Title: Elucidation of β-oxidation Pathways in *Ralstonia eutropha* H16 by Examination of Global Gene Expression

Running title: Gene Expression Microarray Analysis of *Ralstonia eutropha* H16

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Abstract

Ralstonia eutropha H16 is capable of growth and polyhydroxyalkanoate production on plant oils and fatty acids. However, little is known about the triacylglycerol and fatty acid degradation pathways of this bacterium. We compare whole-cell gene expression of *R. eutropha* H16 during growth and polyhydroxyalkanoate production on trioleate and fructose. Trioleate is a triacylglycerol that serves as a model for plant oils. Among the genes of note, two potential fatty acid β-oxidation operons and two putative lipase genes were shown to be upregulated in trioleate cultures. The genes of the glyoxylate bypass also exhibit increased expression during growth on trioleate. We observed that single β-oxidation operon deletion mutants of *R. eutropha* could grow using palm oil or crude palm kernel oil as the sole carbon source, regardless of which operon was present in the genome, but a double mutant was unable to grow under these conditions. A lipase deletion mutant did not exhibit a growth defect in emulsified oil cultures, but did exhibit a phenotype in cultures containing non-emulsified oil. Mutants of the glyoxylate shunt gene isocitrate lyase were able to grow in the presence of oils, while a malate synthase (*aceB*) deletion mutant grew more slowly than wild-type. Gene expression under polyhydroxyalkanoate storage conditions was also examined. Many findings of this analysis confirm results from previous studies by our group and others. This work represents the first examination of global gene expression involving triacylglycerol and fatty acid catabolism genes in *R. eutropha*.

Introduction

Polyhydroxyalkanoate (PHA) carbon storage polymers produced by numerous microorganisms are biodegradable, biocompatible alternatives to petroleum-based plastics. The model organism of PHA biosynthesis is the Gram negative β-proteobacterium *Ralstonia eutropha*. *R. eutropha* can store PHA up to 80% of its cell dry weight as a result of nutrient limitation (31). During nutrient starvation, wild-type *R. eutropha* produces short chain length PHA (scl-PHA), such as polyhydroxybutyrate (PHB) and poly(hydroxybutyrate-*co*-hydroxyvalerate) (P(HB-*co*-HV)) (45, 52, 53). Other bacterial species such as the pseudomonads produce medium chain length PHA (mcl-PHA), derived mainly from fatty acid β-oxidation intermediates (23). Some species are capable of producing a combination of scl- and mcl-PHA during nutrient starvation (45, 52, 53). These copolymers comprised of scl- and mcl- monomers exhibit thermal and mechanical properties similar to petroleum-based plastics (12, 53), and are thus desirable for use as substitutes for petrochemical polymers in household, medical, and industrial goods.

Many groups have explored production of PHA from renewable carbon sources such as plant oils. These studies include examination of recombinant strains of *R. eutropha* containing heterologous synthase genes, whose products exhibit broad substrate specificity, thus producing PHA with a combination of scl- and mcl- monomers (27, 30). Plant oils are a suitable carbon source for this endeavor as 3-hydroxyacyl-CoA PHA precursors can be produced from intermediates in the fatty acid degradation pathway (23, 58).

Plant oils consist of triacylglycerols (TAGs), in which three fatty acids are joined to a glycerol backbone. Recently, plant oils have been explored as a possible alternative feedstock to petroleum for chemical production (7). These oils can also be used as sources of carbon for bioplastic production by bacteria such as *R. eutropha*. The oil palm tree (*Elaeis giuneensis*), an important agricultural product in Africa and Southeast Asia, is the most productive oilseed crop (3, 61). In Malaysia, the palm oil yield in 2009 was 4 tonnes/hectare (http://econ.mpob.gov.my/economy/EID_web.htm). Palm fruits yield two different oils: palm oil from the flesh of the fruit and palm kernel oil from the seed. Palm oil is composed of several fatty acids with palmitic (C16:0), oleic (C18:1), and linoleic acids (C18:2) comprising more than 90% of the total fatty acid content (13). Palm kernel oil is comprised mostly of lauric (C12:0), myristic (C14:0) and oleic acids (13). *R. eutropha* has been shown to grow on these oils as carbon sources (32).

R. eutropha must therefore employ a fatty acid degradation pathway to consume oils and fatty acids. In the model for microbial fatty acid catabolism, free fatty acids within the cell are first ligated to coenzyme-A, by action of the FadD enzyme. The newly formed acyl-CoA molecules are converted to an enoyl-CoA by action of an acyl-CoA dehydrogenase. The enoyl-CoA is converted to (*S*)-3-hydroxyacyl-CoA by an enoyl-CoA hydratase. Next, a 3-ketoacyl-CoA molecule is formed by action of a 3-hydroxyacyl-CoA dehydrogenase. The last step is the cleavage of the 3-ketoacyl-CoA by a 3-ketoacyl-CoA thiolase to produce a shorter length fatty acyl-CoA and one acetyl-CoA molecule. The pathway acts in a cyclic fashion, with each complete “turn” of the cycle decreasing the length of the substrate by two carbon atoms through the release of acetyl-CoA (18) (see also, Figure 1). The fatty acid β-oxidation pathway in *R. eutropha* is uncharacterized in the literature. Most studies of microbial fatty acid β-oxidation have been conducted with *E. coli* and *B. subtilis* (18, 29), although some information is available regarding fatty acid degradation in *Pseudomonas* species (9, 14). Both the *E. coli* and *B. subtilis* pathways are similar, producing the same types of intermediates and yielding acetyl-CoA as the final product (18, 29). The main difference between the two systems is that *B. subtilis* has the ability to break down branched chain fatty acids (18). A search of the *R. eutropha* H16 genome reveals many potential β-oxidation pathway gene homologs (38). For example, 50 genes in the *R. eutropha* H16 genome are annotated as enoyl-CoA hydratases and 46 genes are annotated as acyl-CoA dehydrogenases. However, it is not known which of these homologs actually play a role in fatty acid breakdown.

In order to better understand oil and fatty acid metabolism in *R. eutropha*, we performed gene expression microarray experiments using custom designed chips with cultures containing either fructose or trioleate as the sole carbon source. Gene expression was examined during both the growth phase and PHB production phase of the cultures. Utilizing the results of these transcriptional studies, we identified lipase genes and potential fatty acid β-oxidation genes in the *R. eutropha* H16 genome, and demonstrated their roles in metabolism of plant oils by growing gene/operon deletion mutant strains on palm oil and crude palm kernel oil (CPKO). We also examined genes involved in the glyoxylate bypass of *R. eutropha* H16, and their roles in oil and fatty acid utilization.

Comparison of gene expression under growth and PHB production conditions confirms results from previous studies by our group and others (24, 37, 39, 44, 46, 62-65). In addition, we determined that deletion of fatty acid metabolism and glyoxylate bypass genes do not affect PHB production or utilization in *R. eutropha*.

Materials and Methods

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). *Pfu* DNA polymerase and other DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Natural red palm oil was purchased from Wilderness Family Naturals (Finland, MN). CPKO and the plasmid pBBR1MCS-2 were generous gifts from Dr. K. Sudesh Kumar (Universiti Sains Malaysia, Penang, Malaysia).

Design of custom *Ralstonia eutropha* H16 microarray chips. Probes representing 6626 protein-encoding genes and 3 rRNA genes from the *R. eutropha* H16 genome, as annotated per Pohlmann, et al (38), were printed on an 11 µm array (49-5241 format, Affymetrix, Santa Clara, Calif.). Probe sets for each open reading frame include 15 exact match 25-mer probes and 15 mismatch 25-mer probes (8, 47). After submission of design parameters, custom *R. eutropha* H16 gene expression microarray chips were constructed according to the quality control guidelines outlined by the manufacturer (www.affymetrix.com).

Cell growth and total cellular RNA isolation procedure. Four individual colonies of *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. Aliquots of 0.5 mL of overnight culture were inoculated into 250 mL shake flasks containing 50 mL of minimal medium, modified from (36), containing 0.1 % NH_4Cl and either 2 % (w/v) fructose or 1 % (w/v) trioleate, emulsified with 0.3% (w/v) gum arabic. 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 % NH_4Cl and either 2 % (w/v) fructose or 1 % (w/v) trioleate, emulsified with 0.3% (w/v) gum arabic. Cultures were grown for 12 h. Cultures for sampling were inoculated to an initial OD_{600} of 0.05 in 250 mL shake flasks containing 50 mL of minimal medium with 0.05 % NH_4Cl and either 2 % (w/v) fructose or 1 % (w/v) trioleate, emulsified with 0.3% (w/v) gum arabic. 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. The concentration of NH_4^+ in the growth medium was monitored using an Ammonia Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. An aliquot of cells (OD_{600} equivalent = 2.5) was harvested at an NH_4^+ concentration of 0.025%, and another aliquot of cells (also an OD_{600} equivalent = 2.5) was harvested 2 h after depletion of nitrogen in the media. 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.

For RNA isolation, frozen cell pellets were thawed at room temperature. Cells were incubated with a lysozyme and Proteinase K solution for 10 minutes on ice, and cell suspensions were vortexed every 2 minutes. RNA was then isolated from cells using the RNEasy Mini Kit (QIAgen) following the manufacturer's instructions. Total RNA was quantified by A_{260}, and analyzed for quality using an Agilent 2100 BioAnalyzer, where RNA was quantified and quality was confirmed. Only RNA samples with an RNA Integrity Number of 9.0-10.0 (10.0 is highest quality) were used for microarray analysis (15). 100 ng of total RNA from triplicate samples was amplified and labeled using the MessageAmp II-Bacteria prokaryotic RNA Kit (Ambion-AM1790) and hybridized to *R. eutropha* H16 custom Affymetrix arrays. Samples were hybridized for 16 hours at 45°C and scanned according to platform specifications. Array chips were scanned using an Affymetrix 7G scanner.

Microarray data analysis. Microarray data was extracted using Affymetrix GCOS v.1.4. All data were normalized by Robust Microchip Average (RMA, ArrayStar software, Madison, WI) with quantile normalization. Statistically significant gene expression changes between two triplicate sets of samples were determined using the unpaired, two-tailed, equal variance Student's *t*-test (ArrayStar) and confirmed using ANOVA (ArrayStar). The FDR (Benjamini Hochberg) method was used to restrict the false discovery rate. Annotation of genes in the final output was performed based on Pohlmann, et al. (38). Genes of interest with a statistically significant change in expression ($p < 0.01$) were selected for further study.

Cloning and construction of deletion strains. Oligonucleotide primers used in this work are listed in Supplemental Table 1. Plasmid vectors (see Table 1) for cleanly deleting operons from the *R. eutropha* genome were made by first constructing stretches of DNA in which the regions directly upstream and directly downstream of a given gene or operon were connected. The initial step in this vector construction was the amplification of two ~500 bp sequences, one directly upstream of the gene or operon of interest, and another directly downstream of the gene or operon. Primers were designed such that the two fragments had identical 16 bp sequences at the ends that were to be connected. A single DNA fragment containing the upstream and downstream DNA fragments was created by overlap extension PCR (48). Primers used in the overlap PCR were designed so that the product had BamHI restriction sites at each end. The product of the overlap PCR was isolated and purified using QIAquick Gel Extraction kit (QIAgen, Valencia, Calif.), digested with BamHI, and then ligated into the backbone of pGY46 (see Table 1). The plasmid pGY46 had been used previously to delete *R. eutropha phaC1* (62), so it was digested with BamHI and the backbone fragment was separated from the Δ*phaC1* fragment using QIAquick Gel Extraction kit. Plasmids for deletion of individual genes were constructed following a similar procedure, except the gene deletion fragments (consisting of two connected ~250 bp stretches of DNA upstream and downstream of the gene) were synthesized directly by Integrated DNA Technologies. Newly constructed gene and operon deletion plasmids (see Table 1) were transformed into *E. coli* S17-1 (50) and introduced into *R. eutropha* by a standard mating procedure (50, 51). *R. eutropha* strains with the desired mutation were selected and the deletion was confirmed using diagnostic PCR. Details of each gene and operon deletion can be found in the Supplemental Material 1.

<u>Construction of complementation plasmids and introduction into *R. eutropha* deletion mutants.</u> The following genes and operons were cloned via PCR and inserted into pBBR1MCS-2 (Table 1): *aceB*, lipase gene A1322, β-oxidation operon A0459-A0464, and β-oxidation operon A1526-A1531. Genes and operons were amplified by PCR using primers listed in Supplemental Table 1. PCR products were purified using QIAquick Gel Extraction Kit. The *aceB*, A1322, and A1526-A1531 operon DNA inserts were digested with KpnI and HindIII and ligated into KpnI/HindIII cut pBBR1MCS-2 to produce pCJB200, pCJB201, and pCJB203 (Table 1) respectively. The A0459-A0464 operon DNA insert was digested with KpnI and EcoRV and ligated into KpnI/EcoRV cut pBBR1MCS-2 to create plasmid pCJB202 (Table 1). Plasmids were introduced into *E. coli* S17-1 by electroporation and selected by growing on LB agar plates with the addition of 50 µg/mL kanamycin. Plasmids were introduced into *R. eutropha* by mating with *E. coli* S17-1 (50).

<u>Growth of *R. eutropha* strains in medium containing plant oils or fatty acids.</u> Individual colonies of wild-type and mutant *R. eutropha* grown on a TSA plate were inoculated into 5 mL of TSB and grown overnight at 30°C with agitation. Overnight cultures were washed and diluted 1:10 in sterile saline. Aliquots of 50 µL of a 1:10 dilution of overnight culture were inoculated into 250 mL shake flasks containing 50 mL of minimal medium, modified from (36), containing 0.1 % NH_4Cl and 1 % (w/v) palm oil, CPKO, or

oleic acid, emulsified with 0.3% (w/v) gum arabic. These cultures were grown for up to 72 h at 30°C with agitation (200 rpm). Aliquots of cells were removed at 0, 4, 8, 12, and 24 h, serially diluted in 0.85 % saline, and plated onto TSA. Dilution plates were incubated for 24 h at 30°C, after which time viable colonies were counted.

Quantitation of polyhydroxybutyrate. Aliquots of 5-10 mL of culture were transferred to preweighed borosilicate glass tubes at various time points during the PHB production cycle. Cells were pelleted, washed with a mixture of 5 mL of cold water and 2 mL cold hexane for removal of residual oil, pelleted again and dried *in vacuo* at 80°C. Cells grown on fructose were harvested as above, except hexane was not included in the wash step. The PHB content and CDW were determined from the dried samples using established methods (5, 21).

Microarray data accession number. The microarray data discussed in this work have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and can be accessed through the GEO series accession number GPL10276.

Results

Microarray analysis of *R. eutropha* H16 gene expression in trioleate cultures compared to fructose cultures. Studies have shown that *R. eutropha* is capable of accumulating large amounts of PHA using plant oils as the sole carbon source (25, 27). Our research group is interested in producing PHA from palm oil and CPKO using *R. eutropha* as the production organism. A better understanding of this bacterium's fatty acid metabolism is important for achieving this goal. While it is well established that *R. eutropha* grows robustly using plant oils (25, 27, 30, 32), we do not yet know what specific genes and proteins play important roles in oil metabolism.

To begin to understand the changes that occur in the *R. eutropha* transcriptome when the cells are grown on oils as the sole carbon source, we isolated total cellular RNA from *R. eutropha* strain H16 grown in minimal medium using either 2% fructose or 1% trioleate as the carbon source (see Materials and Methods). We decided to use trioleate as a representative triacylglycerol, as trioleate is a uniform, defined carbon source, as opposed to plant oils, which may contain contaminating compounds that could add unwanted complexity to the analysis of the microarray data. We monitored the concentration of NH_4Cl in the cultures so that samples from each culture were taken at approximately the same phase of growth. To represent the logarithmic growth phase, we took samples when cultures had utilized approximately half of the NH_4Cl in the media (~250 µg/mL). Samples were also taken ~2 h after all NH_4Cl in the culture was depleted, representing the PHB production phase.

We focused our analysis on genes that exhibited at least 2-fold altered expression at the 99% confidence level between growth phase samples for the two carbon sources. Expression levels of genes in this analysis are reported on a scale of 1-15, which represents the base 2 logarithm of the measured expression values from the hybridized microarray chip readings. Genes in which the expression level was below 6 under all conditions were considered to be unexpressed, and thus excluded from further analysis. A total of 787 genes from the *R. eutropha* genome are differentially expressed according to this analysis: 418 are upregulated during growth on trioleate, and 369 are upregulated during growth on fructose. A breakdown of the differentially expressed genes into functional groups is summarized in Table 2. Notably, a higher percentage of lipid metabolism genes demonstrate increased expression when *R. eutropha* H16 is grown on trioleate, compared to fructose (Table 2). Alternatively, a higher percentage of carbohydrate metabolism genes have increased transcript levels when *R. eutropha* H16 is grown on fructose, compared to trioleate (Table 2). While these results were not surprising, they did provide an early indication that our data captured the differences in gene expression arising from growth on the two carbon sources.

Analysis of the individual genes that exhibited increased expression under trioleate growth conditions, compared to fructose growth conditions (i.e. genes upregulated in the presence of trioleate), revealed several potential genes and gene clusters that could be involved in lipid metabolism (Table 3, Figure 1A). The greatest change in expression is associated with a cluster of genes beginning with A3736 that appear to encode outer membrane related proteins. (Note that the nomenclature "A*xxxx*" and "B*xxxx*" refer to the locus tags of genes discussed in this work, where A indicates the gene is on chromosome 1 and B indicates the gene is on chromosome 2). The reason for the extremely high increases in expression of these genes is partially due to the fact that their expression levels on fructose were very low. A deletion was constructed of gene cluster

A3736-A3732 using *R. eutropha* H16 as the parental strain, but the resulting deletion strain grew similarly to wild type in all conditions tested (data not shown). Therefore, this strain was not studied further. Two potential operons (A0459 – A0464 & A1526 – A1531) each appear to contain genes that encode the enzymes necessary for fatty acid *β*-oxidation (Table 3, Figure 1A), including acyl-CoA dehydrogenases (A0460 and A1530), 2-enoyl-CoA hydratases (A0464 and A1526), 3-hydroxyacyl-CoA dehydrogenases (A0461 and A1531), and 3-ketoacyl-CoA thiolases (A0462 and A1528), as well as other proteins of unknown function (A0463, A1527, and A1529). Figure 1B illustrates a schematic of fatty acid β-oxidation in *R. eutropha* H16, indicating which gene products are believed to catalyze each reaction. Three acyl-coA ligase (*fadD*) homologs are present in the *R. eutropha* H16 chromosome: *fadD1* and *fadD2* (PHG398 and PHG399), are present on the pHG1 megaplasmid, while *fadD3* (A3288) is found on chromosome 1. Only *fadD3* exhibits a significant increase in expression during growth on trioleate, compared to fructose (Table 3). Genes A1322 and A3742, both of which are upregulated in trioleate cultures, encode putative lipases for cleaving fatty acids from triacylglycerols at the interface of the insoluble substrate and water (43). Interestingly, the potential operon A2507 – A2509 encodes proteins that catalyze the first steps in glycerol metabolism. These genes may be upregulated in response to the appearance of glycerol in the medium that occurs as trioleate is metabolized. Other genes of interest that are upregulated during growth on trioleate include the malate synthase gene *aceB* (A2217) and the isocitrate lyase genes *iclA* (A2211) and *iclB* (A2227), which provides evidence that the glyoxylate bypass plays a role in triacylglycerol metabolism (Table 3). Previous studies have shown that expression of isocitrate lyase is significantly induced when *R.*

eutropha is grown on acetate, in contrast to malate synthase expression (59). Our analysis shows that while malate synthase is upregulated in the presence of trioleate compared to fructose, both isocitrate lyase genes are upregulated to a much greater degree. Products of the glyoxylate bypass are normally converted to phosphoenolpyruvate (PEP), which is an important cellular intermediate. This can occur either by conversion of oxaloacetate to PEP by a PEP carboxykinase, or by conversion of malate to pyruvate via the malic enzyme, followed by conversion of pyruvate to PEP by a PEP synthetase (4, 6). No genes encoding these enzymes appear to be upregulated during growth on trioleate. Malate dehydrogenase, A2634 is upregulated only 1.26-fold in trioleate cultures, and malic enzyme genes *maeA* and *maeB*, A3153 and A1002, are downregulated 2.89-fold and upregulated 1.14-fold in trioleate cultures, respectively. These results make this an interesting area for further investigation.

Changes in expression of other genes in *R. eutropha* H16 grown in trioleate, compared to cells grown in fructose, and comparison of gene expression in the presence and absence of nitrogen. Further discussions of gene expression changes discovered in our microarray analysis can be found in the Supplemental Material.

Growth of β-oxidation mutant strains of *R. eutropha* in the presence of plant oils and fatty acids. Microarray analysis revealed the presence of two potential fatty acid β-oxidation operons in *R. eutropha*. To investigate the roles of these operons during growth on plant oils, strains containing clean deletions of each gene cluster were constructed. The resulting mutant strains (see Table 1) were then grown in minimal medium with palm oil, CPKO, or oleic acid as the sole carbon source. After 24 h of growth, the A0459-A0464 deletion strain (Re2300), and the A1526-A1531 deletion strain (Re2302), reached similar cell densities compared to wild-type (Figure 2). These results suggest that, even in the absence of one of the putative β-oxidation operons, the expression and activity from the other intact operon is sufficient to allow for normal cell growth on plant oils. The double β-oxidation operon deletion strain, Re2303, did not grow in the oil or fatty acid media (Figure 2), suggesting that at least one β-oxidation operon is needed for catabolism of long chain fatty acids. We were able to complement the growth defect of strain Re2303 on oils by introduction of plasmids containing either the A0459-A0464 or A1529-A1531 gene clusters (Supplemental Figure 2). Since *fadD3* showed a significant increase in expression during growth on trioleate compared to fructose (Table 3), we decided to examine *fadD3* further. A *fadD3* deletion mutant strain, Re2312, was constructed and grown in the presence of palm oil and CPKO. Growth of Re2312 was similar to that of wild-type (Figure 2), suggesting another *R. eutropha* gene product also provides FadD activity in this mutant strain.

All β-oxidation mutant strains were tested for growth defects in rich medium and minimal medium with fructose as the sole carbon source. All strains grew similarly to wild-type in rich medium and fructose minimal medium (data not shown), indicating that the growth phenotype observed with Re2303 is specific for growth on plant oils.

<ins>Growth phenotype of a lipase mutant strain in the presence of plant oils.</ins> Two genes encoding putative lipases were discovered to be upregulated during growth on trioleate.

One gene, A1322, encoding a putative triacylglycerol lipase, is located upstream of a lipase chaperone gene (A1323). This arrangement is similar to a lipase/chaperone gene cluster found in the genome of *Ralstonia* sp. M1 (43). The expression of both the lipase gene and the lipase chaperone are upregulated significantly in trioleate cultures (Table 3). A primary sequence comparison of the putative lipase encoded by A1322 and the *Ralstonia* M1 lipase shows that both proteins are classified as "true lipases" according to the classification system of bacterial lipolytic enzymes, and are similar to the well-characterized *Pseudomonas* lipases (1, 19). We created a clean deletion of A1322 using H16 as the parental strain, to create strain Re2313. In medium containing emulsified palm oil or crude palm kernel oil, Re2313 grew similarly to wild-type (Figure 3). We also examined growth of Re2313 in medium with non-emulsified palm oil as the carbon source. When grown in this manner, wild-type *R. eutropha* metabolizes the oil, and within ~24 h the unconsumed oil in the culture becomes emulsified. The lipase deletion mutant, in contrast to wild-type, was not able to break down the oil significantly and emulsify it. However, the cells of the mutant strain did exhibit some growth on palm oil in this experiment. Introduction of the A1322 gene expressed on a plasmid reversed the palm oil emulsification phenotype of Re2313 (Supplemental Figure 1). This suggests that the lipase gene A1322 is necessary for optimal growth in non-emulsified plant oil media.

<u>Growth phenotype of glyoxylate bypass mutants.</u> Our gene expression studies have shown that the genes of the glyoxylate bypass are upregulated when *R. eutropha* is grown on trioleate. For utilization of fatty acids, which are primarily metabolized to acetyl-CoA,

the presence of a functional glyoxylate bypass is important (59). We constructed in-frame deletions of each gene in the glyoxylate bypass, and grew the mutant strains in the presence of palm oil and crude palm kernel oil. One strain, Re2304 (Δ*aceB*) exhibited a decreased growth rate in the presence of oils (Figure 4). Wang, et al. (59) also observed a slow growth phenotype of an *aceB* mutant when the strain was grown on acetate. The *aceB* gene is the only gene in the *R. eutropha* H16 genome annotated as a malate synthase gene. However, when the *aceB* gene was knocked out, malate synthase activity was decreased, but not eliminated (59), suggesting the presence of another enzyme with malate synthase activity in *R. eutropha* H16. This slow growth phenotype in the presence of oils was reversed when *aceB* was introduced to Re2304 expressed on a plasmid (Supplemental Figure 3).

Isocitrate lyase gene deletion mutant strains Re2306 (Δ*iclA*) and Re2307 (Δ*iclB*) both exhibited growth on oil cultures similar to that of wild-type (Figure 4). One possible explanation for this finding is that, in either mutant, the activity of the other isocitrate lyase enzyme present is capable of compensating for the loss of *iclA* or *iclB*. Our data differs from a previous study, which showed that an *iclA* mutant of *R. eutropha* HF39 was unable to grow on acetate as the sole carbon source (59). All glyoxylate cycle mutant strains grew similarly to wild-type in rich media and minimal media containing fructose as the sole carbon source (data not shown), indicating that the growth defect of Re2304 was dependent on the carbon source.

PHB production and utilization in mutant strains. We examined the ability of mutants generated in this study to produce and mobilize PHB. All mutant strains were able to produce PHB in similar quantities as the wild-type strain, using palm oil, CPKO, or fructose as the carbon source (data not shown). These results are in contrast to previous published results, where *iclA* and *iclB* mutant strains exhibited PHB production defects during growth on gluconate and acetate (59). PHB utilization was also examined in our mutant strains. After accumulation of PHB in fructose minimal medium, cells were washed and incubated in PHB utilization medium (62). After 24 h, it was found that all strains utilized PHB to the same extent as the wild-type strain (Table 4). Table 4 also shows that all strains grew as they mobilized PHB, based on the increase in viable cell counts after 24 h.

Discussion

Comparison of gene expression of *R. eutropha* H16 grown in fructose or trioleate cultures revealed several interesting genes involved in breakdown of plant oils and fatty acids. Two fatty acid β-oxidation operons were highly upregulated in the presence of trioleate, compared to fructose. Each individual operon was found to contain all of the genes necessary for the entire β-oxidation cycle (Figure 1), excluding the *fadD* gene (encoding the fatty acyl CoA ligase). Operon deletions and subsequent growth studies revealed that growth in the presence of plant oils was unaffected if either individual operon was deleted, but growth on oils or oleic acid was not possible if both operons were deleted. The individual roles of each operon remain to be elucidated. In Eukaryotes, there exist multiple enzymes for each step of the β-oxidation pathway, with different sets of enzymes for short-, medium-, and long-chain fatty acid degradation (2). Given that strains Re2300 and Re2302 can both utilize palm and palm kernel oil for growth, it is likely that the gene products of both β-oxidation operons can utilize long chain (C12 and longer) fatty acids as substrates.

In addition to the β-oxidation-related genes, one operon (A0459-A0464) contains a gene encoding a hypothetical membrane-associated protein (A0463, Figure 1A). Further primary and secondary structure analysis (http://www.sbg.bio.ic.ac.uk/phyre) shows that the gene product of A0463 is similar to DegV-like proteins found in several *Bacillus* species. The functions of DegV and DegV-like proteins are not completely understood, however, a structural study of DegV showed that it is a fatty acid binding protein found only in bacteria (33). It is tempting to speculate that A0463 encodes a DegV-like protein involved in binding fatty acid substrates for β-oxidation. Further study is necessary to determine the importance of this gene product in *R. eutropha* fatty acid degradation. The other β-oxidation operon contains a gene encoding a potential bifunctional pyrazinamidase/nicotinamidase (A1527, *pncA*). Sequence analysis demonstrates that the putative gene product of *pncA* contains all of the highly conserved amino acid residues found in the previously-characterized PncA from *Mycobacterium tuberculosis* (66). PncA is known to function in NAD^+ recycling pathways in many organisms (17, 66). It is possible the *R. eutropha* PncA enzyme contributes to regulation of NAD^+/NADH levels during fatty acid β-oxidation. Another gene, A1529, encodes a product annotated as having homology to a thioesterase involved in phenylacetic acid degradation. Previous studies in *E. coli* revealed a novel thioesterase III that hydrolyzes degradation-resistant metabolites resulting from β-oxidation (34, 35). It is possible A1529 may carry out a similar role in *R. eutropha.* Recently, several *R. eutropha* β-ketothiolases were studied by creating multiple β-ketothiolase gene knockout strains and examining their ability to produce PHB and poly(3-mercaptopropionate). It was determined that a deletion mutation of the A1528 β-ketothiolase gene did not have an effect on acetoacetyl-CoA biosynthesis, and thus PHB production. Based on these findings, the authors of this study postulate astutely that the A1528 gene product may be involved in fatty acid degradation (26).

Three predicted genes in the *R. eutropha* genome are annotated as encoding fatty acyl CoA ligase (FadD) homologs. Of these, the *fadD3* gene was examined to determine its role in fatty acid β-oxidation. The *fadD3* gene was chosen because it was the only *fadD* homolog whose expression was upregulated in trioleate-grown cells. However, it is likely that other *fadD* homologs can play a role in β-oxidation, as the *fadD3* mutant grew similarly to wild-type in palm oil and CPKO cultures. We also found that *fadD1* and *fadD2* were expressed in *R. eutropha*, although transcript levels did not change significantly under different culture conditions. Sequence analysis shows that there are other genes not annotated as *fadD* in the *R. eutropha* H16 genome that potentially encode fatty acyl-CoA ligases (38). One gene, A2794, shows increased expression when cells are grown on trioleate, compared to fructose. It is possible that the A2794 gene product plays a role in fatty acid β-oxidation. Future studies are needed to confirm this hypothesis.

Because fatty acids are converted to 2-carbon units by β-oxidation, there must be a pathway that provides 3- and 4-carbon compounds necessary for biosynthesis of cellular components. In most bacteria, synthesis of these larger molecules from TCA cycle intermediates is mediated by the glyoxylate bypass (10). Previously, growth of *R. eutropha* glyoxylate bypass mutant strains had been examined on acetate and gluconate as the sole carbon sources (59). We created gene deletions of each individual glyoxylate bypass gene in *R. eutropha* H16 and examined the growth of the resulting mutant strains on oils. The *aceB* mutant strain, Re2304, exhibited a slower growth phenotype on oils, when compared to wild-type (Figure 4). Consistent with previous data (59), the *aceB* mutant strain also exhibited slower growth on acetate as a carbon source (data not shown). Both *icl* mutant strains grew similar to the wild-type strain on palm oil and CPKO (Figure 4). These results are in contrast to previous results, where an *iclA* knockout strain of *R. eutropha* was unable to grow on minimal medium containing acetate as the sole carbon source (59). It is possible that gene expression in *R. eutropha* varies when acetate is used as a carbon source, as opposed to TAGs.

Gene expression data also revealed two putative lipase genes (A1322 and A3742) that are both upregulated in the presence of trioleate. The A1322 gene deletion mutant, while still able to grow on plant oils (Figure 3), exhibited an interesting phenotype. When grown on non-emulsified palm oil, Re2313 (the ΔA1322 mutant) was not able to create a stable emulsion of oil droplets, even after 72 hours of growth (Supplemental Figure 1). These results suggest that the A1322 lipase gene product plays a critical role in *R.*

eutropha's ability to emulsify plant oils. We suggest that the action of lipases from *R. eutropha* produces free fatty acids that in turn emulsify the oil in the media. We hypothesize that Re2313 can grow on oil emulsified with gum arabic because this strain secretes other esterases that do not efficiently release fatty acids from unemulsified TAGs, but that are more efficient at breaking down the tiny oil droplets present in an emulsion. It is possible that the A1030 or A3742 gene products, both putative lipases/esterases, could carry out this reaction. Both genes are upregulated in trioleate cultures, although A1030 is upregulated less than 2-fold (for A3742 expression increase, see Table 3). Further study of *R. eutropha* H16 lipases is ongoing. Recently, the genome sequence of another *R. eutropha* strain, JMP134, was published (28). The genome of this strain does not appear to contain genes for either of the lipase homologs mentioned in this work, which suggests that *R. eutropha* JMP134 may not able to grow on TAGs as the sole carbon source.

Previous studies concluded that the *phaC1-phaA-phaB1* operon is constitutively expressed in *R. eutropha* H16 (24). In our microarray studies, expression of the *phaCAB* operon and of the *bktB* gene is high under all conditions tested, indicating that these genes are indeed constitutively expressed during growth and PHB production, using either carbon source. A previous study showing that a *phaC1* deletion mutant of *R. eutropha* H16 does not produce PHB (62), suggests that the *phaC2* gene present in the *R. eutropha* H16 genome is not expressed. We detected only low levels of *phaC2* transcript under all conditions. These expression values were so low ($\log_2$(expression) = 3-4), that they were within background levels and suggest that *phaC2* is unexpressed. Expression of the *phaR* regulator gene does not change significantly during growth or PHB production (data not shown), whereas *phaP1* expression increases during PHB production (Supplemental Table 2). These results agree with the current model for PhaP1 expression, in which *phaR* is constitutively expressed. As PHB storage begins, PhaR protein binds to nascent PHB granules, thus allowing expression of the *phaP1* gene (39, 65). Expression of *phaZ1* increases 4-fold during nitrogen limitation (Supplemental Table 2), according to our studies. This increase in expression during PHB production is not surprising, given that PhaZ1 is associated with the PHB granule (20, 57). A previous RT-PCR study showed that the *phaZ2* gene is upregulated significantly upon the cells' entry into PHB production (24). Our microarray data confirm this finding. It was also previously shown that expression of *phaZ2* is not dependent on the production of PHB, as increased expression of *phaZ2* occurred in a *phaC1* mutant strain (24). Further study of this gene is required to determine its role in PHB homeostasis. The *phaZ3*, *phaZ5*, and *phaZ6* genes are also significantly upregulated ($p < 0.01$) in the absence of nitrogen (Supplemental Table 2). It remains to be seen whether their gene products are associated with PHB granules. It has been shown that PHB turnover occurs during PHB accumulation in *R. eutropha* batch cultures. The molecular weight of PHB decreases during PHB production and also decreases after cessation of polymer accumulation (54). These phenomena may be due to expression of PHA depolymerases during PHB production.

With the help of gene expression analysis, we have begun to elucidate the roles of lipid and fatty acid degradation genes in *R. eutropha* H16. We can manipulate both the β-oxidation pathway and the PHB production pathway to produce novel and useful PHAs from plant oils. Also, by improving the rate at which *R. eutropha* breaks down lipids, we can potentially create a useful strain for industrial scale PHA production.

Acknowledgements

We thank Dr. Stuart Levine, Ms. Manlin Luo, and the rest of the MIT BioMicro Center for hybridization and processing of microarray samples. We thank Robert Dorkin and Jingnan Lu for assistance with *R. eutropha* growth, PHB production, and PHB utilization experiments. Lastly, we thank Dr. Daniel MacEachran for critical review of this manuscript prior to submission. This work was funded by the Malaysia/MIT Biotechnology Partnership Program, which is a collaborative effort 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.

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Table 1: Bacterial strains and plasmids used in this work

Strains:

Strain name	Relevant genotype	Reference
R. eutropha		
H16	Wild-type *R. eutropha*, gentamicin resistant (Gent-r)	(60)
Re2300	H16Δ(A0459-A0464), Gent-r	This work
Re2302	H16Δ(A1526-A1531), Gent-r	This work
Re2303	Re2300Δ(A1526-A1531), Gent-r	This work
Re2304	H16Δ*aceB*, Gent-r	This work
Re2306	H16Δ*iclA*, Gent-r	This work
Re2307	H16Δ*iclB*, Gent-r	This work
Re2312	H16Δ*fadD3*, Gent-r	This work
Re2313	H16ΔA1322, Gent-r	This work
E. coli		
S17-1	Strain for conjugative transfer of plasmids into *R. eutropha*	(50)

Plasmids:

Name	Description	Reference
pGY46	pJQ200Kan with Δ*phaC1* allele inserted into BamHI restriction site, confers kanamycin resistance	(42, 62)
pCJB4	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with a Δ(A0459-A0464) allele	This work
pCJB5	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with a Δ(A1526-A1531) allele	This work
pCB86	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with Δ*aceB* allele	This work
pCB94	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with Δ*iclA* allele	This work
pCB95	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with Δ*iclB* allele	This work
pCB96	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with Δ*fadD3* allele	This work
pCB97	pGY46 with Δ*phaC1* allele removed by BamHI digestion, and replaced with ΔA1322 allele	This work
pBBR1MCS-2	Broad host range cloning vector, confesr kanamycin resistance	(22)
pCJB200	pBBR1MCS-2 with *R. eutropha aceB* gene and surrounding region inserted into the multiple cloning site	This work
pCJB201	pBBR1MCS-2 with *R. eutropha* A1322 lipase gene and surrounding region inserted into the multiple cloning site	This work
pCJB202	pBBR1MCS-2 with *R. eutropha* A0459-A0464 operon DNA fragment inserted into the multiple cloning site	This work
pCJB203	pBBR1MCS-2 with *R. eutropha* A1526-A1531 operon DNA fragment inserted into the multiple cloning site	This work

Table 2: Summary of the functional annotations of genes differentially expressed during growth on either fructose or trioleate.

Code[a]	Functional group	Upregulated on trioleate		Upregulated on fructose	
		2-4-fold[b]	>4-fold[b]	2-4-fold[b]	>4-fold[b]
Information storage and processing					
J	Translation, ribosomal structure, and biogenesis	1 (0.6)	0 (0.0)	4 (2.2)	1 (0.6)
K	Transcription	32 (4.1)	9 (1.1)	10 (1.3)	6 (0.8)
L	DNA replication, recombination, and repair	5 (3.1)	0 (0.0)	1 (0.6)	1 (0.6)
Cellular processes					
D	Cell division and chromosomal partitioning	3 (10.8)	0 (0.0)	3 (10.8)	0 (0.0)
O	Post-translational modification, protein turnover, chaperones	8 (5.1)	2 (1.3)	7 (4.5)	2 (1.3)
M	Cell envelope biogenesis, outer membrane	11 (4.7)	4 (1.7)	9 (3.9)	2 (0.9)
N	Cell motility and secretion	0 (0.0)	0 (0.0)	16 (8.9)	12 (6.7)
P	Inorganic ion transport and metabolism	17 (16.7)	5 (4.9)	18 (17.6)	9 (8.8)
T	Signal transduction mechanisms	9 (1.1)	5 (0.6)	2 (0.2)	1 (0.1)
Metabolism					
C	Energy production and conversion	20 (4.1)	7 (1.4)	45 (9.3)	31 (6.4)
G	Carbohydrate metabolism and transport	11 (7.4)	4 (2.7)	20 (13.3)	14 (9.4)
E	Amino acid metabolism and transport	8 (2.7)	2 (0.7)	30 (10.1)	13 (4.4)
F	Nucleotide metabolism and transport	0 (0.0)	0 (0.0)	3 (4.0)	0 (0.0)
H	Coenzyme metabolism	3 (1.9)	2 (1.3)	2 (1.3)	1 (0.6)
I	Lipid metabolism	27 (8.2)	19 (5.7)	11 (3.3)	6 (1.8)
Q	Secondary metabolite biosynthesis, transport, and catabolism	3 (3.1)	2 (2.0)	2 (2.0)	1 (1.0)
Uncharacterized or poorly characterized					
R	General function prediction only	32 (4.3)	9 (1.2)	26 (3.5)	10 (1.3)
S	Function unknown	117 (6.4)	41 (2.2)	35 (1.9)	15 (0.8)
	TOTAL GENES	307	111	244	125

[a] Functional group annotations follow Tatusov, *et al.* (55)

[b] Numbers in parentheses indicate percentage of genes in a given functional group that are differentially expressed. Percentages are based on the total number of genes in that functional group present in the *R. eutropha* H16 genome.

Table 3: Genes and potential operons upregulated in expression during growth on trioleate.

Gene locus tag	GeneID Numbers[b]	Description	Fold increase
A3736-A3732	4246691, 4247741, 4247742, 4247743	Function unknown, likely outer membrane-related gene products	184[a]
A0459-A0464	4247875, 4247128, 4247876, 4247877, 4247878, 4247879	Fatty acid β-oxidation operon	36[a]
A1526-A1531	4249355, 4250030, 4249356, 4249357, 4249358, 4249320	Fatty acid β-oxidation operon	5[a]
A2507-A2509	4247547, 4247548, 4247471	First steps in glycerol metabolism	4[a]
A1322	4249488	Triacylglycerol lipase	7
A1323	4249489	Lipase chaperone	8
A3742	4249675	Lipase	4
A2217	4247136	Malate synthase, *aceB*	9
A2211	4250181	Isocitrate lyase, *iclA*	36
A2227	4250182	Isocitrate lyase, *iclB*	40
A3288	4246987	Acyl-CoA synthetase, *fadD3*	6

[a] Increase in expression of gene clusters is represented as an average fold increase in expression of all genes in a cluster.

[b] NCBI GeneID numbers are listed according to the corresponding locus tags, in ascending order (for gene clusters; i.e. A3732, A3733, A3734...)

Table 4. PHB utilization of β-oxidation and glyoxylate cycle mutant strains.

Strain	PHB content, production[a]	PHB content, utilization[b]	cfu/mL (× 10^5) 0 h[c]	cfu/mL (× 10^5) 24 h[c]
H16	75.5 ± 3.5	33.2 ± 3.8	2.2 ± 0.4	85 ± 5
Re2300	75.3 ± 1.1	33.1 ± 0.2	4.9 ± 2.6	135 ± 60
Re2302	70.6 ± 5.4	34.7 ± 0.3	2.2 ± 0.8	90 ± 20
Re2303	72.2 ± 1.5	27.9 ± 3.1	6.2 ± 2.3	90 ± 30
Re2304	67.5 ± 3.8	41.9 ± 1.5	3.1 ± 1.0	150 ± 50
Re2306	68.5 ± 1.4	24.9 ± 3.0	1.2 ± 0.2	160 ± 60
Re2307	67.5 ± 2.7	23.8 ± 5.0	2.1 ± 0.1	100 ± 25
Re2312	66.9 ± 0.1	33.8 ± 3.0	2.3 ± 0.3	120 ± 30
Re2313	71.5 ± 2.6	33.0 ± 1.5	2.8 ± 0.2	95 ± 15

[a] Intracellular PHB produced (% of cell dry weight) after 72 h incubation at 30°C in minimal medium with 2% fructose and 500 µg/mL NH_4Cl

[b] Intracellular PHB remaining after 24 h incubation at 30°C in minimal medium with 1 mg/mL NH_4Cl and no extracellular carbon source.

[c] Cell viable counts before incubation in PHB utilization media (0 h) and after 24 h incubation in PHB utilization media (24 h). Data are averages of 3 separate experiments.

Figures.

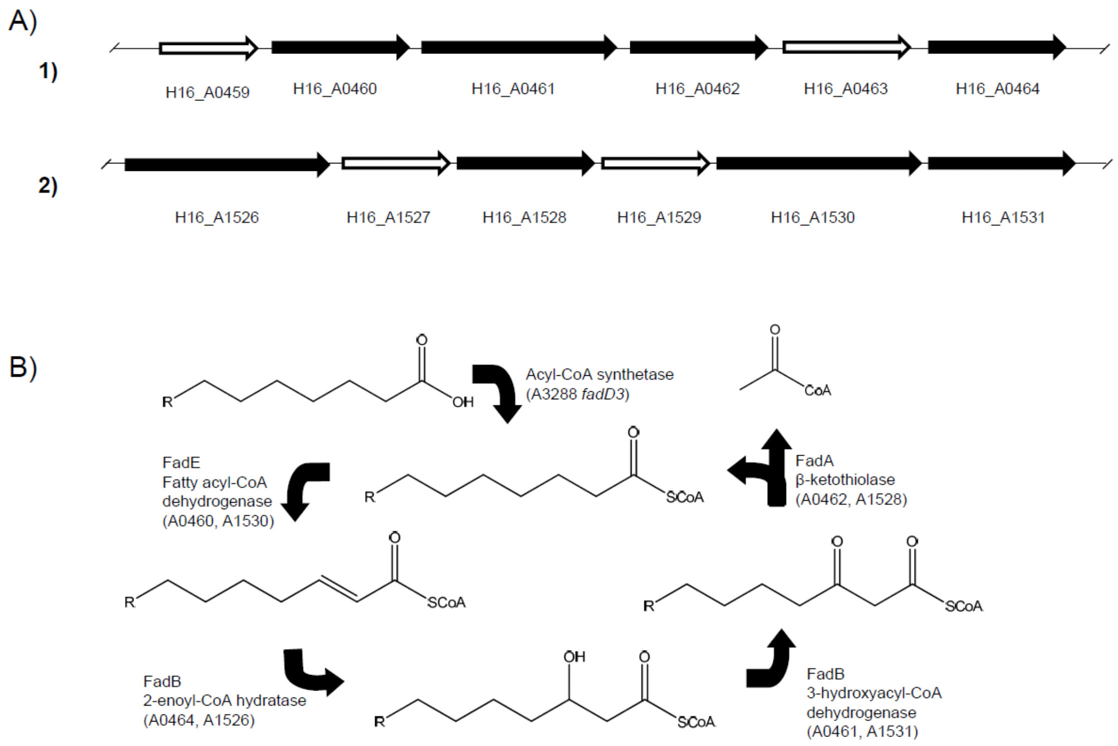


Figure 1. A) Two putative fatty acid β-oxidation operons were upregulated in expression when *R. eutropha* H16 was grown in the presence of trioleate. (1) and (2) are two distinct gene clusters, both containing genes encoding enzymes for all reactions in the β-oxidation cycle. B) Schematic of fatty acid β-oxidation in *R. eutropha*. The *R. eutropha* H16 gene locus tags indicate which gene products perform each step in the β-oxidation cycle. The products of four genes (A0459, transcriptional regulator; A0463, hypothetical DegV family protein; A1527, bifunctional pyrazinamidase/nicotinamidase; A1529, phenylacetic acid degradation protein PaaI) were not assigned roles in (B), and are denoted by white arrows in (A).

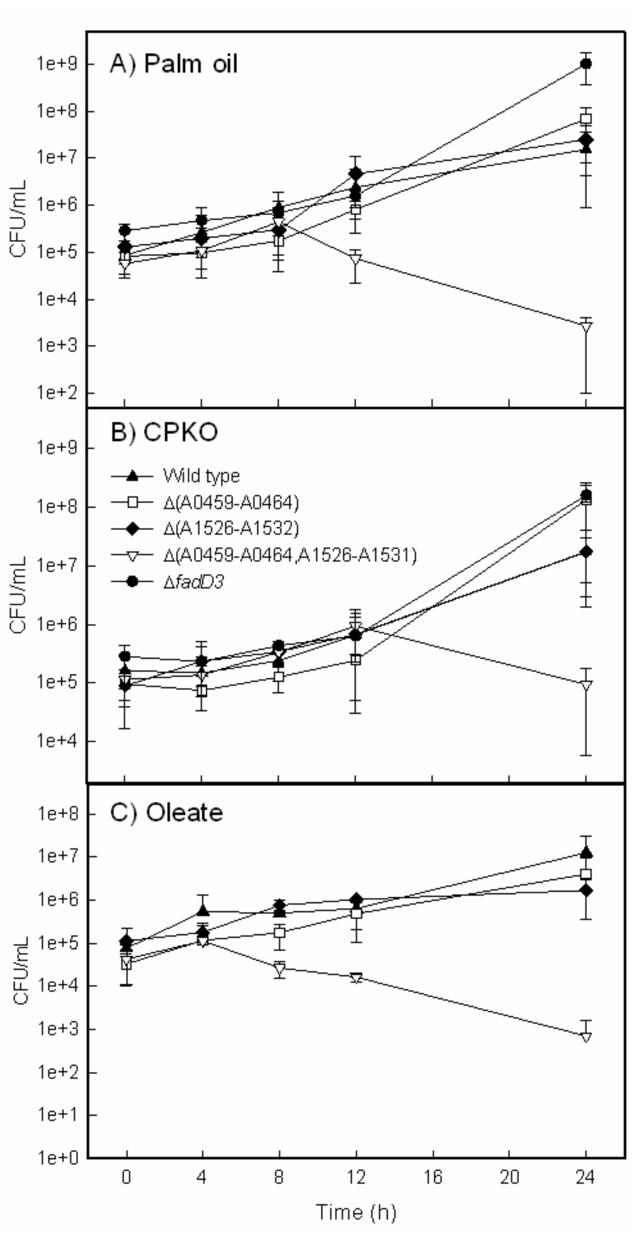


Figure 2. Growth of *R. eutropha* wild type (H16, filled triangles), β-oxidation mutants Re2300 (ΔA0459-A0464, open squares), Re2302 (ΔA1526-A1531, filled diamonds), Re2303 (ΔA0459-A0464, A1526-A1531, open inverted triangles) , Re2312 (Δ*fadD3*, filled circles) in minimal media with emulsified palm oil (A), CPKO (B), or oleic acid (C) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each point based on 3 separate experiments.

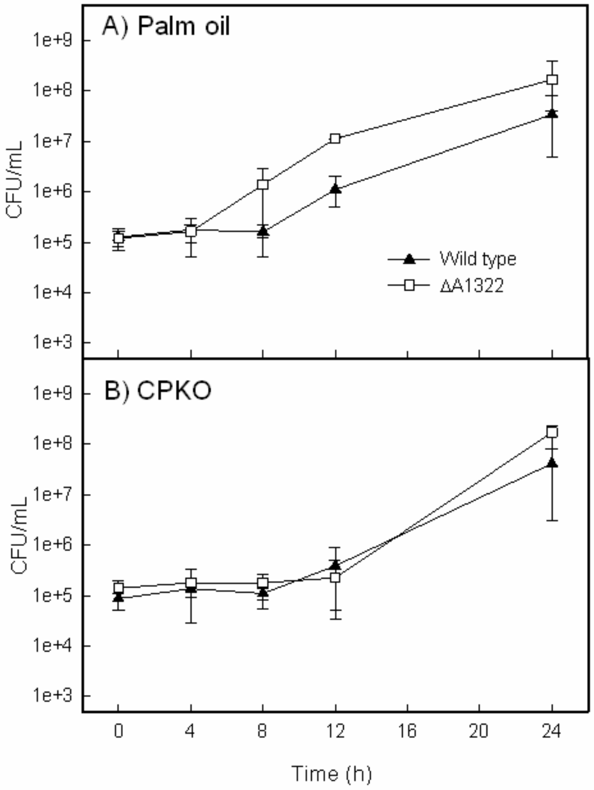


Figure 3. Growth of *R. eutropha* wild type (H16, filled triangles) and A1322 lipase gene deletion mutant Re2313 (ΔA1322, open squares) in minimal media with emulsified palm oil (A) or CPKO (B) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each point based on 3 separate experiments.

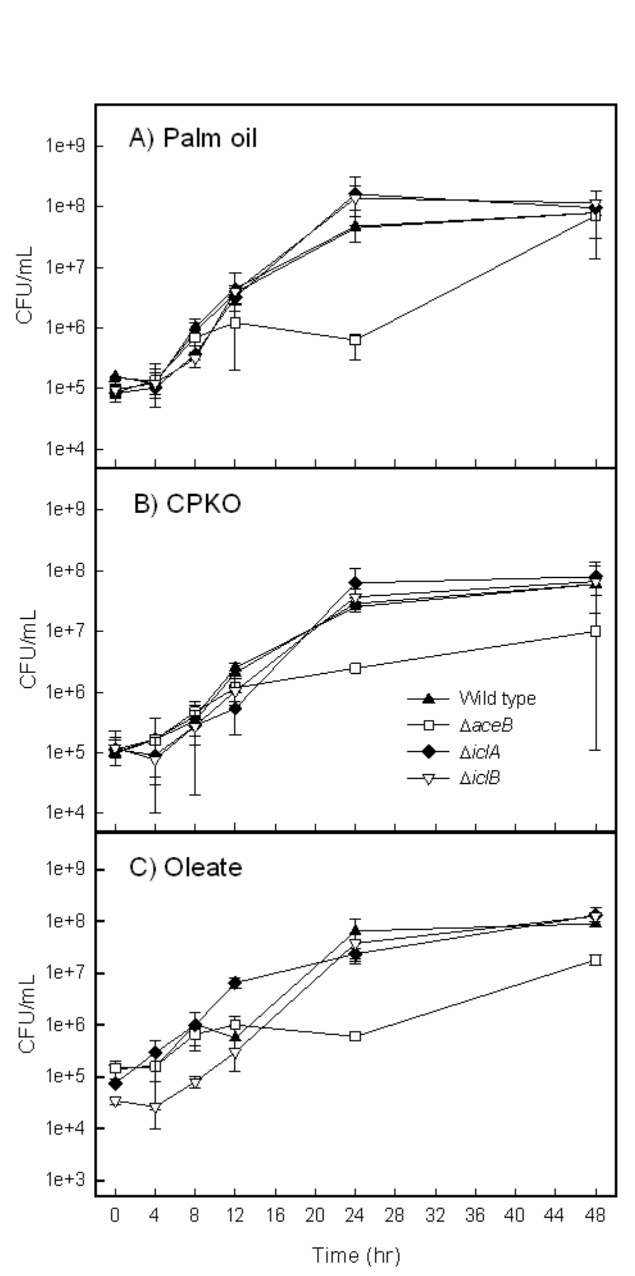


Figure 4. Growth of *R. eutropha* wild type (H16, filled triangles), glyoxylate cycle mutants Re2304 (Δ*aceB*, open squares), Re2306 (Δ*iclA*, filled diamonds), Re2307 (Δ*iclB*, open inverted triangles) in minimal media with emulsified palm oil (A), CPKO (B), or oleic acid (C) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each point based on 3 separate experiments.

Figure Legends.

Figure 1. A) Two putative fatty acid β-oxidation operons were upregulated in expression when *R. eutropha* H16 was grown in the presence of trioleate. (1) and (2) are two distinct gene clusters, both containing genes encoding enzymes for all reactions in the β-oxidation cycle. B) Schematic of fatty acid β-oxidation in *R. eutropha*. The *R. eutropha* H16 gene locus tags indicate which gene products perform each step in the β-oxidation cycle. The products of four genes (A0459, transcriptional regulator; A0463, hypothetical DegV family protein; A1527, bifunctional pyrazinamidase/nicotinamidase; A1529, phenylacetic acid degradation protein PaaI) were not assigned roles in (B), and are denoted by white arrows in (A).

Figure 2. Growth of *R. eutropha* wild type (H16, filled triangles), β-oxidation mutants Re2300 (ΔA0459-A0464, open squares), Re2302 (ΔA1526-A1531, filled diamonds), Re2303 (ΔA0459-A0464, A1526-A1531, open inverted triangles) , Re2312 (Δ*fadD3*, filled circles) in minimal media with emulsified palm oil (A), CPKO (B), or oleic acid (C) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each point based on 3 separate experiments.

Figure 3. Growth of *R. eutropha* wild type (H16, filled triangles) and A1322 lipase gene deletion mutant Re2313 (ΔA1322, open squares) in minimal media with emulsified palm oil (A) or CPKO (B) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each point based on 3 separate experiments.

Figure 4. Growth of *R. eutropha* wild type (H16, filled triangles), glyoxylate cycle mutants Re2304 (Δ*aceB*, open squares), Re2306 (Δ*iclA*, filled diamonds), Re2307 (Δ*iclB*, open inverted triangles) in minimal media with emulsified palm oil (A), CPKO (B), or oleic acid (C) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each point based on 3 separate experiments.

Elucidation of β-oxidation Pathways in *Ralstonia eutropha* H16 by Examination of Global Gene Expression

Supplemental Text

Supplemental Material 1: Construction of gene deletion strains and operon deletion strains of *Ralstonia eutropha*.

In-frame deletions of two β-oxidation operons in *R. eutropha* were constructed in the wild-type strain H16. The deletion of operon A0459-A0464 was constructed by allelic exchange between the chromosomal A0459-A0464 operon and the Δ(A0459-A0464) locus of the plasmid pCJB4 (Table 1). The pCJB4 plasmid was constructed as follows. A fragment, consisting of 504 bp upstream of the chromosomal A0459 gene was amplified using the oligonucleotides A0459upstreamF and A0459upstreamR (Supplemental Table 1) as primers. A second fragment, consisting of 507 bp downstream of the A0464 gene was amplified using A0459downstreamF and A0459downstreamR (Supplemental Table 1) as primers. The respective products were purified using a QIAquick PCR Purification Kit (QIAgen), and used for another round of amplification. The upstream and downstream PCR products were used for overlap PCR amplification (45), along with the A0459upstreamF and A0459downstreamR primers. The ~1 kb product was purified using a QIAquick PCR Purification Kit (QIAgen). The Δ(A0459-A0464) insert DNA was digested with *Bam*HI and then ligated into a *Bam*HI-digested pGY46 to create the Δ(A0459-A0464) allelic exchange plasmid pCJB4. The digested pGY46 plasmid had previously been gel purified using the QIAquick Gel Extraction Kit (QIAgen) to remove the *phaC* clean deletion allele. The deletion plasmid was then transferred to *R. eutropha* H16 by conjugation with *E. coli* strain S17-1. Precise deletions of the A0459-A0464 operon were constructed in *R. eutropha* by a standard procedure (39, 48, 59, 60), and confirmed by colony PCR using A0459diagF and A0459diagR (Supplementary Table 1) as primers to determine the presence of a deletion allele.

The deletion of operon A1526-A1531 was constructed by allelic exchange between the chromosomal A1526-A1531 operon and the Δ(A1526-A1531) locus of the plasmid pCJB5 (Table 1). The pCJB5 plasmid was constructed similarly to pCJB4. Briefly, a fragment, consisting of 569 bp upstream of the chromosomal A1526 gene was amplified using the oligonucleotides A1526upstreamF and A1526upstreamR (Supplementary Table 1) as primers. A second fragment, consisting of 577 bp downstream of the A1531 gene was amplified using A1526downstreamF and A1526downstreamR (Supplemental Table 1) as primers. The respective products were purified as described above, and the upstream and downstream PCR products were used for overlap PCR amplification, along with the A1526upstreamF and A1526downstreamR primers. The ~1.1 kb product was purified and ligated into a *Bam*HI-digested pGY46 as described above to create the Δ(A1526-A1531) allelic exchange plasmid pCJB5. The deletion plasmid was then transferred to *R. eutropha* H16 by conjugation with *E. coli* strain S17-1. Precise deletions of the A1526-A1531 operon were constructed in *R. eutropha* by a standard procedure (39, 48, 59, 60), and confirmed by colony PCR using A1526diagF and A1526diagR (Supplemental Table 1) as primers to determine the presence of a deletion allele.

DNA sequences for deletion of individual genes were ordered from Integrated DNA Technologies. Each sequence consisted of ~250 bp upstream of a given gene connected to ~250 bp downstream of the gene. BamHI restriction sites were present at both ends of each sequence so that the gene deletion fragments could be ligated into the pCB46 backbone. The sequences to delete each gene are given below.

aceB deletion sequence

GGATCCGCCGCCTGTACAGGAGCATTGTAGTGCGCAAGCGATGCGTGTGGCCCCCGTCATCCAGCGCC
CGTAAGTTGGCGGCTTCGACGCAGAAGGCTTACGTTCTGCGCACATTTGGAGGCTGTGCCAGTGAGTG
ACCGCCTTCCTGAACGACTCCAACAGTTGAGTGGACACTCGATCGGCATCCCAAAAGAAAAAACCGCA
GCCAAAGCTGCGGTTTTTTCATCCTGCTAGCACCCTACCCGCTTAATTAACAGTCTTCTCCTGTGATCG
ATAGCGGTAAGGCTTGGTTGAAGGGACCCTGCTACACCGCCACGCCACGGACGCGGACGAATTCAAG
CAGGTCGTTCATGTCGTGCCCGGTGCCGGAGGGGGCTACGTCGAGCCGCTCCGCCGGATGGCCGGCAC
GGTTGATCCAGAACGTGGTGTAGCCGAACCACGTGGCGCCGCAGGCGTCCCAGCCGTTGGACGAGAC
AAAGAGCATCTCTTCGGCGGGATCC

iclA deletion sequence

GGATCCGTGGAATTTGCTCCGGCTTTCCCCCGGCGCTATCTTTCGTTCCTTGAAAATGCTTTCCACATTG
CGGAATTTAGTGTTTATCTCATTGAAAAATAAGGAAAAATCAACTCTTATGTCTTATATAAGACTTTGC
CGCGACGCATCAAGAACGCCCTACAATGAACCCACGCTGCTCGCCTCGACATCCGCCAGCACACTCAA
CTATTTTTTTCAAACGCGCTTTCTTCTTAGGAAATTCCCTTAATTAATACCAGTAATACCCCAAGCAAA
ACGCCAACCGATACGGGTTGGCGTTTTTTTGTCCGGCGAGATCGCTCGCGGGGTTCTCATCAAACATAT
TCTTGTGCGCTTCCATGCAGAAGCTGTACGTGTGGCTTGAAATCAGCGTGCTGGGTTGAGACGCGGAT
GCGTCTCCTCTCCCCAGTGATACAGTACAAGGCCAGACAGTCCCATCGCGATGGCGACGAACCAGAAT
GCACTCTCGACGCGGATCC

iclB deletion sequence

GGATCCATGGAAGATCATTTCCCGCATCGTGAAATAAAATCTATAAGCCATTGAATTATTGTAAGAAA
AAAACTAAGCGAGGACTGCAGATCAATGTTGTTGCAACGCGGGAGGGTTCTCTACACTCTTATATAAG
ACTCATGACATGAGGCGCGAAAAACAGCAGGCGACGCAGCCACCGCGACGTAGCCCAGGTCAGGTGA
TCAACAGATTCTCAGCCATCCCCGCATCATCAGGAGAGTGACCTTAATTAACTCTCCTGGTCACCCCCG
CCGGAACGTGGGTCCGGCGGTTAAGGAAGGCGTGCGACCATCACGCCTTCTGATTTAGGCGCCGCGCC
GTTCTCTCTCAGGGACGGCGCGTTTTTTTATTTTCCAATGCTGGGGGCGGGTTAGCGGGTAGCTGTTCA
ACGGGTGTTCAAAACTCAAACTCCATGCAGAGTTACCGCCCAAGCCTGGCCGGGTTCACGCCGGCTAT
TTAGAAGCCTGACGCGGGGATCC

fadD3 deletion sequence

GGATCCGCAGGAGCTTGCCGCCAGCATGATGAAACTGGCGCTGCCCAGCGGCGGCTGAGTGTGGATTT
GCCTCACTAGGGGTTATCCCCCTACTTGCGCCCGATCGGTGGCGCATAATGCCCGTCAGGGGCACCAA
GGCCTGTCCGGTGGCCCCGAGGACTTCGTCTAAGTTGTTGTTTTAACCGGGGAGCACGTTTAGCATCTC
GGCAAAATCGAACGACCATTCAGAAATCAGGAGACGGTGCTTTAATTAAGCGGCAGCCGCTGCCAAC
GACAACGGCCTCCCGAGTGGAGGCCGTTGTGCTTTCAGGCTGCGCCGCGGCGGCTCAGCACTCGTTCG
ATGGCGCCAGGCGGGCTTCCTGGCTGCCGTCGGGGTAGATCACCCGCACGCACGCGCCTGGCTCCAGC
CCCGGCGCCGCCGGCGCGTTGGCCGCCACGATCGTGCCGTCCATGGTTCGCACCTTGTACTGGAACAG
GTCCACCTGCTGCACCGGGGATCC

A1322 (lipase) deletion sequence

GGATCCCGCAGGTTGCCTTGCGCGTCGAGTTCGGGGCCCGCGGGCACGTCGACGCCGGACAGCGATGG
CAAGGCAGCCACGCCGGCGGGTCCGGCGTCAGCCGATGTCGCCGAAGGACTCGCAGCGGTGGTGATC
GCTGCCGGGCGCTGCGGCACGGCAACCGGGCCAGACGGCATCGTCAGCCAGTACACCGCGGCCGCCG
CCGCGCCGGCCGGCAGCAAGGCCAGCCAGGGCCGCGGCGAGCGCTTAATTAACTTGTCTCCTCGGTGG
TGGGTCTTTTTATGTTGGAAGCAGTAAAGACTGGCCGCCCCGACGAGACAATCAAAACATTAGCGGAT
TCCCTCTAAACGGCCGCTACAGCCGCGGGCTGCAAGGGCATGCGGGTGCTGGAAAGCAGCTGTTCGTA
GATGTAGCACTGCAGCAGCGTCAGCTCGCGCTCGGCCAGCACGTCGAAGGCCACGCCGAAGGCCGGA
AAGTCGGCATCGTCGCTGGCCGGATCC

Gene deletions were performed by allelic exchange similar to the procedure mentioned above, and gene deletions were confirmed by PCR using forward and reverse diagnostic primers listed in Supplemental Table 1.

Supplemental Material 2: Changes in expression of other genes in *R. eutropha* H16 grown in the presence of trioleate, compared to cells grown in the presence of fructose.

A putative fructose metabolism operon (B1497-B1505) (37) is increased in expression an average of 15-fold when grown on fructose, compared to trioleate. Interestingly, a large number of the genes upregulated during growth on fructose are annotated as being involved in energy metabolism (Table 2), partially because many of the genes on megaplasmid pHG1 involved in hydrogen oxidation (*hox* and *hyp* genes) (11,48) are upregulated during growth on fructose (data not shown). A study on *R. eutropha* autotrophic metabolism enzyme activities during heterotrophic growth provides an interesting insight into this phenomenon (16). The authors of this study show that hydrogen oxidation enzyme activity can be found in extracts of cells grown on fructose as the main carbon source, but not in cells grown on acetate, pyruvate, and succinate. In the case of fructose growth, the presence of molecular hydrogen was not necessary for hydrogenase enzyme formation. In fact, the authors suggest that growth rate may be more of a contributing factor to hydrogenase gene expression in *R. eutropha*. Wild-type *R. eutropha* grows at an intermediate rate on fructose as a carbon source, and thus may activate autotrophic growth genes as a safeguard until a more readily usable carbon source can be found, such as acetate, succinate, or formate (16).

Interestingly, a gene (A2172) encoding phasin homolog PhaP3 (39-41) and a gene (A2171) encoding an acetoacetyl-CoA reductase homolog PhaB3*,* exhibited a 24-fold and a 21-fold decrease in gene expression, respectively, suggesting that expression of both these genes is repressed in the presence of plant oil. This agrees with recent work in which we showed that a *R. eutropha* strain harboring clean deletions of *phaB1* and *phaB2* homologs produces PHB when grown on fructose, but produces very little PHB when grown on palm oil (Budde, et al., manuscript submitted for publication). This suggests that PhaB3 is the only active PhaB homolog in this particular strain, and that its expression is repressed when cells are grown on oils as the sole carbon source. Given our results, it is also likely that the neighboring *phaP3* gene is repressed during growth on oils. Expression of both the *phaP3* and *phaB3* genes were also downregulated during PHB production on both carbon sources (Table 4). This contradicts previous reports that suggested *phaP3* is *phaR* regulated and should therefore increase in expression during PHB production (41).

Supplemental Material 3: Comparison of gene expression of cells in cultures in the presence and absence of nitrogen.

We also examined differences in gene expression in cultures before and after nitrogen was depleted using microarray analysis. Genes known to be involved in PHB biosynthesis (i.e. the *phaCAB* operon) were all highly expressed under all culture conditions tested. The gene encoding the predominant phasin protein, *phaP1,* was upregulated 8-fold in the absence of nitrogen. This finding confirms a recent gene expression study that shows an increase in expression of *phaP1* upon *R. eutropha*'s entry into stationary phase (37). This observation indicates that *R. eutropha* H16 is producing PHB under these conditions, as expected (56). Most genes encoding PHA depolymerases, including all known intracellular PHB depolymerases, were upregulated in the absence of nitrogen. Notably, the *phaZ2* gene was upregulated significantly during PHB production conditions (Supplemental Table 2). This phenomenon had been documented in quantitative RT-PCR studies of *R. eutropha* H16 cells grown in fructose (24). Also, in a recent gene expression study (37), expression of several intracellular PHA depolymerase genes were noted to have increased in *R. eutropha* H16 during the stationary phase of growth, presumably during nitrogen limitation. Other genes influenced by low nitrogen levels include a potential nitrogen scavenging gene cluster and putative nitrogen-responsive two-component system genes (Supplemental Table 2).

Elucidation of β-oxidation Pathways in *Ralstonia eutropha* H16 by Examination of Global Gene Expression

Supplemental Tables

Supplemental Table 1: List of primers used in this work

Name	Sequence[a]
A0459upstreamF	5'-CGGATCCAACGTCGCCGTTTATGATGCGG-3'
A0459upstreamR	5'-GCCGTTAATTAAGCCGGTTTTTCTCCGTTCTGCGG-3'
A0459downstreamF	5'-CGGCTTAATTAACGGCGGCCCACGTCTGTCCAATCC-3'
A0459downstreamR	5'-CGGATCCACCACACACCGTCATGAACGCTCC-3'
A0459diagF	5'-CGAGTATGCCGAGAGCTTCC-3'
A0459diagR	5'-GCGCGATTGTCGCAGAGTTC-3'
A1526upstreamF	5'-CGGATCCCTTGAGCCTGCGCTTGAGGTG-3'
A1526upstreamR	5'-GCCGTTAATTAAGCCGGCTGGTTCCTTTGGTGTCAA-3'
A1526downstreamF	5'-CGGCTTAATTAACGGCCGGATTGCCCTCAAACTTGG-3'
A1526downstreamR	5'-CGGATCCCGGCTTGAATACTGCGTCGGGA-3'
A1526diagF	5'-GCTGTAGGTGACGAAGGAGC-3'
A1526diagR	5'-GCGCTTGAACGTTTTGGACA-3'
aceBdiagF	5'-GCTGCTGTCGGTCATCTGGA-3'
aceBdiagR	5'-CTTTGTCTCGTCCAACGGCTGG-3'
iclAdiagF	5'-CGTCTGCCATTGGCATCTACC-3'
iclAdiagR	5'-GACGATCATCCCATCCGTGC-3'
iclBdiagF	5'-GGACACATGGACTGCGCTGA-3'
iclBdiagR	5'-CAACGCGGGAGGGTTCTCTAC-3'
fadD3diagF	5'-GCTTTGCCTTCGACCTGAGC-3'
fadD3diagR	5'-TCCTCTACACCTGGCATGAACC-3'
A1322diagF	5'-GTCACCATCGATGGCTGGATC-3'
A1322diagR	5'-CGGATATCGTCGTACACCAGCC-3'
aceBregionF	5'-GCTTGCGGTACCGCATTGTGCAGGTCGTTCAG-3'
aceBregionR	5'-GCTTGCAAGCTTGTACAGGAGCATTGTAGTGCGCAAGC-3'
A1322regionF	5'-GCTTGCGGTACCGTTTCGCTACCTGGTGCTGT-3'
A1322regionR	5'-GCTTGCAAGCTTCAAGGATCCGACCCAGAACATCGTCTG-3'
A0459opF	5'-GCTTGCGGTACCGCCAACGTCGCCGTTTATGA-3'
A0459opR	5'-GCTTGCGATATCCCGTACCTCGATCACGGTGTCGG-3'
A1526opF	5'-GCTTGCGGTACCCTTGAGCCTGCGCTTGAGGT-3'
A1526opR	5'-GCTTGCAAGCTTCTGTTCTGGTGGCGCTTCTCGATCG-3'

[a]Restriction sites underlined

Supplemental Table 2. Select genes and operons differentially regulated in cultures with and without nitrogen[a].

Gene locus tag	GeneID Number	Gene name	Expression[b] (with nitrogen)	Expression[b] (without nitrogen)	Fold change[c]
A1381	4250158	Phasin gene, *phaP1*	12.7	15.7	8.0
PHG202	2656644	Phasin gene, *phaP2*	7.1	8.1	2.0
A2172	4250159	Phasin gene, *phaP3*	12.0	10.1	(4.0)[d]
B2021	4456981	Phasin gene, *phaP4*	9.5	12.0	5.0
A2171	4250155	Acetoacetyl-CoA reductase gene, *phaB3*	10.5	6.9	(12.5)[d]
A1150	4250163	PHB depolymerase gene, *phaZ1*	9.2	11.1	4.0
A2862	4250164	PHB depolymerase gene, *phaZ2*	4.7	12.7	265.6
B1014	4455259	PHB depolymerase gene, *phaZ3*	5.4	8.6	9.0
B0339	4457024	PHB depolymerase gene, *phaZ5*	7.5	8.8	2.5
B2073	4456176	PHB depolymerase gene, *phaZ7*	6.9	8.0	2.0
A2251	4250161	3HB oligomer hydrolase gene, *phaY1*	7.1	9.5	5.2
B0078	4455951	Signal transduction histidine kinase gene	3.2	8.0	29.0
B0079	4455312	Response regulator gene, *narL*	3.5	9.3	54.0
A2332	4250208	Response regulator gene, *ntrC*	8.3	14.1	55.0
A2333	4250207	Signal transduction histidine kinase gene, *ntrB*	8.9	10.3	3.0
A1075-A1087	4248690–4249128	Potential urea scavenging gene cluster	5.1[e]	11.5[e]	316[e]

[a] Nitrogen source = 0.05% (initial concentration) NH_4Cl

[b] Expression values are base 2 logarithms of measured values.

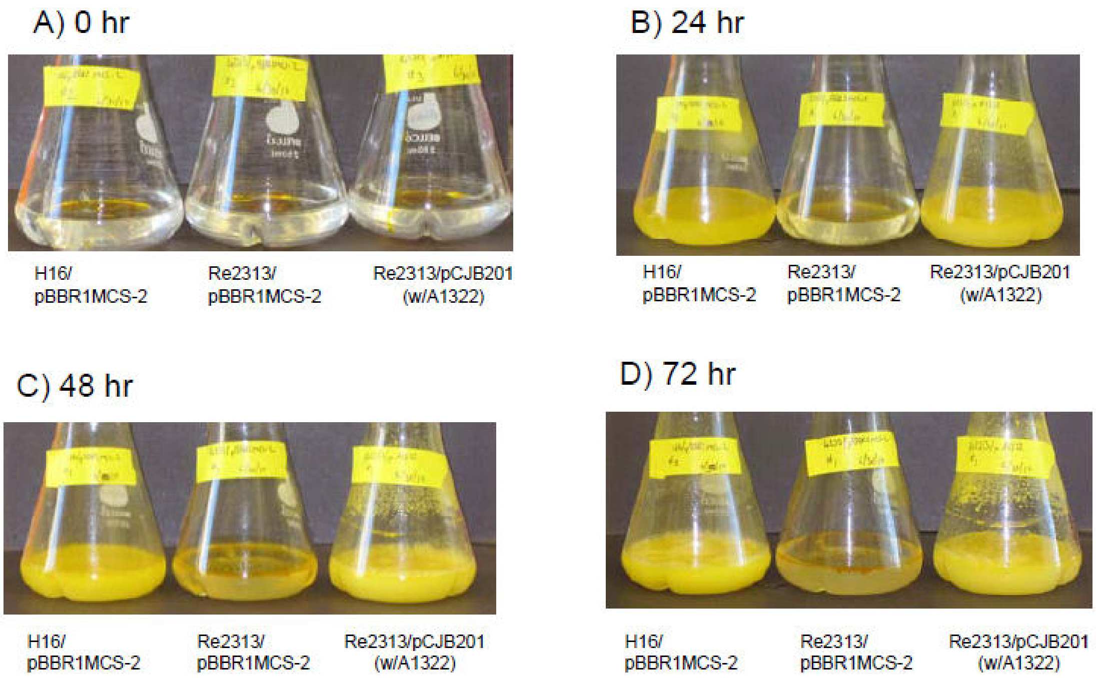
[c] All gene expression changes represented in this table have a *p* value less than or equal to 0.01.

[d] Values in parentheses indicate a decrease in gene expression

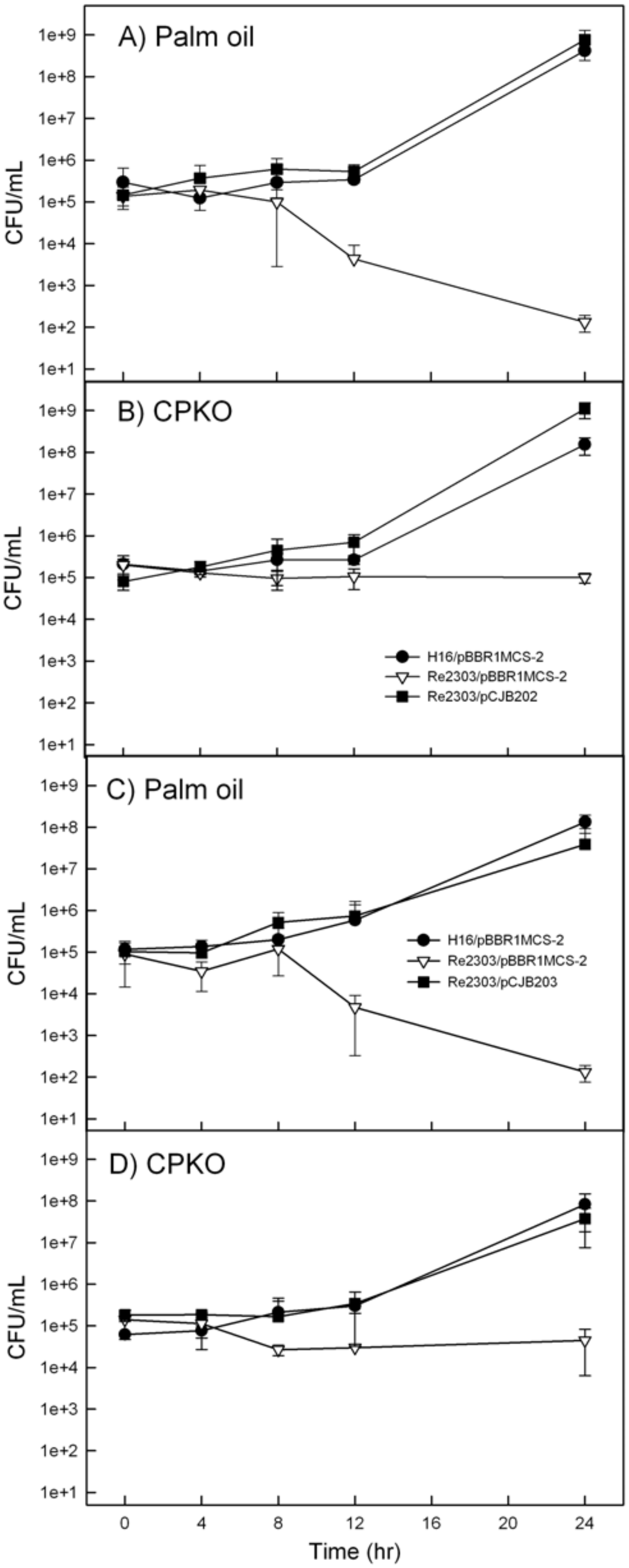
[e] Values listed are averages from 13 individual genes.

Elucidation of β-oxidation Pathways in *Ralstonia eutropha* H16 by Examination of Global Gene Expression

Supplemental Figures:

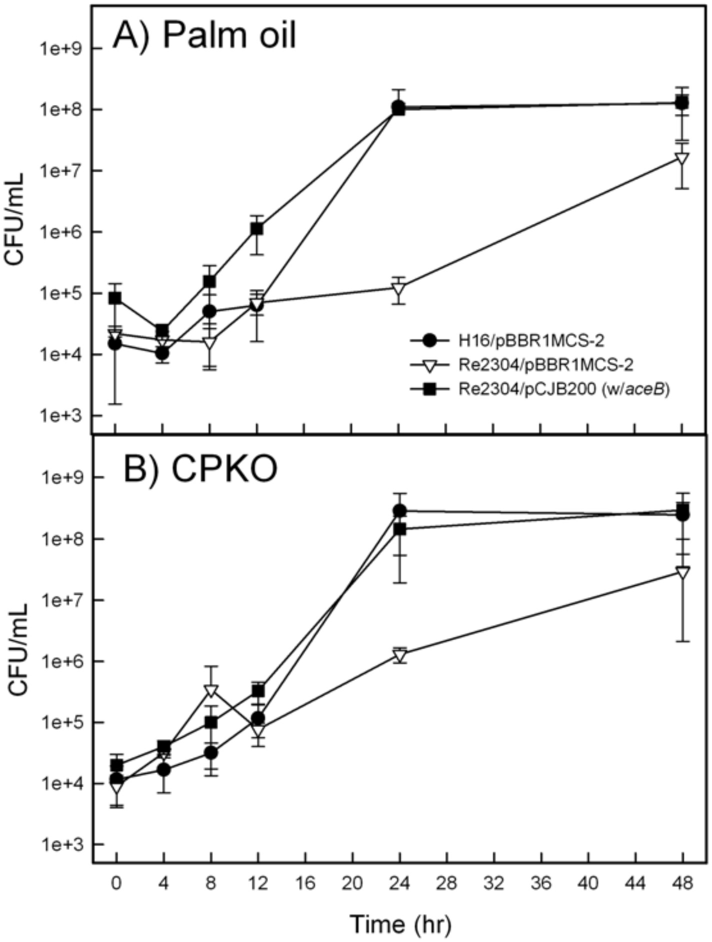


Supplemental Figure 1. Growth of *R. eutropha* H16/pBBR1MCS-2 (wild-type with empty vector), Re2313/pBBR1MCS-2 (A1322 lipase gene deletion mutant with empty vector), and Re2313/pCJB201 (A1322 deletion mutant with plasmid containing the A1322 gene) in minimal media with non-emulsified palm oil as the carbon source: A) 0 h growth, B) 24 h growth, C) 48 h growth, D) 72 h growth. In (A-D), the H16/pBBR1MCS-2 culture is pictured on the left, the Re2313/pBBR1MCS-2 culture is pictured in the middle, and the Re2313/pCJB201 culture is pictured on the right. This figure is representative of 3 separate experiments.



Supplemental Figure 2

Supplemental Figure 2. Growth of *R. eutropha* wild type containing empty vector (H16/pBBR1MCS-2, filled circles), β-oxidation double mutant Re2303 (ΔA0459-A0464, ΔA1526-A1531) containing empty vector (Re2303/pBBR1MCS-2, open inverted triangles), Re2303 containing a plasmid expressing the A0459-A0464 β-oxidation operon (A and B, Re2303/pCJB202, filled boxes), and Re2303 containing a plasmid expressing the A1526-A1531 β-oxidation operon (C and D, Re2303/pCJB203, filled boxes) in minimal media with emulsified palm oil (A and C) or CPKO (B and D) as the sole carbon source. Data points are the averages of 3 separate experiments, and error bars represent the maxima and minima of each data set based on 3 separate experiments.



Supplemental Figure 3. Growth of *R. eutropha* wild type with empty vector (H16/pBBR1MCS-2, filled circles), glyoxylate cycle mutants Re2304 (Δ*aceB*) with empty vector (Re2304/pBBR1MCS-2, open inverted triangles), and Re2304 with *aceB* expressed on a plasmid (Re2304/pCJB200, +*aceB,* filled boxes) in minimal media with emulsified palm oil (A) or CPKO (B) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima and minima of each data set based on 3 separate experiments.