assayed as described in Materials and Methods (n = 4). (B) Induction of stringent response by DL-norvaline and its effect on PHB accumulation during early exponential growth (OD$_{600}$ ≈ 0.7), while nitrogen is still present in the medium. DL-norvaline was added (at time = 20 min, indicated by the arrow) to a final concentration of 0.1% (w/v) (n = 3).

**Figure 2.** Reduced cofactor quantitation in *Ralstonia eutropha* wild-type (H16) and spoT2 deletion (Re2411) strains. In (A), NADH is quantified in H16 and Re2411 cells during growth (6 h) and nitrogen starvation (24 h). In (B), NADPH is quantified in H16 and Re2411 cells during growth (6 h) and nitrogen starvation (24 h). *R. eutropha* cells were prepared and reduced cofactors were quantified as described in Materials and Methods (n = 3).

**Figure 3.** Expression of β-ketothiolases (A), acetoacetyl-CoA reductases (B), PHB synthases (C), phaP genes (D), phaR and phaM genes (E), intracellular and putative intracellular phaZ genes (F), and putative extracellular phaZ genes (G) under growth, PHB production, and PHB utilization conditions. In (A), the expression profile of *phaA* and *bktB* is shown. In (B), the expression of *phaB1*, *phaB2*, and *phaB3* is shown. In (C), the PHB synthase gene *phaC1* and the synthase homolog *phaC2* are shown. For all values represented in (A) and (C), p > 0.05. In (B), p > 0.05, except for expression changes in *phaB3*, for which p < 0.01. In (D), p values of *phaP1*, *phaP3*, and *phaP4* are <0.01 and for *phaP2*, 0.05 > p ≥ 0.01. In (E), the p value for *phaM* expression is >0.05 and for *phaR*, 0.05 > p ≥ 0.01. In (F), the p value of all
genes under all conditions is less than 0.01. In (G), the $p$ value of $phaZ6$ is $<0.01$, and the $p$ value for $phaZ4$ and $phaZ7$ are $>0.05$.

**Figure 4. Intracellular PHB content of Ralstonia eutropha strains incubated in PHB utilization media.**

*R. eutropha* strain H16/pBBR1MCS-2 (wild type with empty vector, boxes), Re2424/pBBR1MCS-2 ($rpoS$ deletion strain with empty vector; diamonds), and Re2424/pASCB1 ($rpoS$ deletion strain complemented with the $rpoS$ gene in trans; triangles), all containing intracellular PHB, were incubated in PHB utilization medium for 72 h. Intracellular PHB content was assayed as described in Materials and Methods (n = 3).