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

3 Running Title: Microarray analysis of *Ralstonia eutropha* H16

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27 **Abstract**

28 PHB production and mobilization in *Ralstonia eutropha* is well studied, but in only a small
29 number of instances has PHB production been explored in relation to other cellular processes.
30 We examined global gene expression of wild-type *R. eutropha* throughout the PHB cycle;
31 growth on fructose, PHB production using fructose following ammonium depletion, and PHB
32 utilization in the absence of exogenous carbon after ammonium was resupplied. Our results
33 confirm or lend support to previous results regarding expression of PHB-related genes and
34 enzymes. Additionally, genes for many different cellular processes, such as DNA replication,
35 cell division and translation, are selectively repressed during PHB production. In contrast,
36 expression of genes under control of the alternative sigma factor σ^{54} increases sharply during
37 PHB production and is repressed again during PHB utilization. The global gene regulation
38 during PHB production is strongly reminiscent of the gene expression pattern observed during
39 stringent response in other species. Furthermore, a ppGpp synthase deletion mutant did not show
40 accumulation of PHB and chemical induction of the stringent response with DL-norvaline caused
41 increased accumulation of PHB in the presence of ammonium. These results indicate that the
42 stringent response is required for PHB accumulation in *R. eutropha*, helping to elucidate a thus
43 far unknown physiological basis for this process.

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44 **Introduction**

45 Many organisms have been reported to accumulate storage compounds when encountering
46 nutrient stress (10). One of the best studied examples of such a storage compound is poly(3-
47 hydroxybutyrate) (PHB). Accumulation of PHB has been observed in a wide range of organisms
48 and stress conditions (2, 47). PHB accumulation has been intensively studied for both its
49 industrial potential and its role in survival of environmental stress (36, 49, 59). PHB
50 accumulation is regulated, in part, by PhaR, which represses transcription of the phasin gene
51 instrumental in PHB granule formation, *phaP1* (42, 73). When PHB starts accumulating in the
52 cell, PhaR binds to the polymer instead of its DNA binding site, thus removing repression of
53 *phaP1* expression (32, 71). High expression of *phaP1* subsequently results in proper formation
54 of PHB granules, coated predominantly by PhaP1, PhaR, the PHB synthase PhaC1 and the PHB
55 depolymerase PhaZ1 (24, 48). Although this process is well studied, thus far it is unclear what
56 governs the threshold accumulation to set this regulatory network in motion.

57 One possibility is that initiation of PHB accumulation occurs as a result of the stringent response.
58 This process is a mechanism providing protection against nutrient stress in a wide range of
59 organisms (for recent reviews, see (33, 41)). The stringent response is governed by the
60 nucleotide guanosine-tetraphosphate (ppGpp), which destabilizes the RNA polymerase-σ^{70}
61 holoenzyme and thus strongly reduces transcription of genes under control of σ^{70} or its homologs
62 in other organisms (22, 33). This inhibition of σ^{70} controlled genes results in strong induction of
63 genes under control of alternative σ-factors, such as σ^{54} (6). Additionally, ppGpp has been shown
64 to directly inhibit translation (28, 60). This process could potentially reduce alternative carbon
65 sinks, thus triggering PHB accumulation.

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67 To determine if the physiological basis for PHB carbon storage could be found in the stringent
68 response, we examine the model organism for polymer accumulation, *Ralstonia eutropha* H16.
69 *R. eutropha* H16 is probably the most widely used model organism for the study of PHB
70 accumulation. A large number of scientific works have been published about the PHB production
71 and utilization of *R. eutropha* (referred to in this work as the "PHB Cycle"), including a number
72 of reviews (47, 48, 58, 59). The genome sequence of *R. eutropha* H16 has, in the past decade,
73 become available (40, 52), further establishing it as the organism of choice for the study of PHB
74 accumulation. However, the stringent response has not been studied in *R. eutropha* previously.

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

84 In this study we used custom designed *R. eutropha* microarray chips to compare gene expression
85 under three conditions: cell growth (with ammonium as nitrogen source) in the presence of
86 fructose as the sole carbon source, PHB production (after ammonium has been depleted) in the
87 presence of fructose as the sole carbon source and utilization of PHB as the sole carbon source
88 (after ammonium has been resupplied). The emerging global gene expression pattern was further
89 studied through promoter identification of the regulated genes. The physiological origin of the

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90 observed global expression changes was studied in more detail using a *R. eutropha* strain lacking
91 the *relA* homolog and chemically inducing the stringent response using DL-norvaline. The role
92 of the σ^S gene, *rpoS*, in the PHB cycle was also examined.

93 **Materials and Methods**

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

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

105 Cell growth and total cellular RNA isolation procedure. Individual colonies of *R. eutropha* H16
106 grown on a tryptic soy agar (TSA) plate were inoculated into 5 mL of dextrose-free tryptic soy
107 broth (TSB, Becton Dickinson, Sparks, MD) and grown for 24 h. Cultures for microarray
108 analysis were all performed in triplicate. Aliquots of 0.5 mL of overnight culture were inoculated
109 into 250 mL shake flasks containing 50 mL of minimal medium, modified from (35), containing
110 0.1 % NH_4Cl and 2 % (w/v) fructose. These cultures were grown for 24 h. Overnight cultures
111 were inoculated to an initial OD_{600} of 0.1 into 250 mL shake flasks containing 50 mL of minimal

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112 medium containing 0.05 % NH_4Cl and 2 % (w/v) fructose. Cultures were grown for 12 h.
113 Cultures for sampling were inoculated to an initial OD_{600} of 0.05 in 250 mL shake flasks
114 containing 50 mL of minimal medium with 0.05 % NH_4Cl and 2 % (w/v) fructose. All flask
115 cultures were grown at 30 °C with agitation (200 rpm). Unless otherwise mentioned, all growth
116 media in this study contained 10 μg/mL gentamicin. In selecting culture conditions for
117 microarray analysis, we focused on logarithmic growth (when the nitrogen source in the culture
118 was depleted by 50%), PHB production (when nitrogen in the culture was depleted), and PHB
119 utilization (when cells were actively utilizing intracellular PHB stores as a carbon and energy
120 source). The concentration of NH_4^+ in the growth medium was monitored using an Ammonia
121 Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. An aliquot of cells (OD_{600}
122 equivalent = 2.5) was harvested at an NH_4^+ concentration of approximately 0.025 %, and another
123 aliquot of cells (also an OD_{600} equivalent = 2.5) was harvested 2 h after depletion of nitrogen in
124 the media. For PHB utilization samples, cells were grown in PHB production medium for 48 h,
125 washed and transferred to PHB utilization medium, which was free of extracellular carbon and
126 contained 0.1% NH_4Cl, allowing cells to utilize PHB stores as the main carbon source.
127 Intracellular PHB content was monitored (see below) and, after 6 h of incubation, cells were
128 harvested and later used for RNA isolation. All culture aliquots were treated with 2 volumes of
129 RNA Protect reagent (QIAgen, Valencia, CA). Cells were centrifuged at 5000 rpm, growth
130 medium was removed, and cell pellets were stored at -80°C until RNA extraction.

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

133 Microarray data analysis. Microarray data analysis was performed as described previously (9),
134 using Affymetrix GCOS v.1.4 for data extraction and robust multichip average (RMA; ArrayStar

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135 Software, Madison, WI) with quantile normalization for further analysis. Annotation of genes in
136 the final output was performed based on Pohlmann, et al. (40). Genes of interest with a
137 statistically significant change in expression ($p < 0.01$) were selected for further study. Gene
138 expression values are denoted as log(2) of actual expression values, similar to previous work (9).

139 Growth of strains and PHB quantitation. A *relA* homolog (Supplemental Figure S1) (*spoT2*,
140 locus tag H16_A1337) was identified in the *R. eutropha* H16 genome using the genome
141 information broker (GIB) (19). A deletion mutant strain, denoted Re2411 (Table 1), was
142 constructed using methods described previously (9) using primer pairs listed in Supplemental
143 Table 1. Four cultures of H16 and Re2411 were grown in 50 mL minimal medium containing 0.1
144 % NH_4Cl and 2 % fructose for 70 h at 30 °C with agitation (200 rpm). Aliquots of culture (5 mL)
145 were removed at 0, 12, 20, 44 and 69 h for intracellular PHB quantitation.

146 An *rpoS* homolog (H16_A2373) was also identified using GIB (19). A deletion mutant strain,
147 denoted Re2424 (Table 1), was constructed using methods described previously (9), using primer
148 pairs listed in Supplemental Table 1. An *rpoS* complementation plasmid, pASCB1 (Table 1), was
149 constructed by inserting the *rpoS* gene into pBBR1MCS-2, as follows. The *R. eutropha rpoS*
150 gene was amplified by PCR using primers rpoScompFW and rpoScompFW (Supplemental Table
151 1). The PCR product was cut using SalI and XbaI restriction endonucleases and ligated into a
152 SalI/XbaI-cut pBBR1MCS-2. Three cultures of H16/pBBR1MCS-2, Re2424/pBBR1MCS-2, and
153 Re2424/pASCB1 were grown in 50 mL minimal medium containing 0.1 % NH_4Cl and 2 %
154 fructose for 72 h at 30 °C with agitation (200 rpm). Aliquots of culture (5 mL) were removed at
155 0, 24, 48 and 72 h for intracellular PHB quantitation. The remaining cultures were washed twice
156 with sterile 0.85% saline solution and the cells were resuspended in PHB utilization medium

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157 containing 0.2 % NH_4Cl for 72 h at 30 °C with agitation (200 rpm). Aliquots of culture (5 mL)

158 were removed at 0, 24, 48 and 72 h for intracellular PHB quantitation.

159 For quantitation of intracellular PHB, 5 mL aliquots of culture were transferred to pre-weighed

160 borosilicate glass tubes at various time points during the PHB production cycle. Cells were

161 pelleted, washed with 5 mL of cold water and pelleted again and dried *in vacuo* at 80°C. The

162 PHB content and cell dry weight (CDW) were determined from the dried samples using

163 established methods (7, 26).

164 Enzyme assays. Cultures of H16 and Re2411 were grown in 50 mL minimal medium containing

165 0.1 % NH_4Cl and 2 % fructose for 72 h at 30 °C with agitation (200 rpm). Cells were grown for

166 up to 24 h, then pelleted and stored at -80°C. Pellets were thawed on ice and resuspended in 20

167 mM Tris-HCl buffer (pH 7.5) for PHB synthase and malate dehydrogenase assays, 150 mM

168 EPPS buffer (pH 8.0) for β-ketothiolase assays, 50 mM KH_2PO_4 (pH 6.0) for Acetoacetyl-CoA

169 dehydrogenase assays, and 100 mM Tris-HCl (pH 8.0) for citrate synthase assays. One milliliter

170 of suspended cells was placed, along with ~100 μL 0.1-mm zirconia/silica beads (Biospec

171 products, Bartlesville, OK), in a 2-mL microcentrifuge tube and sealed. Tubes were loaded onto

172 a FastPrep-24 machine (MP Biomedicals, Solon, OH) and treated twice at 6.0 m/s for 30 sec,

173 with a 5-min rest period in between treatments. Following lysis, samples were centrifuged for 5

174 min at 4°C to pellet cell debris. Clarified cell extracts were removed to a clean tube and enzyme

175 assays were performed.

176 Discontinuous PHB synthase (PhaC) activity assays were performed as described previously

177 (15), using 3HB-CoA as the substrate. The 3HB-CoA sample was a generous gift from Dr. Mimi

178 Cho and Professor JoAnne Stubbe (Department of Chemistry, MIT). Activities in cell extracts

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179 were monitored for 5 min for each assay. β-ketothiolase (PhaA) activity assays were performed
180 as described previously (55). Acetoacetyl-CoA reductase (PhaB) assays were performed as
181 described previously (12, 23). Citrate synthase assay was performed according to (18, 56), using
182 acetyl-CoA and oxaloacetate as the substrates. Malate dehydrogenase activity was monitored by
183 following reduction of NAD^+ to NADH during production of oxaloacetate from malate. The
184 assay mixture was composed of 600 μL 100 mM Tris-HCl (pH 7.6), 100 μL 8 mM $MnSO_4$, 100
185 μL 5 mM NAD^+, 100 μL 8 mM malic acid. The reaction was started upon addition of 100 μL
186 cell extract, and activity was monitored by following A_{340} over time. In all cases, one unit (U) of
187 enzyme activity is the amount of enzyme needed to convert 1 μmol substrate to product per min
188 at 25°C.

189 Reduced pyridine nucleotide cofactor quantitation. Intracellular levels of NADH and NADPH
190 were quantified in *R. eutropha* H16 and Re2411 cells during growth (6 h culture) and nitrogen
191 limitation (24 h culture) in minimal medium containing 0.1 % NH_4Cl, 2 % fructose, and 10
192 μg/mL gentamicin. A volume of cells equivalent to an OD_{600} of 5.0 were harvested, centrifuged
193 for 5 min at 2800 × *g*, washed in 5 mL of 0.85% saline, and stored at -80°C. Reduced pyridine
194 nucleotides (NADH and NADPH) were extracted from cells according to (20). Intracellular
195 NADH concentrations were measured using an alcohol dehydrogenase assay coupled to the
196 reduction of phenazine ethosulfate (PES) and 3-(4,5-dimethylthiazolyl-2)-2,5-
197 diphenyltetrazolium bromide (MTT), as described previously (20, 66). The substrate was 3%
198 (v/v) ethanol. Intracellular NADPH concentrations were measured by coupling with glucose 6-
199 phosphate dehydrogenase coupled to reduction of PES and MTT, as previously described (20,
200 74). The substrate was 25 mM glucose 6-phosphate. NADH and NADPH quantitations were
201 performed by comparison with a standard curve of each nucleotide cofactor.

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202 Chemical induction of the stringent response. Six individual cultures of H16 were grown on 50
203 mL minimal medium containing 0.1 % NH_4Cl and 2 % fructose at 30 °C with agitation (200
204 rpm). During exponential growth ($OD_{600} \approx 0.7$), 5 mL aliquots of culture were sampled for CDW
205 determination and PHB quantitation. After 20 min, another 5 mL sample was taken and DL-
206 norvaline was added to three cultures of H16 to a final concentration of 0.1 % (w/v). At the time
207 of addition, 5 mL of culture was sampled for CDW determination and PHB quantitation.
208 Sampling was repeated every 20 min for 100 min after addition of DL-norvaline. PHB content
209 and CDW were determined as described above.

210 Promoter analysis. Upstream sequences of strongly regulated genes were retrieved from the GIB
211 and further analyzed using MEGA 5 (61). Potential σ^{54} promoters were manually identified
212 based on the consensus sequence published previously (3). Potential σ^{70} promoters were
213 identified using BPROM (Softberry).

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

217 **Results**

218 *R. eutropha* H16 fructose cultures – Gene expression in the presence or absence of nitrogen.
219 We have examined global gene expression in *R. eutropha* H16 under growth conditions in the
220 presence of nitrogen, under nitrogen depletion, and upon reintroduction of nitrogen. A total of
221 2263 genes exhibited a greater than 2-fold change in expression ($p < 0.01$) when nitrogen was
222 depleted in fructose cultures. Of these 2263 genes, roughly half were observed to be upregulated

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223 and half were downregulated after nitrogen depletion. The magnitude of this change suggests
224 that major physiological changes occur in *R. eutropha* upon entrance into PHB production.

225 Table 2 breaks down these 2263 genes into functional groups, categorized as per Tatusov, *et al.*
226 (62). Transcription of several of these groups of genes was shown to be generally repressed
227 during PHB production. The downregulated functional groups include genes involved in
228 nucleotide metabolism (55.7% down), DNA replication (32.2% down), cell envelope biogenesis
229 (28% down), cell division (40.6% down), and translation (67.6% down) (Table 2). Additionally,
230 cell motility and secretion genes were observed to be generally downregulated (30.6% down) in
231 the absence of nitrogen (Table 2). An earlier study has shown that *R. eutropha* stops producing
232 flagella and that the amount of flagellin decrease in cells during nitrogen starvation (45), The *fliC*
233 gene was also shown to be downregulated in stationary phase in a previous *R. eutropha* gene
234 expression study (37). Our results show that the flagellin gene, *fliC* (H16_B2360), is indeed
235 downregulated, 9.3-fold, during PHB production compared to growth. Furthermore, genes of the
236 TCA cycle were all observed to be downregulated during PHB production (Table 3), although
237 some exhibited more of a decrease in expression than others. These data suggest that there is a
238 change in the flux of acetyl-CoA in the cell when *R. eutropha* enters the PHB production phase,
239 providing a potential additional level of control for PHB production, since the precursor of PHB
240 is produced using two molecules of acetyl-CoA.

241 Expression of a large group of genes involved in inorganic ion or carbohydrate transport and
242 metabolism was shown to be increased during nitrogen starvation. The upregulated genes
243 involved in inorganic ion transport and metabolism can largely be accounted for by genes
244 involved in nitrogen uptake and metabolism. For example, an operon encoding potential nitrogen
245 scavenging transporters and enzymes (locus tags H16_A1075 through H16_A1087) is

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246 upregulated an average of 491-fold during nitrogen starvation. The upregulated genes involved
247 in carbohydrate metabolism were most notably involved in fructose transport and metabolism
248 and, surprisingly, the two *cbb*-operons encoding Calvin cycle enzymes for CO_2 fixation.

249 The pattern of this expression change is similar to those observed during the stringent response
250 in various other species (11, 14, 34, 63) suggesting a central role for the nucleotide guanosine
251 tetraphosphate (ppGpp) in regulation of PHB production. As mentioned above, inhibition of σ^{70}
252 controlled genes by action of ppGpp results in a strong induction of genes under control of
253 alternative σ-factors, such as σ^{54} (6). To further examine this possibility, we have analyzed the
254 upstream sequences of the most strongly regulated genes. σ^{54}-family promoter elements were
255 identified in the upstream sequences of 79 out of 96 genes with a greater than 50-fold increase in
256 expression (Supplemental Figure S2). In the upstream sequences of most strongly downregulated
257 genes the 'housekeeping' σ^{70}-family promoter was identified. In agreement with earlier
258 observations in *E. coli* (21), a guanine is conserved at the -5 position of rRNA promoters and
259 promoters of the most strongly repressed genes under nitrogen starvation (data not shown).

260 Involvement of RelA (SpoT2) in PHB biosynthesis. To ascertain that changes in gene expression
261 mentioned above can be attributed to a stringent response, we created a ppGpp synthase (*spoT2,*
262 locus tag H16_A1337) deletion strain, H16Δ*spoT2,* denoted Re2411 (Table 1). Under nitrogen
263 starvation, Re2411 accumulated no detectable PHB (Figure 1A), confirming the hypothesis that
264 the stringent response is required for PHB accumulation. To obtain further evidence that the lack
265 of PHB accumulation could be attributed to a lack of stringent response, DL-norvaline was used
266 to chemically induce a stringent response during exponential growth in *R. eutropha* H16.
267 Following addition of DL-norvaline, wild-type *R. eutropha* cultures accumulated more PHB than
268 an untreated control group (Figure 1B).

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269 It has been suggested that expression of PHB production genes (*phaCAB*) is constitutive
270 throughout the PHB cycle (29). This suggestion was confirmed by our gene expression data (see
271 below). Thus, it is likely that the stringent response in *R. eutropha* is controlling PHB production
272 at a level other than transcription, such as enzyme activity. To investigate whether SpoT2 (RelA)
273 affects activities of key PHB biosynthetic enzymes, we examined activities in extracts of the
274 wild-type and *spoT2* mutant strains during nitrogen limitation (Table 4). No significant
275 difference in PhaA or PhaC activities was seen in H16 or Re2411, but the *spoT2* mutant strain
276 did exhibit a small (<2 fold), but significant, decrease in PhaB activity. It has been shown in *R.*
277 *eutropha* that both PhaB1 and PhaB3 enzymes exhibit acetoacetyl-CoA reductase activity in
278 minimal medium cultures with fructose as the carbon source (12). It is unclear whether one or
279 both PhaB enzymes are responsible for the decrease in activity in strain Re2411. Since TCA
280 cycle genes were shown to be downregulated during PHB production in *R. eutropha* H16, we
281 examined some TCA cycle enzyme activities in strains H16 and Re2411, to determine if the lack
282 of *relA* affected the activity, and thus the flow of carbon, through the TCA cycle. Table 4 shows
283 that malate dehydrogenase activity was not significantly altered in the presence or absence of an
284 intact RelA enzyme, suggesting that these activities were not modulated by the stringent
285 response. Citrate synthase activity, on the other hand, was lower in extracts of strain Re2411
286 compared to those of the wild type suggesting expression or activity of citrate synthase is
287 occurring in the *relA* mutant. Given the reduction in PhaB activity, we hypothesized that this
288 would have an effect on intracellular concentrations of reduced cofactors, both of which were
289 shown to be able to act as substrates in acetoacetyl-CoA reductase reactions (12). Quantitation of
290 NADH and NADPH levels in strains H16 and Re2411 revealed an increase in intracellular
291 NADH concentration of Re2411 cells during nitrogen starvation in minimal medium (24 h). No

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292 significant differences in NADPH levels were detected in either strain during growth or nitrogen
293 limitation (Figure 2). The results shown here suggest a link to RelA activity, PhaB activity, and
294 reduced cofactor levels in the *R. eutropha* cell during PHB production.

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

302 *R. eutropha* H16 gene expression changes of cells in PHB utilization, compared to growth on
303 fructose. Although PHB utilization in *R. eutropha* has been less well-studied than PHB
304 production, several important physiological changes occur when the culture resumes growth
305 (PHB utilization in the presence of nitrogen and absence of exogenous carbon). Consistent with
306 past works in the literature regarding changes in expression of PHB-related genes like *phaP* and
307 *phaZ* during transition from PHB production to PHB utilization (29, 42, 44, 72), our results show
308 that expression patterns of these genes are linked to and generally follow the production of
309 intracellular polymer (see below). Furthermore, we observed that the global gene expression
310 pattern returns to that observed during growth on fructose. To study the differences between the
311 physiology of extracellular carbon source utilization versus intracellular carbon source utilization
312 we have compared gene expression during growth and PHB utilization.

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313 Table 4 shows a breakdown of genes exhibiting a change in expression during growth with PHB
314 utilization (cells containing intracellular PHB grown in the presence of no extracellular carbon
315 and abundant nitrogen), compared to growth on fructose. Expression of a total of 1166 genes
316 changed greater than 2-fold between growth on fructose and PHB utilization. Of these genes, 745
317 are repressed during PHB utilization relative to growth on fructose. Table 4 categorizes these
318 genes in functional groups according to Tatusov, *et al.* (62). A large group of genes repressed
319 (relative to growth on fructose) during PHB utilization were also seen to be repressed during
320 PHB production. These genes mostly classify within the functional groups cell motility (15%
321 down), amino acid biosynthesis (26.7% down), carbohydrate metabolism (34.1% down), and
322 translation (19.5% down).

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

330 The genes that were most strongly induced during PHB utilization encode hypothetical proteins
331 or proteins with a general predicted function only, reflecting the limited knowledge on this
332 process. Interestingly, expression of four (out of six predicted in the genome) genes encoding
333 cold-shock family proteins increases an average 9-fold, suggesting a role for these gene products
334 during PHB utilization.

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335 Finally, a group of genes induced during PHB production remained induced during PHB
336 utilization. Most notably, the periplasmic nitrate reductase (locus tag H16_PHG209 –
337 H16_PHG213) is induced an average 20-fold. Additionally, genes encoding a *bo*$_3$ quinol oxidase
338 (locus tag H16_B1025 – H16_B1028) and a *bb*$_3$ cytochrome c oxidase (locus tag H16_B2058 –
339 H16_B2062) were upregulated an average 15-fold and 10-fold respectively.

340 Changes in expression of PHB-related genes throughout the PHB cycle. Figure 3 illustrates the
341 changes in expression of all PHB production pathway and related genes present in *R. eutropha*
342 H16. The main PHB production gene cluster in *R. eutropha*, *phaCAB,* the regulator gene *phaR*,
343 and recently-discovered granule-associated nucleation factor gene *phaM* (39) did not appear to
344 exhibit dramatic changes in expression under PHB production or utilization conditions (Figure
345 3A, B, C, and E), confirming that these genes are constitutively expressed throughout the cell
346 cycle and PHA production as hypothesized previously (29). One caveat regarding this result is
347 that the *p*-value of each gene expression comparison is high, suggesting high variation among
348 these probe sets. However, this is still an interesting result, because it suggests that the regulation
349 of PHB production in *R. eutropha* may not be achieved through the regulation of *phaCAB*
350 expression alone, given the irregular changes in expression pattern of the genes in that operon.
351 Also of note is the increase in expression of the *phaZ* genes under nitrogen limitation. The *phaZ1*
352 gene increased in expression slightly when cells produce PHB. This gene was observed to
353 maintain a similar level of expression in PHB utilization compared to PHB production (Figure
354 3F). This makes sense, as PhaZ1 has been shown to play an integral role in PHB utilization (65,
355 72). The most dramatic increase in expression is the *phaZ2* gene, encoding an intracellular PHB
356 depolymerase, which increases 256-fold (Figure 3F). These results are similar to quantitative
357 RT-PCR results examining the amount of *phaZ1* and *phaZ2* transcription in the H16 cell,

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358 published previously (29). The *phaZ2* results also agree with a trend seen in a previous
359 transcriptome analysis of *R. eutropha* (37). The *phaZ2* gene also exhibited a dramatic decrease in
360 expression from PHB production to PHB utilization conditions. As PhaZ2 has also been shown
361 to play a role in PHB utilization (72), the gene expression pattern suggests a role for PhaZ2 that
362 is much different than PhaZ1 in the PHB cycle. Three genes in the *R. eutropha* genome are
363 predicted to encode extracellular PhaZ enzymes, albeit no extracellular PHB depolymerase
364 activity has ever been detected in *R. eutropha* cultures. One of these putative depolymerase
365 genes, *phaZ6,* was shown to be upregulated during PHB production (Figure 3G). These results
366 agree with a trend observed previously (37).

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

376 Besides the genes discussed in Figure 3, there are many other genes whose putative products
377 have been associated with PHB production (48). Aside from *phaA* and *bktB*, there are 13 other β-
378 ketothiolase homologs present in *R. eutropha* (31, 37, 48). According to recent transcriptome
379 analysis, 6 of the 15 total β-ketothiolase homologs (including *phaA* and *bktB*) were actually
380 expressed during growth and PHB production using gluconate as a carbon source (31).

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381 Examination of our array data revealed that 7 β-ketothiolase homologs (including *phaA* and
382 *bktB*) were observed to be expressed under growth and PHB production on fructose
383 (Supplemental Table 2). Four of the 5 additional active β-ketothiolase homologs in our study are
384 the same as those that were discovered previously (31), the one exception being H16_B0759,
385 which our data has shown to be expressed during PHB production (Supplemental Table 2).
386 Several acetoacetyl-CoA reductase genes and homologs are also present in the *R. eutropha*
387 genome (48). Two acetoacetyl-CoA reductases, PhaB1 and PhaB3, have been shown to be
388 responsible for most of the 3HB-CoA production in the cell, using fructose as the extracellular
389 source of carbon (12). Of the other acetoacetyl-CoA reductase homologs, only 8 are shown to be
390 expressed in the *R. eutropha* cell at any time during this study (Supplemental Table 3).

391 A role for RpoS (σ^S) in the PHB cycle. In *E. coli*, the *rpoS* gene is upregulated in response to
392 many stresses, among them carbon starvation (5). Since PHB utilization conditions represent a
393 form of extracellular carbon starvation, we postulated that RpoS in *R. eutropha* may play a role
394 in intracellular PHB mobilization. To address this possible relationship, we constructed an *rpoS*
395 deletion mutant strain of *R. eutropha* and observed the behavior of the resulting strain, Re2424,
396 under different stress conditions. As is common in *rpoS* mutant strains of other bacterial species
397 (25, 50), *R. eutropha* Re2424 exhibited decreased resistance to hydrogen peroxide, as compared
398 to wild type (Supplemental Figure S4). Also, no change in PHB production was seen between
399 the wild-type and *rpoS* mutant strains (data not shown). During PHB utilization, however,
400 Re2424 exhibited an increased rate and an increased overall level of polymer mobilization, as
401 compared to strain H16. Strain Re2424 was complemented with *rpoS* gene *in trans* on plasmid
402 pASCB1 (Table 1), and the resulting strain utilized intracellular PHB at a similar rate and to a
403 similar extent as wild type (Figure 4).

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404 **Discussion**

405 In recent years, genome wide expression microarray studies have been conducted to shed new
406 light on the metabolism of *R. eutropha,* solidly establishing the technique in this organism (9,
407 37). Although a previous microarray study aimed at providing new insights in the PHB cycle in
408 *R. eutropha* has been published (37), the resolution of that study was much lower, showing only
409 297 genes changing expression between growth and PHB production (as opposed to 2267 genes
410 in this study). Moreover, no explanation for the global changes in gene expression was provided.
411 In contrast, this study suggests that the bacterial alarmone, guanosine tetraphosphate (ppGpp),
412 plays a significant role in the production of PHB in *R. eutropha* and can account for the global
413 expression pattern observed in both studies. However, the set of genes from the previous work
414 (37) included *accC2* (encoding biotin carboxylase), *fabG* (encoding 3-oxoacyl-[ACP] reductase),
415 *fabZ* (encoding 3-hydroxymyristoyl-[ACP] dehydratase), and H16_A3307 (encoding a putative
416 enoyl-CoA hydratase). Our microarray data has confirmed these trends seen in the previous
417 study, as transcription of all these genes decreased in PHB production compared to growth (data
418 not shown).

419 Some connections between ppGpp and the PHB cycle have been reported previously, but to date
420 few works have shown evidence of a relationship between PHB synthesis and the stringent
421 response. The PHB negative phenotype, similar to that observed in Re2411 in this study, has
422 previously been observed in a *Rhizobium etli rsh*-negative mutant (13). However, the phenotype
423 received little attention in that study and no follow-up work has been reported. Evidence for
424 stringent response control of PHB production in *R. eutropha* is observed in how strain Re2411
425 stores its reducing equivalents when it is unable to synthesize PHB. Typically, reducing
426 equivalents are stored in the PHB polymer during times of stress, through the reduction of

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427 acetoacetyl-CoA by NADPH-dependent action of PhaB enzymes (12, 17). Since NADH levels in
428 the cell are higher than NADPH levels and that the electrons of NADH mainly get transferred to
429 oxygen for respiration (2, 51), reducing power is most likely stored by *R. eutropha* during
430 growth in laboratory and fermentative cultures as NADH. Transhydrogenase gene clusters are
431 present in the *R. eutropha* genome that could potentially convert NADH to NADPH for
432 supplying to PhaB activity during PHB production. In the *relA* mutant strain Re2411, NADH
433 levels remain high during nitrogen limitation conditions (Figure 2), thus implicating RelA and
434 ppGpp in the distribution of reducing potential by affecting PHB production during nitrogen
435 starvation. This increase in cellular NADH levels could potentially result in the decreased citrate
436 synthase activity observed in extracts of strain Re2411 (Table 4). This was also suggested in a
437 previous study where the presence of increasing reduced nucleotide cofactor concentrations were
438 shown to have inhibitory effects on *R. eutropha* citrate synthase activity (30).

439 Furthermore, Ruiz *et al.* (49) have shown a correlation between ppGpp accumulation and PHB
440 degradation in *Pseudomonas oleovorans*, but ppGpp levels during PHB accumulation were not
441 reported. A more recent study has shown statistically significant correlations of PHA production
442 in acetate-fed, mixed microbial consortia with ppGpp levels in the biomass (1). The findings of
443 the aforementioned works, combined with the results of our study, suggest an integral regulatory
444 role for ppGpp throughout the PHB cycle. The absolute PHB negative phenotype observed in
445 Δ*relA* strain Re2411 (Figure 1A) suggests that nitrogen shortage causes amino acid deprivation,
446 thus activating RelA-dependent ppGpp synthesis and PHB accumulation (68) (Supplemental
447 Figure S5). In addition to ppGpp synthase activity of RelA, ppGpp synthase activity of SpoT has
448 been reported in response to stimuli different than amino acid deprivation (53, 67, 70). Recently,
449 SpoT has been shown to modulate the stringent response during fatty acid shortage, sensing the

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450 availability of fatty acids through an interaction with ACP (4) It is tempting to speculate that
451 SpoT, in a similar fashion, interacts with a heretofore uncharacterized sensor of products of PHB
452 utilization, triggering the observed ppGpp spike coinciding with a commencement of PHB
453 degradation (49) (Supplemental Figure S5). SpoT-dependent ppGpp accumulation during PHB
454 utilization could explain, for example, the continued repression of genes involved in translation,
455 amino acid biosynthesis and cell motility during PHB utilization observed in this study. An
456 integral role of ppGpp throughout the PHB cycle is further supported by a recent study showing
457 that different intracellular concentrations of ppGpp result in expression of different (sub)sets of
458 genes in *E. coli* (64). This finding suggests the possibility that, likewise, gene expression during
459 PHB production and utilization is governed by different intracellular ppGpp levels. Although we
460 have not identified specific regulators that are directly influenced by ppGpp, the magnitude and
461 variation in intensity of the expression changes throughout the PHB cycle suggests the action of
462 a multitude of effectors.

463 It is clear, however, that RpoS is playing a role in PHB utilization. Deletion of *rpoS* results in
464 increased PHB mobilization, suggesting that one of the roles of σ^S is repression of over-
465 utilization of intracellular polymer. The exact role of the master regulator remains to be
466 elucidated, but given the PHB utilization phenotype of the *rpoS* mutant strain (Figure 4), it is
467 clear that σ^S is regulating nutrient homeostasis during the PHB cycle. RpoS has been shown to
468 have a role in polyhydroxyalkanoate (PHA) biosynthesis in *Pseudomonas oleovorans* (16, 50).
469 However, in *Pseudomonas putida*, an *rpoS* mutation resulted in an increased PHA degradation
470 rate (46), similar to what was observed in this study. The *rpoS* mutant strain in the
471 aforementioned study also demonstrated reduced survival under hydrogen peroxide stress,

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472 similar to our observations with *R. eutropha.* This suggests that PHB accumulation and survival
473 during stress conditions are intertwined and regulated, at least in part, by RpoS in *R. eutropha*.

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

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

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720 **Tables**

721 **Table 1: Strains and plasmids used in this study.**

Strain	Genotype	Reference
R. eutropha		
H16	Wild type, Gen^R	(69)
Re2411	H16 Δ(H16_A1337) (Δ*relA*)	This study
Re2424	H16 Δ(H16_A2373) (Δ*rpoS*)	This study
Re2061	H16Δ*phaCAB*	This study
E. coli		
S17-1	Strain for conjugative transfer of plasmids into *R. eutropha*	(54)
Plasmid	**Characteristics**	**Reference**
pJV7	*phaC* deletion plasmid, confers Kanamycin resistance	(12)
pBBR1MCS-2	Broad host range cloning vector, confers kanamycin resistance	(27)
pDS1	*spoT2* deletion plasmid, confers Kanamycin resistance	This study
pCB300	*rpoS* deletion plasmid, confers Kanamycin resistance	This study
pCJB-CAB	*phaCAB* deletion plasmid, confers Kanamycin resistance	This study
pASCB1	pBBR1MCS-2 with *R. eutropha rpoS* gene inserted into the multiple cloning site	This study

722

723

724 **Table 2: *R. eutropha* H16 gene expression microarray experiment comparing samples of**
725 **cells growing in the presence of nitrogen (+ N) to samples in PHB production (- N).**

Code	Functional group[1]	No. (%) of genes			
		Up[2]		Down[2]	
		2-4 fold	> 4 fold	2-4 fold	> 4 fold
Information storage and processing					
J	Translation, ribosomal structure and biogenesis	3 (1.7)	2 (1.1)	20 (11.2)	101 (56.4)
K	Transcription	42 (5.3)	21 (2.7)	30 (3.8)	21 (2.7)
L	DNA replication, recombination and repair	5 (3.1)	3 (1.9)	27 (16.7)	25 (15.5)
Cellular processes					
D	Cell division and chromosomal partitioning	1 (3.6)	1 (3.6)	10 (36.1)	4 (14.5)
O	Posttranslational modification, protein turnover, chaperones	9 (5.7)	25 (15.9)	24 (15.3)	24 (15.3)
M	Cell envelope biogenesis, outer membrane	4 (1.7)	6 (2.6)	26 (11.2)	39 (16.8)
N	Cell motility and secretion	8 (4.4)	6 (3.3)	23 (12.8)	32 (17.8)
P	Inorganic ion transport and metabolism	12 (11.7)	34 (33.2)	14 (13.7)	32 (31.3)
T	Signal transduction mechanisms	9 (1.1)	29 (3.7)	13 (1.7)	7 (0.9)
Metabolism					
C	Energy production and conversion	35 (7.2)	82 (16.9)	13 (2.7)	53 (10.9)
G	Carbohydrate metabolism and transport	13 (8.7)	57 (38.1)	16 (10.7)	26 (17.4)
E	Amino acid metabolism and transport	28 (9.5)	53 (17.9)	35 (11.8)	66 (22.3)
F	Nucleotide metabolism and transport	2 (2.7)	12 (16.0)	19 (25.3)	23 (30.7)
H	Coenzyme metabolism	3 (1.9)	7 (4.4)	21 (13.3)	15 (9.5)
I	Lipid metabolism (includes genes in PHB cycle)	20 (6.0)	16 (4.8)	21 (6.4)	23 (7.0)
Q	Secondary metabolite biosynthesis, transport, and catabolism	8 (8.2)	8 (8.2)	5 (5.1)	9 (9.2)
Uncharacterized or poorly characterized					
R	General function prediction only	51 (6.9)	105 (14.5)	85 (11.5)	66 (9.0)
S	Function unknown	121 (6.6)	266 (14.5)	87 (4.7)	101 (5.5)
Total		374	733	489	667

726 [1]Functional group categories are based on those of Tatusov, *et al* (62).

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727 [2]Genes in the "Up" category have undergone an upregulation of expression in PHB production
728 compared to growth using fructose as a carbon source. Genes in the "Down" category have
729 undergone a downregulation of genes in PHB production compared to growth using fructose as a
730 carbon source. The percentage genes affected per functional group was based on the number of
731 genes in each group exhibiting significant changes in expression as a percentage of the total
732 number of *R. eutropha* genes in a given functional group based on (40). For all genes represented
733 in this table, $p < 0.01$.

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735 **Table 3: Changes in expression of TCA cycle genes during nitrogen depletion in minimal**
736 **medium cultures with fructose.[a]**

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

737 [a]All p values < 0.01, except *odhB* (0.04)

738

739

740 **Table 4. Key PHB production and citric acid cycle enzyme activities in extracts of nitrogen-**
741 **limited[a] *R. eutropha* cells.**

Strain	PhaA[b]	PhaB[b]	PhaC[b]	MDH[b]	CS[b]
H16	25 ± 5	26 ± 2	68 ± 17	5.2 ± 1.5	2.5 ± 0.8
Re2411	30 ± 6	15 ± 4	45 ± 25	6.0 ± 2.0	1.4 ± 0.1

742 All enzyme activities defined in U/mg protein; One unit (U) is equivalent to the amount of
743 enzyme needed to convert 1 μmol substrate to product per min at 25°C, except for MDH activity,
744 which is defined as the amount of enzyme needed to convert 1 μmol substrate to product per h at
745 25°C. All enzyme assays were performed in replicates of three or more.

746 [a]Cells were grown in minimal medium containing 0.1 % (w/v) NH_4Cl and 2 % (w/v) fructose as
747 described in Materials and Methods. Nitrogen limitation occurred at 12-14 h of culture time.
748 Cells were harvested for enzyme activity determinations 6-8 h after the onset of nitrogen
749 limitation.

750 [b]PhaA = β-ketothiolase; PhaB = acetoacetyl-CoA reductase; PhaC = PHB synthase; MDH =
751 malate dehydrogenase; CS = citrate synthase.

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752 **Table 5:** ***R. eutropha*** **H16 gene expression during growth on fructose compared to gene**
753 **expression during growth on intracellular PHB**

Code	Functional group[1]	No. (%) of genes			
		Up[2]		Down[2]	
		2-4 fold	> 4 fold	2-4 fold	> 4 fold
Information storage and processing					
J	Translation, ribosomal structure and biogenesis	7 (3.9)	3 (1.7)	30 (16.7)	5 (2.8)
K	Transcription	26 (3.3)	5 (0.6)	34 (4.3)	10 (1.3)
L	DNA replication, recombination and repair	2 (1.2)	1 (0.6)	22 (13.6)	3 (2.7)
Cellular processes					
D	Cell division and chromosomal partitioning	1 (3.6)	0 (0.0)	6 (21.7)	1 (3.6)
O	Posttranslational modification, protein turnover, chaperones	8 (5.1)	3 (1.9)	11 (7.0)	5 (3.2)
M	Cell envelope biogenesis, outer membrane	3 (1.3)	0 (0.0)	21 (9.0)	19 (8.2)
N	Cell motility and secretion	3 (1.7)	2 (1.1)	18 (10.0)	9 (5.0)
P	Inorganic ion transport and metabolism	7 (6.8)	11 (10.7)	19 (18.6)	3 (2.9)
T	Signal transduction mechanisms	5 (0.6)	10 (1.3)	16 (2.0)	4 (0.5)
Metabolism					
C	Energy production and conversion	17 (3.5)	15 (3.1)	24 (4.9)	28 (5.8)
G	Carbohydrate metabolism and transport	5 (3.3)	5 (3.3)	24 (16.1)	27 (18.0)
E	Amino acid metabolism and transport	10 (3.4)	8 (2.7)	52 (17.6)	27 (9.1)
F	Nucleotide metabolism and transport	8 (10.7)	0 (0.0)	15 (20.0)	2 (2.7)
H	Coenzyme metabolism	6 (3.8)	3 (1.9)	18 (9.1)	5 (3.2)
I	Lipid metabolism (includes genes in PHB cycle)	12 (3.6)	8 (2.4)	30 (6.4)	10 (7.0)
Q	Secondary metabolite biosynthesis, transport, and catabolism	1 (1.0)	4 (4.1)	6 (6.1)	4 (4.1)
Uncharacterized or poorly characterized					
R	General function prediction only	38 (5.2)	19 (2.6)	86 (11.7)	30 (4.1)
S	Function unknown	89 (4.9)	76 (4.1)	88 (4.8)	33 (1.8)
Total		248	173	520	225

754 [1]Functional group categories are based on those of Tatusov, *et al* (62).

755 [2]Genes in the "Up" category have undergone an upregulation of expression in PHB utilization
756 compared to growth using fructose as a carbon source. Genes in the "Down" category have

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757 undergone a downregulation of genes in PHB utilization compared to growth using fructose as a
758 carbon source. The percentage genes affected per functional group was based on the number of
759 genes in each group exhibiting significant changes in expression as a percentage of the total
760 number of *R. eutropha* genes in a given functional group based on (40). For all genes represented
761 in this table, $p < 0.01$.

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763 **Figure legends:**

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

771 **Figure 2. Reduced cofactor quantitation in *Ralstonia eutropha* wild-type (H16) and *spoT2***
772 **deletion (Re2411) strains.** In (A), NADH is quantified in H16 and Re2411 cells during growth
773 (6 h) and nitrogen starvation (24 h). In (B), NADPH is quantified in H16 and Re2411 cells
774 during growth (6 h) and nitrogen starvation (24 h). *R. eutropha* cells were prepared and reduced
775 cofactors were quantified as described in Materials and Methods (n = 3).

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

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785 genes under all conditions is less than 0.01. In (G), the *p* value of *phaZ6* is <0.01, and the *p* value

786 for *phaZ4* and *phaZ7* are > 0.05.

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

788 **utilization media.** *R. eutropha* strain H16/pBBR1MCS-2 (wild type with empty vector, boxes),

789 Re2424/pBBR1MCS-2 (*rpoS* deletion strain with empty vector; diamonds), and

790 Re2424/pASCB1 (*rpoS* deletion strain complemented with the *rpoS* gene *in trans*; triangles), all

791 containing intracellular PHB, were incubated in PHB utilization medium for 72 h. Intracellular

792 PHB content was assayed as described in Materials and Methods (n = 3).

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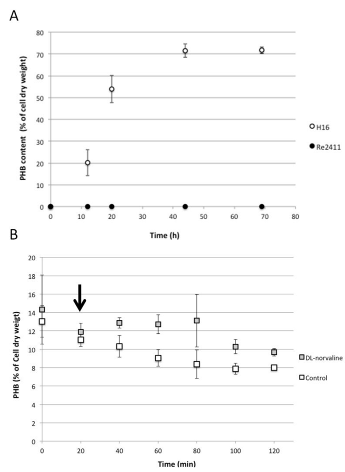

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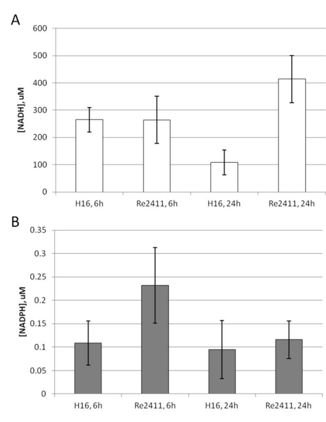

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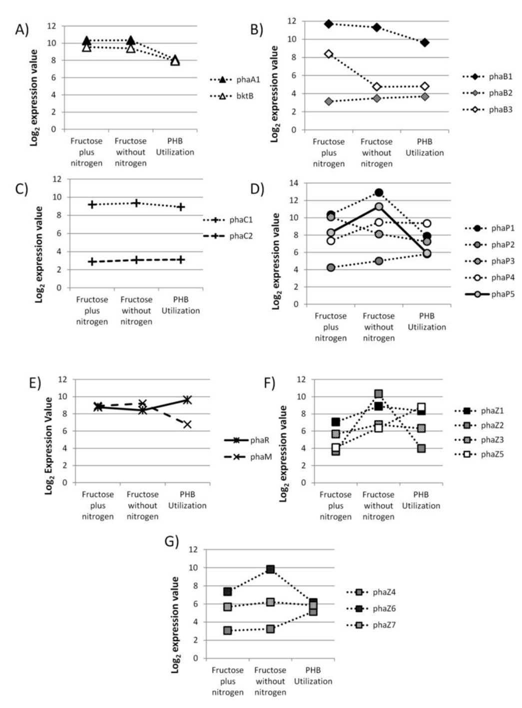

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