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Biochemical and Biophysical Research Communications 366 (2008) 142–148
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Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes

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Received 16 November 2007
Available online 4 December 2007

Abstract

The sulfate-reducing strain AK-01 activates alkanes via addition of the subterminal carbon to the double bond of fumarate. This reaction is similar to the action of the glycyl radical enzyme benzylsuccinate synthase (Bss). It was hypothesized that strain AK-01 possesses a similar enzyme. Degenerate *bssA* primers and inverse PCR were used to amplify two unlinked genes *(assA1* and *assA2*), which encode catalytic subunits of glycyl radical type enzymes. Subsequent genome sequencing of AK-01 revealed two *ass* operons. SDS–PAGE analysis of AK-01 grown on *n*-hexadecane revealed a 95-kDa protein which is absent in hexadecanoate-grown cells. LC-MS/MS data obtained from a tryptic digest of this protein match the deduced amino acid sequence encoded by *assA1*, thus confirming AssA1's involvement in alkane metabolism. This report is the first description of a gene involved in anaerobic *n*-alkane metabolism in a sulfate-reducer and provides evidence for a novel glycyl radical enzyme.

Keywords: Glycyl radical enzyme; Alkylsuccinate synthase; Alkane; Anaerobic; Benzylsuccinate synthase; Bioremediation; Anaerobic alkane degradation

Alkanes comprise a large component of petroleum [1]. Understanding the environmental fate of alkanes in marine and groundwater systems contaminated with crude and refined petroleum is important because the degradation of alkanes, as well as other hydrocarbons, is often hindered by the chemical inertness of the carbon-hydrogen bonds. In the absence of catalysts [2], biotransformation is one of the most important removal processes in the environment. Under aerobic conditions, *n*-alkanes are activated by oxygenase enzymes, in which oxygen serves as a key reactant [3–6]. Oxygen's key role in these reactions led to the belief for many years that anaerobes were unable to activate hydrocarbons. Research over the past two decades, however, has shown that anaerobic biodegradation of hydrocarbons does occur and proceeds via unique mechanisms by physiologically and phylogenetically distinct microorganisms utilizing a range of hydrocarbon substrates [7]. However, little is known about the enzymes catalyzing these reactions.

One of the most well-studied anaerobic enzyme mechanisms is that of the glycyl radical enzyme, benzylsuccinate synthase (Bss). Bss catalyzes the reaction of fumarate addition to monoaromatic hydrocarbons in several aromatic hydrocarbon-degrading anaerobes [7]. The dominant features of the catalytic subunits of glycyl radical enzymes, including Bss, are the conserved glycine motif (I/V-R-I/V-X-G-F/W/Y) located near the C-terminus and the conserved cysteine residue(s) located in the middle of the

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doi:10.1016/j.bbrc.2007.11.094

polypeptide chain [8–10]. These conserved sites are involved in transferring a radical from the glycine residue to a cysteine residue. It is the thiyl radical that initiates the abstraction of a hydrogen atom from the substrate. The Bss glycyl radical mechanism provides a distinct alternative for anaerobic microorganisms to activate hydrocarbon substrates in the absence of oxygen. Interestingly, several anaerobic microorganisms appear to use fumarate addition as the initial mechanism in activating *n*-alkanes [11–15]. Mechanistically, there is an obvious parallel between the anaerobic activation of aromatic hydrocarbons and the anaerobic activation of *n*-alkanes via fumarate addition. In both cases, the reaction proceeds with the retention of the abstracted H atom (from the *n*-alkane or the aromatic hydrocarbon) in the succinyl moiety of the first intermediate [7,11,12,15]. This similarity in activation mechanisms prompted a closer look at whether anaerobic alkane degraders possess glycyl radical enzymes.

The sulfate-reducing strain AK-01 was isolated from the Arthur Kill waterway (NJ/NY) and is capable of growth on C_{13}–C_{18} alkanes [16]. Previous work has shown that strain AK-01 initiates alkane degradation via fumarate addition to the subterminal carbon [15]. We hypothesized that strain AK-01 possesses an enzyme similar to Bss. The work described in this paper not only identifies such an enzyme, but furthermore identifies the genes involved. To our knowledge, this is the first report of a gene involved in anaerobic alkane biotransformation in a sulfate-reducer. These findings shed significant insight on anaerobic alkane activation and provide a basis for examining the enzyme mechanisms and genes of other anaerobic, alkane-degrading microorganisms.

Methods

Growth conditions. AK-01 was grown as previously described [15]. Cultures (10% inoculum) were amended with either 30 μl of hexadecane or 1 ml of a 1 M stock solution of sodium hexadecanoate (1 mM final concentrations) (Sigma Aldrich, St. Louis, MO). Cultures were incubated horizontally in the dark at 30 °C for several months. Growth was monitored via measuring sulfate loss by ion chromatography [16].

DNA extraction, PCR, and inverse PCR. Genomic DNA was extracted from frozen cell pellets by a method adapted from [17]. The degenerate primers *bssA* 1230F (5′-GACATGACCGAYGCCATYCT-3′) and *bssA* 2000R (5′-TCRTCGTCRTTGCCCCAYTT-3′) were used to amplify *bssA*-like genes in 50 μl reaction mixtures containing 5–7 ng template DNA, 20 pmol of each primer, and 45 μl of PCR SuperMix (Invitrogen, Carlsbad, CA). Cycling conditions were: 95 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The amplicon was sequenced and served as the template to design AK-01-specific inverse PCR primers. Inverse PCR reactions were performed using 25 ng of genomic DNA which had been digested with BamHI, EcoRI, BglII, HindIII, XbaI, SacI, ApaI, NcoI, or PstI (Invitrogen, Carlsbad, CA) and circularized with 6 units of T4 Ligase (Invitrogen, Carlsbad, CA) at 37 °C for 3 h. Inverse PCR reactions (50 μl) contained 5 ng of DNA template, 50 pmol of each primer, 2.5 mM $MgCl_2$, 400 μM of each dNTP, and 5 units of *Taq* polymerase (Invitrogen, Carlsbad, CA). Cycling parameters were: 95 °C for 3 min, 40 cycles at 95 °C for 1 min, annealing temperature specific to the primers for 1 min, and a final extension step at 72 °C for 10 min. All PCR and inverse PCR products were either sequenced directly or cloned into pCR®4-TOPO (Invitrogen, Carlsbad CA) before sequencing. Gene sequences were verified by re-amplifying the complete region from genomic DNA and sequencing the resulting PCR products in both directions. Concomitantly, the genome of strain AK-01 is being sequenced in collaboration with the Department of Energy's Joint Genome Program. Analysis of the available draft genome assembly allowed the identification of two different operons containing the *assA1* and *assA2* genes. No additional *bssA*-like genes were detected in the draft assembly.

Phylogenetic analysis. DNA sequences were assembled using SeqMan (DNAStar, Madison, WI). A representative set of related protein sequences, including the closest matches in GenBank as determined by BlastN and BlastP [18] were obtained and aligned using the ClustalW algorithm and manually adjusted to correct obvious misalignments introduced by the algorithm. Neighbor-joining dendrograms were generated using MEGA3 [19].

Nucleotide sequence accession numbers. DNA sequences of *assA1* and *assA2* of strain AK-01 are found in GenBank under the Accession Nos. DQ826035 and DQ826036.

Protein extraction and electrophoresis. Cells were grown on either 1 mM sodium hexadecanoate or 1 mM *n*-hexadecane. Protein was harvested from 1 L of each culture condition using the ProteoPrep Universal Extraction Kit (Sigma Aldrich, St. Louis, MO). The protocol was modified to include a 50 μl amendment of protease inhibitor (100 μM benzamidine, 1 mM amino-caproic acid, 1 mM EDTA) to 5 ml of the ProteoPrep soluble extraction reagent. Protein was concentrated using a Millipore Ultra-Free CL Centrifugal Filter Kit (Billerica, MA) and assayed using the BioRad Protein Dye reagent (Hercules, CA) according to the manufacturer's instructions. Protein samples (25 μg) were applied to an 8% polyacrylamide SDS–PAGE gel. Electrophoretic conditions were as follows: equilibration for 20 min at 20 mA/gel and electrophoresis for 2.25 h at 30 mA/gel in Tris–Glycine buffer (196 mM glycine, 0.1% SDS, and 25 mM Tris–HCl, pH 8.3). The gel was stained with 0.08% Colloidal Coomassie Brilliant Blue G (Sigma Aldrich, St. Louis, MO) in 20/1.6/8.0% methanol/phosphoric acid/ammonium sulfate. A replicate gel was transferred to a PVDF membrane (Immobilon P_{SEQ}, Millipore). Transfer conditions were as follows: 3 h at 37 V in Tris glycine buffer (25mm Tris-Base, 192 mM glycine, 20% methanol). After electrophoresis, the gel was stained with 0.1% amido black. Protein bands were excised and sent to the Eastern Quebec Proteomics Center (CHUL Research Center, Ste-Foy, Québec, Canada) for analysis. Trypsin digests of protein samples were conducted on a MassPrep liquid handling station (Micromass Ltd, Manchester, UK) following the manufacturer's instructions. MS/MS spectra of peptides were acquired by microcapillary reverse-phase chromatography coupled to a quadrupole ion trap mass spectrometer with a nanospray interface (LC-MS/MS LCQ Deca XP, ThermoFinnigan, San Juan, P.R.). Spectra were analyzed using the MASCOT algorithm and software [20,21] (Matrix Science Ltd, London, England).

Results

PCR and inverse PCR

PCR primers were designed based on conserved regions of known *bssA* genes. The *bssA* gene codes for the catalytic subunit of Bss. Total genomic DNA from strain AK-01 was PCR amplified using the degenerate primers *bssA* 1230 F and *bssA* 2000 R. The 750 base-pair PCR product was sequenced and the deduced encoded protein found to be related to several different BssA sequences in the GenBank database. Inverse PCR was used to amplify and sequence the flanking regions of the putative alkylsuccinate synthase (*assA1*) gene. Sequencing of inverse PCR products revealed a second related gene (*assA2*) located elsewhere in the genome. The deduced amino acid sequences

encoded by the *assA1* and *assA2* genes are 80.9% identical to each other. The predicted sizes of the *assA1* and *assA2* gene products are 833 (Nominal mass: 95,194 Da) and 834 (Nominal mass: 95,292 Da) amino acids, respectively.

The translated amino acid sequences of *assA1* and *assA*2 were compared to the sequences of known and suspected catalytic subunits of glycyl radical enzymes (Fig. 1). The AssA1 and AssA2 amino acid sequences cluster and form a distinct and deep branch that represents a new family of glycyl radical enzymes. Their closest relatives (34 to 36% identity) are in the family of known Bss enzymes from *Thauera* (CAA05052, AAC38454, BAC05501), *Azoarcus (*AAK50372, YP_158060)*, Magnetospirillum* (BAD42366) and *Geobacter* (EAM79462). The branch depth and separation between Ass and Bss are likely due to evolutionary constraints imposed by differences in the enzymatic structure required for the addition of either an aromatic or alkane substrate to fumarate. AssA1 and AssA2 are more distantly related to known or suspected pyruvate formate-lyase subunits found in a variety of anaerobic bacteria.

Consistent with the catalytic subunits of other glycyl radical enzymes, AssA1 and AssA2 have two conserved regions thought to be involved in catalysis (Fig. 2). A conserved glycine residue is located at position 810 in AssA1 and position 809 in AssA2. A single conserved cysteine residue is located at position 473 in AssA1 and 472 in AssA2. The PFL family of enzymes typically has two cysteines next to each other in this region of the protein [22–24]. Comparison of the Bss and Ass catalytic subunits revealed significant similarity around the conserved glycine and cysteine residues.

Genome sequencing of strain AK-01 allowed identification of the complete operons containing the *assA1* and *assA2* genes. A comparison of these operons to the gene organizations of known *bss* operons is shown in Fig. 3. Subunits were named based on similarity to genes of the *bss* operon in *Azoarcus* sp. EbN1. Both operons contain homologs to *bssC*, *bssD,* and *bssE.* Their predicted amino acid sequences are between 30% and 58% identical to the respective genes in EbN1. Homologs to *bssB* are not readily apparent. Both operons of AK-01 do contain several

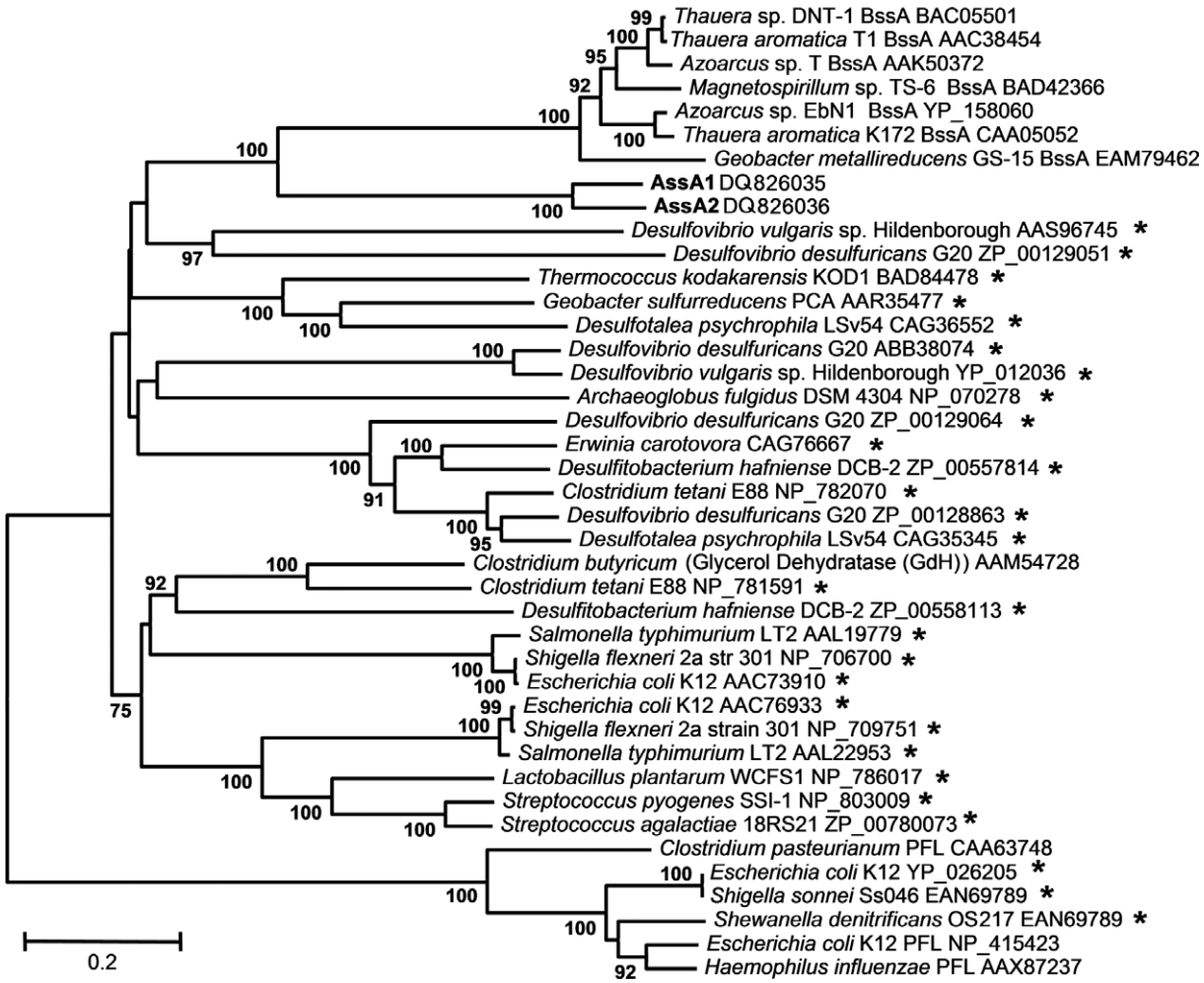

Fig. 1. Neighbor-joining dendrogram of amino acid sequences of AssA1 and AssA2 and sequences of the catalytic subunits of well characterized and putative glycyl radical enzymes. A star (*) designates putative glycyl radical enzymes identified by genome annotation. One-thousand bootstraps were performed. Bootstrap values below 65 are not shown.

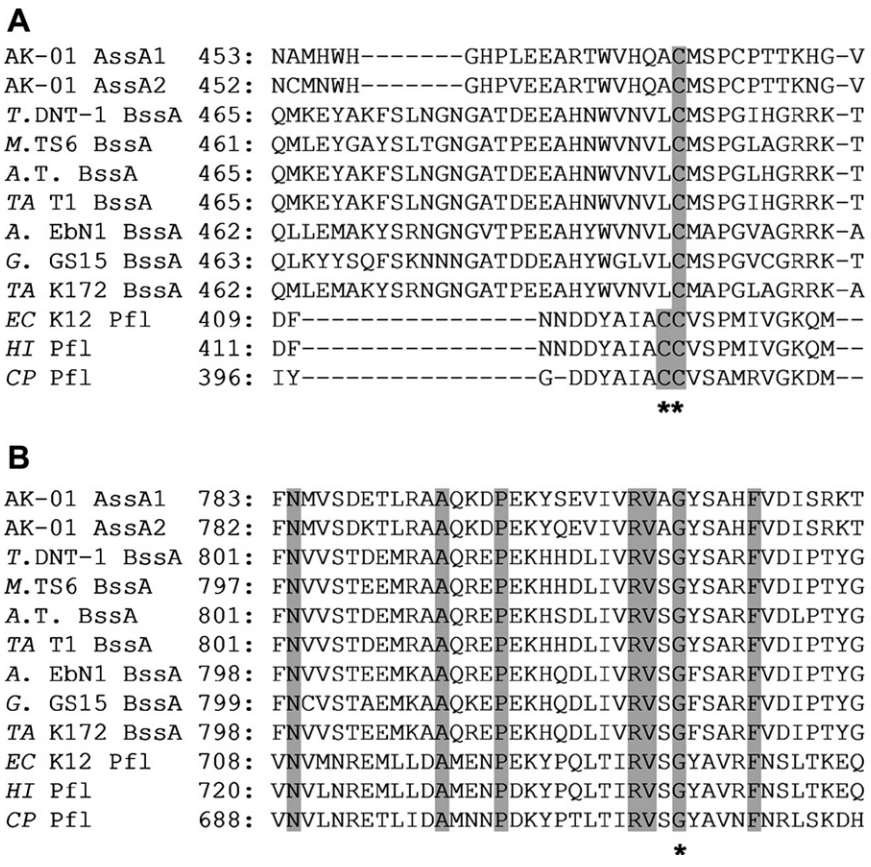

Fig. 2. Partial sequence alignments of AssA1 and AssA2 and other known and predicted catalytic subunits of glycyl radical enzymes. (A) Alignment of the region containing the conserved cysteine residue. (B) Alignment of the region containing the conserved glycine residue. Conserved regions are shaded; numbers refer to the amino acid positions. The abbreviated names are as follows: *T.* DNT-1, *Thauera* sp DNT-1 (BAC05501); *M.* TS6, *Magnetospirillum* sp. TS6 (BAD42366); *A*.T., *Azoarcus* sp. strain T (AAK50372); *TA* T1, *Thauera aromatica* T1 (AAC38454); *A*. EbN1, *Azoarcus* sp. strain EbN1 (YP_158060); *G*. GS15, *Geobacter metallireducens* GS-15 (EAM79462); *TA K172*, *Thauera aromatica K172* (CAA05052); *EC* K12, *Escherichia coli* K12 (NP_415423); *HI*, *Haemophilus influenza*e (AAX87237), and *CP, Clostridium pasteurianum* (CAA63748).

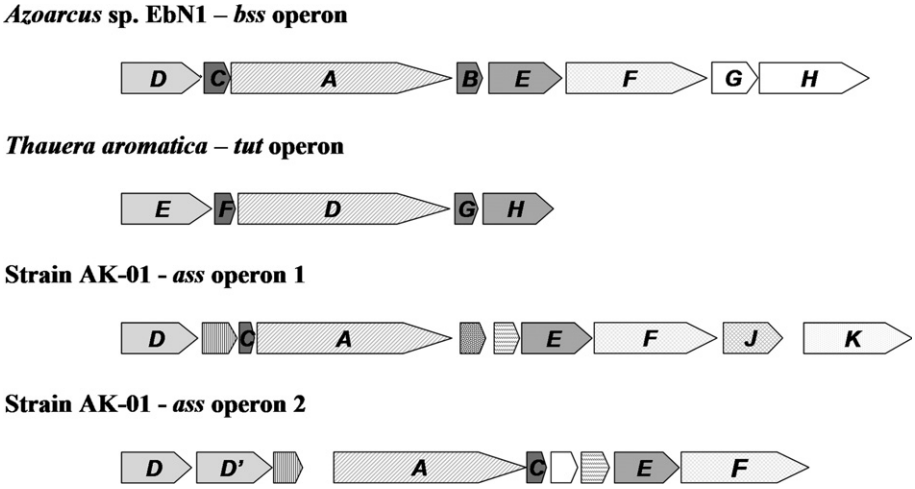

Fig. 3. Gene organization of the benzylsuccinate synthase operons in *Azoarcus* sp. EbN1 (*bss* operon) and *Thauera aromatica* (*tut* operon) in comparison with the two AK-01 alkylsuccinate synthase (*ass*) operons. Genes in AK-01 were labeled based on similarity to and gene organization in *Azoarcus sp.* EbN1. Shading and patterns indicate genes encoding similar proteins. The operon containing *assA1* also includes genes encoding proteins similar to long-chain fatty acid CoA-ligases (*assK*) and enoyl-CoA hydratases (*assJ*) not found in any of the other known glycyl radical enzyme operons.

additional ORFs with strong ribosome binding sites, but their predicted amino acid sequences are all <8% identical to BssB. The operon containing *assA1* also contains a gene encoding a protein similar to BssF (AssF; 30% amino acid identity). In addition, this operon contains genes encoding two enzymes involved in beta-oxidation:

a long-chain fatty acid CoA-ligase (AssK is 37% identical to this protein in *Ralstonia eutropha*) and an enoyl-CoA hydratase (AssJ is 36% identical to this protein in *Bdellovibrio bacteriovorus*). The operon of *assA2* contains two genes (labeled *assD2A* and *assD2B*) encoding proteins similar to the activating enzyme of benzylsuccinate synthase (BssD, 33% and 29% amino acid identity, respectively). All predicted AssD protein sequences of AK-01 (D1, D2A, and D2B) contain two cysteine clusters matching the ferredoxin consensus sequence CxxCxxCxxxC. This feature is unique to alkyl- and benzylsuccinate synthases and is not found in the related activating enzymes of pyruvate formate lyase and anaerobic ribonucleotide reductase [25].

Proteomic analysis

A prominent protein band was identified in the SDS PAGE gel of protein extracts from strain AK-01 grown on *n*-hexadecane that was not observed in extracts from hexadecanoate-grown cells (data not shown). Tryptic digestion and LC-MS/MS analysis of this ~95 kDa protein resulted in the identification of several significant matches to predicted tryptic fragments of AssA1 and AssA2. The MASCOT software was used to identify MS/MS peptide signatures and match the data to internal peptide fragments of the AssA1 and AssA2 proteins (Fig. 4). In general, a MASCOT MS/MS ion score greater than 49 is considered a significant match ($p < 0.05$). For AssA1, four MS/MS signatures matched predicted tryptic fragments with a score above 49, fourteen matched with a score between 25 and 49, and one matched with a score less than 25. For AssA2, two MS/MS signatures matched with a score above 49 (both peptide fragments are identical to the AssA1 matches), eight matched with a score between 25 and 49 (4 identical and 4 similar to AssA1 matches), and two matched with a score below 25. AssA1 thus had more unique matches with higher scores than AssA2. The only matches with significant scores for AssA2 are also identical to AssA1. These data clearly show that AssA1 is produced by strain AK-01 during growth on hexadecane. However, the identification of AssA2 is inconclusive.

Discussion

Here we report the identification of two complete operons in strain AK-01 that encode glycyl radical-type enzymes. The predicted molecular weights of their encoded alpha subunits (AssA1 and AssA2) are consistent with a 95-kDa protein band that is observed when AK-01 is grown on hexadecane, but is not seen in hexadecanoate-grown cells. Proteomic analysis of this 95-kDA protein band indicates that AssA1 is produced by cells growing on hexadecane. Analysis of the *assA1*-containing operon indicates the presence of genes encoding a long-chain fatty acid CoA-ligase and an enoyl-CoA hydratase. The encoded enzymes are directly involved in the activation and hydration of fatty acids during beta-oxidation, providing further support for the interpretation that the *assA1* operon is involved in the activation of hexadecane and further metabolism of the first metabolite, methylpentadecylsuccinate. The operon containing *assA2* does not contain genes involved in beta-oxidation, and AssA2 is not implicated by proteomic analysis. These data suggest that the *assA2* operon is likely involved in the activation of a substrate other than hexadecane.

Although the function of *assA*2 remains unclear, multiple genes of related glycyl radical enzymes in a single organism are not uncommon. Examples have been found in the complete genome sequences of *Desulfovibrio desulfuricans* strain G20 (NC_007519), *Desulfovibrio vulgaris* (NC_002937), and *Escherichia coli* K12 (NC_000913). Although the biochemical functions of related glycyl radical enzymes in a single organism have yet to be fully elucidated, several hypotheses can be proposed. For example, differential expression of multiple catabolic gene copies for alkane degradation has been observed in aerobic organisms and has been attributed to different substrate ranges and growth conditions [26–29]. The expression of *assA1* and *assA2* in strain AK-01 may thus be governed by several factors. Strain AK-01 utilizes C_{13}–C_{18} alkanes, alkenes (C_{15} and C_{16}), 1-alkanols (C_{15} and C_{16}), and fatty acids [16]. It is possible that expression of *assA2* is linked to an alkane substrate other than hexadecane or is linked to a different point in the growth phase.

Phylogenetic analysis, based on the catalytic subunits, indicates that AssA1 and AssA2 form a distinct and separate cluster when compared to other catalytic subunits of glycyl radical enzymes (Fig. 1). We conclude, therefore, that the Ass enzymes belong to a novel class of glycyl radical enzymes. The conserved cysteine and glycine motifs that have been shown to be involved in catalysis in other glycyl radical enzymes [8–10] are observed in both AssA1 and AssA2. Except for PFL and TdcE, which contain neighboring cysteine residues [22], all other characterized glycyl radical enzymes have only one conserved cysteine residue (including AssA1 and AssA2). The locations of the conserved motifs in AssA1 and AssA2 are consistent with the sequences of other glycyl radical enzymes whose tertiary structures have been solved and in which the cysteine and glycine conserved motifs are in close proximity to each other, allowing the transfer of the radical [8–10,30].

To our knowledge, this is the first report of a gene involved in anaerobic *n*-alkane biodegradation for a sulfate-reducer. We provide evidence of a previously unknown class of glycyl radical enzyme involved in anaerobic alkane metabolism. The identification of this new glycyl radical enzyme provides significant insight into enzymes governing anaerobic alkane degradation and will serve as a basis for studying other anaerobic alkane degraders.

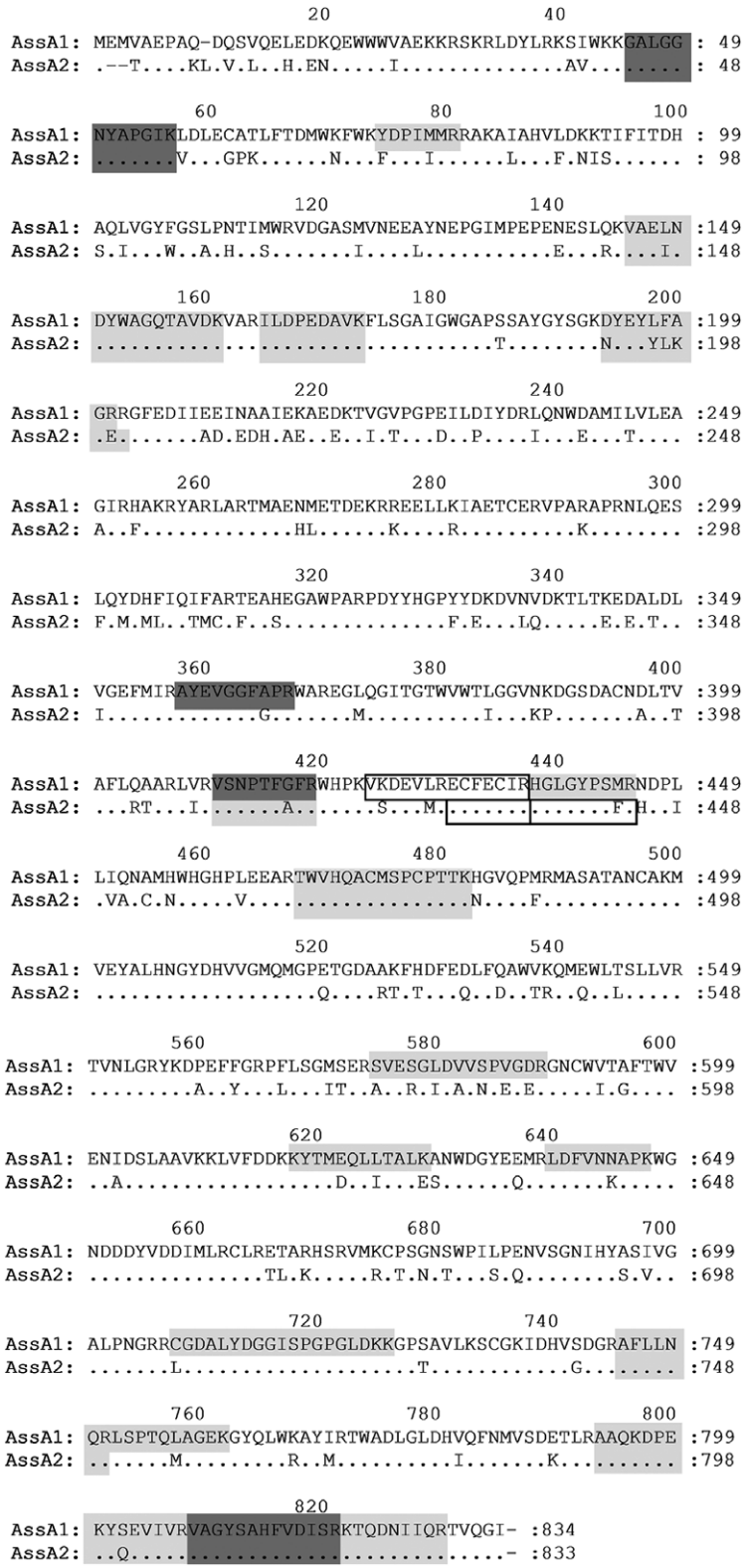

Fig. 4. Amino acid sequence alignment of AssA1 and AssA2. The periods designate identical amino acids and the dashes designate gaps. MASCOT ion scores greater than 49 are shown in dark gray; scores between 25 and 49 are shown in light gray; scores below 25 are shown in clear boxes.

Acknowledgments

This research was supported by grant NSF CHE-0221978 (L.Y.Y. and G.J.Z.), NIH grant ES012824 (G.J.Z.), and by a U.S. Department of Education GAANN fellowship to A.V.C. (Grant P200A010808).

The authors wish to thank Dr. Sylvie Bourassa and Isabelle Kelly from the Eastern Quebec Proteomics Center

for protein sequencing, Laurie Seliger for DNA sequencing, and Dr. Dan Bost for technical advice. We also thank Dr. Rachel Austin for discussion regarding alkane chemistry.

References

[1] A.A. Petrov, Petroleum Hydrocarbons, Springer-Verlag, New York, 1984.
[2] R.J. Fessenden, J.S. Fessenden, Organic Chemistry, PWS Publishers, Boston, 1982.
[3] M. Ayala, E. Torres, Enzymatic activation of alkanes: constraints and prospective, Appl. Catal. A 272 (2004) 1–13.
[4] J.H. Maeng, Y. Sakai, T. Ishige, Y. Tani, N. Kato, Diversity of dioxygenases that catalyze the first step of oxidation of long-chain *n*-alkanes in *Acinetobacter* sp. M-1, FEMS Microbiol. Lett. 141 (1996) 177–182.
[5] J.H. Maeng, Y. Sakai, Y. Tani, N. Kato, Isolation and characterization of a novel oxygenase that catalyzes the first step in *n*-alkane oxidation in *Acinetobacter* sp. strain M-1, J. Bacteriol. 178 (1996) 3695–3700.
[6] J.B. van Beilen, E.G. Funhoff, Expanding the alkane oxygenase toolbox: new enzymes and applications, Curr. Opin. Biotechnol. 16 (2005) 308–314.
[7] J. Heider, Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms, Curr. Opin. Chem. Biol. 11 (2007) 188–194.
[8] A. Becker, K. Fritz-Wolf, W. Kabsch, J. Knappe, S. Schultz, A.F.V. Wagner, Structure and mechanism of the glycyl radical enzyme pyruvate formate-lyase, Nat. Struct. Biol. 6 (1999) 969–975.
[9] D.T. Logan, J. Andersson, B.-M. Sjöberg, P. Nordlund, A glycyl radical site in the crystal structure of a class III ribonucleotide reductase, Science 283 (1999) 1499–1504.
[10] J.R. O'Brien, C. Raynaud, C. Croux, L. Girbal, P. Soucaille, W.N. Lanzilotta, Insight into the mechanism of the B^{12}-independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization, Biochemistry 43 (2004) 4635–4645.
[11] K.G. Kropp, I.A. Davidova, J.M. Suflita, Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture, Appl. Environ. Microbiol. 66 (2000) 5393–5398.
[12] R. Rabus, H. Wilkes, A. Behrends, A. Armstroff, T. Fischer, A.J. Pierik, F. Widdel, Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism, J. Bacteriol. 183 (2001) 1707–1715.
[13] H. Wilkes, R. Rabus, T. Fischer, A. Armstroff, A. Behrends, F. Widdel, Anaerobic degradation of *n*-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl) succinate via C-skeleton rearrangement, Arch. Microbiol. 177 (2002) 235–243.
[14] C. Cravo-Laureau, V. Grossi, D. Raphel, R. Matheron, A. Hirschler-Réa, Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aliphaticivorans* strain CV2803^T, Appl. Environ. Microbiol. 71 (2005) 3458–3467.
[15] A.V. Callaghan, L.M. Gieg, K.G. Kropp, J.M. Suflita, L.Y. Young, Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium, Appl. Environ. Microbiol. 72 (2006) 4274–4282.
[16] C.M. So, L.Y. Young, Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes, Appl. Environ. Microbiol. 65 (1999) 2969–2976.
[17] F.A. Rainey, N. Ward-Reiney, R.M. Kroppenstedt, E. Stackebrandt, The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: Proposal of *Nocardiopsaceae* fam. nov, Int. J. Syst. Bacteriol. 46 (1996) 1088–1092.
[18] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[19] S. Kumar, K. Tamura, M. Nei, MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment, Briefings Bioinformat. 5 (2004) 150–163.
[20] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, Electrophoresis 20 (1999) 3551–3567.
[21] J.K. Eng, A.L. McCormack, J.R. Yates, III An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database, J. Am. Soc. Mass. Spectrom. 5 (1994) 976–989.
[22] C. Hesslinger, S.A. Fairhurst, G. Sawers, Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in *Escherichia coli* that degrades *L*-threonine to propionate, Mol. Microbiol. 27 (1998) 477–492.
[23] J. Knappe, H.P. Blaschkowski, P. Grobner, T. Schmitt, Pyruvate formate-lyase of *Escherichia coli*: the acetyl-enzyme intermediate, Eur. J. Biochem. 50 (1974) 253–263.
[24] J. Knappe, F.A. Neugebauer, H.P. Blaschkowski, M. Ganzler, Post-translational activation introduces a free radical into pyruvate formate-lyase, Proc. Natl. Acad. Sci. USA 81 (1984) 1332–1335.
[25] B. Leuthner, C. Leutwein, H. Schulz, P. Hörth, W. Haehnel, E. Schiltz, H. Schägger, J. Heider, Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism, Mol. Microbiol. 28 (1998) 615–628.
[26] N. Hamamura, C.M. Yeager, D.J. Arp, Two distinct monooxygenases for alkane oxidation in *Nocardioides* sp. strain CF8, Appl. Environ. Microbiol. 67 (2001) 4992–4998.
[27] M.M. Marín, L. Yuste, F. Rojo, Differential expression of the components of the two alkane hydroxylases from *Pseudomonas aeruginosa*, J. Bacteriol. 185 (2003) 3232–3237.
[28] T.H. Smits, Cloning and functional analysis of bacterial genes involved in alkane oxidation, Swiss Federal Institute of Technology, Zürich, Switzerland, 2001.
[29] A. Tani, T. Ishige, Y. Sakai, N. Kato, Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1, J. Bacteriol. 183 (2001) 1819–1823.
[30] C.V. Parast, K.K. Wong, J.W. Kozarich, J. Peisach, R.S. Magliozzo, Electron paramagnetic resonance evidence for a cysteine-based radical in pyruvate formate-lyase inactivated with mercaptopyruvate, Biochemistry 34 (1995) 5712–5717.