Introduction

Discovery of microbes in extreme environments has always posed challenges to scientists. Their enzymes have been shown to produce biologically active chemicals as well as the ability to degrade and remove waste and hazardous toxic materials (Okeke, 2008; Sorokulova et al., 2009). If we maximize the advantage in utilizing these bacteria, it will be critical to understand the genes that they harbour. One such bacterium is *Exiguobacterium acetylicum*, a member of the family of coryneforms. Critical to survival of these bacteria is the ability to biosynthesize amino acids and small molecules like biotin. One enzyme that mediates such reactions is pyridoxal phosphate (PLP)-dependent acyltransferase, primarily involved in the biosynthesis of amino acids and their derivatives (Salzmann et al., 2000; Mozzarelli and Bettati, 2006). The important metabolic role of PLP-dependent acyltransferase in carrying out a variety of reactions has been extensively studied since it represents about 4% of the enzymes classified by the Enzyme Commission (Mozzarelli and Bettati, 2006). However, the phylogenetic trajectories of PLP-dependent acyltransferase enzymes and protein structures remain inadequate. Earlier, it was observed that the PLP-dependent enzymes are of multiple evolutionary origin and belong to five evolutionarily independent families (Salzmann et al., 2000). PLP-dependent enzymes emerge very early in the evolution conceivably followed by organic cofactors and metal ions (Mehta and Christen, 2000).

Here we set out to search for the presence of PLP-dependent enzymes. This, we did in *E. acetylicum* strain SN as template. We revealed the presence of a PLP-dependent family of enzymes, such as PLP-dependent acyltransferase, and similarity to 8-amino-7-oxononoate synthase. Sequencing analysis and multiple alignment of the thymidine-adenine-cloned PCR amplicon revealed PLP-dependent family enzymes with specific conferring codes and consensus amino acid residues specific to this group of functional proteins. Amino acid residues common to the majority of PLP-dependent enzymes were also revealed by the Lasergene MegAlign software. A phylogenetic tree was constructed. Its analysis revealed a close relationship of *E. acetylicum* to other bacteria isolated from extreme environments suggesting similarities in anabolic adaptability and evolutionary development.

Key words: *Exiguobacterium*, Acyltransferase, Phylogenetics
30 °C, by picking a single colony from a nutrient agar stock plate prepared earlier by the streak-plate technique. 10 mL liquid were placed on a shaker at 200 rpm overnight. Genomic DNA was isolated according to the manufacturer’s protocol with the DNA bactozol kit (Molecular Research Center, Cincinnati, OH, USA). In brief, 1 to 10 μL or 1 to 10 mg of sample were lysed with 0.1 mL of DNAzol Direct (catalog # DN 131) over 15 min of incubation at room temperature. The lysate was thoroughly mixed and a 2- to 5-μL aliquot was transferred directly into 20 to 50 μL of PCR mix. The genomic DNA isolation was confirmed by resolving the bacterial lysate in agarose gel.

**Primers and PCR conditions**

MTF2 (forward) and MTR (reverse) primers (Neilan et al., 1999) as well as 16S rRNA control primers (Duncan et al., 2004) were used. Degenerated primers were synthesized at Integrated DNA Technologies (Coralville, IA, USA). All primers were employed against the genomic DNA of *E. acetylicum* strain SN. The polymerase chain reaction (PCR) was carried out using the PCR protocol as we standardized earlier (Rajendran et al., 2008). The PCR reaction afforded DNA, Premix Taq (catalog # RR003, Takara, Madison, WI, USA), each primer (forward and reverse), and double distilled water. The following PCR conditions were applied in a thermocycler (Eppendorf Mastercycler personal): 95 °C (5 min), 95 °C (1 min), 55 °C (2 min), and 72 °C (3 min) for 30 cycles. The PCR products were resolved by gel electrophoresis. By using the Digidoc-it UVP digital documentation unit, we examined the amplicons and documented the data. PCR amplicons were extracted using the PureLink Quick Gel Extraction kit (catalog # K2100–12, Invitrogen, Carlsbad, CA, USA).

**Subcloning and DNA sequencing**

Amplicons obtained by PCR using the MTF2 and MTR sets of primers were subcloned in *E. coli*, according to the manufacturer’s protocol (Invitrogen). In brief, PCR products were ligated into the pCR4-TOPO vector followed by transformation into chemically competent one shot TOP10 *E. coli*. Positive clones were screened using IPTG/X-Gal (Fermentas, USA) with ampicillin (Sigma). Positive colonies were grown in ampicillin/LB liquid culture followed by plasmid isolation using Pure Yield Plasmid Miniprep System (Promega, Madison, USA). The clones were confirmed by *Eco*R1 restriction followed by sequencing using a T3 and/or T7 primer at the Center for Genetics and Molecular Medicine (CGeMM), DNA Core Facility of the University of Louisville, KY, USA.

**Sequence analysis and construction of phylogenetic tree**

A clean DNA sequence free from vector sequence was confirmed using the NCBI vector contamination software (vecscreen). Homologous nucleotide and amino acid sequence searches were performed using NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). The newly determined partial PLP-dependent transferase nucleotide sequence of *E. acetylicum* strain SN was deposited in GenBank and an accession number (FJ865431) was obtained using BankIT:GenBank (www.ncbi.nlm.nih.gov/BankIT/). To confirm the specificity signature sequences, conserved amino acid residue search was performed during multiple sequence alignment. An alignment of PLP-dependent acyltransferase sequences from significant bacterial species on the basis of highest possible homology was then prepared using ClustalW. A phylogenetic tree of PLP-dependent acyltransferase of *E. acetylicum* along with other microbial sequences, accessible by NCBI blast search, was built using MegAlign (DNAstar).

**Results**

Previously published degenerative nucleotide primers (Neilan et al., 1999), encoding conserved acyladenylation domains of peptide synthetase, were used in the PCR to amplify corresponding regions on genomic DNA of *Exiguobacterium acetylicum* strain SN. The PCR probing of *E. acetylicum* revealed a ~500-bp amplicon as well as another smaller, non-specific band (Fig. 1). The sequence of the 500-bp fragment (Fig. 2) has been submitted to GenBank (accession number FJ865431). NCBI BLAST search against protein data bases revealed the identity of the amplicon as a partial sequence of PLP-dependent acyltransferase. In addition to PLP-dependent acyltransferase, the NCBI BLAST search also revealed similarity to another PLP-dependent family of enzymes, 8-amino-7-oxononoate synthase that catalyzes the first committed step in the biotin biosynthesis (data not shown) (Alexeev et al.,...
In order to confirm the putative identity of the newly cloned PLP-dependent acyltransferase among other bacteria, we compared the sequence by multiple sequence alignment (Fig. 3). The analysis revealed universally conserved amino acids in all proteins compared in the sequence alignment as well as some that are homologous in the majority of the peptides. ClustalW revealed the presence of conserved L-position 29, G-position 41, LSSNYLGL-position 47 to 55, and H-position 89. Other amino acid residues common in the majority of the revealed homologous PLP-dependent acyltransferase include R-position 24, L-position 26, E-position 30, S-position 31, Q-position 33, TID-position 38 to 40, I-position 66, A-position 68, and W-position 72.

Phylogenetic analysis of the partial sequence of PLP-dependent acyltransferase of E. acetylicum along with other microbial sequences, revealed its close relationship with other Exiguobacteria (Fig. 4). In our studies we included PLP-dependent enzyme sequences with homology of the critical functional groups indicated in Fig. 3. Notable are arginine-50 and histidine-89 that have been reported to be critical in binding PLP (Pinon et al., 2005). All bacteria included in the phylogenetic analysis revealed lysine on position 29 and glutamine on position 30 as well as a well-conserved sequence, SNNYL, at position 49 to 53 (Fig. 3). These conserved amino acid residues and the specificity conferring code (SNNYL) may be critical in substrate specificity and type of reaction catalyzed by the PLP-dependent enzyme.

Discussion

Bacteria harbour many enzymes including the PLP-dependent family of enzymes that are essential to cell metabolism. 8-Amino-7-oxononanoate synthase and other PLP-dependent families of enzymes have become significant as targets for chemotherapy (Eliot and Kirsch, 2004). 8-Amino-7-oxononanoate synthase catalyzes the committed step in the biotin biosynthesis pathway (Alexeev et al., 1998; Marquet et al., 2001; Pinon et al., 2005). To our knowledge this is the first report on PLP-dependent acyltransferase and 8-amino-7-oxononanoate synthase in Exiguobacterium acetylicum and an attempt to reveal the phylogenetic position of this bacterium relative to other microbes. In our earlier study we used a degenerated primer set coding for a partially conserved domain sequence of a peptide synthetase, and revealed the tryptophanyl-tRNA synthetases from Actinobacillus actinomycetemcomitans along with its phy-

Fig. 1. Electrophoresis of PCR products. Sets of degenerated primers were employed against the genomic DNA of the newly isolated soil bacterium Exiguobacterium acetylicum under the following conditions: 95 °C (5 min), 95 °C (1 min), 55 °C (2 min), and 72 °C (3 min) for 30 cycles in a mastercycler (Eppendorf). PCR amplicons were resolved via 1% agarose gel electrophoresis. This figure indicates the amplified PCR fragment of interest at the size of 700 kb using primer sets MTF2 and MTR (lane 2), the control primer of 16S (lane 3), and a 100-bp DNA ladder (lane 1).

Fig. 2. Nucleotide sequence (542 bp) of PLP-dependent acyltransferase as sequenced at DNA Core Facility, University of Louisville, KY, USA. The newly determined PLP nucleotide sequence of Exiguobacterium acetylicum strain SN was deposited at GenBank and an accession number (FJ865431) was obtained using BankIT:GenBank (www.ncbi.nlm.nih.gov/BankIT/).
(A) Consensus #1
+ Majority

Majority

Exiguobacterium acetylicum
Alkaliphilus metallireducens QYMf.pro
Alkaliphilus oremlandii OHLAs.pro
Deinococcus geothermalis DSM 1130.pro
Elusimicrobium minutum Pei191.pro
Exiguobacterium AT1b.pro
Exiguobacterium sibiricum 255-15.pro
Geobacillus sp. Y412MC10.pro
Herpetosiphon aurantiacus ATCC 23779.pr
Petrotoga mobilis SJ95.pro
Thermoanaerobacter pseudethanolicus ATC
Thermoanaerobacter sp. X514.pro
Thermotogales bacterium TBF 19.5.1. pro

(B) Consensus #1
+ Majority

Majority

Exiguobacterium acetylicum
Alkaliphilus metallireducens QYMf.pro
Alkaliphilus oremlandii OHLAs.pro
Deinococcus geothermalis DSM 1130.pro
Elusimicrobium minutum Pei191.pro
Exiguobacterium AT1b.pro
Exiguobacterium sibiricum 255-15.pro
Geobacillus sp. Y412MC10.pro
Herpetosiphon aurantiacus ATCC 23779.pr
Petrotoga mobilis SJ95.pro
Thermoanaerobacter pseudethanolicus ATC
Thermoanaerobacter sp. X514.pro
Thermotogales bacterium TBF 19.5.1. pro

(C) Consensus #1
+ Majority

Majority

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Thermoanaerobacter pseudethanolicus ATC
Thermoanaerobacter sp. X514.pro
Thermotogales bacterium TBF 19.5.1. pro
logenetic relationship among other oral bacteria by analyzing the specificity-confirming codes and amino acid residues. In the present study we employed another degenerated primer set, designed earlier (Neilan et al., 1999), coding for a partially conserved domain sequence of peptide synthetase and revealing the PLP-dependent acyltransferase and its family of enzymes. Here we report the probing and cloning of PLP-dependent acyltransferase in *E. acetylicum* using a degenerated primer set derived from the conserved domains of non-ribosomal peptide synthetase (Turgay and Marahiel, 1994; Marahiel et al., 1997; Rajendran, 1999) and the attempt to reveal the potential anabolic mechanism and pathways in this soil bacterium.

The multiple sequence alignment of 8-amino-7-oxononoate synthase and PLP-dependent acyltransferase revealed amino acid residues conserved in all sequences tested (Fig. 3 and supplementary data). The conserved residues are L-position 29, G-position 41, LSSNYLGL-position 47 to 55, and H-position 89 (Turbeville et al., 2007). These functional amino acid residues play critical roles in either binding the cofactor PLP or substrate specificity (Alexeev et al., 1998; Yard et al., 2007; Yoshikane et al., 2006; Mozzarelli and Bettati, 2006). Literature search revealed that the PLP-dependent enzymes we cloned in our studies have conserved functional residues similar to those expressed in plant cells, and these include the N-50 and H-89 (Pinon et al., 2005).

The phylogenetic tree of PLP-dependent acyltransferase of *E. acetylicum* strain SN was constructed. Our studies revealed the acyltransferase type of the PLP-dependent family of enzymes including 8-amino-7-oxononoate synthase. Phylogenetically, the PLP-dependent family of enzymes is a very diverse group of proteins with a wide range of substrate specificity (Mehta and Christen, 2000; Mozzarelli and Bettati, 2006; Pinon et al., 2005; Salzmann et al., 2000; Schulze et al., 2006). The diversity is not only between the different fold types of the PLP-dependent enzymes but also between members of the same group. These pro-
tein sequences, obtained from the NCBI BLAST search, demonstrated similarity in regions and domains overlapping our newly cloned sequence (FJ865431) as well as portions distal to the H-89 in bacteria phylogenetically distant from E. acetylicum (data not shown).

The phylogenetic analysis of these synthetases revealed close relationship of our strain of E. acetylicum to bacteria isolated from extreme environments suggesting similarities in anabolic adaptability and evolutionary development. For example, Alkaliphilus oremlandii OhILAs, formally a Clostridium sp. isolated from Ohio River sediments, may share similar metabolism with E. acetylicum based on the homology of PLP-dependent acyltransferase (Fig. 4) (Stolz et al., 2007). Alkaliphilus oremlandii, an anaerobic, spore-forming, Gram-positive bacterium was shown to metabolize glycerol, fructose, lactate, arsenate and thiosulfate (Stolz et al., 2007). Thermoanaerobacter sp., a metal-reducing bacterium, isolated from the Piceance Basin of Colorado (Roh et al., 2002), and Petrotoga mobilis, an anaerobic, Gram-negative bacterium, isolated from a North Sea oil reservoir (Lien et al., 1998), are able to metabolize elemental sulfur to hydrogen sulfide (Lien et al., 1998) and have homology with E. acetylicum PLP-dependent enzymes. Geobacillus sp. expresses thermostable PLP-dependent enzymes belonging to the beta family (Saavedra et al., 2004). Whether our E. acetylicum strain SN is able to metabolize metals and other environmental pollutants still remains to be investigated, but phylogenetically it shows a close relationship to these bacteria. Other bacteria included in the sequence alignment in Fig. 3, Herpetosiphon aurantiacus, Deinococcus geothermalis and Thermotogales sp. are inhabitants of hot aquatic environments (Liang et al., 2008; Dahle et al., 2008; Miroshnichenko and Bonch-Osmolovskaya, 2006; Urios et al., 2004; L’Haridon et al., 2002; Alain et al., 2002; Hamana et al., 2001; Yernool et al., 2000; Ratnayake et al., 2000; Borneman et al., 1996; Reysenbach et al., 1994; Emond et al., 2008; Filipkowski et al., 2006; Kongpol et al., 2008). Sharing the same environment and similarity in protein sequences have significant phylogenetic implications. These common elements suggest a significant evolutionary relationship between these thermophiles and Exiguobacterium acetylicum particularly in anabolic metabolism since they employ PLP-dependent synthetases.

### Conclusion

We demonstrated the presence of PLP-dependent acyltransferase in Exiguobacterium acetylicum strain SN and its phylogenetic position in relation to other environmental microbes. Multiple sequence alignment of PLP-dependent acyltransferase and 8-amino-7-oxononoate synthase, as identified in this study in E. acetylicum and along with other homologous sequences in microbe databases, revealed well conserved amino acid residues and a specificity conferring codes unique to this class of enzymes. The PLP-dependent acyltransferase phylogenetic tree revealed the relationship with various groups of other bacteria. Our molecular and phylogenetic analysis of PLP-dependent acyltransferase demonstrated the breadth of diversity of microbes that utilize PLP-dependent enzymes. Since this acyltransferase of E. acetylicum is a member of the PLP-dependent family of enzymes that metabolize amino acids and other small molecules, our study has significance in the peptide synthetase study especially in relation to soil microbes.

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