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# Identification and Expression of Benzylsuccinate Synthase Genes in a Toluene-Degrading Methanogenic Consortium[▿]

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**Benzylsuccinate synthase (BSS) initiates anaerobic toluene biodegradation, and BSS genes have been found in several nitrate- and iron-reducing organisms. Here, two new putative *bssA* genes were identified in a methanogenic toluene-degrading culture. Transcription was upregulated with toluene but not with benzoate, consistent with the proposed function. These are the first *bss* sequences from a methanogenic culture.**

Anaerobic toluene biodegradation can be initiated by benzylsuccinate synthase (BSS) (3, 4, 6, 7, 13, 15, 16, 21), and the genes encoding BSS and related enzymes (*bssDCABE*) have to date been sequenced from six denitrifying bacteria (1, 9, 12, 14, 15, 19) and one iron-reducing microorganism (13). No BSS gene sequences from any sulfate-reducing or methanogenic cultures have yet been published. This goal of this study was to identify BSS gene sequences in a highly enriched toluene-degrading methanogenic consortium. We have previously shown that benzylsuccinate is produced by this culture (4) but were unable to amplify BSS genes by the use of published primers (5).

**Identification of putative *bssA* sequences.** The toluene-degrading methanogenic enrichment culture used in this study (4, 10, 11) has been maintained for almost 15 years with toluene as the sole carbon source and electron donor. Genomic DNA was extracted from the culture by use of an UltraClean soil DNA extraction kit (Mo Bio Laboratories Inc., Solana Beach, CA), and PCR amplification of putative *bssA* sequences was performed using a variety of primer sets designed from alignments of known sequences and incorporating degeneracies (Table 1). A partial transcriptional map of the *bss* operon and of the target regions labeled TR1 to TR5, flanked by different forward and reverse primer sets, is shown in Fig. 1. PCR amplifications were performed using Redi*Taq* (New England BioLabs, Mississauga, ON), and conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min (for annealing temperatures, see Table 1), and chain extension for 1.5 min (1 min for primer set 5) at 72°C followed by a final extension step at 72°C for 10 min. All PCRs were carried out in a PTC-200 DNA Engine thermocycler (MJ Research Inc., Waltham, MA). For each primer set, three PCRs were carried out in parallel, and the products were pooled and run on a 1% agarose gel stained with ethidium bromide. Where only one band of the expected length was observed, the PCR products were purified using a QIAquick PCR cleanup kit (QIAGEN, Valencia, CA). Where more than one band was observed, the band of the expected length was excised from the gel and purified using a QIAGEN gel purification kit (QIAGEN, Valencia, CA). Amplicons were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and DNA from 10 to 15 clones was sequenced using vector primers T7f and M13r. To obtain complete sequences of putative *bssA* or *bssABE* clones, internal primers designed with Primer3 software (17) were used. Partial sequences of putative *bssA* or *bssABE* amplicons were aligned and assembled using ChromasPro 1.15 (Technelysium Pty Ltd., Australia), and GeneMark (8) was used to predict start and/or stop codons of the putative *bssA*, *bssB*, and *bssE* sequences identified.

One putative sequence was assembled from amplicons obtained with all of the primer sets listed in Table 1. This contiguous sequence (GenBank: EF134966) includes a putative *bssA* (referred to as *bssA*-1, encoding a protein with 747 amino acids), a putative *bssB* (85 amino acids) and a putative partial *bssE* sequence (113 amino acids). A second putative *bssA* sequence, referred to as *bssA*-2, was only found using primers for TR1 and could not be assembled with any other sequences. BLAST queries of translated *bssA*-1 and *bssA*-2 sequences against the NCBI non-redundant translated database using default parameters (2) revealed 78–84% similarity to the seven previously known BssA sequences, and the expectation value for each hit was 0.0. The translated sequences of *bssA*-1 and *bssA*-2 were 83% similar to each other. As with the previously known BssA sequences, the C-terminal region of BssA-1 was highly conserved, and a conserved glycine residue was present at a position corresponding to residue 825 in the *Thauera aromatica* strain K172 BssA sequence. The conserved glycine residue is characteristic of all glycyl radical enzymes (GREs) (13), and is a part of the glycyl radical fingerprint motif RVxG-(FWY)x6-8(FL)x4Qx2(IV)x2R that is found in most GREs (18) and in all BssA sequences known to date. In addition to the conserved glycine motif in BssA-1, both BssA-1 and BssA-2 contained a conserved cysteine residue at the position corresponding residue 489 of the *T. aromatica* K172 BssA sequence. This conserved residue is also characteristic of GREs (18).

**Differential transcription of *bssA*.** For this part of the study, 60-ml portions of the methanogenic culture were transferred anaerobically into each of six 125-ml glass bottles sealed with Mininert (VICI Precision Sampling, Baton Rouge, LA) caps.

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TABLE 1. Degenerate PCR primers designed to amplify putative *bssA* or *bssABE* sequences in the methanogenic toluene-degrading culture

| Primer set(s) (target region[s]) | Primer[a] | Sequence (5′–3′)[b] | Annealing temp (°C) |
|---|---|---|---|
| 1, 3 | BssA327f | CGAATTCATCNTCGGCTACC | 54 |
| 1 | BssA2004r | GTCGTCRTTGCCCCAYTTNGG | 54 |
| 2 | MBssA1516f[c] | AGACCCAGAAGACCAGGTC | 54 |
| 3 | MBssA2446r[c] | ATGCTTTTCAGGCTCCCTCT | 54 |
| 2, 5 | BssA2524r | ATGATSGTGTTYTGSCCRTAGGT | 54, 46[d] |
| 4, 5 | BssA1985f | CNAARTGGGGCAAYGACGA | 49, 46[d] |
| 4 | BssE347r | TGYTCNGGNCGRTTGATCTCYTC | 49 |

[a] Sequence positions indicated for primers refer to the nucleotide position numbers in the *Azoarcus* sp. strain T *bssA* and *bssE* sequences.
[b] Y = C/T, N = A/C/T/G, R = A/G, S = C/G.
[c] Specific primers designed based on an initial cloning study of the methanogenic consortium.
[d] The annealing temperature used for primer set 5 was 46°C. An annealing temperature of 52°C also amplified DNA of the expected length.

TABLE 2. PCR primers used for amplifying specific genes in the toluene-degrading consortium

| Primer[a] | Sequence (5′–3′) | Target gene |
|---|---|---|
| Eub1-621f | TGAAACCATTGAGCTTGAGG | Eub-1 (16S rRNA gene) |
| Eub1-912r | CCCGTCAATTCCTTTGAGTT | Eub-1 (16S rRNA gene) |
| bssA1-416f | CAGAACACAAAGTATGCCC | *bssA*-1 |
| bssA1-749r | TGGAGTTCCAGAGGTCGATT | *bssA*-1 |
| bssA2-413f | TACCTGCAGAGCAAGTACGC | *bssA*-2 |
| bssA2-713r | ACTTCAGCTTATCGGCGTTC | *bssA*-2 |

[a] The annealing temperature for all primer sets was 59°C. Estimated sequence positions are listed in primer names.

Three bottles were amended with toluene (42 μmol/bottle), while the other three were amended with benzoate (49 μmol/bottle), a downstream metabolite in anaerobic toluene degradation. Toluene and methane concentrations were analyzed as described previously (11). RNA was extracted from all bottles when 50% of the toluene or benzoate was degraded, and all bottles contained approximately the same concentration of methane. Total RNA was extracted and purified from 45 ml of culture as previously described (20) except that culture samples were centrifuged at 15,000 × *g* and 4°C for 30 min. Reverse transcription was performed using random hexamers and Superscript III reverse transcriptase (RT) (Invitrogen, Carlsbad, CA). Each reaction mixture (25 μl total volume) contained 5 μl of 5× First Strand buffer, 250 ng random hexamers (Invitrogen), 50 nmol of deoxynucleoside triphosphates, 2 μmol of dithiothreitol, and approximately 2 μg of RNA. RT reactions were carried out in RNase-free PCR tubes, using a PTC-200 DNA Engine thermocycler (MJ Research). The program was as follows: incubation at 65°C for 5 min and then 25°C for 5 min followed by addition of 1 μl of RT to each sample, annealing at 25°C for 10 min, reverse transcription at 50°C for 2 h, and finally inactivation of the RT at 70°C for 15 min. A no-RT control was run for each RNA sample to ensure that no DNA contamination was present.

PCRs were carried out on all reverse transcription products, including those from the no-RT control reactions. For these PCR experiments, primers with no degenerate bases were designed to specifically target each of the *bssA* sequences found in this study (Table 2). A primer set was also designed to target the 16S rRNA gene for a specific microorganism referred to as Eub-1—likely a *Desulfotomaculum* sp.—found previously to be active in the culture (11). Primer specificity was confirmed by amplifying culture DNA and checking amplicon sequences. Positive or negative amplification of the putative genes was assessed by running PCR products on a 1% agarose gel stained with ethidium bromide and looking for bands of the expected length.

RT-PCRs conducted with RNA from toluene-degrading cultures resulted in the amplification of *bssA*-2, while no amplification was observed in benzoate-degrading cultures (Fig. 2A). Thus, *bssA*-2 transcription was upregulated in the presence of toluene relative to benzoate, consistent with involvement of this gene product in the conversion of toluene to benzoate. Surprisingly, although the *bssA*-1 sequence was readily amplified from culture DNA, transcription of *bssA*-1 in the presence of either toluene or benzoate was not observed (data not shown). To ensure that all RNA extractions and RT reactions were comparable between treatments, cDNA was amplified with 16S rRNA primers for Eub-1. In all cases, bands corre-

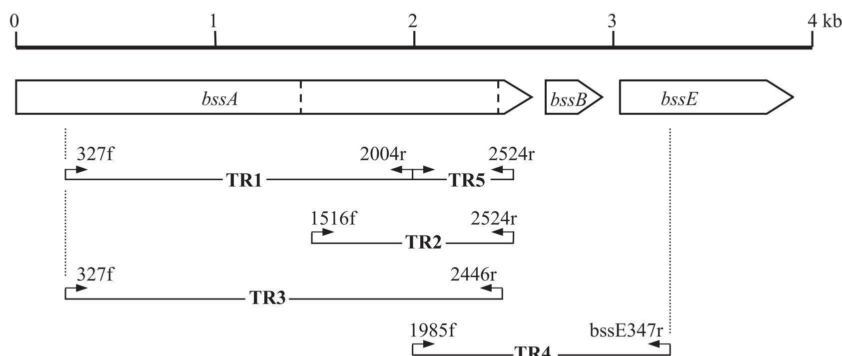


FIG. 1. Partial transcriptional map of the *bss* operon. The target regions (TRs) for PCR amplification and sequencing are shown under the map. The primers used to amplify TR5 were 1985f and 2524r. The two dotted vertical lines shown in the *bssA* box illustrate the approximate positions of nucleotides coding for conserved cysteine (positions 1464 to 1467 in the *Thauera aromatica* K172 *bssA* sequence) and glycine (positions 2472 to 2475 in *T. aromatica* K172) residues in this gene.

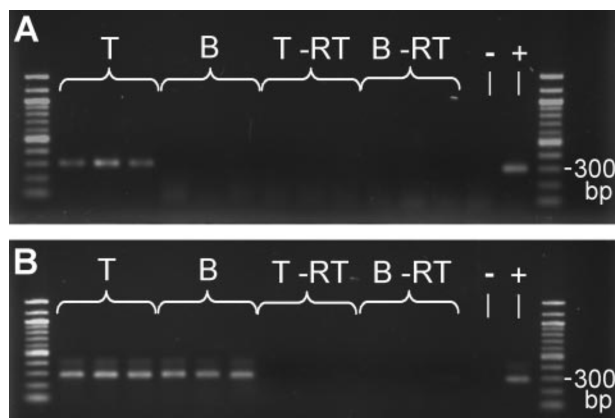


FIG. 2. Agarose gels showing amplicons from RT-PCRs performed using cultures amended with toluene (T) or benzoate (B). Amplification was carried out using specific *bssA*-2 primers (panel A) and Eub-1 (16S rRNA gene) primers (panel B) listed in Table 2. Expected amplicon lengths were ~300 bp for all reactions. Control reactions shown: "T-RT" and "B-RT," controls without reverse transcriptase; +, culture DNA; −, no sample.

sponding to amplicons of expected sizes and of similar intensities were observed by gel electrophoresis (Fig. 2B). No amplification was observed in any of the no-RT control reactions.

The differential transcription of *bssA*-2, combined with the bioinformatic analysis of the sequence, provides strong evidence that *bssA*-2 encodes an enzyme that shares the same catalytic function as those encoded by BssA sequences from the nitrate- and iron-reducing microorganisms discovered to date. Although the transcription of *bssA*-1 was not demonstrated in this study, the strong similarity of BssA-1 sequence to other known BssA sequences and the presence of putative *bssB* and *bssE* sequences on the same operon as *bssA*-1 support the proposed function of this gene. Further studies examining transcription of this gene at very high or low toluene concentrations or in the presence of other alkylbenzenes may help to determine the nature of the role of *bssA*-1 in the culture.

The identification of new *bssA* sequences from the methanogenic culture, combined with the existing sequence data obtained from nitrate-reducing and iron-reducing microorganisms, expands our knowledge of the diversity of *bssA*, *bssB*, and *bssE* genes in the environment. As more *bssA* sequences are discovered, particularly from strict anaerobes such as those found in this study, more comprehensive primers and probes can be designed to track the growth and activity of alkylbenzene-degrading organisms in the environment and confirm biodegradation of toluene and other alkylbenzenes in contaminated soil and groundwater.

**Nucleotide sequence accession numbers.** The putative *bssA* and *bssABE* sequences have been deposited in GenBank under accession numbers EF134965 and EF134966.

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