BadR, a New MarR Family Member, Regulates Anaerobic Benzoate Degradation by *Rhodopseudomonas palustris* in Concert with AadR, an Fnr Family Member

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Received 2 September 1998/Accepted 19 January 1999

A cluster of genes for the anaerobic degradation of benzoate has been described for the phototrophic bacterium *Rhodopseudomonas palustris*. Here we provide an initial analysis of the regulation of anaerobic benzoate degradation by examining the contributions of two regulators: a new regulator, BadR, encoded by the benzoate degradation gene cluster, and a previously described regulator, AadR, whose gene lies outside the cluster. Strains with single mutations in either *badR* or *aadR* grew slowly on benzoate but were relatively unimpaired in growth on succinate and several intermediates of benzoate degradation. A *badR aadR* double mutant was completely defective in anaerobic growth on benzoate. Effects of the regulators on transcriptional activation were monitored with an *R. palustris* strain carrying a chromosomal fusion of *lacZ* to the *badE* gene of the *badDEFG* operon. This operon encodes benzyol-coenzyme A (benzyol-CoA) reductase, an unusual oxygen-sensitive enzyme that catalyzes the benzene ring reduction reaction that is the rate-limiting step in anaerobic benzoate degradation. Expression of *badE::lacZ* was induced 100-fold when cells grown aerobically on succinate were shifted to anaerobic growth on succinate plus benzoate. The *aadR* gene was required for a 20-fold increase in expression that occurred in response to anaerobiosis, and *badR* was responsible for a further 5-fold increase in expression that occurred in response to benzoate. Further studies with the *badE::lacZ* fusion strain grown with various kinds of aromatic acids indicated that BadR probably responds to benzyol-CoA acting as an effector molecule. Sequence information indicates that BadR is a member of the MarR family of transcriptional regulators. These studies expand the range of functions regulated by MarR family members to include anaerobic aromatic acid degradation and provide an example of a MarR-type protein that acts as a positive regulator rather than as a negative regulator, as do most MarR family members. AadR resembles the *Escherichia coli* Fnr regulator in sequence and contains cysteine residues that are spaced appropriately to serve in the capacity of a redox-sensing protein.

The anaerobic degradation of aromatic compounds by bacteria has been studied in a sustained way only in the last decade. A major theme that has emerged is that diverse aromatic compounds, including aromatic hydrocarbons, chlorinated aromatics, phenols, and plant-derived lignin monomers, are metabolized to form benzoate or, more often, benzyol-coenzyme A (benzyol-CoA) as a common intermediate (12, 13). Benzoate and benzyol-CoA are then degraded to central biosynthetic intermediates by a series of reactions that involve aromatic ring reduction, ring modification, and ring cleavage as critical steps. The two organisms that have been most studied with respect to anaerobic benzoate degradation are *Rhodopseudomonas palustris*, a photoheterotrophic bacterium, and *Thauera aromatica*, a denitrifier. These two species have slightly different benzoate degradation pathways that diverge in the steps following ring reduction (12, 19). Studies of benzoate degradation enzymes have been aided by the recent cloning and sequencing of clusters of benzoate degradation genes from each organism (2, 8).

In spite of good progress on the enzymology of anaerobic benzoate degradation, very little is known about how this process is regulated in either *R. palustris* or *T. aromatica*. Both organisms are facultative anaerobes that can degrade some aromatic compounds under aerobic as well as anaerobic conditions. In the presence of oxygen, aromatic rings are hydroxylated by oxygenases and, hence, aerobic degradation proceeds by a biochemical strategy completely different from that used anaerobically. Thus, *R. palustris* and *T. aromatica* must regulate the expression of aromatic compound degradation genes in response not only to aromatic carbon source availability but also to oxygen levels.

To date, just one gene, *aadR* from *R. palustris*, has been identified as being involved in the regulation of anaerobic aromatic acid degradation. Strains with mutations in *aadR* grow extremely slowly on benzoate and not at all on 4-hydroxybenzoate. The *aadR* mutation has little, if any, effect on growth on nonaromatic carbon sources and no obvious effect on other aspects of metabolism (6). AadR is similar in its deduced amino acid sequence to Fnr, a well-studied regulator of anaerobic respiration in *Escherichia coli* (36), and this similarity has led to the suggestion that the role of AadR is to regulate the expression of anaerobic aromatic compound degradation genes in response to oxygen. However, this notion has not been shown directly, and it is equally possible, based on available evidence, that AadR is primarily a sensor of aromatic compounds.

In this study, we evaluated the effect of *aadR* on anaerobic benzoate degradation at the transcriptional level by monitoring the expression of the *badDEFG* operon by using a *badE::lacZ* fusion as a reporter. The *badDEFG* operon encodes benzyol-CoA reductase, an unusual iron-sulfur flavoprotein that catalyzes the ring reduction step that is critical for the anaerobic degradation of benzoate (8) (Fig. 1). The reaction catalyzed is a strongly endergonic two-electron reduction that is energized...
by the hydrolysis of two molecules of ATP (1). Benzoyl-CoA reductase is extremely sensitive to oxygen. Because of this fact and because the reductase catalyzes an energy-requiring reaction, one would expect the badDEFG operon to be tightly regulated.

We also examined the contribution of badR, a recently described gene (8), to the expression of benzoyl-CoA reductase and characterized the effects of various carbon sources on badDEFG operon expression.

FIG. 1. Anaerobic benzoate degradation pathway and reactions involved in the conversion of 4-hydroxybenzoate (4-OHBen) and cyclohexanecarboxylate (Chc) to intermediates of benzoate degradation by R. palustris (8). The product of benzoyl-CoA reduction is uncertain but is probably one or more of three cyclohexadiene-carboxyl-coenzyme A isomers (10, 17), just one of which is shown. SCoA, coenzyme A.

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 defined mineral medium (16) prepared as described previously (11). Carbon sources were added at the time of inoculation to a final concentration of 3 mM, except for succinate, which was added to 10 mM. Cultures were incubated at 30°C and illuminated with a 40-W incandescent light bulb. E. coli cultures were grown in Luria broth at 37°C. The growth of the cultures was monitored by measuring the A_{660} spectrophotometrically. Antibiotics were used at the following concentrations (in micrograms per milliliter): for R. palustris, gentamicin at
100 and kanamycin at 100; and for *E. coli*, ampicillin at 100, gentamicin at 10, and kanamycin at 100.

Cloning and DNA manipulation. Standard protocols were used for cloning and transformations (31). Plasmid DNA was purified with a QIAprep spin miniprep kit (QIAGEN Inc., Chatsworth, Calif.). DNA fragments were purified from agarose gels with a GeneClean spin kit (Bio 101, Inc., La Jolla, Calif.). Chromosomal DNA was purified by a variation of the method of Saito and Miura (30).

Mutant construction. A *badR* mutant was generated by gene replacement with a cloned copy of *badR* that had been interrupted with a gentamicin resistance (*Gmr*) cassette. To generate this construct, a 1,873-bp fragment containing *badR* was PCR amplified from cosmID pPE304 (8) with primers BADR1 (5'-CCGATCCTTGGATCAGGTCTTCGAACTGC-3') and BADR2 (5'-GATACTGATCAGGTCTTCGAACTGC-3'). The resulting PCR product was then cloned into the BamHI site of pUC19 to generate pPE600. The cloned *badR* gene was then interrupted at a unique *MscI* site with the *Gmr* cassette from pGM1 (32). The resulting plasmid, pPE602, was mated into *R. palustris*, and excgonogans were described as described previously (7) to identify recombinants harboring the disrupted *badR* gene. A *badC* mutant was constructed similarly. Briefly, *badC* was PCR amplified and cloned, and the *Gmr* cassette from pGM1 (32) was cloned into an *XmuI* site within *badC*. The resulting construct was then cloned into a *recA*-containing vector and mated into *R. palustris*. *R. palustris* strains with the *badC*::*Gmr* genotype were constructed similarly by mating pPE408 (8) into *badC* mutant CGA101 (6). Southern hybridization with a *Genius* kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and PCR analysis were carried out to verify mutants.

Primer extension. Primer extension analysis was used to determine the start site for the transcription of *badR*. An avian myeloblastosis virus reverse transcriptase primer extension system was used according to the protocol supplied by the manufacturer (Promega Corp.). The primer used to map the *badR* start site (5'-CAACCAATATTTGCGCATTGGAATG-3') was complementary to nucleotides 81 to 103 of *badR*. Primer extension products were analyzed on a 6% polyacrylamide gel next to a sequence ladder generated with the same primer. Sequencing reactions were performed with an fmol DNA sequencing system from Promega. RNA was prepared with TRI-REAGENT (Molecular Research Center Inc., Cincinnati, Ohio) according to the protocol supplied by the manufacturer. To improve cell lysis, ground glass was added to cells resuspended in TRI-REAGENT, and the cell suspensions were sonicated.

Sequence analysis. DNA sequences were analyzed with GENE Inspector, version 1.0.1 (Textco Inc., West Lebanon, N.H.). Similar sequences were identified from the SWISS-PROT and GENPEPT databases by use of the BLAST network service at the National Center for Biotechnology Information (Bethesda, Md.) and the BLOCK SEARCH program (14). The GAP and PILEUP programs from the University of Wisconsin Genetics Computer Group software package, version 8.0, were used to make sequence comparisons and alignments. Sequences were aligned with the aid of a variation of the method of Miller (23). Clusters in the logarithmic phase of growth were harvested, washed in Z buffer, and sonicated. Cell extract and Z buffer were combined to a volume of 1 ml, and 0.2 ml of a 4 mg/ml solution of 5-nitrophenylgalactopyranoside was added to start the reactions. The rate of increase in the 

### Results

#### Molecular characteristics of *badR*.

The benzoic acid degradation (*bad*) gene cluster (Fig. 2A) from *R. palustris* includes a 528-bp open reading frame, designated *badR* (8), that is predicted to encode a protein with similarity to members of the MarR family of transcriptional regulators (35). When the BLOCK SEARCH program (14) was applied to the *badR* protein, the helix-turn-helix motif associated with the MarR family (5, 35) was evident. GAP alignments of *BadR* to characterized members of the MarR family is shown in Fig. 3.

The transcriptional start site of *badR* was located by primer extension to 27 bp upstream of the first of the three possible ATG initiation codons (Fig. 2). The *badR* promoter region contained elements typical of a σ^70_ promoter. These include a sequence (TATATC) 6 bp upstream of the +1 transcriptional start site that matched the *E. coli* σ^70_–10 consensus sequence (TATAAT) at four of six positions and a sequence (CTGG
CA) starting 31 bp upstream of the +1 site that matched the E. coli σ^70 −35 consensus sequence (TTGACA) at four of six positions.

Characterization of a badR mutant. To determine whether badR is involved in anaerobic benzoate degradation, a badR mutant of R. palustris was constructed by gene replacement as described in Materials and Methods. The mutant grew slowly on benzoate relative to the wild-type parent but was unimpaired in growth on carbon sources, including cyclohex-1-ene-1-carboxylate, which are metabolized via the lower part of the benzoate pathway (Fig. 1 and Table 2). These results suggested that BadR might play a role in regulating an early step of benzoate degradation.

The possibility that the phenotype of the badR mutant was

FIG. 2. (A) Map of the bad (benzoic acid degradation) gene cluster from R. palustris. Arrows indicate probable transcriptional units. The genes encode enzymes of benzoate degradation, as indicated in Fig. 1. The location on the R. palustris chromosome of aadR with respect to the bad gene cluster has not been determined but is at least 30 kb away. (B) Nucleotide sequence of the badR promoter region showing the start site of badR transcription (+1) and the putative −10 and −35 regions. Three possible ATG start codons for badR are underlined. (C) Mapping of the badR transcriptional start site by primer extension. RNA was isolated from benzoate-grown cells (lane 1). A sequence ladder generated with the same primer is shown. The position of the primer extension product is indicated by the arrow.
role in anaerobic aromatic compound degradation has not yet been determined.

**Effect of BadR on gene expression.** We tested the possible role of BadR in regulating one or both of the first two steps of anaerobic benzoate degradation by examining the effect of the *badR* mutation on benzoate-coenzyme A (benzoate-CoA) ligase synthesis and expression of the benzoyl-CoA reductase operon. Immunoblot analysis showed that the *badR* mutant synthesized wild-type levels of benzoate-CoA ligase, indicating that BadR is not required for the expression of *badA*, the gene that encodes benzoate-CoA ligase (data not shown). Expression of the benzoyl-CoA reductase operon was tested by measuring the levels of β-galactosidase activity from *R. palustris* strains carrying a chromosomal transcriptional fusion of *badE* to *lacZ* (*badE::lacZ*) (Fig. 4A). In a wild-type background (strain CGA606), the activity of the *badE::lacZ* fusion was induced about fivefold by anaerobic growth in the presence of benzoate (Fig. 4B). In a *badR* background (strain CGA610), however, benzoate did not induce increased *lacZ* expression from the *badE::lacZ* fusion. The levels of β-galactosidase activity in the *badR* mutant *badE::lacZ* strain were approximately the same in cells grown on benzoate plus succinate as in cells grown on succinate alone (Fig. 4B). This result suggests that BadR regulates gene expression in response to benzoate as a carbon source.

Although benzoate was the best inducer of the *badE::lacZ* fusion, growth in the presence of 4-hydroxybenzoate, which is also processed through benzoyl-CoA (Fig. 1), also elicited induced levels of β-galactosidase activity (6,838 nmol of product/min/mg of protein). The nonmetabolizable compound 3-chlorobenzoate did not serve as an inducer (3,016 nmol of product/min/mg of protein) when it was supplied in combination with succinate.

**AadR, a second regulator required for full expression of the benzoyl-CoA reductase operon.** To test the possible involvement of AadR in the expression of benzoyl-CoA reductase, we introduced the *badE::lacZ* fusion into an *aadR* mutant strain of *R. palustris* to generate strain CGA607. The *aadR* mutation caused a reduction in *badE::lacZ* expression regardless of the carbon source used for growth (Fig. 4B). Moreover, the level of *badE* expression in the *aadR* mutant grown anaerobically on succinate was reduced to the level seen for wild-type cells grown aerobically on succinate (Fig. 4B). Although the introduction of the *aadR* mutation caused an overall reduction in the level of *badE* expression in cells grown anaerobically, the cells still retained their ability to induce *badE* expression when grown anaerobically in the presence of benzoate, in comparison with cells grown on succinate only. These results are consistent with the idea that AadR mediates gene expression in response to anaerobiosis.

**BadR and AadR have additive effects on benzoyl-CoA reductase expression.** A *badR* *aadR* double mutant (strain CGA611) grown anaerobically on succinate had the same low levels of *badE::lacZ* expression as an *aadR* single mutant grown under the same conditions. The double mutant differed from the *aadR* single mutant, however, in being unable to activate *badE::lacZ* expression in response to benzoate, since β-galactosidase activities in cells grown on benzoate plus succinate were just as low as those in cells grown on succinate alone (Fig. 4B). This result indicates that BadR and AadR have independent effects on *badE* expression and that these effects are additive. The importance of these two regulators is underscored by the finding that a *badR* *aadR* mutant of *R. palustris*, in contrast to a *badR* or an *aadR* single mutant, was completely defective in anaerobic growth on benzoate (Table 2).

![Phylogenetic tree of members of the MarR family of regulatory proteins constructed with the AllAll program from the Computational Biochemistry Research Group (4a). The proteins included, their accession numbers, their sources, and the systems regulated are as follows: BadR (U75363), *R. palustris*, this study; NhhD (D67027), *Rhodococcus rhodochrous*, multidrug resistance (27); MexR (U23763), *Pseudomonas aeruginosa*, multidrug resistance (27); SlyA (P40676), *Salmonella typhimurium*, hemolysin production (24); T. orf1 (A001830), *T. aromatica*, open reading frame adjacent to genes encoding 4-hydroxybenzoate-coenzyme A reductase (3); EmrR (P27245), *E. coli*, protease production and sporulation (25); HecR (P27245), *E. coli*, multiple antibiotic resistance (4); CinR (U64802), *Butyrivibrio fibrisolvens*, release of cinnamate from plant material (5); Hpr (P11065), *Bacillus subtilis*, protease production and sporulation (25); MarR (P27245), *E. coli*, multiple antibiotic resistance (4); CinR (U64802), *Butyrivibrio fibrisolvens*, release of cinnamate from plant material (5); Hpr (P11065), *Bacillus subtilis*, protease production and sporulation (25). All proteins on the tree, with the exception of T. orf1, have been directly demonstrated to have a regulatory function.](http://jb.asm.org/)

**FIG. 3.** Phylogenetic tree of members of the MarR family of regulatory proteins constructed with the AllAll program from the Computational Biochemistry Research Group (4a). The proteins included, their accession numbers, their sources, and the systems regulated are as follows: BadR (U75363), *R. palustris*, this study; NhhD (D67027), *Rhodococcus rhodochrous*, multidrug resistance (27); MexR (U23763), *Pseudomonas aeruginosa*, multidrug resistance (27); SlyA (P40676), *Salmonella typhimurium*, hemolysin production (24); T. orf1 (A001830), *T. aromatica*, open reading frame adjacent to genes encoding 4-hydroxybenzoate-coenzyme A reductase (3); EmrR (P27245), *E. coli*, protease production and sporulation (25); HecR (P27245), *E. coli*, multiple antibiotic resistance (4); CinR (U64802), *Butyrivibrio fibrisolvens*, release of cinnamate from plant material (5); Hpr (P11065), *Bacillus subtilis*, protease production and sporulation (25). All proteins on the tree, with the exception of T. orf1, have been directly demonstrated to have a regulatory function.

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**TABLE 2.** Anaerobic rates of growth of *badR* and *aadR* mutants

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Wild type</th>
<th><em>badR</em> (min/mg of protein)</th>
<th><em>aadR</em> (min/mg of protein)</th>
<th><em>badR</em> <em>aadR</em> (min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>12 (1)</td>
<td>29 (6)</td>
<td>58 (18)</td>
<td>NG</td>
</tr>
<tr>
<td>Cyclohex-1-ene-1-carboxylate</td>
<td>8 (2)</td>
<td>7 (1)</td>
<td>28 (1)</td>
<td>18 (2)</td>
</tr>
<tr>
<td>Succinate</td>
<td>6 (1)</td>
<td>10 (3)</td>
<td>11 (3)</td>
<td></td>
</tr>
</tbody>
</table>

* Reports doubling times in hours. Numbers are the averages of two or more growth experiments; standard deviations are given in parentheses. NG, no growth.
DISCUSSION

Our results indicate that the regulatory protein BadR is required for the induction of benzoyl-CoA reductase expression in response to aromatic compounds and that AadR modulates the levels of expression in response to anaerobiosis. Together, these two regulators account for the approximately 100-fold induction of \textit{badE}::\textit{lacZ} expression that occurs when \textit{R. palustris} cells grown aerobically on succinate are shifted to anaerobic growth with benzoate.

BadR is most closely related to members of the MarR family of bacterial regulatory proteins (Fig. 3). MarR, the best-characterized member of this family, negatively regulates the expression of the antibiotic resistance genes \textit{marAB} (4). An inducer of these genes, 2-hydroxybenzoate (salicylate), has been shown to bind to MarR and, by inhibiting its ability to bind to the \textit{marAB} operator region, to allow transcription of the \textit{marAB} operon (21). In addition to MarR, several other proteins in this family are likely to respond to aromatic compounds (35). These include other regulators of antibiotic resistance, such as MexR (27) and EmrR (20), which has also been shown to respond to salicylate; HpcR, which regulates genes involved in the metabolism of homoprotocatechuate.

FIG. 4. (A) Map of the \textit{badDEFG} chromosomal region of strain CGA606 containing the \textit{badE}:\textit{lacZ} fusion. The \textit{badDEFG} start site of transcription (+1), putative –10 region, and Fnr consensus binding sequence are indicated. (B) Expression of \textbeta-galactosidase from a chromosomally encoded \textit{badE}:\textit{lacZ} fusion in wild-type (strain CGA606), \textit{badR} mutant (strain CGA610) and \textit{aadR} mutant (strain CGA607), and \textit{badR aadR} double-mutant (strain CGA615) backgrounds. Cells were grown on 10 mM succinate in the presence or absence of 3 mM benzoate. Activities are expressed as nanomoles of product formed per minute per milligram of protein. Values are the averages of data obtained from three or more separately prepared cell extracts. Standard deviations are represented by error bars.
4-hydroxybenzoate, that have been found to induce monitor gene expression (8); (ii) several compounds, such as benzoyl-CoA accumulated to induce the expression of the benzoyl-CoA reductase operon. Of the two compounds, benzoyl-CoA is the more likely effector because (i) benzoyl-CoA accumulated to high levels in the badE::lacZ reporter strain that was used to monitor gene expression (8); (ii) several compounds, such as 4-hydroxybenzoate, that have been found to induce badE expression are metabolized by R. palustris to form benzoyl-CoA, but not free benzoate, as an intermediate; and (iii) aromatic compounds, such as 3-chlorobenzoate, that are structurally similar to benzoate but that are not metabolized to benzoyl-CoA do not induce badE::lacZ expression.

BadR differs from many members of the MarR family in that it activates, rather than represses, gene expression. However, SlyA (24) and NhhD (18) are two other MarR family members that have also been shown to regulate gene expression in a positive manner. MexR has both positive and negative regulatory effects on expression (27). No work has yet been reported on the mechanism responsible for transcriptional activation by any of these proteins. It is likely that BadR binds directly to the badD promoter region to activate gene expression, but this notion has yet to be demonstrated.

The activation of badDEFG operon expression mediated by AadR in response to anaerobiosis makes sense because the AadR protein has the signature characteristics of a redox-sensing protein. It has the four cysteine residues, three at its N terminus and one near the center of the protein, that are conserved among most Fnr family members and that are required for the E. coli Fnr protein to be active (6, 22). Recent data indicate that the cysteines coordinate a 4Fe-4S center in Fnr that disassembles on exposure to oxygen, rendering the protein inactive, and then reassembles when the protein is reoxygenated (15). The reduced form of the protein forms dimers that bind DNA. The promoter region of the badDEFG operon includes an Fnr consensus binding sequence (TTGAT-N4-ATCAA) (34) centered at −39.5 relative to the transcriptional start site (Fig. 4A) (8). It is likely that the AadR protein responds to anaerobiosis by assuming an active conformation that is proficient in binding to the R. palustris badDEFG promoter to activate transcription. We cannot rule out, however, the possibility that the effect of AadR on transcription is indirect and occurs through the activation of yet another regulatory protein.

AadR is the first example of an Fnr family member reported to be involved in regulating aromatic compound degradation in response to oxygen. From what is known so far, AadR is quite specialized in the target genes that it regulates relative to other Fnr family members (37). It does not appear, for example, to control genes required for photosynthesis, as does the FnrL protein from the related phototroph, Rhodobacter sphaeroides (40), a species that does not grow on aromatic compounds. Many Fnr proteins, including those from Rhodobacter sphaeroides and another phototroph of the same genus, Rhodobacter capsulatus, regulate genes for anaerobic respiration (39). The wild-type strain of R. palustris used in our work is unable to grow by anaerobic respiration in the dark with a variety of electron acceptors, including nitrate and dimethyl sulf Oxide.

This study has provided the first information about how anaerobic benzoate degradation is regulated in one organism. It has also provided an initial analysis of a new regulatory protein, BadR, and has improved understanding of the role of AadR in the physiology of R. palustris. However, it has also shown that understanding of the regulation of anaerobic benzoate degradation is far from complete. For example, several portions of the anaerobic benzoate degradation pathway, including the initial benzoate activation step and the lower portion of the pathway, do not appear to be regulated by BadR. We know, however, that the production of enzymes in addition to benzoyl-CoA reductase is regulated in R. palustris to aromatic compounds. Benzoate-CoA ligase is synthesized in 10-fold higher amounts by cells grown anaerobically in the presence of aromatic acids than by cells grown on succinate (7, 16). Levels of the three enzymes that catalyze the conversion of cyclohex-1-ene-1-carboxyl-coenzyme A to pimelyl-coenzyme A are also higher in benzoate-grown cells (26). How the genes encoding these other benzoate degradation enzymes are regulated is an open question since, based on sequence analysis, the bad gene cluster does not include other genes that seem likely to have a regulatory function.

ACKNOWLEDGMENTS

This work was supported by the Division of Energy Biosciences, Department of Energy (grant DE-FG02-95ER20184), and by the U.S. Army Research Office (grant DAAG55-98-1-0188). We thank Jane Gibson, Rebecca Parales, and Abel Ferrández for helpful discussions.

REFERENCES