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 anaerobic photosynthetic 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, methylnaphthols, 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 α3β2γ2 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 glycol radicals, suggested that benzylsuccinate synthase may be a glycol radical enzyme. In addition, recent electron paramagnetic resonance studies have shown the presence of a glycol radical in samples of active benzylsuccinate synthase purified from *Azoarcus* sp. strain T, demonstrating experimentally that benzylsuccinate synthase is a glycol 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 *tutCIB1* (9, 10, 21). Based on N-terminal amino acid sequences of benzylsuccinate synthase,
purified from \textit{T. aromatica} strain K172, \textit{bssDCAB} and \textit{tdiSR} were cloned and sequenced in this microorganism (22). The \textit{bssCAB} genes show similarity to the \textit{tutFDG} genes, which encode the /H9253, /H9251, and /H9252 subunits of benzylsuccinate synthase, respectively. The predicted amino acid sequence of \textit{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 \textit{bssD} and \textit{tutE} show homology to these activases and have been proposed to perform a similar function. \textit{tutCB}, \textit{tutC1B1}, and \textit{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 \textit{T. aromatica} strains K172 and T1 are almost identical, several differences exist in the organization of the genes in the \textit{bss/tut} regions in these two strains (Fig. 2B). In \textit{T. aromatica} strain T1, the \textit{tdiSR} homologs \textit{tutC1B1} are separated from the \textit{bssDCAB} homologs, \textit{tutE} and \textit{tutFDG}, by genes encoding another sensor kinase/response regulator pair, called \textit{tutCB} (21). \textit{tutCB} is transcribed divergently from both \textit{tutC1B1} and \textit{tutE}. The gene products of \textit{tutCB} more closely resemble the sensor kinases/response regulators believed to control aerobic toluene oxidation, including TodST, than the gene products of \textit{tdiSR} (21). Since \textit{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, \textit{tutCB} homologs are not observed in the vicinity of the \textit{bss} operon in \textit{T. aromatica} strain K172. Instead, \textit{tdiSR} and \textit{bssDCAB} are transcribed in the same direction and are not separated by any additional open reading frames (21).

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

In contrast to \textit{T. aromatica} strains K172 and T1, \textit{Azoarcus} sp.
strain T is able to mineralize both toluene and m-xylene anaerobically. Furthermore, the specific activity of purified benzylsuccinate synthase from \textit{Azoarcus} sp. strain T is relatively high compared to the enzyme purified from \textit{T. aromatica} strain K172 (5). As a result, several studies of the benzylsuccinate synthase from \textit{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 glycol 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 \textit{Azoarcus} sp. strain T.

In this study, we report the cloning and sequencing of the \textit{bssDCAB} and \textit{tdiSR} homologs from \textit{Azoarcus} sp. strain T. Operon structure determinations were performed 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 \textit{\Delta bssA} mutant demonstrated that BssA is essential for both toluene and m-xylene mineralization in \textit{Azoarcus} sp. strain T.

**MATERIALS AND METHODS**

**Growth conditions.** The bacterial strains, plasmids, and phages used in this study are described in Table 1. \textit{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). \textit{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: \textit{E. coli}, oxytetracycline, 25 µg/ml; carbenicillin, 100 µg/ml; and kanamycin, 50 µg/ml; \textit{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 \textit{E. coli} (29). The DNA packaging kit from Boehringer-Mannheim, Indianapolis, Ind., was used to prepare the cosmid library. Plasmids were introduced into \textit{Azoarcus} sp. strain T by electroporation. Briefly, cells of \textit{Azoarcus} sp. strain T were grown aerobically to an optical density at 600 nm (\textit{OD}_{600}) of ~0.3, centrifuged at 4°C, washed twice with cold distilled water, and then resuspended to an \textit{OD}_{600} of >2.0. 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 \textit{Azoarcus} sp. strain T was partially digested with Sau3A 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-SKII digested with BamHI. The ligated DNA was packaged into \lambda bacteriophage with the DNA packaging kit obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The phage particles were transduced into \textit{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 \textit{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 HindIII fragment of p810F in pBluescript II KS. pGA6 is a deletion subclone of pGA1 containing the 3' end of \textit{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 \textit{bssA} null mutant.** An in-frame deletion of \textit{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 \textit{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' CCAATCCACTAAAATTTAATA 3', and bssCi, 5' TGTTTAAGTTTAGTG CCCCCCAT 3'. The PCR product was sequenced to confirm the in-frame deletion and then cloned into pBl113. The internal primers were designed so that only 116 bp of the 2.6-kb \textit{bssA} gene remain in the final \textit{\Delta bssA} mutant. The final PCR product was digested with HindIII and cloned into pBluescript KS+ to form pGA5. The disrupted region was sequenced to confirm the in-frame deletion and then cloned into pBl113. pBl113 contains a positive-negative KG (Km'/galK) cassette for creating the two-step integration-excision events during gene replacement (32). pGA6 was introduced into \textit{Azoarcus} sp. strain T by electroporation, and cells were plated on kanamycin selective plates. One of the Km' colonies determined
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 begins at Gly67 in *T. aromatica* from strain T and between 60 and 80% identity to *B. subtilis* (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* strain 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 T 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 G2623TG and ends at T4523AA. *BssD* has a predicted molecular mass of 37,764 Da and shows approximately 70% identity to *B. subtilis* and *T. terrae* strains K172 and T1, respectively. The motif C5X5C5X5C5, 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 Cys749 in *Azoarcus* sp. strain T. These proteins also contain two additional cysteine clusters of the form CX5C5X5C5, C5X5VTGCGCAC, and C5X5QRCRCVAA 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 A7499TG and end at T8357AA. *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 A8369TG. There is a potential ribosome-binding site before the translational start site of *orf2*, T8335AAGG. 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 A3021TG and A1311TG and end at T1366AG and T660GA, 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*...
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, A1325GGAGG and A1325GGAGG, respectively, and are transcribed in the direction opposite that of the genes. The predicted ATG start codon of bssE and orf2. Odd-numbered lanes are controls without reverse transcriptase. Numbers on the left represent sizes of markers (in base pairs).

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. Od numbered lanes are controls without reverse transcriptase. Numbers on the left represent sizes of markers.

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 initiation codon of bssD at a cytosine base (Fig. 4). The bssDCABE promoter region contains elements of a likely σ70 promoter, including two sequences, TTAATT and TAAATT, which lie 5 and 6 bp upstream of the +1 transcriptional start site, respectively, and match the *E. coli* σ70 −10 consensus sequence (TATAAT) at four of the six positions. A sequence (TGGTCA) starting at −29 matches the *E. coli* σ70 −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 benzene-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 σ70 promoter, including a sequence (TAACAC) 8 bp upstream of the +1 transcriptional start site that matched the *E. coli* σ70 −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 benzene-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.
obvious −35 sequence. No extension product was observed when a primer (tdiRp) 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 σ70 −10 consensus sequence (TATAAT) at three of the six positions. A sequence (GTGACG) starting at −34 matches the E. coli σ70 −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 benzene-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 /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 benzyl-CoA and 3-methylbenzoyl-CoA, respectively, occurs by similar pathways (16). Notably, the addition of both toluene and m-xylene to lumarate 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 /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 HindIII 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...
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 σ70 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 ΔbssA 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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