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Benzylsuccinate Synthase of *Azoarcus* sp. Strain T: Cloning, Sequencing, Transcriptional Organization, and Its Role in Anaerobic Toluene and *m*-Xylene Mineralization

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Biochemical studies in *Azoarcus* sp. strain T have demonstrated that anaerobic oxidation of both toluene and *m*-xylene is initiated by addition of the aromatic hydrocarbon to fumarate, forming benzylsuccinate and 3-methyl benzylsuccinate, respectively. Partially purified benzylsuccinate synthase was previously shown to catalyze both of these addition reactions. In this study, we identified and sequenced the genes encoding benzylsuccinate synthase from *Azoarcus* sp. strain T and examined the role of this enzyme in both anaerobic toluene and *m*-xylene mineralization. Based on reverse transcription-PCR experiments and transcriptional start site mapping, we found that the structural genes encoding benzylsuccinate synthase, *bssCAB*, together with two additional genes, *bssD* and *bssE*, were organized in an operon in the order *bssDCABE*. *bssD* is believed to encode an activating enzyme, similar in function to pyruvate formate-lyase activase. *bssE* shows homology to *tutH* from *Thauera aromatica* strain T1, whose function is currently unknown. A second operon that is upstream of *bssDCABE* and divergently transcribed contains two genes, *tdiS* and *tdiR*. The predicted amino acid sequences show similarity to sensor kinase and response regulator proteins of prokaryotic two-component regulatory systems. A chromosomal null *bssA* mutant was constructed (the *bssA* gene encodes the α-subunit of benzylsuccinate synthase). This *bssA* null mutant strain was unable to grow under denitrifying conditions on either toluene or *m*-xylene, while growth on benzoate was unaffected. The growth phenotype of the Δ*bssA* mutant could be rescued by reintroducing *bssA* in *trans*. These results demonstrate that benzylsuccinate synthase catalyzes the first step in anaerobic mineralization of both toluene and *m*-xylene.

Azoarcus sp. strain T is a facultative microorganism capable of mineralizing both toluene and *m*-xylene anaerobically with nitrate as the electron acceptor. Based on biochemical studies, a pathway for anaerobic oxidation of toluene has been proposed (2, 6). In this pathway, a novel enzyme, benzylsuccinate synthase, catalyzes the addition of the methyl group of toluene to fumarate to form benzylsuccinate (Fig. 1). Benzylsuccinate is then oxidized to benzoyl-coenzyme A (CoA), a central intermediate in anaerobic aromatic hydrocarbon metabolism. Other studies have shown that this fumarate addition reaction is found in a wide range of microorganisms capable of anaerobic toluene mineralization, including other denitrifying bacteria, *Thauera aromatica* strain K172 and strain EbN1 (6, 27), several sulfate-reducing bacteria (3, 27), an anoxygenic phototrophic bacterium (35), and a methanogenic mixed culture (1). Detection of benzylsuccinate in cultures of the toluene-degrading *Azoarcus tolulyticus* Tol-4 and *Thauera aromatica* strain T1 suggests that a fumarate addition reaction may also be involved in anaerobic toluene mineralization in these microorganisms (7, 13). In addition, recent work has suggested that *m*-xylene (16), *m*-cresol (24), *p*-cresol (25), and the aliphatic hydrocarbons *n*-hexane (28) and *n*-dodecane (18) are also activated anaerobically by a fumarate addition reaction. Thus, it appears that the formation of benzylsuccinate, or a corresponding succinate derivative, is a common mode for initiating anaerobic mineralization of methylbenzenes, methylphenols, and long-chain *n*-alkanes.

Benzylsuccinate synthase has been purified from *Thauera aromatica* strain K172 and *Azoarcus* sp. strain T and shown to have an $\alpha_2\beta_2\gamma_2$ composition (5, 22). The enzyme was shown to be irreversibly inactivated in the presence of molecular oxygen by oxygenolytic cleavage of the α subunit of benzylsuccinate synthase (22). Furthermore, toluene addition to fumarate is believed to occur by a radical mechanism because the H atom abstracted from the methyl group of toluene during addition to fumarate is retained in the succinyl moiety of benzylsuccinate (2). These observations, in conjunction with the sequence similarity of the α subunit of benzylsuccinate synthase to glycyl radical proteins, suggested that benzylsuccinate synthase may be a glycyl radical enzyme. In addition, recent electron paramagnetic resonance studies have shown the presence of a glycyl radical in samples of active benzylsuccinate synthase purified from *Azoarcus* sp. strain T, demonstrating experimentally that benzylsuccinate synthase is a glycyl radical enzyme (17).

The genes encoding benzylsuccinate synthase have been independently identified in two microorganisms, *T. aromatica* strains T1 and K172, by a genetic and a reverse genetic approach, respectively. In *T. aromatica* strain T1, mutants defective in toluene utilization and benzylsuccinate formation were isolated (10). Complementation studies with these mutants led to the identification of several open reading frames, including *tutE*, *tutFDGH*, *tutCB*, and *tutC1B1* (9, 10, 21). Based on N-terminal amino acid sequences of benzylsuccinate synthase

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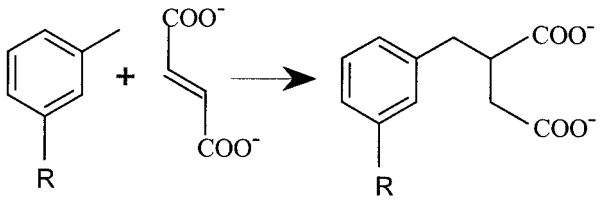

FIG. 1. Addition of toluene and *m*-xylene to fumarate catalyzed by benzylsuccinate synthase. R = H for toluene and R = CH_3 for *m*-xylene.

purified from *T. aromatica* strain K172, *bssDCAB* and *tdiSR* were cloned and sequenced in this microorganism (22). The *bssCAB* genes show similarity to the *tutFDG* genes, which encode the γ, α, and β subunits of benzylsuccinate synthase, respectively. The predicted amino acid sequence of *bssA* shows similarity to the anaerobic glycyl radical enzymes pyruvate formate-lyase (PFL) and anaerobic ribonucleotide reductase (ARNR) (9, 22). The glycyl radical in PFL and ARNR is post-translationally generated by PFL activase and ARNR activase, respectively. The predicted translation products of *bssD* and *tutE* show homology to these activases and have been proposed to perform a similar function. *tutCB*, *tutC1B1,* and *tdiSR* encode proteins with homology to sensor kinase and response regulator proteins of bacterial two-component regulatory systems.

Although the predicted amino acid sequences of the benzylsuccinate synthases from *T. aromatica* strains K172 and T1 are almost identical, several differences exist in the organization of the genes in the *bss*/*tut* regions in these two strains (Fig. 2B). In *T. aromatica* strain T1, the *tdiSR* homologs *tutC1B1* are separated from the *bssDCAB* homologs, *tutE* and *tutFDG*, by genes encoding another sensor kinase/response regulator pair, called *tutCB* (21). *tutCB* is transcribed divergently from both *tutC1B1* and *tutE*. The gene products of *tutCB* more closely resemble the sensor kinases/response regulators believed to control aerobic toluene oxidation, including TodST, than the gene products of *tdiSR* (21). Since *T. aromatica* strain T1 is capable of both aerobic and anaerobic toluene oxidation, Leuthner et al. have proposed that TutC1B1 may be responsible for control of anaerobic toluene oxidation, while TutCB controls aerobic toluene oxidation (21). Notably, *tutCB* homologs are not observed in the vicinity of the *bss* operon in *T. aromatica* strain K172. Instead, *tdiSR* and *bssDCAB* are transcribed in the same direction and are not separated by any additional open reading frames (21).

The transcriptional organization of the *bss*/*tut* genes also appears to be different in *T. aromatica* strains K172 and T1. In *T. aromatica* strain K172, Northern blot studies of toluene-grown cells showed that *bssDCAB* are cotranscribed. No *bssDCAB* mRNA was observed when the cells were grown on benzoate (22). In contrast, in *T. aromatica* strain T1, Northern blot and primer extension studies suggested that the *bssD* homolog *tutE* is transcribed independently from the *bssCAB* homologs *tutFDG. tutFDG* are cotranscribed with *tutH*, a gene downstream of *tutG* whose predicted amino acid sequence is similar to that of the NorQ/NirQ family of proteins. No *tutFDGH* mRNA was observed when cells were grown on pyruvate (8). No sequence data downstream of *bssDCAB* have been reported for *T. aromatica* strain K172, and it is unknown if a *tutH* homolog exists in *T. aromatica* strain K172.

In contrast to *T. aromatica* strains K172 and T1, *Azoarcus* sp.

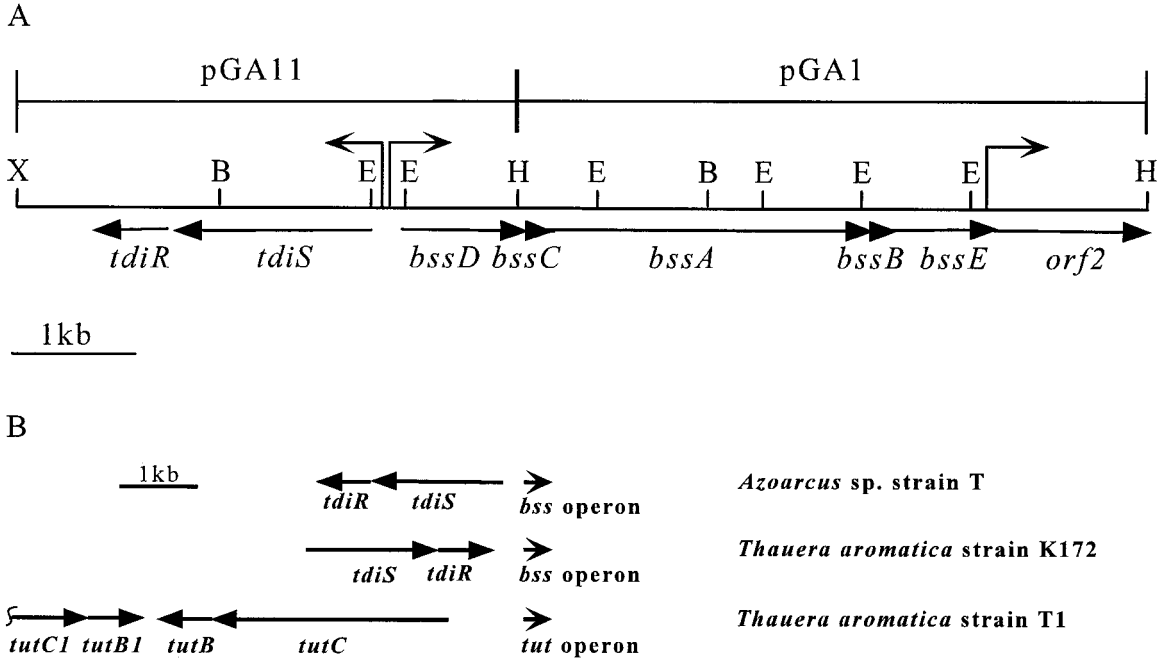

FIG. 2. (A) Restriction map of the *tdiSR*/*bssDCABE* chromosomal region of *Azoarcus* sp. strain T. The eight identified open reading frames are indicated with arrows below the map. Arrows above the map indicate the major transcriptional start sites identified. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. (B) Gene organization of *tut* and *tdi*/*bss* operons in *Azoarcus* sp. strain T, *T. aromatica* strain K172, and *T. aromatica* strain T1. Accession numbers are U57900 and AF036765 for *T. aromatica* T1 and AJ001848 for *T. aromatica* K172.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Azoarcus sp. strain T	Wild type	12
AST2	Δ*bssA*	This study
AST3	AST2 containing pGA14	This study
E. coli		
DH10B	F$^-$ *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL* λ$^-$	Bethesda Research Laboratories
TG1*recO* 1504::Tn*5*	K-12 *supE thi* Δ(*lac-proAB*) *hsd*Δ5 F′ [*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15]	Novagen
Plasmids		
pBluescript II KS	ColE1 ori, *lacZ*, Ap^r	Stratagene
pLAFR5-SK1	oriV, cos, Tet^r	15
pBJ113	pUC118 containing Km^r and *galK*; plasmid used for generating gene replacements, derived from pKG2	14, 32
pRK415	oriV, oriT, Tet^r	15
p810F	25-kb *Sau*3A fragment of partially digested chromosomal DNA of *Azoarcus* sp. strain T in pLAFR5-SK1	This study
pGA1	5.3-kb *Hin*dIII fragment of 25-kb insert of p810F in pBluescript-II KS	This study
pGA6	2.6-kb PCR deletion of pGA1 in pBJ113; Δ*bssA*	This study
pGA11	4.2-kb *Xba*I/*Hin*dIII fragment of 25-kb insert of p810F in pBluescript-II KS	This study
pGA14	5.3-kb insert of pGA1 in pRK415	This study

strain T is able to mineralize both toluene and *m*-xylene anaerobically. Furthermore, the specific activity of purified benzylsuccinate synthase from *Azoarcus* sp. strain T is relatively high compared to the enzyme purified from *T. aromatica* strain K172 (5). As a result, several studies of the benzylsuccinate synthase from *Azoarcus* sp. strain T have led to insights into this enzyme's reaction mechanism, including H atom retention in the benzylsuccinate product (2), stereoselectivity and substrate specificity of the benzylsuccinate synthase reaction (4, 5), and demonstration of a glycyl radical signal (17). However, the genes encoding benzylsuccinate synthase, their genetic organization, regulation, and function in anaerobic toluene and *m*-xylene mineralization have not been studied in *Azoarcus* sp. strain T.

In this study, we report the cloning and sequencing of the *bssDCAB* and *tdiSR* homologs from *Azoarcus* sp. strain T. Operon structures were determined by reverse transcription-PCR (RT-PCR) and primer extension studies. Transcriptional start sites were used to determine putative promoter regions. Analysis of the growth phenotype of a Δ*bssA* mutant demonstrated that BssA is essential for both toluene and *m*-xylene mineralization in *Azoarcus* sp. strain T.

MATERIALS AND METHODS

Growth conditions. The bacterial strains, plasmids, and phages used in this study are described in Table 1. *Azoarcus* sp. strain T (DSM 9506) was grown at 30°C aerobically with benzoate (12) or at room temperature under denitrifying conditions with benzoate, toluene, or *m*-xylene, as described before (16). *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium. Growth was monitored as absorbance at 600 nm. Antibiotics were used at the following concentrations: *E. coli*, oxytetracycline, 25 μg/ml; carbenicillin, 100 μg/ml; and kanamycin, 50 μg/ml; *Azoarcus* sp. strain T, kanamycin, 50 μg/ml; and oxytetracycline, 10 μg/ml.

Cloning and DNA manipulations. Standard protocols were used for cloning and transformations of *E. coli* (29). The DNA packaging kit from Boehringer-Mannheim, Indianapolis, Ind., was used to prepare the cosmid library. Plasmids were introduced into *Azoarcus* sp. strain T by electroporation. Briefly, cells of *Azoarcus* sp. strain T were grown aerobically to an optical density at 600 nm (OD_{600}) of ≈0.3, centrifuged at 4°C, washed twice with cold distilled water, and then resuspended to an OD_{600} of >200. Cells were mixed with DNA in a cold cuvette and exposed to 2 μF, 1.5 kV, and 200 Ω. Time constants were about 4.5 ms. Cells were outgrown at 30°C for 10 h in aerobic growth medium (12) augmented with 0.2% yeast extract and 0.5% Casamino Acids (see reference 11) before plating on selective medium.

Construction of a DNA library. Chromosomal DNA from *Azoarcus* sp. strain T was partially digested with *Sau*3A and size fractionated by ultracentrifugation in a linear 10 to 40% sucrose gradient. Fractions containing DNA fragments in the size range of 18 to 28 kb were pooled and dephosphorylated. DNA fragments were then ligated to pLAFR5-SK1 digested with *Bam*HI. The ligated DNA was packaged into λ bacteriophage with the DNA packaging kit obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The phage particles were transduced into *E. coli* DH10B, and colonies were grown on LB agar containing oxytetracycline (25 μg/ml). Microtiter wells were filled with LB and inoculated with single colonies, and after an overnight incubation, they were augmented with 20% glycerol and stored at −80°C.

Plasmid constructions. p810F is a cosmid clone containing an approximately 25-kb insert of *Azoarcus* sp. strain T chromosomal DNA, of which 9.5 kb was sequenced in this study. Subclones of this cosmid were constructed in pBluescript II KS+. pGA1 contains a 5.3-kb *Hin*dIII insert encoding the 3′ end of *bssD*, *bssCABE*, and the 5′ end of *orf2*. pGA11 contains a 4.2-kb *Hin*dIII/*Xba*I fragment encoding *tdiSR* and the 5′ end of *bssD*. pGA5 is a deletion subclone of pGA1 (see below). pGA6 contains the insert of pGA5 in pBJ113. pGA14 contains the insert of pGA1 in pRK415 (15).

DNA sequencing and sequence analysis. Plasmid DNA for sequencing was purified using Qiagen Plasmid Kits (Qiagen Inc., Chatsworth, Calif.). The insert of pGA1 was sequenced by Bio 101, La Jolla, Calif. All other DNA sequencing was performed by the Stanford University Protein and Nucleic Acid (PAN) facility using Big Dye terminator cycle sequencing (Perkin Elmer, Foster City, Calif.). DNA sequences were analyzed with the University of Wisconsin Genetics Computer Group software package, version 10.0. Similar sequences were identified using the Blast network service at the National Center for Biotechnology Information (NCBI; version 2.1.2, 13 November 2000) (Bethesda, Md.).

Construction of a *bssA* null mutant. An in-frame deletion of *bssA* was constructed by the method of Link et al. (23). PCR deletion products were constructed in two steps. In the first step, two different PCRs generated fragments to the left and right of the *bssA* sequence targeted for deletion. In the second step, the left and right fragments were annealed at the overlapping region included in the internal primers and amplified by PCR as a single fragment using external primers. Internal primers were bssNi, 5′CCCATCCACTAAACTTAA ACACARCAYTTNCCYTTRTARTC3′, and bssCi, 5′TGTTTAAGTTTAGTG GATGGGAAYACNAUHAUHGCNCG3′ (H is A, C, or T; N is A, C, G, or T; R is A or G; and Y is C or T). Universal primers were used as external primers. The internal primers were designed so that only 116 bp of the 2.6-kb *bssA* gene remain in the final Δ*bssA* mutant. The final PCR product was digested with *Hin*dIII and cloned into pBluescript KS+ to form pGA5. The disrupted region was sequenced to confirm the in-frame deletion and then cloned into pBJ113. pBJ113 contains a positive-negative KG (Km^r/*galK*) cassette for creating the two-step integration-excision events during gene replacement (32).

pGA6 was introduced into *Azoarcus* sp. strain T by electroporation, and cells were plated on kanamycin selective plates. One of the Km^r colonies determined

by Southern blot and PCR analysis to contain both *bssA* and Δ*bssA* was grown in nonselective liquid medium and plated on 1% galactose basal medium. Several segregants were isolated. PCR and Southern blot analyses of these segregant strains were used to distinguish between the wild-type and deletion allele by testing for the presence of Δ*bssA* and the absence of *bssA*. One Δ*bssA* strain was selected for further studies and designated AST2. The Δ*bssA* mutant was complemented with pGA14, which was introduced by electroporation.

RT-PCR. Total RNA was prepared from cells in mid-log phase by the hot phenol method of von Gabain et al. (33). DNA was removed from the RNA by two treatments with RNase-free DNase (Boehringer-Mannheim, Indianapolis, Ind.). RT-PCRs were performed using the Access RT-PCR kit from Promega Corp. (Madison, Wis.). Primers were chosen to produce fragments less than 500 bp in length. Negative control reactions in which reverse transcriptase was omitted from the reaction mixture ensured that DNA products resulted from amplification of cDNA rather than of chromosomal DNA contamination.

Primer extension of mRNA transcripts. The avian myeloblastosis virus reverse transcriptase primer extension system was used to determine the transcriptional start sites of *tdiSR*, *bssDCABE*, and *orf2* (Promega Corp., Madison, Wis.). Primer extension products were resolved on a 6% polyacrylamide gel containing 7 M urea next to a DNA sequence generated with the same primer (*fmol* DNA sequencing system; Promega Corp., Madison, Wis.). The oligonucleotides used in these studies included bssDpe (5′TGAACCTCTGTATTTCGGTAACAACA 3′), orf2pe2 (5′ACCTTAGGCGGCAATGTACTGAACGT3′), tdiSpe (5′TCC ACCGCGACCACGTCATTCTTCAT3′), tdiRpe (5′TCATCGACGACGAAC ACGGTCGGCGA3′), orf1pe (5′ACAAGCCATTTGTGCTAGGAGAGGT 3′), orf1pe2 (5′ATATCTTGGCGAATTTATCGAGAAGCT3′), orf1pe3 (5′AT GATCTATCGTCAACGCGGT3′), bssbpe (5′CCGCTTCCATGTTATGG3′), and bssCpe (5′CCAAAGGTATCTACTCAG3′).

Northern blotting. Hybridization was performed in RapidHyb buffer at 70°C according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, N.J.). An RNA probe for the 3′ end of *bssD*, approximately 800 bp in size and called bssD3, was produced using the Riboprobe T3 system from Promega Corp. (Madison, Wis.). pGA10 digested with *Sma*I was the template.

Nucleotide sequence accession number. All sequence data have been assigned GenBank accession number AY032676.

RESULTS AND DISCUSSION

Cloning of benzylsuccinate synthase and neighboring genes. In a genetic approach to identifying genes involved in anaerobic toluene utilization in *T. aromatica* strain T1, several mutants defective in anaerobic toluene mineralization and benzylsuccinate formation were identified (P. W. Coschigano, Abstr. 97th Annu. Meet. Am. Soc. Microbiol. 1997, p. 493). One of the mutations mapped to a gene, *tutD*, whose predicted amino acid sequence showed homology to pyruvate formate-lyase. Based on two regions of amino acid identity in TutD and pyruvate formate-lyase, GNDDD (Gly^{568} in *E. coli* pyruvate formate-lyase) and RVSGY (Gly^{734} in *E. coli* pyruvate formate-lyase), we designed degenerate primers, and a 162-bp fragment, called bss1, was amplified from *Azoarcus* sp. strain T chromosomal DNA.

The bss1 fragment was cloned and sequenced. The predicted amino acid sequence of bss1 showed homology to both pyruvate formate-lyase and TutD (data not shown). Using the bss1 fragment to probe a colony blot containing approximately 1,050 clones of a cosmid library of chromosomal DNA from *Azoarcus* sp. strain T identified a single hybridizing cosmid clone, p810F. A 5.3-kb *Hin*dIII fragment and a 4.2-kb *Xba*I/*Hin*dIII fragment from p810F were cloned into pBluescript KS+ and sequenced. Eight open reading frames were identified and, based on similarity to genes identified in *T. aromatica* strain K172 (22), were named *tdiR*, *tdiS*, *bssD*, *bssC*, *bssA*, *bssB*, *bssE*, and *orf2* (Fig. 2A).

The open reading frames designated *bssC*, *bssA*, and *bssB* from *Azoarcus* sp. strain T, encoding the γ, α, and β subunits of benzylsuccinate synthase, respectively, have predicted translational start codons at $A^{4344}TG$, $A^{4550}TG$, and $A^{7221}TG$, respectively. The start codons of *bssC*, *bssA*, and *bssB* are preceded by likely ribosome-binding sites, $A^{4331}GGAG$, $A^{4539}GGAG$, and $T^{7209}GGAG$, respectively. *bssC*, *bssA*, and *bssB* have predicted stop codons at $T^{4524}AA$, $T^{7142}GA$, and $T^{7449}GA$, respectively. The encoded proteins BssA, BssB, and BssC have calculated molecular masses of 97,483 Da, 8,808 Da, and 6,964 Da, respectively. These masses are similar to published data on the benzylsuccinate synthase subunits from *Azoarcus* sp. strain T and *T. aromatica* K172 (5, 22). The predicted translation products of *bssC*, *bssA*, and *bssB* show about 90% identity to TutF, TutD, and TutG, respectively, from *T. aromatica* strain T1 and between 60 and 80% identity to BssC, BssA, and BssB, respectively, from *T. aromatica* strain K172. Sequence analysis using Terminator (University of Wisconsin Genetics Computer Group software package, version 10.0) shows a potential weak rho-independent terminator 22 bp downstream of the predicted translational stop site for *bssB*.

The *bssD* gene, predicted to encode the activating enzyme for benzylsuccinate synthase, begins at $G^{3327}TG$ and ends at $T^{4323}AA$. BssD has a predicted molecular mass of 37,764 Da and shows approximately 70% identity to BssD and TutE from *T. aromatica* strains K172 and T1, respectively. The motif CX_3CX_2C, proposed to coordinate a [4Fe-4S] cluster at the N termini of pyruvate formate-lyase-activating enzymes (19) and anaerobic ribonucleotide reductase activating-enzymes (31), is present, with sequence CPLRCPWC, at the N termini of all three benzylsuccinate synthase-activating proteins, beginning at Cys^{29} in *Azoarcus* sp. strain T. These proteins also contain two additional cysteine clusters of the form $CX_2CX_2CX_3C$, C^{55}VGCGRCMAVC, and C^{89}QRCMRCVAAC in the predicted amino acid sequence of BssD in *Azoarcus* sp. strain T, a motif conserved in ferredoxins with two [4Fe-4S] clusters (34). This ferredoxin motif is not found in either the pyruvate formate-lyase activating enzyme or the anaerobic ribonucleotide reductase activating enzyme.

The *bssE* gene of *Azoarcus* sp. T is predicted to start at $A^{7499}TG$ and end at $T^{8357}AA$. BssE has a predicted molecular mass of 31,818 Da and is 97% identical to the gene product of *tutH* from *T. aromatica* strain T1. Currently, the function of TutH is unknown, although it shows homology to the NorQ/NirQ family of proteins. No obvious ribosome-binding site is observed upstream of the predicted translational start of *bssE*. *orf2* is predicted to start at $A^{8368}TG$. There is a potential ribosome-binding site before the translational start site of *orf2*, $T^{8357}AAGG$. No translational stop for *orf2* was identified in the present nucleotide sequence, but the predicted gene product would be larger than 59,578 Da. An NCBI Blast search in the nonredundant GenBank, PDB, SwissProt, PIR, and PRF databases revealed no significant similarity between the deduced incomplete amino acid sequence of *orf2* and any other known protein.

The predicted gene products of *tdiSR* show homology to sensor kinase and response regulator proteins of bacterial two-component regulatory systems. *tdiS* and *tdiR* are predicted to start at $A^{3021}TG$ and $A^{1314}TG$ and end at $T^{1386}AG$ and $T^{660}GA$, respectively. TdiS and TdiR have predicted molecular masses of 61,694 Da and 24,220 Da, respectively, and show approximately 95% identity to TdiS and TdiR of *T. aromatica*

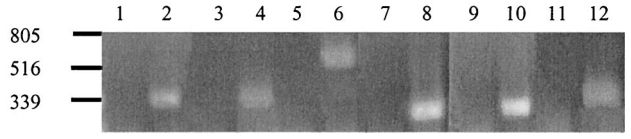

FIG. 3. Agarose gel electrophoresis of RT-PCR products. RT-PCR products observed using primers to amplify intergenic regions between (lanes 1 and 2) *tdiS* and *tdiR*, (3 and 4) *bssD* and *bssC*, (5 and 6) *bssC* and *bssA*, (7 and 8) *bssA* and *bssB*, (9 and 10) *bssB* and *bssE*, and (11 and 12) *bssE* and *orf2*. Odd-numbered lanes are controls without reverse transcriptase. Numbers on the left represent sizes of markers (in base pairs).

strain K172 and about 80% identity to TutC1 and TutB1 from *T. aromatica* strain T1, respectively. The *tdiSR* genes are oriented in the direction opposite that of the *bssDCABE* and *orf2* genes. The predicted ATG start codon of *tdiS* is 305 bp upstream of the predicted GTG start codon of *bssD*. *tdiS* and *tdiR* are preceded by excellent ribosome-binding sites, A^{3036}GGAGG and A^{1325}GGAGG, respectively. There is a potential strong rho-independent terminator 22 bp downstream of the predicted translational stop site of *tdiR*.

Since benzylsuccinate synthase is a glycyl radical-containing enzyme which is irreversibly inactivated in the presence of molecular oxygen, we considered that its expression might be controlled by regulatory elements similar to those that control expression of pyruvate formate-lyase. Expression of *pfl* is controlled by several regulators, including FNR (fumarate-nitrate reduction regulator), ArcA, NarL, and IHF (integration host factor) in response to oxygen, nitrate, and pyruvate (see review in reference 30). We searched the sequenced region for consensus binding sites for these regulatory molecules, but no perfect matches were found. Further studies will be necessary to determine if any of these regulatory proteins play a role in the control of expression of the *bss* operon.

The organization of the *bss* and *tdi* genes in *Azoarcus* sp. strain T is quite different from the organization of homologous genes in *Thauera aromatica* strains. In *T. aromatica* strains K172 and T1, *tdiSR* and *tutB1C1* are transcribed in the same direction as *bssDCAB* and *tutEFGH*, respectively (21) (Fig. 2B). In contrast, in *Azoarcus* sp. strain T, a putative sensor kinase/response regulator system, closely related to *tdiSR* and *tutB1C1*, is encoded upstream of the *bssDCABE* genes, but is transcribed divergently to *bssDCABE*.

Transcriptional organization of *tdiSR* and *bssDCABE* operons. RT-PCR experiments using total RNA harvested from toluene-grown cells of *Azoarcus* sp. strain T were used to determine which open reading frames are cotranscribed. Amplification products were obtained using primers complementary to neighboring open reading frames to amplify the intergenic regions between *tdiS* and *tdiR* (expected size, 320 bp), *bssD* and *bssC* (expected size, 340 bp), *bssC* and *bssA* (expected size, 553 bp), *bssA* and *bssB* (expected size, 240 bp), *bssB* and *bssE* (expected size, 265 bp), and *bssE* and *orf2* (expected size, 310 bp). Controls without reverse transcriptase were negative, indicating the absence of chromosomal DNA (Fig. 3). The RT-PCR fragments obtained were of the expected sizes. The presence of these RT-PCR products suggests that the *tdiS* and *tdiR* genes, as well as the *bssDCABE* genes, are each cotranscribed as an operon.

Primer extension studies were performed to determine the transcriptional start sites of the two operons. The transcriptional start site of *bssDCABE* was mapped to 98 bp upstream of the putative GTG intiation codon of *bssD* at a cytosine base (Fig. 4). The *bssDCABE* promoter region contains elements of a likely σ^{70} promoter, including two sequences, TTAAAT and TAAATT, which lie 5 and 6 bp upstream of the +1 transcriptional start site, respectively, and match the *E. coli* σ^{70} −10 consensus sequence (TATAAT) at four of the six positions. A sequence (TGGTCA) starting at −29 matches the *E. coli* σ^{70} −35 consensus sequence (TTGACA) at four of the six positions. The same transcriptional start site was observed when total RNA from *m*-xylene-grown cells was used. No primer extension product was observed when total RNA from benzoate-grown cells was used as the template for reverse transcription reactions. Therefore, *bssDCABE* is probably controlled in the same way when *Azoarcus* sp. strain T is growing in the presence of either toluene or *m*-xylene. No extension product was observed when primers (names in parentheses) were used from *bssC* (bssCpe), *bssB* (bssBpe), or *bssE* (orf1pe, orf1pe2, and orf1pe3).

The transcriptional start site of the *tdiSR* operon was mapped 95 bp upstream of the proposed ATG initiation codon of *tdiS* at a cytosine base (Fig. 4). The *tdiSR* promoter region contained elements of a σ^{70} promoter, including a sequence (TAACAC) 8 bp upstream of the +1 transcriptional start site that matched the *E. coli* σ^{70} −10 consensus sequence (TATAAT) at three of the six positions. However, there is no

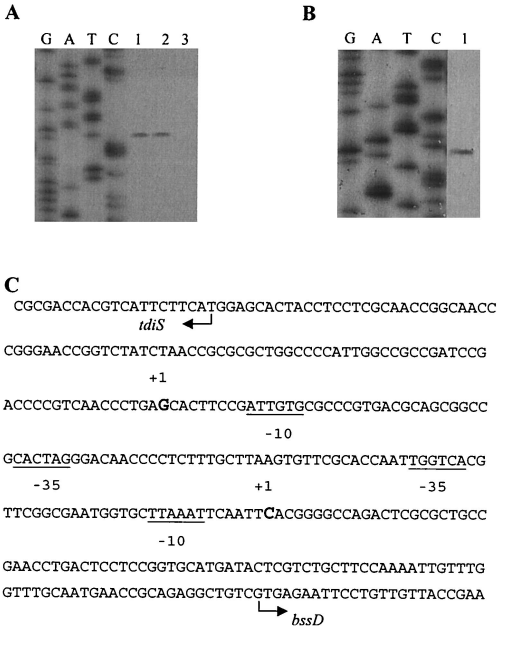

FIG. 4. Mapping of transcriptional start sites of *bssDCABE* and *tdiSR* by primer extension. (A) Sequencing and primer extension reactions with bssDpe primer. (B) Sequencing and primer extension reactions with tdiSpe primer. RNA was isolated from toluene-grown (lane 1), *m*-xylene-grown (lane 2), and benzoate-grown (lane 3) cells. (C) Nucleotide sequence of the *tdiSR*/*bssDCABE* promoter regions. Transcription start sites are indicated by +1, and putative −10 and −35 regions are underlined. Note that only one of the potential −10 regions of the *bssDCABE* promoter is underlined.

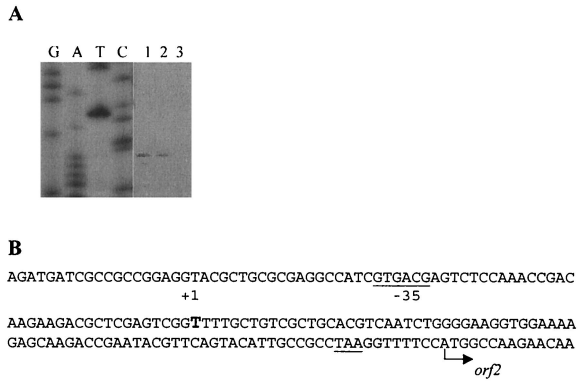

FIG. 5. Mapping of transcriptional start site of *orf2* by primer extension. (A) RNA was isolated from toluene-grown cells (lane 1), *m*-xylene-grown cells (lane 2), and benzoate-grown cells (lane 3). A sequence ladder generated with the same primer is shown. (B) Nucleotide sequence of the *orf2* promoter region, showing the start site of *orf2* transcription (+1) and a potential −35 region. The stop codon for *bssE* is underlined.

obvious −35 sequence. No extension product was observed when a primer (tdiRpe) from within the *tdiR* gene was used.

Although RT-PCR experiments are consistent with *orf2* being cotranscribed with *bssDCABE*, a transcriptional start site of *orf2* was mapped 84 bp upstream of the putative ATG initiation codon of *orf2* at a thymine residue (Fig. 5). Further inspection of this region revealed that the putative *orf2* promoter region contains a sequence (AAGAAG) 13 bp upstream of the +1 transcriptional start site that matches the *E. coli* σ^{70} −10 consensus sequence (TATAAT) at three of the six positions. A sequence (GTGACG) starting at −34 matches the *E. coli* σ^{70} −35 consensus sequence (TTGACA) at four of the six positions. While there is some sequence similarity, the spacing of these potential promoter sequences is not ideal. Typically, the canonical −10 sequence begins 5 to 9 bp before the transcriptional start, and the −35 sequence begins 17 bp after the −10 region. The same transcriptional start site was observed when total RNA from *m*-xylene-grown cells was used. No primer extension product was observed when total RNA from benzoate-grown cells was used.

The discovery of a primer extension product with *orf2pe* suggests that *orf2* may be transcribed independently of *bssDCABE*, while RT-PCR results suggest that *orf2*, or its 5′ region, is cotranscribed with *bssDCABE*. In the former case, the RT-PCR result can be explained by a transcriptional readthrough of the *bssDCABE* transcript and termination of this transcript more than 202 bp downstream of the *bssE* stop codon. The RT-PCR product could not arise from the independent *orf2* transcript because the PCR primer used to amplify the reverse-transcribed intergenic region between *bssE* and *orf2* is complementary to the DNA sequence upstream of the start of the *orf2* transcript. No factor-independent termination sites were found in this area.

Northern blot analysis using total RNA obtained from toluene- and *m*-xylene-grown cells of *Azoarcus* sp. strain T were conducted (data not shown). A 5.2-kb band was observed when total RNA from cells grown on toluene or *m*-xylene was probed with an RNA probe, bssD3, derived from the 3′ end of *bssD*. A minimum mRNA length of 5.1 kb is necessary to accommodate the *bssDCABE* genes. No hybridization was observed with RNA harvested from benzoate-grown cells, indicating that the presence of toluene or *m*-xylene is necessary to induce transcription of the *bssDCABE* operon under anaerobic conditions.

The data presented suggest that the *bssDCABE* genes are cotranscribed in *Azoarcus* sp. strain T. The downstream *orf2* gene is transcribed independently of the *bssDCABE* genes, although it is also transcribed in the presence of toluene and *m*-xylene, but not in the presence of benzoate. In *T. aromatica* strain K172, *bssC* and *bssB* were reported to be located on a transcript of 4.6 kb, just 0.1 kb longer than the minimum size necessary to accommodate the *bssDCAB* genes (22). No sequence data downstream of *bssDCAB* have been reported for *T. aromatica* strain K172. In *T. aromatica* strain T1, *tutE*, a *bssD* homolog, is transcribed as a single gene, while *tutFDGH* are transcribed as an operon. In contrast, in *Azoarcus* sp. strain T, *bssD* and *bssE* are cotranscribed on the *bssDCABE* operon.

Phenotype of a Δ*bssA* mutant. *Azoarcus* sp. strain T is able to grow anaerobically with either toluene or *m*-xylene as the sole carbon source and electron donor. Biochemical studies have established that the conversion of toluene and *m*-xylene to benzoyl-CoA and 3-methylbenzoyl-CoA, respectively, occurs by similar pathways (16). Notably, the addition of both toluene and *m*-xylene to fumarate has been observed at almost equal rates in studies with permeabilized cells of *Azoarcus* sp. strain T, regardless of whether the cells were grown on toluene or *m*-xylene (16). Furthermore, only a single copy of a *bssA*-like gene was observed in Southern blot studies of the chromosomal DNA of *Azoarcus* sp. strain T (data not shown). These observations suggested that the benzylsuccinate synthase and 3-methylbenzylsuccinate synthase reactions may be catalyzed by the same enzyme in vivo. In order to test this hypothesis, we constructed a Δ*bssA* mutant and analyzed its growth phenotype.

We constructed an in-frame deletion in the chromosomal copy of *bssA* in *Azoarcus* sp. strain T, generating strain AST2, as described in Materials and Methods. In AST2, 96% of the original 2.6-kb *bssA* gene was removed. AST2 was tested for anaerobic growth with toluene and *m*-xylene and found to be defective in growth on either methylbenzene (Fig. 6), while growth on benzoate was unaffected. This phenotype demonstrates that *bssA* is required for growth on both toluene and *m*-xylene. In Northern blot studies, total RNA harvested from AST2 cells grown with benzoate in the presence of toluene was probed with bssD3, a probe derived from *bssD*. A hybridizing band approximately 2.2 kb in size was observed, indicating that transcription of the *bssDCABE* operon can be induced by toluene in the presence of benzoate (data not shown). The observed size difference from the previously observed band is consistent with a 2.5-kb deletion of the 5.2-kb wild-type *bssDCABE* mRNA.

The mutant phenotype of AST2 could be rescued by introduction of the *bssA* gene in *trans*. A 5.6-kb *Hin*dIII fragment containing *bssA*, which was the insert of pGA1, was cloned into pRK415 to create pGA14. pGA14 was introduced into AST2 by electroporation to form AST3. AST3 cells were able to grow on both toluene and *m*-xylene, although the growth rates did not reach wild-type levels (Fig. 6). The slower growth on toluene and *m*-xylene may be caused by lower expression levels of

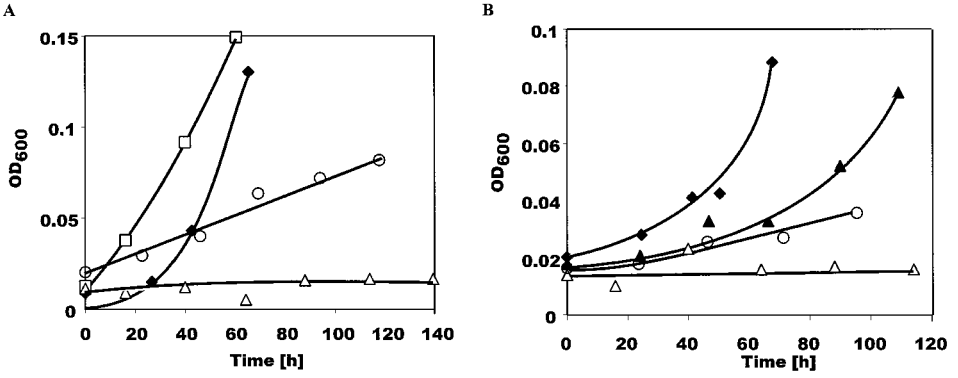

FIG. 6. Role of *bssA* in anaerobic growth on (A) toluene and (B) *m*-xylene in *Azoarcus* sp. strain T. Growth of the wild-type strain on toluene or *m*-xylene (solid diamonds); AST2 (Δ*bssA*) on benzoate (open squares) and toluene or *m*-xylene (open triangles); and AST3 (AST2 + pGA14) on benzoate (solid triangles) and toluene or *m*-xylene (open circles). Note that wild-type *Azoarcus* sp. strain T has been observed to grow with a range of doubling times: 16 to 25 h on benzoate, 16 to 27 h on *m*-xylene, and 14 to 20 h on toluene (C. J. Krieger, unpublished data).

bssA from the pRK415 *lac* promoter. The reduced growth rate observed on benzoate may be a result of the metabolic stress of tetracycline resistance, an effect that has been observed in *E. coli* (20, 26).

In summary, the transcriptional organization of the region encoding the genes for benzylsuccinate synthase in *Azoarcus* sp. strain T was determined. The genes *bssDCABE* and *tdiSR* form operons, for which transcriptional start sites were identified. The promoter regions of both of these operons show similarity to the *E. coli* σ^{70} consensus sequence. Primer extension studies and Northern blots show that the expression pattern of the *bssDCABE* operon is the same under toluene and *m*-xylene growth conditions. The growth phenotype of a Δ*bssA* mutant, AST2, provided the first genetic evidence in *Azoarcus* sp. strain T that benzylsuccinate synthase is required for growth on both toluene and *m*-xylene, but not on benzoate.

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