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13. ABSTRACT (Maximum 200 words) The anaerobic degradation of aromatic compounds is important for environmental bioremediation because substantial amounts of toxic aromatic compounds are produced industrially and make their way into anoxic groundwaters and sediments. Although the biodegradation of aromatic compounds under anaerobic conditions has great practical significance, investigators have studied the degradation of only a very few different kinds of aromatics. In this era of microbial genomics it is now technically possible to take a comprehensive look at the complete biodegradation potential of an individual microbial species. The purple nonsulfur phototrophic bacterium <i>Rhodospseudomonas palustris</i> is an ideal organism for this. Its genome has recently been completely sequenced and it can be manipulated genetically. <i>R. palustris</i> also has great metabolic versatility and can grow on many different kinds of aromatic compounds. In the project period we have completed and published our description of the <i>R. palustris</i> genome sequence. We have completed a study of the lower pathway of benzoate degradation, characterized an enzyme involved in chloroaromatic degradation and initiated studies of the degradation of a green plant associated compound; 4-hydroxycinnamate.			
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A GENOMIC ANALYSIS OF ANAEROBIC AROMATIC DEGRADATION.

1) Publications supported under this grant:

a. Peer-reviewed:

Gibson, J. and C.S. Harwood. 2002. Metabolic diversity in aromatic compound utilization by anaerobic microbes. *Annu. Rev. Microbiol.* **56**: 345-369.

Larimer, F. W., P. Chain, J. Lamerdin, S. Stilwagen Malfatti, L. Do, M. Land, L. Hauser, D. A. Pelletier, J. T. Beatty, A. S. Lang, F. R. Tabita, J. L. Gibson, T. E. Hanson, C. Bobst, J. Torres Y Torres, C. Peres, F. Harrison, J. Gibson and C. S. Harwood. 2004. The genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. *Nature Biotech.* **22**: 55-61.

Harrison, F. H. and C. S. Harwood. Identification of a fatty acid β -oxidation operon from *Rhodopseudomonas palustris* that is involved in the latter stages of anaerobic benzoate degradation. Submitted.

b. Papers presented at meetings. (MS in preparation).

Samanta, S. K. and C. S. Harwood. 2002. The anaerobic degradation of 3-chlorobenzoate by *Rhodopseudomonas palustris* strain RCB100 requires a CoA ligase that differs by just one amino acid from a ligase present in a strain that cannot grow on 3-chlorobenzoate. Amer Soc. Microbiol. Ann. Meet.

2) SCIENTIFIC PERSONNEL SUPPORTED BY THIS PROJECT:

Faith Harrison: Graduate student

Sudip Samanta: Research Scientist

3) INVENTIONS: none

4) SCIENTIFIC PROGRESS AND ACCOMPLISHMENTS:

Objectives and significance. Anaerobic biodegradation is important for environmental bioremediation because substantial amounts of toxic aromatic compounds are produced industrially and make their way into anoxic groundwaters and sediments. Although the biodegradation of aromatic compounds under anaerobic conditions has great practical significance, investigators have studied the degradation of only a very few different kinds of aromatics. In this era of microbial genomics it is now technically possible to take a comprehensive look at the complete biodegradation potential of an individual microbial species. The purple nonsulfur phototrophic bacterium *Rhodopseudomonas palustris* is an ideal organism for this. Its genome has recently been completely sequenced and it can be manipulated genetically. *R. palustris* also has great metabolic versatility and can grow on many different kinds of aromatic compounds.

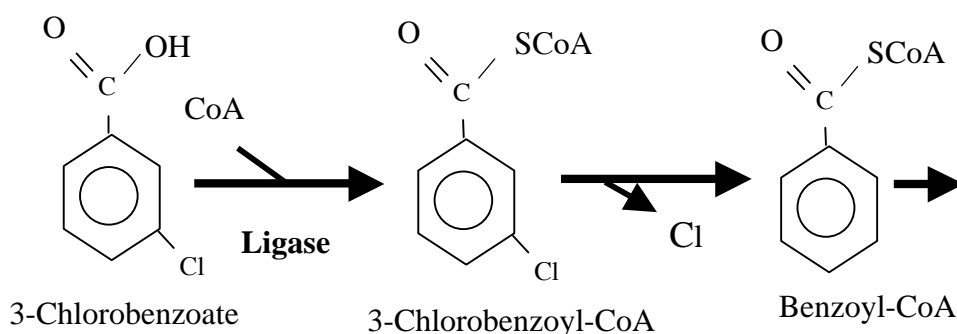
Summary of the most important results.

a. We carried out an *in silico* analysis of the *Rhodopseudomonas* genome sequence. Genes for key metabolic enzymes and regulatory proteins are easily identified in the 5.49 Mb *Rhodopseudomonas* genome. *R. palustris* has a large cluster of photosynthesis genes. It has genes for the metabolism of diverse kinds of carbon sources, including chlorinated compounds and plant lignin monomers. A surprising finding is that *R. palustris* has five completely different and distinct pathways for the degradation of aromatic compounds. This is the largest number of ring fission pathways that has been seen in any one organism. Another surprising finding is that *R. palustris* encodes three different nitrogen fixation enzymes, each with a different transition metal at its

active site. Nitrogenases catalyze the conversion of nitrogen gas to ammonia and hydrogen. *R. palustris* can convert sunlight to ATP and derive electrons by biodegrading plant material. ATP and electrons so generated can theoretically then be used to fix nitrogen, with accompanying hydrogen production. We recently published the description of the *R. palustris* genome sequence in Nature Biotechnology.

b. During the project period we completed work defining the broad outlines of how the model chlorinated aromatic compound 3-chlorobenzoate (3CBA) is degraded anaerobically. We determined that suspensions of anaerobic 3CBA-grown cells of *R. palustris* convert ^{14}C -radiolabeled 3CBA to products that co-migrate with 3-chlorobenzoyl-CoA and benzoyl-CoA. From this we concluded that *R. palustris* grows on 3CBA by i) converting it to 3-chlorobenzoyl-CoA, ii) reductively dehalogenating 3-chlorobenzoyl-CoA to benzoyl-CoA, and iii) degrading benzoyl-CoA to acetyl-CoA and carbon dioxide via the benzoate pathway.

We went on to purify the 3-CBA-CoA ligase that is responsible for the first step of 3CBA degradation. By referring to the recently completed *R. palustris* genome sequence, we were able to identify the gene that encoded this ligase.



Only some strains of *R. palustris* degrade 3CBA. We compared the 3-CBA-CoA ligase from a 3CBA-degrading strain of *R. palustris* with a closely related ligase from the sequenced strain. This allowed us to **identify a single amino acid residue in the 3-CBA-CoA ligase that was responsible for the activity of this enzyme with chlorinated substrates.** This story is a bit complicated and can be summarized as follows. (This is the abstract of a paper that we are preparing for publication).

The strain of *Rhodospseudomonas palustris* (CGA009) that has recently been sequenced does not grow anaerobically with 3-chlorobenzoate as a sole carbon source, but *R. palustris* strain RCB100 has this capability. We purified from strain RCB100 a coenzyme A ligase that is active with 3-chlorobenzoate and found its N-terminal amino acid sequence to be identical to that of a cyclohexanecarboxylate-CoA ligase encoded by the *aliA* gene of the sequenced strain. The *aliA* gene from the 3-chlorobenzoate degrading strain differed by a single nucleotide from the *aliA* gene from strain CGA009, resulting in the substitution of a serine for a threonine at position 208. The AliA enzymes were purified as His-tagged fusion proteins and found to have comparable activities with cyclohexanecarboxylate. However, the enzyme from the sequenced strain was less active with 3-chlorobenzoate (k_{cat}/K_m $0.32 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$) than the enzyme from the 3-CBA degrading strain (k_{cat}/K_m of $4.26 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$ for 3-CBA). The CGA009 enzyme was not sufficiently active with 3-chlorobenzoate to complement an RCB100 *aliA* mutant for growth on this compound. In this study whole genome sequence information helped us to identify a single nucleotide among 5.4 million nucleotides that contributes to the substrate preference of a coenzyme A ligase.

c. We identified a cluster of genes for pimelate (a dicarboxylic acid) degradation. This cluster of eight genes encodes the expected enzymes needed for the β -oxidation of fatty acids. We obtained evidence that enzymes encoded by the *pim* operon are active with dicarboxylic acids of between 4 and 14 carbons in length and with fatty acids up to 16 carbons in length. These genes are also required for optimal growth on the aromatic compound benzoate under anaerobic conditions. A manuscript that describes these results has been submitted for publication.

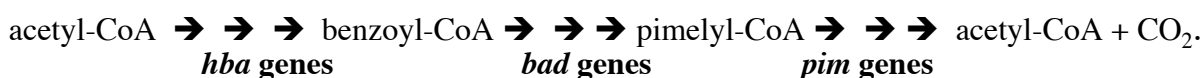
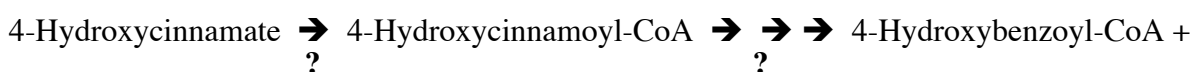
Pimelyl-CoA is a key intermediate in anaerobic benzoate degradation. It is the seven carbon aliphatic compound that is formed when the alicyclic ring derived from benzoate is cleaved. As such, the pimeloyl-CoA degradation sequence comprises the “lower” portion of the benzoate degradation pathway. The free dicarboxylic acid pimelate is a good growth substrate for *R. palustris* as well as for many other microorganisms, yet the molecular basis for pimelate degradation had yet to be examined in any organism.

We purified one of the expected enzymes of pimelate degradation (pimeloyl-CoA dehydrogenase) and used the N-terminal sequence from the purified protein to identify the corresponding gene in the *Rhodopseudomonas* genome. It is located in a cluster of genes that we have named *pim* (for pimelate) genes. We expressed and purified a His-tagged version of an enzyme encoded by another gene in the cluster, a CoA ligase. The CoA ligase was active with pimelate as well as with a series of other dicarboxylic acids. The pimelate CoA ligase (PimA) is also active with fatty acids. We used reverse transcriptase PCR to determine that the *pim* genes are organized as an operon, We used a *lacZ* reporter to show that *pimA* was induced about two fold by growth on pimelate. We constructed a mutant deleted in the entire *pim* operon and found that it grew more slowly on pimelate and benzoate than the wild type.

d. We initiated studies to identify genes required for the anaerobic degradation of the lignin-derived aromatic compound 4-hydroxycinnamate. Our specific goal was to see if we could use DNA microarray technology as a tool for pathway discovery.

A whole genome glass slide microarray of *R. palustris* has been constructed in the laboratory of Dr. Jizhong Zhou at Oak Ridge National Laboratory and made available to us as part of a joint DOE Microbial Genome Project grant that he and I have. We also used the microarray as part of this ARO project to identify genes involved in biodegradation of 4-hydroxycinnamate.

We decided to try to identify genes for anaerobic 4-hydroxycinnamate degradation because it is a model aromatic compound that is widely distributed in nature. *R. palustris* grows well on this compound and we can predict from known principles of metabolism that its pathway of degradation is probably something like the pathway shown below. Genes for the degradation of the acyl side chain portion of 4-hydroxycinnamate are not easily identified by sequence analysis alone because they likely involve a subset of the many β -oxidation enzymes that are encoded by *R. palustris*. Pathways for the degradation of a particular kind of compound are, however, usually induced by growth on that compound. Thus we hoped that just one or a few sets of β -oxidation genes would be highly expressed and visible in the whole genome microarrays after growth on 4-hydroxycinnamate.



We would like to fill in the question marks.

We have previously identified and characterized *hba* genes, *bad* genes and *pim* genes involved in anaerobic 4-hydroxybenzoyl-CoA degradation. These comprise a total of about 50 genes. In our microarray experiments all of the expected *hba-bad-pim* genes were up regulated by growth with 4-hydroxycinnamate. We also observed significant up-regulation of genes *rpa0674-rpa0678*. These five genes are located next to the *hba* genes on the *R. palustris* chromosome. We hypothesize that *rpa0674*, *rpa0675* and *rpa0676* together catalyze the conversion of 4-hydroxycinnamoyl-CoA to 4-hydroxybenzoyl-CoA and acetyl-CoA. We have expressed these genes in *E. coli* and purified them. We will follow the conversion of 4-hydroxycinnamoyl-CoA by HPLC to confirm the we have successfully used microarray technology to elucidate a new metabolic pathway.

5) INVENTIONS: none. (DD From 882 submitted under separate cover from U of Iowa Office of Sponsored Programs.)