Molecular and Phylogenetic Analysis of Pyridoxal Phosphate-Dependent Acyltransferase of *Exiguobacterium acetylicum*

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The pyridoxal-5'-phosphate (PLP)-dependent family of enzymes is a very diverse group of proteins that metabolize small molecules like amino acids and sugars, and synthesize cofactors for other metabolic pathways through transamination, decarboxylation, racemization, and substitution reactions. In this study we employed degenerated primer-based PCR amplification, using genomic DNA isolated from the soil bacterium *Exiguobacterium 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 confering 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

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 that has potential relationship to a large community of soil-inhabiting coryneform bacteria. It is an effort to better understand the microbial phylogeny of the PLP-dependent family of enzymes given the critical role that they play. In this study, we carried out molecular cloning and sequence analysis. The comprehensive data and annotated sequences in the NCBI-linked databases enabled us to construct the evolutionary heritage of this versatile group of enzymes.

Material and Methods

Bacterial culture methods and DNA isolation

A nutrient broth culture (10 mL) of *Exiguobacterium acetylicum* strain SN was grown at

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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 as16S 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 ºC (5 min), 95 ºC (1 min), 55 ºC (2 min), and 72 ºC (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*.,

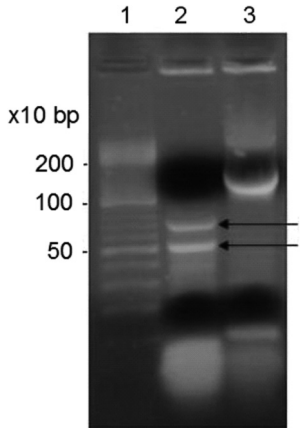


Fig. 1. Electrophoresis of PCR products. Sets of degenerated primers were employed against the genomic DNA of the newly isolated soil bacterium *Exiguobacterium acetylicum* under the following conditions: 95 ºC (5 min), 95 ºC (1 min), 55 ºC (2 min), and 72 ºC (3 min) for 30 cycles in a mastercycler (Eppendorf). 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).

ACGTCAGTTACGATTCGCCTTGCGGTGGTGCGTAT
GTGCCCATCGTCCTTAAGAACCGCCCATGACGACG
ATGCCCGTCCGTGCCTCCTGCGTTTCTTGTATCGC
TGCTTCGGACACGCAAAGTCAACGTGATGATGAAT
ACGGCGTTTCGGATTTGTGGACGGATCCCTCCTCC
GATGCGTGATTGGGACGGCCTCTTCGATATGGGAT
TCATCTCCTTCGGTCTGACGGGCGGCTACTGGTCG
ATTGCATGCAAGCGAGGAGAACCGACGTCTGCGG
GACTACTATGCTTGATGGTCATCAGTAACTGGTCCG
AACGATGGGACTACTTGCGTGTTCCTCCCATCCGT
TTCGACGATGCAGTTTGAGAGCCTGAAATAACTTTT
GCTTCCATATGGAGTCCGCATGAATGAAAAAGATG
AGAGAGAGTCCGAGGTAGTTGTTCGACGACAGTTG
GATCAGTTCCTTCCCGTCAATCGTCACGCGATTGT
GTTGCGCGCTCTCGAGTGCTACAAGGTTGCGGAAC
GTAAGCACCACCGGCA

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/).

1998; Eliot and Kirsch, 2004; Pinon *et al*., 2005; Webster *et al*., 1998).

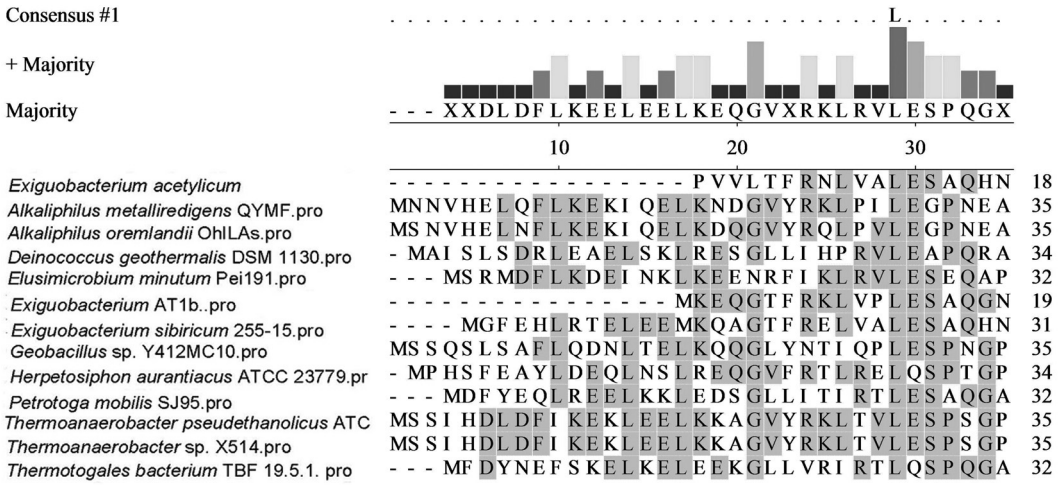
In order to confirm the putative identity of the newly cloned PLP-dependent acyltransferase among other bacteria, we compared the sequence by multiple sequence alignment (Fig. 3). The analysis revealed universally conserved amino acids in all proteins compared in the sequence alignment as well as some that are homologous in the majority of the peptides. ClustalW revealed the presence of conserved L-position 29, G-position 41, LSSNNYLGL-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.

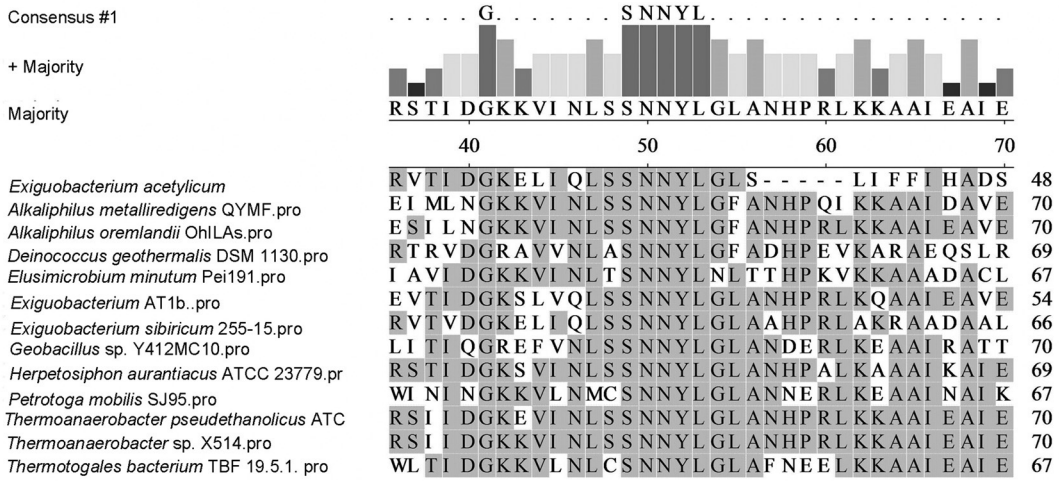
Discussion

Bacteria harbour many enzymes including the PLP-dependent family of enzymes that are essential to cell metabolism. 8-Amino-7-oxononoate synthase and other PLP-dependent families of enzymes have become significant as targets for chemotherapy (Eliot and Kirsch, 2004). 8-Amino-7-oxononoate 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-oxononoate 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-

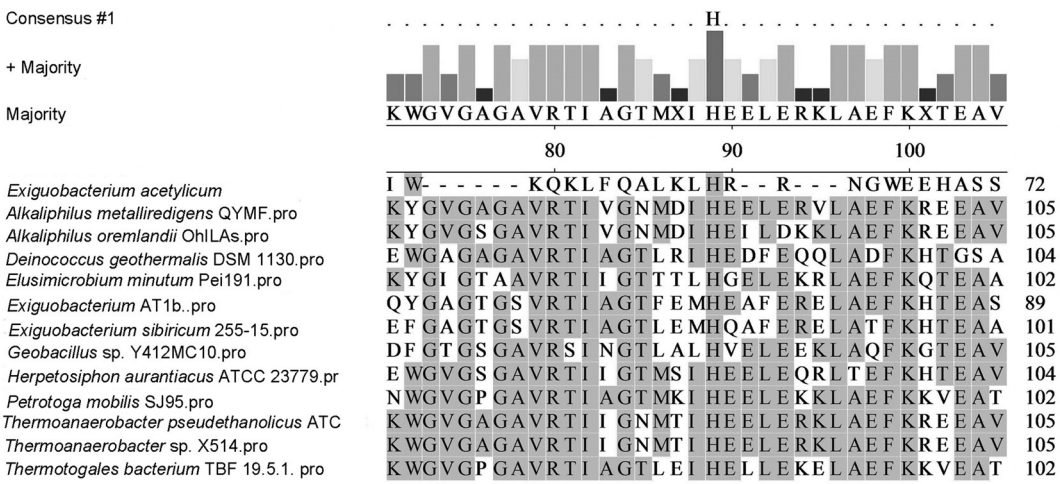
(A)


Consensus #1
+ Majority
Majority
- - - XXDLDFLKEELEELKEQGVXRKLRVLESPQGX
10
20
30
Exiguobacterium acetylicum - - - - - - - - - - - - - - - - - - PVVLTFRNLVALESAQHN 18
Alkaliphilus metalliredigens QYMF.pro MNNVHELQFLKEKIQELKNDGVYRKLPILEGPNEA 35
Alkaliphilus oremlandii OhILAs.pro MSNVHELNFLKEKIQELKDQGVYRQLPVLEGPNEA 35
Deinococcus geothermalis DSM 1130.pro - MAISLSDRLEAELSKLRESGLLIHPRVLEAPQRA 34
Elusimicrobium minutum Pei191.pro - - - MSRMDFLKDEINKLKEENRFIKLRVLESEQAP 32
Exiguobacterium AT1b..pro - - - - - - - - - - - - - - - - MKEQGTFRKLVPLESAQGN 19
Exiguobacterium sibiricum 255-15.pro - - - - MGFEHLRTELEEMKQAGTFRELVALESAQHN 31
Geobacillus sp. Y412MC10.pro MSSQSLSAFLQDNLTELKQQGLYNTIQPLESPNGP 35
Herpetosiphon aurantiacus ATCC 23779.pr - MPHSFEAYLDEQLNSLREQGVFRTLRELQSPTGP 34
Petrotoga mobilis SJ95.pro - - - MDFYEQLREELKKLEDSGLLITIRTLESAQGA 32
Thermoanaerobacter pseudethanolicus ATC MSSIHDLDFIKEKLEELKKAGVYRKLTVLESPSGP 35
Thermoanaerobacter sp. X514.pro MSSIHDLDFIKEKLEELKKAGVYRKLTVLESPSGP 35
Thermotogales bacterium TBF 19.5.1. pro - - - MFDYNEFSKELKELEEKGLLVRIRTLQSPQGA 32

(B)


Consensus #1
+ Majority
Majority
RSTIDGKKVINLSSNNYLGLANHPRLKKAAIEAIE
40
50
60
70
Exiguobacterium acetylicum RVTIDGKELIQLSSNNYLGLS- - - - - LIFFIHADS 48
Alkaliphilus metalliredigens QYMF.pro EIMLNGKKVINLSSNNYLGFANHPQIKKAAIDAVE 70
Alkaliphilus oremlandii OhILAs.pro ESILNGKKVINLSSNNYLGFANHPRLKKAAIEAVE 70
Deinococcus geothermalis DSM 1130.pro RTRVDGRAVVNLASNNYLGFADHPEVKARAEQSLR 69
Elusimicrobium minutum Pei191.pro IAVIDGKKVINLTSNNYLNLTTHPKVKKAAADACL 67
Exiguobacterium AT1b..pro EVTIDGKSLVQLSSNNYLGLANHPRLKQAAIEAVE 54
Exiguobacterium sibiricum 255-15.pro RVTVDGKELIQLSSNNYLGLAAHPRLAKRAADAAL 66
Geobacillus sp. Y412MC10.pro LITIQGREFVNLSSNNYLGLANDERLKEAAIRATT 70
Herpetosiphon aurantiacus ATCC 23779.pr RSTIDGKSVINLSSNNYLGLANHPALKAAAIKAIE 69
Petrotoga mobilis SJ95.pro WININGKKVLNMCSNNYLGLANNERLKEAAINAIK 67
Thermoanaerobacter pseudethanolicus ATC RSIIDGKEVINLSSNNYLGLANHPRLKKAAIEAIE 70
Thermoanaerobacter sp. X514.pro RSIIDGKKVINLSSNNYLGLANHPRLKKAAIEAIE 70
Thermotogales bacterium TBF 19.5.1. pro WLTIDGKKVLNLCSNNYLGLAFNEELKKAAIEAIE 67

(C)


Consensus #1
+ Majority
Majority
KWGVGAGAVRTIAGTMXIHEELERKLAEFKXTEAV
80
90
100
Exiguobacterium acetylicum IW- - - - - - KQKLFQALKLHR- - R- - - NGWEEHASS 72
Alkaliphilus metalliredigens QYMF.pro KYGVGAGAVRTIVGNMDIHEELERVLAEFKREEAV 105
Alkaliphilus oremlandii OhILAs.pro KYGVGSGAVRTIVGNMDIHEILDKKLAEFKREEAV 105
Deinococcus geothermalis DSM 1130.pro EWGAGAGAVRTIAGTLRIHEDFEQQLADFKHTGSA 104
Elusimicrobium minutum Pei191.pro KYGIGTAAVRTIIGTTTLHGELEKRLAEFKQTEAA 102
Exiguobacterium AT1b..pro QYGAGTGSVRTIAGTFEMHEAFERELAEFKHTEAS 89
Exiguobacterium sibiricum 255-15.pro EFGAGTGSVRTIAGTLEMHQAFERELATFKHTEAA 101
Geobacillus sp. Y412MC10.pro DFGTGSGAVRSINGTLALHVELEEKLAQFKGTEAV 105
Herpetosiphon aurantiacus ATCC 23779.pr EWGVGSGAVRTIIGTMSIHEELEQRLTEFKHTEAV 104
Petrotoga mobilis SJ95.pro NWGVGPGAVRTIAGTMKIHEELEKKLAEFKKVEAT 102
Thermoanaerobacter pseudethanolicus ATC KWGVGAGAVRTIIGNMTIHEELERKLAEFKREEAV 105
Thermoanaerobacter sp. X514.pro KWGVGAGAVRTIIGNMTIHEELERKLAEFKREEAV 105
Thermotogales bacterium TBF 19.5.1. pro KWGVGPGAVRTIAGTLEIHELLEKELAEFKKVEAT 102

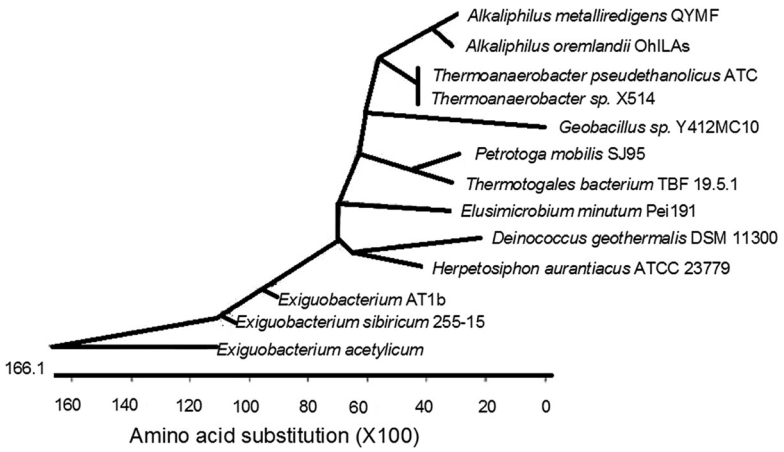


Fig. 4. Phylogenetic tree of PLP-dependent acyltransferases. Sequences of the query *Exiguobacterium acetylicum* and other related microbes aligned in Fig. 3 were compared and used in the phylogenetic tree construction. The tree is unrooted and each node and length of arm represents sequence divergence estimated as number of amino acid substitution as the tree branches. The phylogenetic tree reveals that PLP-dependent acyltransferase of our strain *E. acetylicum* (at the bottom) is closely related to *E. sibiricum and Exiguobaterium* sp. AT1b.

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 nonribosomal 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, LSSNNYLGL-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-

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Fig. 3. Amino acid sequence alignment for PLP-dependent acyltransferase. The results of the BLASTX sequences were aligned as given in A, B and C. These sequence alignments, compared on the basis of highest percentage of homologous amino acid sequences from various bacterial species, reveal the presence of conserved L-position 29, G-position 41, LSSNNYLGL-position 47 to 55, and H-position 89. There are also other amino acid residues conserved in the majority of the revealed homologous PLP-dependent acyltransferase. These 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.

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 confering 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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