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BadM Is a Transcriptional Repressor and One of Three Regulators That Control Benzoyl Coenzyme A Reductase Gene Expression in *Rhodopseudomonas palustris*▽

Caroline M. Peres[1] and Caroline S. Harwood[2]*

Department of Microbiology, University of Washington, Seattle, Washington 98195-7242,[2] and Danisco Genencor, 925 Page Mill Road, Palo Alto, California 94304[1]

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The rate-limiting enzyme of anaerobic benzoate degradation by *Rhodopseudomonas palustris*, benzoyl coenzyme A (CoA) reductase, is highly sensitive to oxygen, and its synthesis is tightly regulated. We determined that a previously unknown gene in the benzoate gene cluster, *badM*, encodes a transcriptional repressor of benzoyl-CoA reductase gene expression. BadM controls gene expression from the benzoyl-CoA reductase promoter in concert with two previously described transcriptional activators.

Under anaerobic conditions some bacteria catabolize structurally diverse aromatic compounds through peripheral pathways to form benzoate or benzoyl coenzyme A (CoA), which then enters a central pathway of aromatic ring reduction and ring cleavage (12). A novel oxygen-sensitive enzyme, benzoyl-CoA reductase, is critical for the operation of the pathway, because it relieves the resonance stability of the benzene ring (5, 11). The photosynthetic bacterium *Rhodopseudomonas palustris* is one of several species that have served as model organisms for studies of anaerobic benzoate degradation (2, 15). The *R. palustris* benzoate degradation gene cluster is unique in that it encodes a small protein, named BadM, which belongs to the Rrf2 family of transcriptional regulators (PFAM: PF02082 and Interpro: IPR000944) (16) (Fig. 1). Several Rrf2 family members have been shown to function as repressors, and we present evidence here that BadM (RPA0663) represses the transcription of benzoate degradation genes. A *badM* mutation resulted in constitutive expression of the *badDEFGAB* operon, encoding benzoyl-CoA reductase and benzoate-CoA ligase. We had previously shown that transcription of the benzoyl-CoA reductase operon is activated by the regulators AadR and BadR in response to anaerobiosis and benzoate, respectively (6, 9). BadM is thus a third regulator that acts at the *badDEFGAB* promoter.

A *badM* mutant expresses the first two enzymes of anaerobic benzoate degradation constitutively. To examine the possible contribution of BadM to benzoate degradation, we used standard techniques (14) to construct a mutant (CGA131) in which the *badM* gene had been deleted from the chromosome (Table 1). Southern hybridization experiments verified that the expected deletion had occurred. The *badM* deletion mutant grew as well as its wild-type parent in minimal medium with 3 mM benzoate under anaerobic conditions in light, and it also grew normally in minimal medium with 10 mM succinate both aerobically and anaerobically in light (17). The enzymes of anaerobic benzoate degradation are induced when cells are grown anaerobically on benzoate and are not present at significant levels in succinate-grown cells (9, 22). Rabbit polyclonal antisera were raised against purified polyhistidine-tagged subunits of benzoyl-CoA reductase (His-BadD and His-BadG) at Covance Research Products Inc. (Denver, PA) and used to screen for a *badM* mutant phenotype. Cell extracts (20 μg protein) of *R. palustris* were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and immunoblotted as described previously (22). Immunoblot analysis indicated that the *badM* mutant differed from the wild type in that it synthesized the BadD subunit of benzoyl-CoA reductase when it was grown anaerobically with succinate as well as when it was grown with benzoate (Fig. 2). Immunoblots probed with antisera against either the BadG subunit of benzoyl-CoA reductase or benzoate-CoA ligase (BadA) (17) showed a similar pattern of constitutive protein production in the *badM* mutant (data not shown). In all cases anaerobic growth was prerequisite for constitutive enzyme production. The *badM* mutation did not cause constitutive production of three enzymes for anaerobic benzoate degradation that function downstream of the benzoyl-CoA reductase step (e.g., BadK, cyclohex-1-ene-1-carboxyl-CoA hydratase; BadH, 2-hydroxycyclohexane carboxyl-CoA dehydrogenase; and BadI, 2-ketocyclohexane carboxyl-CoA hydrolase) (15) as determined by Western blot analysis (data not shown). Constitutive expression of benzoate-CoA ligase activity was observed in succinate-grown cells of the *badM* mutant (Table 2), confirming the results from immunoblot analyses. When BadM was expressed in *trans* in the *badM* mutant, repression of benzoate-CoA ligase activity in succinate-grown cells was restored. Immunoblot analysis indicated that BadM expressed in *trans* also complemented the constitutive expression phenotype of benzoyl-CoA reductase (data not shown).

BadM represses at the level of transcription. Total RNA was isolated from *R. palustris* cells using the High Pure RNA isolation kit (Roche Diagnostics Corp., Indianapolis, IN), and 10 μg RNA was used per lane for Northern blot analysis. The analysis was performed with the NorthernMax-Gly kit (Ambion, Austin, TX) according to the manufacturer's instructions.

* Corresponding author. Mailing address: Department of Microbiology, Box 357242, 1959 N. E. Pacific Street, University of Washington, Seattle, WA 98195-7242. Phone: (206) 221-2848. Fax: (206) 221-5041. E-mail: csh5@u.washington.edu.

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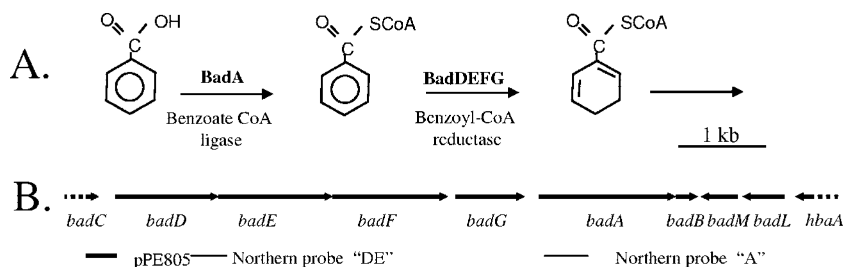

FIG. 1. (A) The first two steps of anaerobic benzoate degradation in *R. palustris.* (B) The organization of some of the benzoate degradation genes in *R. palustris*. Arrows indicate the direction of transcription. The DNA fragment used to construct the promoter fusion plasmid pPE805 is indicated, as are the fragments used as probes for the Northern hybridizations.

A 440-bp PCR product containing the junction of *badD* and *badE* and a 522-bp PCR product targeting *badA* (Fig. 1) were labeled with [α-^{32}P]dCTP using Ready-To-Go DNA labeling beads (Amersham, Arlington Heights, IL), purified with a ProbeQuant G-50 Microcolumn (Pharmacia Biotech, Piscataway, NJ), and used as probes. A single transcript of about 7,000 bp was present at much higher levels in succinate-grown *badM* cells than in wild-type cells (data not shown). The predicted size of a *badDEFGAB* transcript is 6,758 bp (19). BadB encodes a ferredoxin that likely functions with benzoyl-CoA reductase. This gene is separated from *badA* by just 16 bp. Together our results indicate that BadM acts at the level of transcription to repress expression of a *badDEFGAB* operon. Consistent with this we found that in a wild-type background benzoate induced the expression of a *badA*::*lacZ* chromosomal transcriptional fusion 12-fold above the level seen in succinate-grown cells (Fig. 3). When the *badM* gene was mutated, there was no significant difference in *lacZ* expression levels between induced and noninduced cells and the levels of β-galactosidase were much higher than the noninduced wild-type level. Significant levels of *badA* expression were seen only under anaerobic conditions.

BadM represses transcription from the *badD* promoter. We constructed a *badD* promoter-*lacZ* transcriptional fusion plasmid according to methods described previously (10) and introduced it into *Pseudomonas aeruginosa* together with a plasmid expressing *badM* to test whether BadM was sufficient to regulate gene expression in a heterologous background. BadM repressed P*badD-lacZ* expression in *P. aeruginosa* cells grown anaerobically in Luria broth under denitrifying conditions (Fig. 4). Addition of benzoate to the growth medium did not cause an increase in P*badD-lacZ* expression. The repressive effect of BadM was also seen but was much less pronounced in aerobically grown cells. Previously we have shown that the *badD* promoter is regulated by an Anr/Fnr homologue called AadR in response to anaerobiosis (9). It is possible that the transcription factor Anr activates P*badD* expression in anaerobically grown *P. aeruginosa* cells in the absence of BadM.

Possible mechanism of BadM activity. Our description of the BadM protein expands the range of functions regulated by

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant property[a]	Source or reference
Strains		
Escherichia coli		
DH5α	Host strain for cloning	Gibco-BRL
S17-1	Donor strain for transferring broad-host-range plasmids	26
P. aeruginosa PAO1	Wild-type strain	A. Kropinsky
R. palustris		
CGA009	Wild-type strain; spontaneous Cm^r derivative	19
CGA131	*badM* (257-bp deletion mutant)	This study
CGA901	*badA*::*'lacZ*; Km^r	This study
CGA301	*badM badA*::*'lacZ*; Km^r	This study
Plasmids		
pBBR1MCS-2	Broad-host-range vector; Km^r	18
pBBR1MCS-5	Broad-host-range vector; Gm^r	18
pJQ200mp18	Mobilizable suicide vector; *sacB* Gm^r	23
pHRP309	Reporter plasmid, contains promoterless *'lacZ*	21
pCP230	pJQ200mp18 with *badA*::*'lacZ*; Km^r	This study
pCP439	pBBR1MCS-2 with the *badM* gene inserted; Km^r	This study
pCP561	pBBR1MCS-5 with the *badM* gene inserted; Gm^r	This study
pPE805	pHRP309 with *badD* promoter region fused to *'lacZ*; Sp^r Gm^r	This study

[a] Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Sp^r, spectinomycin resistance. Antibiotic concentrations were as described previously (24).

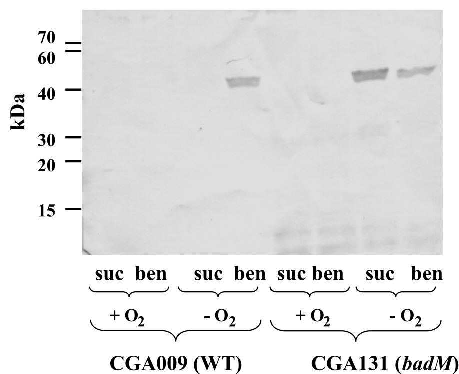

FIG. 2. Western immunoblot of cell extracts of *R. palustris* wild type (WT; CGA009) and a *badM* mutant (CGA131) grown under different conditions and probed with BadD (first subunit of benzoyl-CoA reductase) antiserum. + O_2, grown in aerobic conditions; − O_2, grown in anaerobic conditions. Cells were grown with 10 mM succinate (suc) or with 10 mM succinate plus 3 mM benzoate (ben). Each lane contains 20 μg protein. Size markers are indicated on the left in kilodaltons.

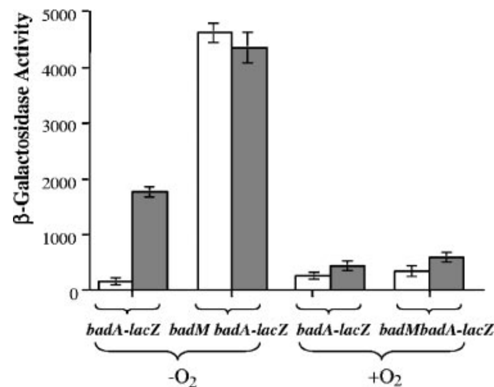

FIG. 3. Expression of β-galactosidase activity from a *badA*::*'lacZ* chromosomal fusion in *R. palustris* wild-type and *badM* mutant cells. Cells were grown with succinate (open bars) or on succinate plus benzoate (filled bars). Data are averages from three different experiments, plus or minus standard deviations. β-Galactosidase activity is shown as specific activity (nanomoles *o*-nitrophenol formed per min per mg protein) (9).

Rrf2 proteins to include anaerobic biodegradation. Other characterized Rrf2 family regulators are repressors of genes involved in nitrite, nitric oxide, or iron metabolism (3, 4, 13, 25, 27). The best-studied Rrf2 protein, IscR, contains a Fe-S cluster and represses the expression of several Fe-S-cluster-containing anaerobic respiration enzymes in addition to regulating Fe-S cluster formation genes (13, 25). Three cysteine residues that are conserved in the C-terminal portion of IscR and other Rrf2 regulators have been proposed to be involved in the formation of its Fe-S cluster (4, 25). Although BadM has the Rrf2-type helix-turn-helix domain signature presumed to be involved in DNA binding, it does not have the conserved cysteines that are found in many members of this family. In a transcriptome analysis using *R. palustris* Affymetrix gene chips we found that the expression of only a few genes in addition to the benzoyl-CoA reductase operon (expressed at 20-fold-higher levels in a *badM* mutant grown anaerobically on succinate than in wild-type cells grown the same way) was affected by more than fivefold by *badM*. These included a dicarboxylic acid transporter gene (RPA2448) and a gene for a conserved hypothetical protein (RPA3401) that were expressed at about sevenfold- and sixfold-higher levels in a *badM* mutant, respectively. *badM* was required for full expression of a possible cytochrome P450 gene (RPA1009; eightfold effect) as well as an operon of conserved hypothetical genes (RPA1209-1212; sixfold effect). None of these genes appear to encode Fe-S proteins or to be involved in iron-related metabolism.

We were surprised to find that BadM had such a large effect in repressing benzoyl-CoA reductase expression because we had previously described two regulators, AadR (which senses anaerobiosis) and BadR (which senses benzoate or benzoyl-CoA), that appeared to account for the full range of benzoate-induced expression of the *badDEFG* genes (9). A *badR aadR* double mutant is completely defective in growth on benzoate, indicating an absolute requirement for these regulators (9). The anaerobic benzoate degradation genes of *Azoarcus* sp. strain CIB, a denitrifying bacterium, are induced by AcpR, a regulator that is related to AadR, in response to oxygen (7), and by BzdR, a transcriptional repressor that is not related to BadM (1), in response to benzoyl-CoA. It is logical to hypoth-

TABLE 2. Effect of *badM* on benzoate-CoA ligase activity

Strain[b]	Benzoate-CoA ligase activity for cells[a]	
	Benzoate grown	Succinate grown
CGA009	30.2	6.0
CGA131	27.8	55.9
CGA009(pCP561)	27.7	7.5
CGA131(pCP561)	28.7	8.5

[a] Expressed as nmol product per min per mg protein in crude cell extracts (8). The concentration of benzoate added to the reaction mixtures was 10 μM. Values are the averages of activities from two independently prepared extracts.
[b] CGA009, wild type; CGA131, *badM* mutant.

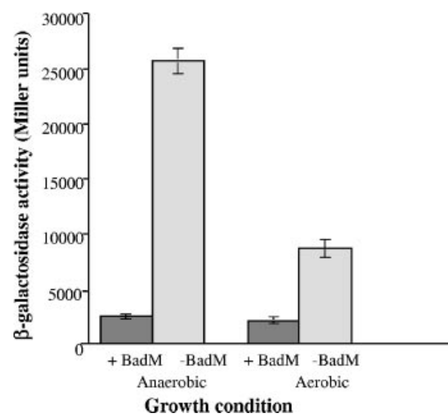

FIG. 4. Expression of β-galactosidase activity in *P. aeruginosa* cells containing the P*badD-'lacZ* reporter plasmid pPE805 and either the BadM-expressing plasmid pCP439 (+ BadM) or the control vector pBBR1MCS-2 (−BadM). Cells were grown aerobically in Luria broth or anaerobically in Luria broth plus 0.2% glucose plus 10 mM potassium nitrate. Data are averages from three different experiments, plus or minus standard deviations. β-Galactosidase activity is in Miller units (20).

esize that BadM represses benzoate degradation by binding to the *badD* promoter and that benzoate binds to BadM to cause it to come off DNA and derepress gene expression in *R. palustris*, but we have not been able to show this. The addition of benzoate to *P. aeruginosa* cells harboring BadM and a *badD-lacZ* promoter fusion did not have an effect on *lacZ* expression. This could be because the effector of BadM is a compound (such as benzoyl-CoA) other than benzoate, or it could be that BadM interacts with BadR or AadR or their DNA binding sites in a more complex way to modulate gene expression. It will be important to work with purified regulators in vitro to determine their DNA binding sites and define possible physical interactions between regulatory proteins and RNA polymerase in order to fully understand the mechanism of oxygen- and benzoate-mediated regulation at the *badDEFGAB* promoter.

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