JOURNAL OF BACTERIOLOGY, Nov. 1995, p. 6545–6551 Vol. 177, No. 22
0021-9193/95/$04.00+0

Benzoate-Coenzyme A Ligase, Encoded by *badA*, Is One of Three Ligases Able To Catalyze Benzoyl-Coenzyme A Formation during Anaerobic Growth of *Rhodopseudomonas palustris* on Benzoate

PAUL G. EGLAND,[1] JANE GIBSON,[2] AND CAROLINE S. HARWOOD[1]*

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242,[1] and Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853[2]

Received 26 May 1995/Accepted 8 September 1995

The first step of anaerobic benzoate degradation is the formation of benzoyl-coenzyme A by benzoate-coenzyme A ligase. This enzyme, purified from *Rhodopseudomonas palustris*, is maximally active with 5 μM benzoate. To study the molecular basis for this reaction, the benzoate-coenzyme A ligase gene (*badA*) was cloned and sequenced. The deduced amino acid sequence of *badA* showed substantial similarity to other coenzyme A ligases, with the highest degree of similarity being that to 4-hydroxybenzoate-coenzyme A ligase (50% amino acid identity) from *R. palustris*. A *badA* mutant that was constructed had barely detectable levels of ligase activity when cell extracts were assayed at 10 μM benzoate. Despite this, the mutant grew at wild-type rates on benzoate under laboratory culture conditions (3 mM benzoate), and mutant cell extracts had high levels of ligase activity when assayed at a high concentration of benzoate (1 mM). This suggested that *R. palustris* expresses, in addition to BadA, a benzoate-activating enzyme(s) with a relatively low affinity for benzoate. A possible role of 4-hydroxybenzoate-coenzyme A ligase (encoded by *hbaA*) in this capacity was investigated by constructing a *badA hbaA* double mutant. Although the double mutant grew more slowly on benzoate than *badA* cells, growth rates were still significant, suggesting the involvement of a third enzyme in benzoate activation. Competition experiments involving the addition of a small amount of cyclohexanecarboxylate to ligase assay mixtures implicated cyclohexanecarboxylate-coenzyme A ligase as being this third enzyme. These results show that wild-type *R. palustris* cells synthesize at least three enzymes that can catalyze the initial step in anaerobic benzoate degradation during growth on benzoate. This observation supports previous suggestions that benzoyl-coenzyme A formation plays a central role in anaerobic aromatic compound biodegradation.

Benzoyl-coenzyme A (benzoyl-CoA) is the first intermediate formed during anaerobic benzoate degradation, and it also figures prominently in the anaerobic catabolism of toluene, phenylalkane carboxylates, phenol, and many other aromatic compounds that are ultimately funneled into the benzoate pathway to achieve complete degradation (15, 19). The benzoate pathway, although still not worked out in detail, is characterized by an initial oxygen-sensitive reduction of benzoyl-CoA to a cyclohexadienecarboxyl-CoA intermediate (20, 25). This is followed by a series of β-oxidation-like modifications to generate a cyclic β-keto thioester which is cleaved to give a medium-chain dicarboxylic acid (pimelyl-CoA or a related compound) as a product (11, 25, 33). This sequence of reactions has been most thoroughly studied as it occurs in the denitrifying strain K172 (*Thauera aromatica* [2]) and in the purple nonsulfur phototroph *Rhodopseudomonas palustris*, although selected aspects of the pathway have been examined with a number of different bacteria.

The best characterized reaction in the anaerobic benzoate pathway is the conversion of benzoate to benzoyl-CoA by benzoate-CoA ligase (AMP forming) (EC 6.2.1.25). This enzyme has been purified from several sources, including a denitrifying pseudomonad (1), a benzoate-degrading syntrophic coculture (4), and *R. palustris* (17). The *R. palustris* enzyme has a narrow substrate range and is highly specific for benzoate. It also has a very high affinity for its substrate, with a reported K_m for benzoate of about 1 μM (17). In addition to catalyzing the first step of benzoate degradation, benzoate-CoA ligase also appears to be responsible for the ability of *R. palustris* cells to take up benzoate with high affinity. Whole cells have an apparent K_m of less than 1 μM for benzoate, and benzoyl-CoA, rather than free benzoate, accumulates intracellularly to high levels during the first 45 s of uptake (21). The importance of benzoate-CoA ligase in anaerobic aromatic compound degradation is also underscored by studies showing that benzoate-CoA ligase expression is induced by a range of different aromatic acids (24).

Although enzymological data indicate that benzoate-CoA ligase is the principal enzyme involved in initiating anaerobic benzoate degradation by *R. palustris*, this organism also has two other enzymes, a 4-hydroxybenzoate-CoA ligase (18) and a cyclohexanecarboxylate-CoA ligase (26), that are active with benzoate. This raises the question of whether *R. palustris* may be able to grow on benzoate by relying on multiple benzoate-CoA ligase activities. Here we have addressed this question by cloning the gene for benzoate-CoA ligase, constructing mutants, and analyzing their phenotypes. Data presented provide a first molecular characterization of a benzoate-CoA ligase gene. Our findings also indicate that although benzoate-CoA ligase is probably required for cells to degrade very low concentrations of benzoate, *R. palustris* has at least two other CoA ligases that allow growth at higher benzoate concentrations.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used are described in Table 1. *R. palustris* cultures were grown anaerobically in

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7783. Fax: (319) 335-9006. Electronic mail address: caroline-harwood@uiowa.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source and/or reference
E. coli strains		
DH5α	F⁻ λ⁻*recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80d*lacZ*Δ*M15*	Gibco-BRL
S17-1	*thi pro hdsR hdsM*⁺ *recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn*7*)	38
S17-1 λpir	S17-1 lysogenized with λpir phage	9
JM109(DE3)	Contains chromosomal copy of T7 RNA polymerase gene	Promega, 39
R. palustris strains		
CGA009	Wild-type strain; spontaneous Cm^r derivative	24
CGA401	*hbaA*::Km^r	18
CGA601	*badA*::Km^r	This study
CGA604	*badA*::Gm^r *hbaA*::Km^r	This study
Plasmids		
pUC1813	Semisymmetrical MCS; Ap^r	22
pGEM-3Z	Cloning vector containing T7 RNA polymerase promoter; Ap^r	Promega
pRK415	IncP1; mobilizable cloning vector; Tc^r	23
pJQ200KS	Mobilizable suicide vector; *sacB* Gm^r	34
pHRP302	Gm^r gene (27) in pUC1318; Ap^r Gm^r	32
pPE202	pUC1813 with 3.4-kb *R. palustris Eco*RI-*Sac*I fragment; Ap^r	This study
pPE204	pGEM-3Z with 3.4-kb *Eco*RI-*Sac*I fragment from pPE202; Ap^r	This study
pPE216	pJQ200KS with 2-kb *Sph*I fragment from pPE202 containing Km^r gene in the *Xho*I site; Gm^r Km^r	This study
pPE212	pRK415 with 2-kb *Sph*I fragment from pPE202 containing a Gm^r gene from pHRP302 in the *Xho*I site; Tc^r Gm^r	This study

[a] Ap, ampicillin; Km, kanamycin; Gm, gentamicin; Tc, tetracycline; Cm, chloramphenicol; MCS, multiple cloning site.

PM medium in either 17-ml screw-cap tubes or 250-ml bottles (24). Carbon sources were added from sterile stock solutions to final concentrations of 3 mM benzoate, 3 mM cyclohexanecarboxylate, or 10 mM succinate. Benzoate and cyclohexanecarboxylate cultures were supplemented with sodium bicarbonate (10 mM), which was added to PM medium as a sterile solution after autoclaving. Cultures were illuminated with 40-W incandescent lightbulbs and incubated at 30°C. *Escherichia coli* strains were grown in Luria broth at 37°C. Growth of the cultures was monitored by measuring A_{660}. Antibiotics were used at the following concentrations (in micrograms per milliliter) for *R. palustris*: gentamicin, 100; kanamycin, 100; and tetracycline, 100. For *E. coli* the antibiotics used and their concentrations (in micrograms per milliliter) were as follows: ampicillin, 100; gentamicin, 5; kanamycin, 100; and tetracycline, 25.

Cloning and DNA manipulations. Plasmid DNA used for cloning and sequencing was purified by the method of Lee and Rasheed (28). *R. palustris* chromosomal DNA was purified by a variation of the method described by Saito and Miura (35). Cells (20 ml) were harvested by centrifugation, washed, resuspended in 5 ml of saline-EDTA (1.5 M NaCl, 0.1 M EDTA [pH 8]), and frozen. Tris-sodium dodecyl sulfate (SDS) buffer (0.1 M Tris, 1% SDS, 0.1 M NaCl) was added, and the frozen cell suspension was thawed at 50°C. The solution was extracted two to three times with buffered phenol, and DNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol. The DNA was collected by centrifugation (10,000 rpm in a Sorvall SA-600 rotor [Dupont Instruments, Wilmington, Del.] for 10 min), washed in 70% ethanol, and resuspended in 200 to 500 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]). DNA fragments were purified from agarose gels by using the GeneClean kit from Bio 101 (La Jolla, Calif.). Standard methods were used for DNA cloning and transformation (5, 36). The vector pUC1813 (22) was used to generate most recombinant plasmids. Plasmids were maintained in *E. coli* DH5α (Gibco-BRL). Southern hybridizations were carried out with the Genius kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Construction and expression of an *R. palustris* DNA library. An *R. palustris* DNA expression library was constructed in the λgt11 cloning vector and expressed as described previously (18). Proteins produced during phage growth were immunoscreened with benzoate-CoA ligase antiserum. Lambda lysogens were prepared and fusion proteins were expressed as described in the *Promega* (Madison, Wis.) *Protocols and Applications Guide* (40).

Mutant construction. The *badA* mutant CGA601 was constructed by gene replacement with a *badA* gene that had been interrupted with a kanamycin resistance Genblock (Pharmacia Biotech Inc., Piscataway, N.J.). The delivery plasmid for the interrupted gene, pPE216, was constructed by using a vector (pJQ200KS [34]) that includes the gene *sacB*. Plating on sucrose, which is toxic to *R. palustris* in the presence of *sacB*, allowed selection of exconjugants in which double recombination, and therefore loss of the *sacB*-containing vector, had occurred. pPE216 was mobilized into *R. palustris* by conjugal transfer as previously described (32) by using the *E. coli* strain S17-1 λpir (9). The mating mixture was plated on PM medium containing succinate, kanamycin, and 5% sucrose. The *badA hbaA* double mutant CGA604 was constructed by using plasmid pPE212 containing *badA* interrupted by a gentamicin resistance gene. This plasmid was then mobilized into the *R. palustris hbaA* mutant CGA401 (18) from *E. coli* S17-1 by patch matings.

Expression of BadA in *E. coli*. The BadA protein was expressed from pPE204 in an *E. coli* strain [JM109(DE3)] carrying an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible gene for T7 RNA polymerase (39). Cultures (25 ml) of *E. coli* cells carrying the cloned *badA* gene were induced with 1 mM IPTG when they reached an A_{660} of 1.0.

Enzyme assays. Benzoate-CoA ligase activity was measured by an isotopic assay as described previously (17). This assay is based on the ATP- and CoA-dependent conversion of ^{14}C-labeled benzoate to benzoyl-CoA, which remains hydrophilic under acidic conditions. This assay system was modified to measure acyl-CoA transferase activities by using 0.25 mM acetoacetyl-CoA, pimelyl-CoA, or succinyl-CoA, instead of reduced CoA (CoASH) as a potential CoA donor (17). Specific activities were expressed as nanomoles of benzoyl-CoA formed per min per mg of protein.

Benzoate uptake and intracellular formation of benzoyl-CoA. Accumulation of [^{14}C]benzoate by whole *R. palustris* cells was measured as previously described (21). Briefly, oxygen-depleted cell suspensions were incubated anaerobically with [7-^{14}C]benzoate, and samples were removed at various time intervals and filtered. The amount of radiolabeled benzoate that accumulated in cells retained by the filters was determined by liquid scintillation counting. In experiments to determine levels of benzoyl-CoA formed intracellularly, collected cells were suspended in boiling water to release the soluble intracellular contents, cell debris was pelleted by centrifugation, and supernatants were lyophilized. The resulting material was then resuspended in 95% ethanol and separated by thin-layer chromatography (30). The accumulation of ^{14}C-labeled intracellular benzoyl-CoA was established by comparison with a benzoyl-CoA standard purchased from Sigma Chemical Co. (St. Louis, Mo.).

DNA sequencing and analysis. DNA sequences were determined by the dideoxy-chain termination method using the *fmol* DNA Sequencing System from Promega and [α-^{33}P]ATP from Amersham Corp (Cleveland, Ohio). The sequences of both strands of DNA were determined by using either universal or custom-synthesized primers (University of Iowa DNA facility). DNA templates included pPE202 (Fig. 1) and several subclones derived from this plasmid. The DNA sequence was analyzed by using DNA Inspector IIe, version 3.15 (Textco, Inc., West Lebanon, N.H.). Similar sequences from the SWISS-PROT (version 26) and Genpep (version 78.0) databases were identified by using the BLAST network service (3) at the National Center for Biotechnology Information (Bethesda, Md.). The GAP and PILEUP programs from the University of Wisconsin Genetics Computer Group software package, version 7.0 (10), were used to make sequence comparisons and alignments.

Other analytical procedures. Western analysis of cell extracts was performed as described previously (18, 24). Protein concentrations were determined by using the Bio-Rad (Richmond, Calif.) protein assay kit.

Nucleotide sequence accession number. The nucleotide sequence of *badA* has been submitted to GenBank and assigned the accession number L42322.

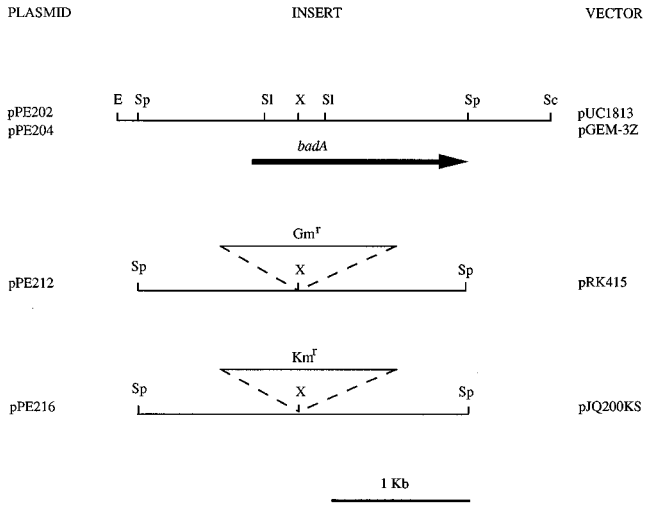

FIG. 1. Restriction map of pPE202 and derivatives carrying *badA*. The insertion of gentamicin and kanamycin resistance genes into *badA* in pPE212 and pPE216, respectively, is shown. Restriction enzyme sites: E, *Eco*RI; Sl, *Sal*I; Sp, *Sph*I; X, *Xho*I; Sc, *Sac*I.

RESULTS

Cloning and expression of *badA*, encoding benzoate-CoA ligase. Immunoscreening of proteins expressed from an *R. palustris* genomic library prepared in λgt11 resulted in the identification of three plaques that reacted with antiserum to purified benzoate-CoA ligase. An *E. coli* λ lysogen prepared from one of these plaques expressed a protein that reacted with benzoate-CoA ligase-specific antiserum in Western blots (immunoblots) and was the same size as purified benzoate-CoA ligase. The gene encoding this protein was subsequently localized to a 3.4-kb *Eco*RI-*Sac*I fragment and named *badA* (Fig. 1). Cell extracts prepared from an *E. coli* strain expressing *badA* from a T7 promoter (pPE204) had high benzoate-CoA ligase activity (130 nmol of benzoyl-CoA formed per min per mg of protein).

Nucleotide sequence of *badA* and sequence comparisons. Nucleotide sequence analysis of the *Eco*RI-*Sac*I fragment encoding benzoate-CoA ligase activity revealed a 1,563-bp open reading frame with an ATG start site preceded by a putative ribosome binding site (Fig. 2). A gene of this size would encode a protein of 521 amino acids with a predicted molecular mass of 56,630 Da. This is close to the estimated size (58,000 Da) of purified benzoate-CoA ligase (17). The amino acid composition deduced from the translated nucleotide sequence of *badA* matched closely that of the purified protein. Although an N-terminal amino acid sequence has been reported for purified benzoate-CoA ligase (17), this sequence is in doubt because more recent work has indicated that this enzyme is blocked at the N terminus (31). *badA* had a G+C content of 66%.

In addition to *badA*, one other gene sequence for a CoA ligase involved in anaerobic degradation of aromatic acids has been reported. This gene, *hbaA*, encodes 4-hydroxybenzoate-CoA ligase in *R. palustris* (18). Sequence comparisons indicated that the *badA* and *hbaA* gene products are 50% identical at the amino acid level, with selected segments of the proteins showing very high degrees of identity (Fig. 3). Lower, but significant, levels of amino acid identity were seen between BadA and aromatic acid CoA ligases that function in aerobic metabolism. For example, the benzoate-CoA ligase sequence was 26% identical to 4-chlorobenzoate-CoA ligases from *Arthrobacter* sp. strain SU (37) and *Pseudomonas* sp. strain CBS-3 (6), as well as to parsley coumarate-CoA ligase (29). The deduced amino acid sequence of the *badA* product had equally significant levels of identity with several fatty acid-CoA ligases, including 30% identity with *E. coli* long-chain fatty acid-CoA ligase (16) and about 25% identity with acetate-CoA ligases from *Neurospora crassa* (8) and *Methanothrix soehngenii* (13).

Construction and characterization of a *badA* mutant. A *badA* mutant was constructed by homologous recombination between the wild-type chromosome and an insertionally inactivated version of the gene carried on the plasmid pPE216 (Fig. 1). Southern hybridization confirmed that the resulting strain,

```
1    GGCGATCAGGAGGAAACCATGAATGCAGCCGCGGTCACGCCGCCACCCGAGAAGTTTAAT   60
                M  N  A  A  A  V  T  P  P  P  E  K  F  N

61   TTTGCCGAGCACCTGCTGCAGACCAATCGCGTGCGGCCGGACAAGACGGCGTTCGTCGAC  120
     F  A  E  H  L  L  Q  T  N  R  V  R  P  D  K  T  A  F  V  D

121  GACATCTCGTCGCTGAGCTTCGCGCAACTCGAAGCTCAGACGCGTCAGCTCGCCGCCGCC  180
     D  I  S  S  L  S  F  A  Q  L  E  A  Q  T  R  Q  L  A  A  A

181  TTACGCGCGATCGGGGTGAAACGCGAAGAGCGCGTGCTGCTGCTGATGCTCGACGGCACG  240
     L  R  A  I  G  V  K  R  E  E  R  V  L  L  L  M  L  D  G  T

241  GATTGGCCGGTGGCGTTTCTCGGCGCAATCTACGCCGGCATCGTGCCGGTCGCGGTCAAT  300
     D  W  P  V  A  F  L  G  A  I  Y  A  G  I  V  P  V  A  V  N

301  ACGCTGCTGACGGCGGACGACTACGCCTACATGCTCGAGCATTCGCGGGCTCAGGCCGTG  360
     T  L  L  T  A  D  D  Y  A  Y  M  L  E  H  S  R  A  Q  A  V

361  CTGGTCAGCGGCGCGCTGCACCCGGTGCTCAAGGCAGCGCTGACCAAGAGCGATCACGAG  420
     L  V  S  G  A  L  H  P  V  L  K  A  A  L  T  K  S  D  H  E

421  GTGCAGCGAGTGATCGTTTCGCGCCCAGCGCTCCGCTGGAGCCGGGCGAGGTCGACTTCG  480
     V  Q  R  V  I  V  S  R  P  A  L  R  W  S  R  A  R  S  T  S

481  CTGAGTCGGCGCACATCGCTTGAGAAGCCTGCCGCTACGCAAGCGGACGATCCGGCGTTC  540
     L  S  R  R  T  S  L  E  K  P  A  A  T  Q  A  D  D  P  A  F

541  TGGCTGTATTCGTCGGGTTCTACCGGGCGGCCGAAGGGCGTGGTGCACACTCACGCCAAT  600
     W  L  Y  S  S  G  S  T  G  R  P  K  G  V  V  H  T  H  A  N

601  CCGTACTGGACCTCGGAGCTGTACGGCCGCAACACGCTGCATCTGCGCGAAGACGACGTC  660
     P  Y  W  T  S  E  L  Y  G  R  N  T  L  H  L  R  E  D  D  V

661  TGCTTTTCGGCGGCCAAACTGTTTTTCGCTTACGGCCTCGGCAACGCGCTGACGTTTCCG  720
     C  F  S  A  A  K  L  F  F  A  Y  G  L  G  N  A  L  T  F  P

721  ATGACGGTCGGCGCGACCACGCTGCTGATGGGCGAGCGACCGACGCCGGACGCGGTGTTC  780
     M  T  V  G  A  T  T  L  L  M  G  E  R  P  T  P  D  A  V  F

781  AAGCGCTGGCTCGGCGGCGTCGGCGGTGTGAAACCGACCGTGTTCTACGGCGCGCCCACC  840
     K  R  W  L  G  G  V  G  G  V  K  P  T  V  F  Y  G  A  P  T

841  GGCTACGCCGGCATGTTGGCCGCGCCGAACCTGCCGTCGCGCGACCAGGTGGCGTTGCGG  900
     G  Y  A  G  M  L  A  A  P  N  L  P  S  R  D  Q  V  A  L  R

901  CTCGCGTCGTCGGCGGGCGAAGCACTGCCGGCGGAGATTGGGCAGCGCTTCCAGCGCCAT  960
     L  A  S  S  A  G  E  A  L  P  A  E  I  G  Q  R  F  Q  R  H

961  TTCGGCCTCGACATCGTCGATGGCATCGGCTCGACCGAGATGCTGCACATCTTTCTGTCG 1020
     F  G  L  D  I  V  D  G  I  G  S  T  E  M  L  H  I  F  L  S

1021 AACCTGCCAGACCGGGTGCGCTACGGCACCACCGGATGGCCGGTGCCGGGCTATCAGATC 1080
     N  L  P  D  R  V  R  Y  G  T  T  G  W  P  V  P  G  Y  Q  I

1081 GAGCTGCGCGGCGACGGCGGCGGACCGGTCGCCGACGGAGAGCCGGGCGATCTCTACATT 1140
     E  L  R  G  D  G  G  G  P  V  A  D  G  E  P  G  D  L  Y  I

1141 CACGGCCCGTCATCGGCGACGATGTATTGGGGCAACCGGGCCAAGAGCCGCGACACCTTC 1200
     H  G  P  S  S  A  T  M  Y  W  G  N  R  A  K  S  R  D  T  F

1201 CAGGGCGGCTGGACCAAGAGCGGCGACAAATACGTCCGCAACGACGACGGCTCCTACACC 1260
     Q  G  G  W  T  K  S  G  D  K  Y  V  R  N  D  D  G  S  Y  T

1261 TATGCGGGCCGCACCGACGACATGCTGAAGGTCAGCGGCATCTATGTCAGCCCGTTCGAG 1320
     Y  A  G  R  T  D  D  M  L  K  V  S  G  I  Y  V  S  P  F  E

1321 ATCGAAGCGACGCTGGTGCAGCATCCCGGTGTGCTCGAAGCCGCAGTGGTCGGGGTCGCC 1380
     I  E  A  T  L  V  Q  H  P  G  V  L  E  A  A  V  V  G  V  A

1381 GACGAACACGGCCTGACCAAACCGAAGGCCTATGTGGTGCCGCGGCCCGGCCAGACCCTG 1440
     D  E  H  G  L  T  K  P  K  A  Y  V  V  P  R  P  G  Q  T  L

1441 TCGGAGACCGAGCTGAAGACCTTCATCAAGGATCGACTGGCGCCGTACAAATATCCGCGC 1500
     S  E  T  E  L  K  T  F  I  K  D  R  L  A  P  Y  K  Y  P  R

1501 AGCACGGTGTTCGTCGCCGAATTGCCGAAGACGGCGACCGGCAAGATTCAGCGCTTCAAG 1560
     S  T  V  F  V  A  E  L  P  K  T  A  T  G  K  I  Q  R  F  K

1561 CTGCGCGAGGGTGTGTTGGGCTGAACCGGGAGGAGGCAACA 1601
     L  R  E  G  V  L  G  *
```

FIG. 2. Nucleotide and deduced amino acid sequences of *badA*. The amino acid sequence proposed to be involved in acyl-adenylate formation (6) is underlined.

```
BadA   1 MNAAAVTPPPEKFNFAEHLLQTN..RVRPDKTAFVDDISSLSFAQLEAQT 48
HbaA   1 .......MPLRDYNAAVDFVDRNVAEGRGGKIAFIDPQRSLSYGELRDAV 43

      49 RQLAAALRAIGVKREERVLLLMLDGTDWPVAFLGAIYAGIVPVAVNTLLT 98
      44 ARVGPMLARLGVEQENRIALVLKDTVDFPILFWGAIRAGIVPVLLNTRLT 93

      99 ADDYAYMLEHSRAQAVLVSGALHPVLKAALTKSDHEVQRVIVSRPALRWS 148
      94 ADQYRYLLEDSRSRVVFASSEFLPVIEEAAADLPH.LRTIIAVGDAPAPT 142

     149 RARSTSLSRRTSLEKPAATQADDPAFWLYSSGSTGRPKGVVHTHANPYWT 198
     143 LQLANLLATEQEGGAPAATCADDIAYWQYSSGTTGMPKGVMHVHSSPRVM 192

     199 SELYGRNTLHLREDDVCFSAAKLFFAYGLGNALTFPMTVGATTLLMGERP 248
     193 AENAGRR.IGYREDDVVFSAAKLFFAYGLGNAMFCPMGIGATSVLYPERP 241

     249 TPDAVFKRWLGGVGGVKPTVFYGAPTGYAGMLAAP.....NLPSRDQVAL 293
     242 TADSVF....DTLRLHQPTLLFAVPTLYAAMLADPRSRTETLPDR....L 283

     294 RLASSAGEALPAEIGQRFQRHFGLDIVDGIGSTEMLHIFLSNLPDRVRYG 343
     284 RLCVSAGEPLPAQVGLNWRNRFGHDIVNGVGSTEMGHLFLTNLPHAVEYG 333

     344 TTGWPVPGYQIELRGDGGGPVADGEPGDLYIHGPSSATMYWGNRAKSRDT 393
     334 TSGVPVDGYRLRLVGDRGQDVADDEIGELLVSGGSSAAGYWNQRDKTRTT 383

     394 FQGGWTKSGDKYVRNDDGSYTYAGRTDDMLKVSGIYVSPFEIEATLVQHP 443
     384 FVGEWTRTGDKYHRRADGVYTYCGRTDDIFKVSGIWVSPFEIEQALMSHA 433

     444 GVLEAAVVGVADEHGLTKPKAYVVPRPGQTLSET....ELKTFIKDRLAP 489
     434 KVLEAAVIPAEDTDGLIKPKAFIVLASRGDIDPGALFDELKEHVKSAIGP 483

     490 YKYPRSTVFVAELPKTATGKIQRFKLREGVLG*..................  522
     484 WKYPRWIQIMDDLPKTSSGKLQRYLLREMTLGGIEATESAPSEPALYGRV 533
```

FIG. 3. Gap alignment of the deduced amino acid sequences of *badA* and *hbaA*.

CGA601, had a kanamycin resistance cassette inserted at the expected location on the chromosome. Construction of the mutant was further verified by a Western immunoblot analysis showing that it failed to produce benzoate-CoA ligase.

As shown in Table 2, the *badA* mutant grew at near-wild-type growth rates when supplied with 3 mM benzoate as the sole organic carbon source. This shows that the *badA* product is not essential for growth on benzoate and suggests that mutant cells either metabolize benzoate by an alternative route or synthesize benzoyl-CoA by using an alternative enzyme. An analysis of labeled metabolites formed intracellularly from [^{14}C]benzoate revealed that whole cells of the *badA* mutant, like those of the wild type, accumulated significant amounts of benzoyl-CoA (data not shown). This indicated that an alternative CoA-activating enzyme was able to compensate for the *badA* defect in mutant cells and catalyze benzoyl-CoA formation at rates close to those of wild-type cells. We searched for, but failed to find, any evidence for involvement of an acyl-CoA transferase activity. No benzoyl-CoA formation was detected when succinyl-CoA, acetoacetyl-CoA, or pimelyl-CoA was tested as a potential CoA donor.

TABLE 2. Growth rates of *badA* mutants

Strain	Anaerobic growth rate[a] with:	
	Benzoate (3 mM)	Succinate (10 mM)
Wild type	14 (1)	8 (1)
badA	18 (2)	6 (2)
badA hbaA	25 (3)	9 (1)

[a] Doubling time in hours. Numbers are averages of results for three or more growth experiments. Standard deviations are reported in parentheses.

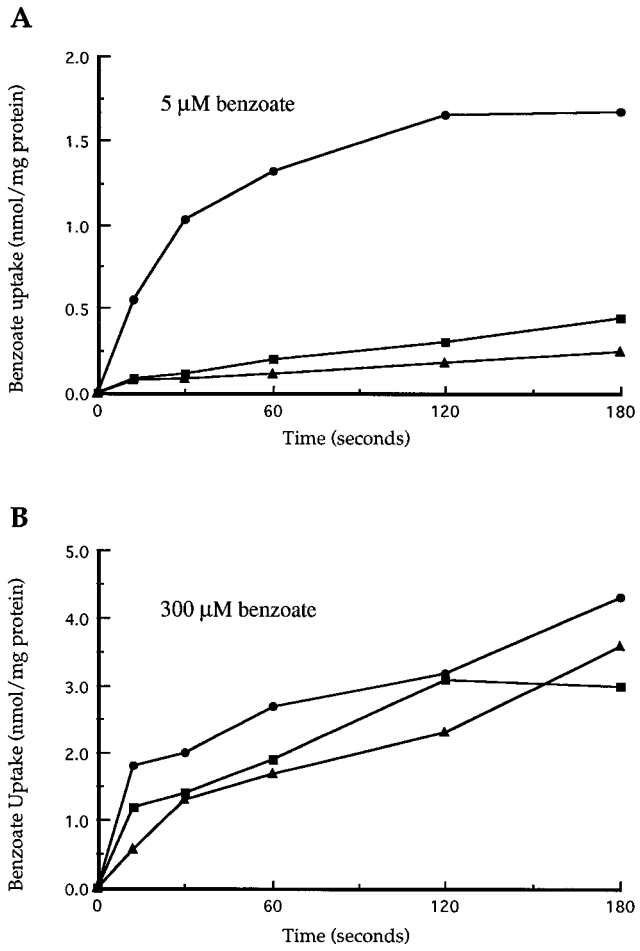

FIG. 4. Uptake of benzoate by whole cells. Results for wild-type (circles), *badA* (squares), and *badA hbaA* (triangles) cells are shown. (A) At 5 μM benzoate, *badA* and *badA hbaA* mutants showed severely reduced uptake. (B) At 300 μM benzoate, uptake by the mutants was comparable to wild-type uptake. Data shown are from one experiment but are representative of four trials.

Two lines of evidence suggested that a ligase with a relatively low affinity for benzoate was responsible for benzoyl-CoA formation by the *badA* mutant. First, rates of [^{14}C]benzoate uptake by whole cells of the *badA* mutant, although nearly normal at a benzoate concentration of 300 μM, were severely de-

TABLE 3. Benzoate-CoA ligase activities of *badA* mutants

Strain	Benzoate-CoA ligase activity[a] at the following benzoate concn[b]:			
	10 μM		1 mM	
	Induced	Uninduced	Induced	Uninduced
Wild type	41.3 (3.1)	4.3 (0.8)	56.8 (2.6)	5.2 (0.2)
badA	1.7 (1.1)	0.6 (0.7)	33.3 (2.8)	1.6 (0.1)
badA hbaA	0.4 (0.2)	<0.1 (0.0)	34.8 (3.8)	1.5 (0.2)

[a] Expressed as nanomoles of product formed per min per mg of protein. Standard deviations are shown in parentheses. Data shown are averages of activities from three or more independently prepared extracts.

[b] Cells were induced by growth on benzoate. Uninduced cells were grown on succinate.

TABLE 4. Effects of exogenous cyclohexanecarboxylate and 4-hydroxybenzoate on benzoate-CoA ligase activities

Strain	Benzoate-CoA ligase activity[a] with the following substrate[b]:		
	Ben	Ben + CHC	Ben + CHC + 4-OH-Ben
Wild type	26.4 (1.5)	14.3 (0.8)	10.4 (0.15)
badA	21.7 (0.1)	11.3 (0.5)	4.50 (0.85)
badA hbaA	17.7 (0.1)	1.5 (0.5)	1.60 (0.17)

[a] Expressed as nanomoles of product formed per min per mg of protein with the standard deviation in parentheses. Values are representative results of one assay with triplicate samples.

[b] Substrate concentrations: benzoate (Ben), 1 mM; cyclohexanecarboxylate (CHC), 50 μM; 4-hydroxybenzoate (4-OH-Ben), 240 μM.

pressed relative to rates exhibited by wild-type cells at a benzoate concentration of 5 μM (Fig. 4). Second, cell extracts of the *badA* mutant had very low benzoate-CoA ligase activity when assayed at 10 μM benzoate, a concentration that gave maximal activity in wild-type cell extracts (Table 3) and also with purified benzoate-CoA ligase (17). When benzoate was supplied at a concentration of 1 mM, the specific activity of benzoate-CoA ligase in *badA* cell extracts approached that measured in wild-type extracts (Table 3). Similar levels of activity were also seen at 300 μM benzoate.

Construction and characterization of a *badA hbaA* double mutant. Two other CoA ligases from *R. palustris* that are active with benzoate have been characterized. These are 4-hydroxybenzoate-CoA (4-OH-ben-CoA) ligase, encoded by the *hbaA* gene, which has a K_m for benzoate of 400 μM (18), and cyclohexanecarboxylate-CoA (chc-CoA) ligase, which has a K_m for benzoate of 500 μM (26). To test the possible involvement of the 4-OH-ben-CoA ligase in anaerobic benzoate degradation, we constructed a *badA hbaA* double mutant as described in Materials and Methods. The genotype of the mutant was verified by Southern hybridization analysis. Western immunoblot analysis confirmed that the double mutant was unable to synthesize the two ligase proteins.

Although the *badA hbaA* mutant grew somewhat more slowly than the *badA* mutant on 3 mM benzoate (Table 2), growth was not severely impaired. The double mutant also had benzoate uptake rates and CoA ligase activities that were only slightly lower than those of the wild type when benzoate was present at a relatively high concentration (0.3 or 1.0 mM) (Fig. 4 and Table 3). When cells or cell extracts were supplied with a low concentration of benzoate, on the other hand, rates of benzoate uptake and benzoyl-CoA formation by the double mutant were very low, even lower than those measured with the *badA* mutant (Fig. 4 and Table 3). These results show that although 4-OH-ben-CoA ligase probably contributes to the ability of *R. palustris* to grow on relatively high concentrations of benzoate, its activity is not sufficient to fully account for the benzoate-CoA ligase activity observed in *badA* cell extracts.

Contribution of chc-CoA ligase. Since the gene for chc-CoA ligase has not been cloned, the possible role of this enzyme in benzoate activation was investigated with competition experiments. As shown in Table 4, addition of cyclohexanecarboxylate to a concentration (50 μM) known to saturate purified chc-CoA ligase (26) reduced the level of benzoate-CoA ligase activity exhibited by the *badA hbaA* double mutant dramatically. A less marked, but significant, effect was seen when extracts of benzoate-grown wild-type and *badA* cells were assayed under the same conditions. These results indicate that the chc-CoA ligase can play a major role in catalyzing benzoyl-CoA formation at high (1 mM) benzoate concentrations. It also appears to be the enzyme that is primarily responsible for catalyzing benzoyl-CoA formation in the absence of the *badA* and *hbaA* products. Inclusion of 4-hydroxybenzoate, in addition to cyclohexanecarboxylate, in extracts of strains that were proficient for 4-OH-ben-CoA ligase synthesis caused a reduction in benzoate-CoA ligase activity compared with that seen with cyclohexanecarboxylate alone (Table 4). This further indicates that 4-OH-ben-CoA ligase can also contribute to the ability of wild-type *R. palustris* cells to activate benzoate with CoA when the benzoate concentration is high.

In related experiments, extracts prepared from wild-type, *badA*, and *badA hbaA* cells grown with cyclohexanecarboxylate all had substantial benzoate-CoA ligase activities when assayed at 1 mM benzoate (13.3, 14.7, and 16.6 nmol of benzoyl-CoA formed per min per mg of protein, respectively). However, activities were very low when extracts were assayed at 10 μM benzoate (2.2, 1.1, and 0.7 nmol of benzoyl-CoA formed per min per mg of protein for wild-type, *badA*, and *badA hbaA* cells, respectively). These results are consistent with previous data indicating that benzoate-CoA ligase and 4-OH-ben-CoA ligase are not induced during growth on cyclohexanecarboxylate (24, 26).

DISCUSSION

The results reported here are a first step in the molecular characterization of the anaerobic benzoate degradation pathway, as *badA* is the first gene which is involved in this metabolic sequence to be characterized. Although it is likely that other benzoate degradation genes are located in close physical proximity to *badA*, the wild-type phenotype of *badA* mutants under laboratory culture conditions does not suggest the presence of benzoate degradation genes downstream of *badA* in the same operon. However, work in progress in our laboratories is aimed at analyzing in detail open reading frames surrounding *badA* for their possible involvement in anaerobic aromatic compound metabolism. The relative positions of *badA* and *hbaA* on the *R. palustris* genome are not yet defined.

Our data show that the wild-type phenotype of the *badA* mutant at high benzoate concentrations can be accounted for by the activities of 4-OH-ben-CoA and chc-CoA ligases, both of which are active with benzoate. Although we have reported previously that 4-OH-ben-CoA ligase is not induced by anaerobic growth on benzoate (18), a careful Western immunoblot analysis that was carried out as part of the present work revealed that the 4-OH-ben-CoA ligase antigen is, in fact, expressed by benzoate-grown cells to a level that is about a fourth of that seen in 4-hydroxybenzoate-grown cells. Succinate-grown cells did not synthesize detectable amounts of 4-OH-ben-CoA ligase antigen. Studies by Küver et al. (26) indicate that chc-CoA ligase activity is fully induced in benzoate-grown cells. The induction of both ligases during growth on benzoate may reflect their role in catalyzing the initial reactions required to funnel 4-hydroxybenzoate or cyclohexanecarboxylate into the anaerobic benzoate degradation pathway. Several lines of evidence indicate that 4-hydroxybenzoyl-CoA is dehydroxylated to yield benzoyl-CoA (7), and more recent work has shown that cyclohexanecarboxyl-CoA is oxidized by *R. palustris* to cyclohex-1-enecarboxyl-CoA, which is a proposed intermediate of the benzoate degradation pathway (26). Redundancy in benzoate-activating enzymes would explain why efforts by our laboratories to obtain benzoate-CoA ligase mutants in screens of randomly mutagenized cells have been unsuccessful.

R. palustris grows on over 20 structurally diverse aromatic and alicyclic carboxylic acids, the degradation of many of which

is initiated by CoA thioesterification. In addition to the ligase enzymes mentioned here, *R. palustris* has also been reported to synthesize two CoA ligases that are active with cinnamate (14), and cells probably synthesize numerous other CoA ligases when growing anaerobically on various aromatic substrates. As more aromatic and alicyclic CoA ligase gene sequences become available, it may be possible to identify "signature" sequences that can be used to screen for additional ligase genes that function in anaerobic aromatic compound degradation. In this regard, it is worth noting that the *badA* and *hbaA* genes from *R. palustris* have extensive regions of nucleotide identity, including one region (from position 646 to position 708) in which 51 of 63 bp are identical. Although the degree of identity between the *badA* and *hbaA* genes is substantial, the proteins that they encode differ markedly in substrate preference and affinity. Also, the *badA* product is active as a monomer, while the *hbaA* product is a homodimer. Amino acid sequence alignments of BadA and HbaA with aromatic acid CoA ligases that operate in aerobic pathways did not reveal extensive regions of similarity. However, the sequences contained a motif proposed by Babbit et al. (6) to be involved in acyl-adenylate formation (Fig. 2).

Because benzoate-CoA ligase, the product of *badA*, is maximally active at very low (micromolar) concentrations of benzoate, it is likely that this is the enzyme that is primarily involved in supporting growth of *R. palustris* cells in soil and water environments. A number of studies have identified several aromatic acids as being present in soils at concentrations ranging from 1 to 10 μM (41, 42). We would expect, on the basis of the results of enzyme assays carried out at 10 μM benzoate, that the *badA* mutant would be markedly impaired in growth at very low concentrations of benzoate. Attempts to demonstrate dependence on BadA at low substrate concentrations by periodic batch feedings (100 μM benzoate) were not successful, and it is probable that continuous culture growth experiments (12) will be needed to fully evaluate the roles of the three known benzoate-CoA ligases in utilization of benzoate under varied, but defined, growth conditions.

The finding that there are at least two other genes that can compensate for the loss of *badA* under some conditions emphasizes the importance of mutant construction and careful phenotypic analysis in providing an accurate picture of anaerobic benzoate degradation by *R. palustris.*

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Department of Energy, Division of Energy Biosciences (DE-FG02-86ER13495), to J.G. and by grants from the U.S. Army Research Office (DAAH04-95-1-0124 and -0315) and the Department of Energy, Division of Energy Biosciences (DE-FG02-95ER20184), to C.S.H.

We thank David T. Evans for help in isolating the λgt11 clone.

REFERENCES

1. **Altenschmidt, U., B. Oswald, and G. Fuchs.** 1991. Purification and characterization of benzoate-coenzyme A ligase and 2-aminobenzoate-coenzyme A ligases from a denitrifying *Pseudomonas* sp. J. Bacteriol. **173:**5494–5501.
2. **Anders, H.-J., A. Kaetzke, P. Kämpfer, W. Ludwig, and G. Fuchs.** 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thaurea*, as *Thaurea aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. Int. J. Syst. Bacteriol. **45:**327–333.
3. **Atschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
4. **Auburger, G., and J. Winter.** 1992. Purification and characterization of benzoyl-CoA ligase from a syntrophic, benzoate-degrading, anaerobic mixed culture. Appl. Microbiol. Biotechnol. **37:**789–795.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1990. Current protocols in molecular biology. Greene Publishing Associates, New York.
6. **Babbit, P. C., G. L. Kenyon, B. M. Martin, H. Charest, M. Slyvestre, J. D. Scholten, K.-H. Chang, P.-H. Liang, and D. Dunaway-Mariano.** 1992. Ancestry of the 4-chlorobenzoate dehalogenase: analysis of amino acid sequence identities among families of acyl:adenyl ligases, enoyl-CoA hydratases/isomerases, and acyl-CoA thioesterases. Biochemistry **31:**5594–5604.
7. **Brackmann, R., and G. Fuchs.** 1993. Enzymes of anaerobic metabolism of phenolic compounds: 4-hydroxybenzoyl-CoA reductase (dehydroxylating) from a denitrifying *Pseudomonas* species. Eur. J. Biochem. **213:**563–571.
8. **Connerton, I. F., J. R. S. Fincham, R. A. Sandeman, and M. J. Hynes.** 1990. Comparison and cross-species expression of the acetyl-CoA synthetase genes of the ascomycete fungi, *Aspergillus nidulans* and *Neurospora crassa*. Mol. Microbiol. **4:**451–460.
9. **De Lorenzo, V., I. Cases, M. Herrero, and K. N. Timmis.** 1993. Early and late responses of TOL promoters to pathway inducers: identification of postexponential promoters in *Pseudomonas putida* with *lacZ-tet* bicistronic reporters. J. Bacteriol. **175:**6902–6907.
10. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
11. **Dutton, P. L., and W. C. Evans.** 1969. The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. Biochem. J. **113:**525–536.
12. **Dykhuizen, D. E.** 1993. Chemostats used for studying natural selection and adaptive evolution. Methods Enzymol. **224:**613–631.
13. **Eggen, R. I. L., A. C. M. Geerling, A. B. P. Boshoven, and W. M. de Vos.** 1991. Cloning, sequence analysis, and functional expression of the acetyl coenzyme A synthetase gene from *Methanothrix soehngenii* in *Escherichia coli*. J. Bacteriol. **173:**6383–6389.
14. **Elder, D. J. E., P. Morgan, and D. J. Kelly.** 1992. Evidence for two differentially regulated phenylpropenoyl-coenzyme A synthetase activities in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. **98:**255–260.
15. **Fuchs, G., M. E. S. Mohamed, U. Altenschmidt, J. Koch, A. Lack, R. Brackmann, C. Lochmeyer, and B. Oswald.** 1994. Biochemistry of anaerobic biodegradation of aromatic compounds, p. 513–553. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
16. **Fulda, M., E. Heinz, and F. P. Wolter.** 1994. The *fad*D gene of *Escherichia coli* K12 is located close to *rnd* at 39.6 min of the chromosomal map and is a new member of the AMP-binding protein family. Mol. Gen. Genet. **242:** 241–249.
17. **Geissler, J. F., C. S. Harwood, and J. Gibson.** 1988. Purification and properties of benzoate-coenzyme A ligase, a *Rhodopseudomonas palustris* enzyme involved in the anaerobic degradation of benzoate. J. Bacteriol. **170:**1709–1714.
18. **Gibson, J., M. Dispensa, G. C. Fogg, D. T. Evans, and C. S. Harwood.** 1994. 4-Hydroxybenzoate-coenzyme A ligase from *Rhodopseudomonas palustris*: purification, gene sequence, and role in anaerobic degradation. J. Bacteriol. **176:**634–641.
19. **Gibson, J., and C. S. Harwood.** 1995. Anaerobic utilization of aromatic carboxylates by bacteria, p. 298–313. *In* R. G. Chaudhry (ed.), Biological degradation and bioremediation of toxic chemicals. Timber Press, Portland, Oreg.
20. **Gibson, K. J., and J. Gibson.** 1992. Potential early intermediates in anaerobic benzoate degradation by *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. **58:**696–698.
21. **Harwood, C. S., and J. Gibson.** 1986. Uptake of benzoate by *Rhodopseudomonas palustris* grown anaerobically in light. J. Bacteriol. **165:**504–509.
22. **Kay, R., and J. McPherson.** 1987. Hybrid pUC vectors for addition of new restriction enzyme sites to the ends of DNA fragments. Nucleic Acids Res. **15:**2778.
23. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
24. **Kim, M.-K., and C. S. Harwood.** 1991. Regulation of benzoate-CoA ligase in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. **83:**199–204.
25. **Koch, J., W. Eisenreich, A. Bacher, and G. Fuchs.** 1993. Products of enzymatic reduction of benzoyl-CoA, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. **211:**649–661.
26. **Küver, J., Y. Xue, and J. Gibson.** Metabolism of cyclohexanecarboxylic acid by the photosynthetic bacterium *Rhodopseudomonas palustris*. Arch. Microbiol., in press.
27. **Labes, M., A. Pühler, and R. Simon.** 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for gram-negative bacteria. Gene **89:**37–46.
28. **Lee, S.-Y., and S. Rasheed.** 1990. A simple procedure for maximum yield of high-quality plasmid DNA. BioTechniques **9:**676–679.
29. **Lozoya, E. H., H. Hoffmann, C. Douglas, W. Schultz, D. Scheel, and K. Hahlbrock.** 1988. Primary structures and catalytic properties of isozymes encoded by the two 4-coumarate:CoA ligase genes in parsley. Eur. J. Biochem. **176:**661–667.
30. **Merkel, S. M., A. E. Eberhard, J. Gibson, and C. S. Harwood.** 1989. Involvement of coenzyme A thioesters in anaerobic metabolism of 4-hydroxybenzoate by *Rhodopseudomonas palustris*. J. Bacteriol. **171:**1–7.

31. **Ngai, K.-L.** Personal communication.
32. **Parales, R. E., and C. S. Harwood.** 1993. Regulation of the *pcaIJ* genes for aromatic acid degradation in *Pseudomonas putida*. J. Bacteriol. **175:**5829–5838.
33. **Perrotta, J. A., and C. S. Harwood.** 1994. Anaerobic metabolism of cyclohex-1-ene-1-carboxylate, a proposed intermediate of benzoate degradation, by *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. **60:**1775–1782.
34. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene **127:** 15–21.
35. **Saito, H., and K. I. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Schmitz, A., K.-H. Gartemann, J. Fiedler, E. Grund, and R. Eichenlaub.** 1992. Cloning and sequence analysis of genes for dehalogenation of 4-chlorobenzoate from *Arthrobacter* sp. strain SU. Appl. Environ. Microbiol. **58:** 4068–4071.
38. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
39. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
40. **Titus, D. E. (ed.).** 1991. Promega protocols and applications guide, 2nd ed. Promega Corporation, Madison, Wis.
41. **Whitehead, D. C.** 1964. Identification of *p*-hydroxbenzoic, vanillic, *p*-coumaric, and ferulic acids in soils. Nature (London) **202:**417–418.
42. **Whitehead, D. C., H. Dibb, and R. D. Hartley.** 1982. Phenolic compounds in soil as influenced by the growth of different plant species. J. Appl. Ecol. **19:**579–588.