JOURNAL OF BACTERIOLOGY, Oct. 2010, p. 5454–5464 Vol. 192, No. 20
0021-9193/10/$12.00 doi:10.1128/JB.00493-10

Elucidation of β-Oxidation Pathways in *Ralstonia eutropha* H16 by Examination of Global Gene Expression▽†

Christopher J. Brigham,[1] Charles F. Budde,[2] Jason W. Holder,[1] Qiandong Zeng,[6] Alison E. Mahan,[1]‡ ChoKyun Rha,[3] and Anthony J. Sinskey[1,4,5]*

Department of Biology,[1] Department of Chemical Engineering,[2] Biomaterials Science and Engineering Laboratory,[3] Division of Health Sciences Technology,[4] and Engineering Systems Division,[5] Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, and Broad Institute, Cambridge, Massachusetts 02139[6]

Received 30 April 2010/Accepted 26 July 2010

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 levels 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 nonemulsified oil. Mutants of the glyoxylate shunt gene for 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*.**

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 betaproteobacterium *Ralstonia eutropha*. *R. eutropha* can store PHA of 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 PHAs (scl-PHAs), 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 PHAs (mcl-PHAs), derived mainly from fatty acid β-oxidation intermediates (23). Some species are capable of producing a combination of scl- and mcl-PHAs during nutrient starvation (45, 52, 53). These copolymers comprised of scl and mcl monomers exhibit thermal and mechanical properties similar to those of 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 PHAs 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 PHAs with a combination of scl and mcl monomers (27, 30). Plant oils are a suitable carbon source for this endeavor as 3-hydroxyacyl coenzyme A (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 feedstock alternative 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 metric tons/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 ($C_{16:0}$), oleic ($C_{18:1}$), and linoleic ($C_{18:2}$) acids comprising more than 90% of the total fatty acid content (13). Palm kernel oil is comprised mostly of lauric ($C_{12:0}$), myristic ($C_{14:0}$), 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

* Corresponding author. Mailing address: Department of Biology, Building 68, Room 370a, Massachusetts Institute of Technology, 31 Ames Street, Cambridge, MA 02139. Phone: (617) 253-6721. Fax: (617) 2534-8550. E-mail: asinskey@mit.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Present address: Harvard University School of Medicine, Boston, MA.

▽ Published ahead of print on 13 August 2010.

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype or description	Reference(s)
Strains		
R. eutropha		
H16	Wild-type *R. eutropha*, gentamicin resistant (Gen^r)	59
Re2300	H16Δ(A0459-A0464), Gen^r	This work
Re2302	H16Δ(A1526-A1531), Gen^r	This work
Re2303	Re2300Δ(A1526-A1531), Gen^r	This work
Re2304	H16Δ*aceB*, Gen^r	This work
Re2306	H16Δ*iclA*, Gen^r	This work
Re2307	H16Δ*iclB*, Gen^r	This work
Re2312	H16Δ*fadD3*, Gen^r	This work
Re2313	H16ΔA1322, Gen^r	This work
E. coli S17-1	Strain for conjugative transfer of plasmids into *R. eutropha*	50
Plasmids		
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, confers 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

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 Fig. 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 *Escherichia coli* and *Bacillus subtilis* (18, 29), although some information is available regarding fatty acid degradation in *Pseudomonas* species (9, 14). 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 the 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 does 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 K. Sudesh Kumar (Universiti Sains Malaysia, Penang, Malaysia).

Design of custom *Ralstonia eutropha* H16 microarray chips. Probes representing 6,626 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, CA). 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 (Affymetrix).

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 reference 36, containing 0.1% NH_4Cl and either 2% (wt/vol) fructose or 1% (wt/vol) trioleate, emulsified with 0.3% (wt/vol) gum arabic. These cultures were grown for 24 h. Overnight cultures were inoculated to an initial optical density at 600 nm (OD_{600}) of 0.1 into 250-ml shake flasks containing 50 ml of minimal medium containing 0.05% NH_4Cl and either 2% (wt/vol) fructose or 1% (wt/vol) trioleate, emulsified with 0.3% (wt/vol) 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% (wt/vol) fructose or 1% (wt/vol) trioleate, emulsified with 0.3% (wt/vol) 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 of 2.5) was harvested at an NH_4^+ concentration of 0.025%, and another aliquot of cells (also an OD_{600} equivalent of 2.5) was harvested 2 h after depletion of nitrogen in the medium. Culture aliquots were treated with 2 volumes of RNA Protect reagent (Qiagen, Valencia, CA). Cells were centrifuged at 5,000 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 min on ice, and cell suspensions were vortexed every 2 min. RNA was then isolated from cells using the RNeasy minikit (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 to 10.0 (10.0 is highest quality) were used for microarray analysis (15). One hundred nanograms 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 h at 45°C and scanned according to platform specifications. Array chips were scanned using an Affymetrix 7G scanner.

Microarray data analysis. Microarray data were 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 *t* test (ArrayStar) and confirmed using analysis of variance (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 the work of 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 Table S1 in the supplemental material. Plasmid vectors (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 the QIAquick gel extraction kit (Qiagen, Valencia, CA), digested with BamHI, and then ligated into the backbone of pGY46 (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 the QIAquick gel extraction kit. Plasmids for deletion of individual genes were constructed following a similar procedure, except that 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 (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 text S1 in the supplemental material.

Construction of complementation plasmids and introduction into *R. eutropha* deletion mutants. 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 Table S1 in the supplemental material. PCR products were purified using the 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 growth 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).

Growth of *R. eutropha* strains in medium containing plant oils or fatty acids. 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 reference 36, containing 0.1% NH_4Cl and 1% (wt/vol) palm oil, CPKO, or oleic acid, emulsified with 0.3% (wt/vol) 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 to 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 that hexane was not included in the wash step. The PHB content and cell dry weight (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 medium (~250 μg/ml). Samples were also taken ~2 h after all NH_4Cl

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

Code[a]	Functional group	No. (%) of genes upregulated on[b]:			
		Trioleate		Fructose	
		2- to 4-fold	>4-fold	2- to 4-fold	>4-fold
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	Posttranslational 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 no. of genes		307	111	244	125

[a] Functional group annotations follow the work of Tatusov et al. (55).
[b] Numbers in parentheses indicate percentages 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.

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 to 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; Fig. 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 "Axxxx" and "Bxxxx" refer to the locus tags of genes discussed in this work, where A indicates that the gene is on chromosome 1 and B indicates that the gene is on chromosome 2.) The reason for the extremely high increases in expression of these genes is partially 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 the wild type under all conditions tested (data not shown). Therefore, this strain was not studied further. Two potential operons (A0459-A0464 and A1526-A1531) each appear to contain genes that encode the enzymes necessary for fatty acid β-oxidation (Table 3; Fig. 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

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

Gene locus tag(s)	Gene ID no.[b]	Description	Fold increase[a]
A3736-A3732	4246691, 4247741, 4247742, 4247743	Function unknown, likely outer membrane-related gene products	184
A0459-A0464	4247875, 4247128, 4247876, 4247877, 4247878, 4247879	Fatty acid β-oxidation operon	36
A1526-A1531	4249355, 4250030, 4249356, 4249357, 4249358, 4249320	Fatty acid β-oxidation operon	5
A2507-A2509	4247547, 4247548, 4247471	First steps in glycerol metabolism	4
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 gene ID numbers are listed according to the corresponding locus tags, in ascending order (for gene clusters, i.e., A3732, A3733, A3734 . . .).

(*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 (60). 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

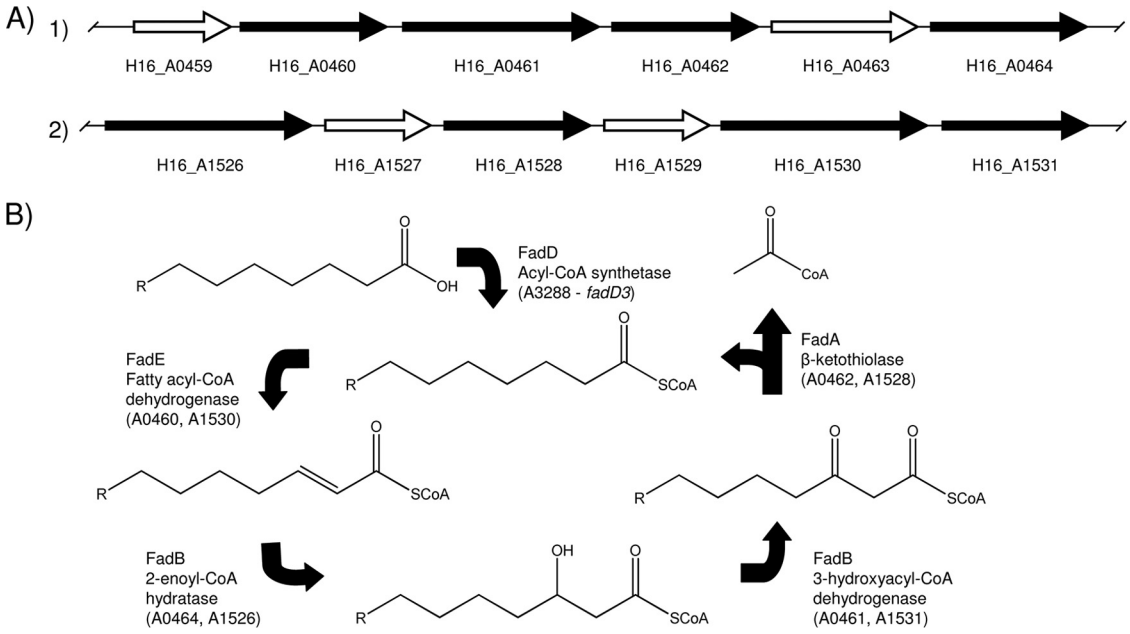

FIG. 1. (A) Two putative fatty acid β-oxidation operons were upregulated in expression when *R. eutropha* H16 was grown in the presence of trioleate. Schematics 1 and 2 are two distinct gene clusters, both containing genes encoding enzymes for all reactions in the β-oxidation cycle. (B) Schematic of 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; and A1529, phenylacetic acid degradation protein PaaI) were not assigned roles in panel B and are denoted by white arrows in panel A.

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 (11, 16, 24, 37–41, 49, 56).

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 (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 cell densities similar to that of the wild type (Fig. 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 are 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 medium (Fig. 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 the A1529-A1531 gene cluster (see Fig. S2 in the supplemental material). 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 (Fig. 2), suggesting that 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 the 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.

Growth phenotype of a lipase mutant strain in the presence of plant oils. 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 is 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 the wild type (Fig. 3). We also examined growth of Re2313 in medium with nonemulsified 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 the 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

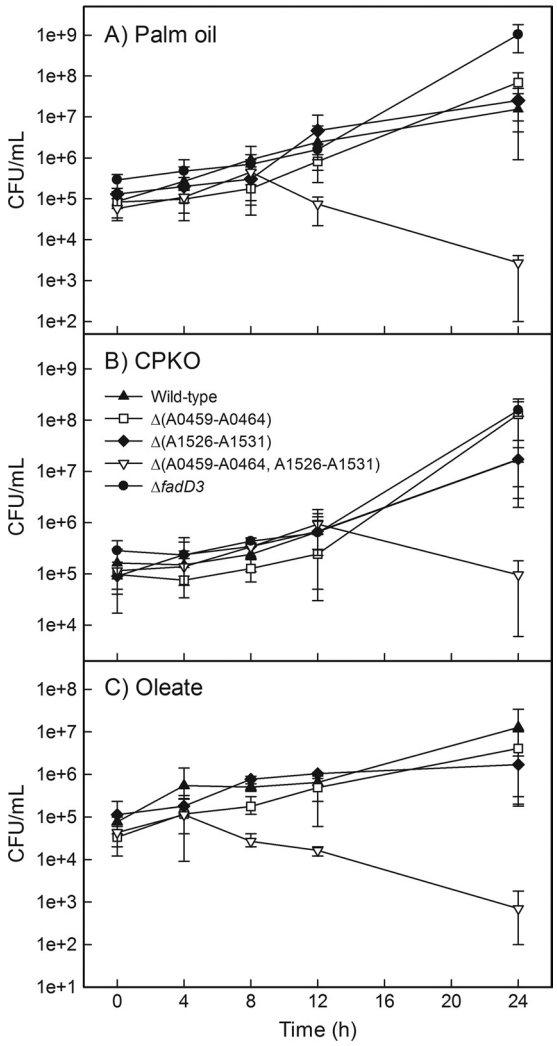

FIG. 2. Growth of *R. eutropha* wild type (H16, filled triangles) and β-oxidation mutants Re2300 (ΔA0459-A0464, open squares), Re2302 (ΔA1526-A1531, filled diamonds), Re2303 (ΔA0459-A0464, A1526-A1531, open inverted triangles), and Re2312 (Δ*fadD3*, filled circles) in minimal medium with emulsified palm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

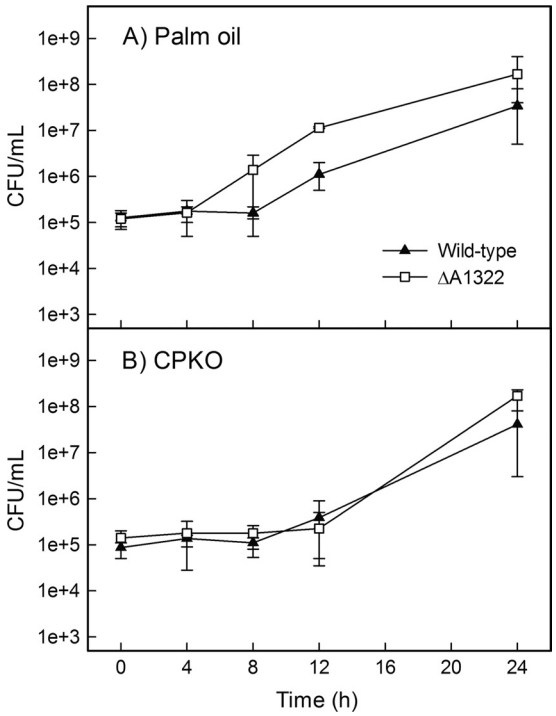

FIG. 3. Growth of *R. eutropha* wild type (H16, filled triangles) and A1322 lipase gene deletion mutant Re2313 (ΔA1322, open squares) in minimal medium with emulsified palm oil (A) or CPKO (B) as the carbon source. Data points are the average of 3 separate experiments, and error bars represent the maxima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

Re2313 (see Fig. S1 in the supplemental material). This suggests that the lipase gene A1322 is necessary for optimal growth in nonemulsified plant oil media.

Growth phenotype of glyoxylate bypass mutants. 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 (60). 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 (Fig. 4). Wang et al. (60) 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 (60), 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 (see Fig. S3 in the supplemental material).

Isocitrate lyase gene deletion mutant strains Re2306 (Δ*iclA*) and Re2307 (Δ*iclB*) both exhibited growth on oil cultures similar to that of the wild type (Fig. 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

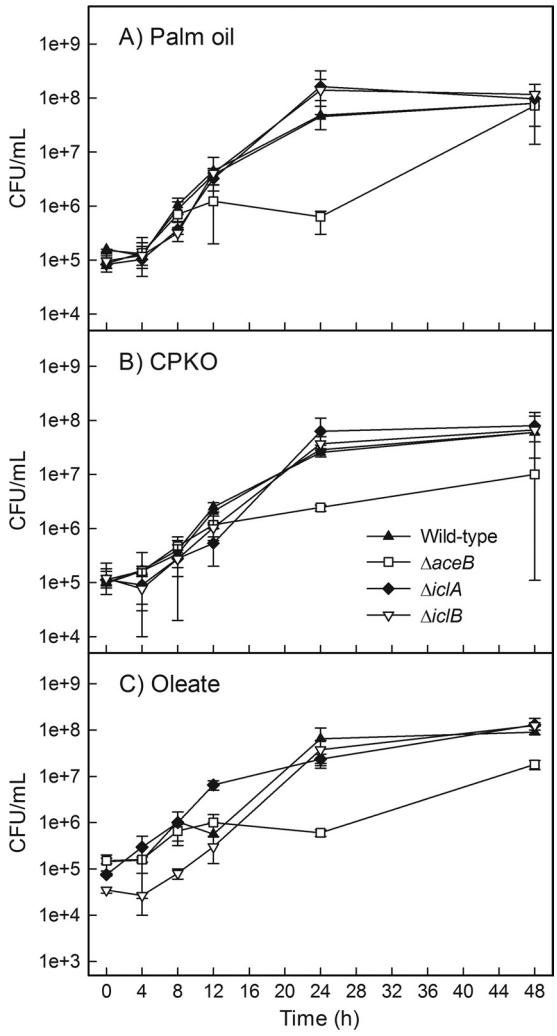

FIG. 4. Growth of *R. eutropha* wild type (H16, filled triangles) and glyoxylate cycle mutants Re2304 (Δ*aceB*, open squares), Re2306 (Δ*iclA*, filled diamonds), and Re2307 (Δ*iclB*, open inverted triangles) in minimal medium with emulsified palm oil (A), CPKO (B), or oleic acid (C) as the carbon source.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

the loss of *iclA* or *iclB*. Our data differ from those of a previous study which showed that an *iclA* mutant of *R. eutropha* HF39 was unable to grow on acetate as the sole carbon source (60). All glyoxylate cycle mutant strains grew similarly to the 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 quantities similar to those of the wild-type strain, using palm oil, CPKO, or fructose as the carbon source (data not shown). These results are in contrast to previously published results, where *iclA* and *iclB* mutant strains exhibited PHB production defects during growth on gluconate and acetate (60). PHB utilization was also examined in our mutant

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

Strain	PHB content		CFU/ml (10^5)[c]	
	Production[a]	Utilization[b]	0 h	24 h
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 of incubation at 30°C in minimal medium with 2% fructose and 500 μg/ml NH_4Cl.

[b] Intracellular PHB (% of cell dry weight) remaining after 24 h of 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 medium (0 h) and after 24 h of incubation in PHB utilization medium (24 h). Data are averages of 3 separate experiments.

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 did 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 (Fig. 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 utilize both palm oil and palm kernel oil for growth, it is likely that the gene products of both β-oxidation operons can utilize long-chain (C_{12} 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, Fig. 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 that 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 that 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 the 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 tricarboxylic acid (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 (60). 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. In comparison to the wild type, the *aceB* mutant strain, Re2304, exhibited a slower-growth phenotype on oils (Fig. 4). Consistent with previous data (60), the *aceB* mutant strain also exhibited slower growth on acetate as a carbon source (data not shown). Both *icl* mutant strains grew similarly to the wild-type strain on palm oil and CPKO (Fig. 4). These results are in contrast to previous results, where an *iclA* knock-

out strain of *R. eutropha* was unable to grow on minimal medium containing acetate as the sole carbon source (60). 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 (Fig. 3), exhibited an interesting phenotype. When grown on nonemulsified palm oil, Re2313 (the ΔA1322 mutant) was not able to create a stable emulsion of oil droplets, even after 72 h of growth (see Fig. S1 in the supplemental material). These results suggest that the A1322 lipase gene product plays a critical role in the ability of *R. eutropha* 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 (A3742 expression increase data are shown in 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 to 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 (see Table S2 in the supplemental material). 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 (see Table S2), 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 reverse transcriptase PCR (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 (see Table S2). 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.

ACKNOWLEDGMENTS

We thank Stuart Levine, 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 Daniel MacEachran for critical review of the manuscript prior to submission.

This work was funded by the Malaysia/MIT Biotechnology Partnership Program, which is a collaborative effort among scientists at MIT, Universiti Sains Malaysia, Universiti Putra Malaysia, and SIRIM Berhad. We thank the members of this program for their collegial collaborations.

REFERENCES

1. **Arpigny, J. L., and K. E. Jaeger.** 1999. Bacterial lipolytic enzymes: classification and properties. Biochem. J. **343:**177–183.
2. **Bartlett, K., and S. Eaton.** 2004. Mitochondrial beta-oxidation. Eur. J. Biochem. **271:**462–469.
3. **Basiron, Y.** 2007. Palm oil production through sustainable plantations. Eur. J. Lipid Sci. Technol. **109:**289–295.
4. **Bramer, C. O., and A. Steinbuchel.** 2002. The malate dehydrogenase of *Ralstonia eutropha* and functionality of the C(3)/C(4) metabolism in a Tn5-induced mdh mutant. FEMS Microbiol. Lett. **212:**159–164.
5. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(beta-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:** 1977–1982.
6. **Bruland, N., I. Voss, C. Bramer, and A. Steinbuchel.** 19 November 2009, posting date. Unravelling the C(3)/C(4) carbon metabolism in *Ralstonia eutropha* H16. J. Appl. Microbiol. doi:10.1111/j.1365–2672.2009.04631.x.
7. **Carlsson, A. S.** 2009. Plant oils as feedstock alternatives to petroleum—a short survey of potential oil crop platforms. Biochimie **91:**665–670.
8. **Chee, M., R. Yang, E. Hubbell, A. Berno, X. C. Huang, D. Stern, J. Winkler, D. J. Lockhart, M. S. Morris, and S. P. Fodor.** 1996. Accessing genetic information with high-density DNA arrays. Science **274:**610–614.
9. **Chung, A., Q. Liu, S.-P. Ouyang, Q. Wu, and G.-Q. Chen.** 2009. Microbial production of 3-hydroxydodecanoic acid by *pha* operon and *fadBA* knockout mutant of *Pseudomonas putida* KT2442 harboring *tesB* gene. Appl. Microbiol. Biotechnol. **83:**513–519.
10. **Cozzone, A. J.** 1998. Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. Annu. Rev. Microbiol. **52:**127–164.
11. **Cramm, R.** 2009. Genomic view of energy metabolism in *Ralstonia eutropha* H16. J. Mol. Microbiol. Biotechnol. **16:**38–52.
12. **Doi, Y., S. Kitamura, and H. Abe.** 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Macromolecules **28:**4822–4828.
13. **Edem, D. O.** 2002. Palm oil: biochemical, physiological, nutritional, hematological, and toxicological aspects: a review. Plant Foods Hum. Nutr. **57:** 319–341.
14. **Fiedler, S., A. Steinbüchel, and B. H. A. Rehm.** 2002. The role of the fatty acid bgr-oxidation multienzyme complex from *Pseudomonas oleovorans* in polyhydroxyalkanoate biosynthesis: molecular characterization of the *fadBA*

operon from *P. oleovorans* and of the enoyl-CoA hydratase genes *phaJ* from *P. oleovorans* and *Pseudomonas putida*. Arch. Microbiol. **178:**149–160.
15. **Flanagan, N.** 15 March 2006. Tissue microarrays reach new markets. Genet. Eng. News, vol. 26, no. 6.
16. **Friedrich, C. G., B. Friedrich, and B. Bowien.** 1981. Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*. J. Gen. Microbiol. **122:**69–78.
17. **Frothingham, R., W. A. Meeker-O'Connell, E. A. Talbot, J. W. George, and K. N. Kreuzer.** 1996. Identification, cloning, and expression of the *Escherichia coli* pyrazinamidase and nicotinamidase gene, *pncA*. Antimicrob. Agents Chemother. **40:**1426–1431.
18. **Fujita, Y., H. Matsuoka, and K. Hirooka.** 2007. Regulation of fatty acid metabolism in bacteria. Mol. Microbiol. **66:**829–839.
19. **Jaeger, K. E., and T. Eggert.** 2002. Lipases for biotechnology. Curr. Opin. Biotechnol. **13:**390–397.
20. **Jendrossek, D.** 2009. Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). J. Bacteriol. **191:**3195–3202.
21. **Karr, D. B., J. K. Waters, and D. W. Emerich.** 1983. Analysis of poly-beta-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Appl. Environ. Microbiol. **46:**1339–1344.
22. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
23. **Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl. Environ. Microbiol. **54:**2924–2932.
24. **Lawrence, A. G., J. Schoenheit, A. He, J. Tian, P. Liu, J. Stubbe, and A. J. Sinskey.** 2005. Transcriptional analysis of *Ralstonia eutropha* genes related to poly-(R)-3-hydroxybutyrate homeostasis during batch fermentation. Appl. Microbiol. Biotechnol. **68:**663–672.
25. **Lee, W. H., C. Y. Loo, C. T. Nomura, and K. Sudesh.** 2008. Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. Bioresour. Technol. **99:**6844–6851.
26. **Lindenkamp, N., K. Peplinski, E. Volodina, A. Ehrenreich, and A. Steinbüchel.** 2 July 2010. Multiple {beta}-ketothiolase deletion mutants of *Ralstonia eutropha* H16: impact on the composition of 3-mercaptopropionic acid-containing copolymer. Appl. Environ. Microbiol. doi:10.1128/AEM.01058–10.
27. **Loo, C. Y., W. H. Lee, T. Tsuge, Y. Doi, and K. Sudesh.** 2005. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. Biotechnol. Lett. **27:**1405–1410.
28. **Lykidis, A., D. Perez-Pantoja, T. Ledger, K. Mavromatis, I. J. Anderson, N. N. Ivanova, S. D. Hooper, A. Lapidus, S. Lucas, B. Gonzalez, and N. C. Kyrpides.** 2010. The complete multipartite genome sequence of *Cupriavidus necator* JMP134, a versatile pollutant degrader. PLoS One **5:**e9729.
29. **Matsuoka, H., K. Hirooka, and Y. Fujita.** 2007. Organization and function of the YsiA regulon of *Bacillus subtilis* involved in fatty acid degradation. J. Biol. Chem. **282:**5180–5194.
30. **Matsusaki, H., H. Abe, K. Taguchi, T. Fukui, and Y. Doi.** 2000. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene *phaC1* from *Pseudomonas* sp. 61-3 Appl. Microbiol. Biotechnol. **53:**401–409.
31. **Merrick, J. M.** 1978. Metabolism of reserve materials, p. 199–219. *In* R. K. Clayton and W. R. Sistrom (ed.), Photosynthetic bacteria. Plenum Publishing Company, New York, NY.
32. **Mifune, J., S. Nakamura, and T. Fukui.** 2008. Targeted engineering of *Cupriavidus necator* chromosome for biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from vegetable oil. Can. J. Chem. **86:**621–627.
33. **Nan, J., Y. Zhou, C. Yang, E. Brostromer, O. Kristensen, and X. D. Su.** 2009. Structure of a fatty-acid-binding protein from *Bacillus subtilis* determined by sulfur-SAD phasing using in-house chromium radiation. Acta Crystallogr. D Biol. Crystallogr. **65:**440–448.
34. **Nie, L., Y. Ren, A. Janakiraman, S. Smith, and H. Schulz.** 2008. A novel paradigm of fatty acid beta-oxidation exemplified by the thioesterase-dependent partial degradation of conjugated linoleic acid that fully supports growth of *Escherichia coli*. Biochemistry **47:**9618–9626.
35. **Nie, L., Y. Ren, and H. Schulz.** 2008. Identification and characterization of *Escherichia coli* thioesterase III that functions in fatty acid beta-oxidation. Biochemistry **47:**7744–7751.
36. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. **264:**15298–15303.
37. **Peplinski, K., A. Ehrenreich, C. Doring, M. Bomeke, F. Reinecke, C. Hutmacher, and A. Steinbüchel.** 15 April 2010. Genome-wide transcriptome analyses of the "Knallgas" bacterium *Ralstonia eutropha* H16 with regard to PHA metabolism. Microbiology. [Epub ahead of print.] doi:10.1099/mic.0.038380-0.
38. **Pohlmann, A., W. F. Fricke, F. Reinecke, B. Kusian, H. Liesegang, R. Cramm, T. Eitinger, C. Ewering, M. Potter, E. Schwartz, A. Strittmatter, I. Voss, G. Gottschalk, A. Steinbüchel, B. Friedrich, and B. Bowien.** 2006. Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat. Biotechnol. **24:**1257–1262.
39. **Pötter, M., M. H. Madkour, F. Mayer, and A. Steinbüchel.** 2002. Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology **148:**2413–2426.
40. **Pötter, M., H. Muller, F. Reinecke, R. Wieczorek, F. Fricke, B. Bowien, B. Friedrich, and A. Steinbüchel.** 2004. The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. Microbiology **150:**2301–2311.
41. **Pötter, M., H. Muller, and A. Steinbüchel.** 2005. Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in *Ralstonia eutropha* H16. Microbiology **151:**825–833.
42. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection of gene replacement in Gram-negative bacteria. Gene **127:**15–21.
43. **Quyen, D. T., T. T. Nguyen, T. T. Le, H. K. Kim, T. K. Oh, and J. K. Lee.** 2004. A novel lipase/chaperone pair from *Ralstonia* sp. M1: analysis of the folding interaction and evidence for gene loss in *R. solanacearum*. Mol. Genet. Genomics **272:**538–549.
44. **Raberg, M., F. Reinecke, R. Reichelt, U. Malkus, S. Konig, M. Pötter, W. F. Fricke, A. Pohlmann, B. Voigt, M. Hecker, B. Friedrich, B. Bowien, and A. Steinbüchel.** 2008. *Ralstonia eutropha* H16 flagellation changes according to nutrient supply and state of poly(3-hydroxybutyrate) accumulation. Appl. Environ. Microbiol. **74:**4477–4490.
45. **Rehm, B. H.** 2003. Polyester synthases: natural catalysts for plastics. Biochem. J. **376:**15–33.
46. **Reinecke, F., and A. Steinbüchel.** 2009. *Ralstonia eutropha* strain H16 as a model organism for PHA metabolism and for biotechnological production of technically interesting polymers. J. Mol. Microbiol. Biotechnol. **16:**91–108.
47. **Rouchka, E. C., A. W. Phatak, and A. V. Singh.** 2008. Effect of single nucleotide polymorphisms on Affymetrix match-mismatch probe pairs. Bioinformation **2:**405–411.
48. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
49. **Schwartz, E., B. Voigt, D. Zuelhke, A. Pohlmann, O. Lenz, D. Albrecht, A. Schwarze, Y. Kohlmann, C. Krause, M. Hecker, and B. Friedrich.** 2009. A proteomic view of the facultatively chemolithoautotrophic lifestyle of *Ralstonia eutropha* H16. Proteomics **9:**5132–5142.
50. **Simon, S., T. Priefer, and A. Puehler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology (NY) **1:**784–791.
51. **Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys.** 1998. Multiple beta-ketothiolases mediate poly(beta-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J. Bacteriol. **180:**1979–1987.
52. **Steinbüchel, A., and H. E. Valentin.** 1995. Diversity of polyhydroxyalkanoic acids. FEMS Microbiol. Lett. **128:**219–228.
53. **Sudesh, K., H. Abe, and Y. Doi.** 2000. Synthesis, structure, and properties of polyhydroxyalkanoates: biological polyesters. Prog. Polymer Sci. **25:**1503–1555.
54. **Taidi, B., D. A. Mansfield, and A. J. Anderson.** 1995. Turnover of poly(3-hydroxybutyrate) (PHB) and its influence on the molecular mass of the polymer accumulated by *Alcaligenes eutrophus* during batch culture. FEMS Microbiol. Lett. **129:**201–205.
55. **Tatusov, R. L., D. A. Natale, I. V. Garkavtsev, T. A. Tatusova, U. T. Shankavaram, B. S. Rao, B. Kiryutin, M. Y. Galperin, N. D. Fedorova, and E. V. Koonin.** 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic Acids Res. **29:**22–28.
56. **Tian, J., A. J. Sinskey, and J. Stubbe.** 2005. Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by transmission electron microscopy. J. Bacteriol. **187:**3814–3824.
57. **Uchino, K., T. Saito, B. Gebauer, and D. Jendrossek.** 2007. Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. J. Bacteriol. **189:**8250–8256.
58. **Vo, M. T., K. W. Lee, Y. M. Jung, and Y. H. Lee.** 2008. Comparative effect of overexpressed *phaJ* and *fabG* genes supplementing (R)-3-hydroxyalkanoate monomer units on biosynthesis of mcl-polyhydroxyalkanoate in *Pseudomonas putida* KCTC1639. J. Biosci. Bioeng. **106:**95–98.
59. **Walde, E.** 1962. Unterschungen uber wachstum und speicherstoffsythese von *Hydrogenomonas*. Arch. Mikrobiol. **43:**109.
60. **Wang, Z.-X., C. O. Brämer, and A. Steinbüchel.** 2003. The glyoxylate bypass of *Ralstonia eutropha*. FEMS Microbiol. Lett. **228:**63–71.
61. **Wu, T. Y., A. W. Mohammad, J. M. Jahim, and N. Anuar.** 2009. A holistic approach to managing palm oil mill effluent (POME): biotechnological advances in the sustainable reuse of POME. Biotechnol. Adv. **27:**40–52.

62. **York, G. M., B. H. Junker, J. A. Stubbe, and A. J. Sinskey.** 2001. Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells. J. Bacteriol. **183:**4217–4226.
63. **York, G. M., J. Lupberger, J. Tian, A. G. Lawrence, J. Stubbe, and A. J. Sinskey.** 2003. *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly[D-(−)-3-hydroxybutyrate] depolymerase genes. J. Bacteriol. **185:**3788–3794.
64. **York, G. M., J. Stubbe, and A. J. Sinskey.** 2001. New insight into the role of the PhaP phasin of *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate. J. Bacteriol. **183:**2394–2397.
65. **York, G. M., J. Stubbe, and A. J. Sinskey.** 2002. The *Ralstonia eutropha* PhaR protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. J. Bacteriol. **184:**59–66.
66. **Zhang, H., J. Y. Deng, L. J. Bi, Y. F. Zhou, Z. P. Zhang, C. G. Zhang, Y. Zhang, and X. E. Zhang.** 2008. Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase. FEBS J. **275:**753–762.