Published in final edited form as:

Anaerobe. 2015 October ; 35(0 0): 33–37. doi:10.1016/j.anaerobe.2015.06.005.

***Clostridium amazonense* sp. nov. an obligately anaerobic bacterium isolated from a remote Amazonian community in Peru**

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Abstract

A strictly anaerobic Gram-stain positive, spore-forming, rod-shaped bacterium designated NE08V^T, was isolated from a fecal sample of an individual residing in a remote Amazonian community in Peru. Phylogenetic analysis based on the 16S rRNA gene sequence showed the organism belonged to the genus *Clostridium* and is most closely related to *Clostridium vulturis* (97.4% sequence similarity) and was further characterized using biochemical and chemotaxonomic methods. The major cellular fatty acids were anteiso C_{13:0} and C_{16:0} with a genomic DNA G + C content of 31.6 mol%. Fermentation products during growth on glucose were acetate and butyrate. Based on phylogenetic, phenotypic and chemotaxonomic information, strain NE08V was identified as representing a novel species of the genus *Clostridium*, for which the name *Clostridium amazonense* sp. nov. is proposed. The type strain is NE08V^T (DSM 23598^T = CCUG 59712^T).

Keywords

Clostridium amazonense; sp. nov.; indigenous community; 16S rRNA phylogeny; Taxonomy

1. Introduction

Molecular inventories based mainly on the use of 16S rRNA gene sequencing have provided tremendous insights into the microbial diversity and the richness of taxa present in the human gastrointestinal tract. Indeed such studies show that approximately 80% of the phylotypes represent uncultured bacteria [1]. However, the majority of these studies have focused on western populations, implementing the use of culture-independent methods [1–3]. In order to truly evaluate the core microbiome, individuals from a variety of geographic

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The 16S rRNA sequence of strain NE08V^T (DSM 23598^T = CCUG 59712^T) have been deposited in GenBank under accession number KP281434.

regions with diverse diets must also be included in these investigations [2,4,5]. Initial studies are now revealing that the microbiome of indigenous communities may be significantly different than those derived from westernized and urbanized communities [6,7]. Culture-dependent approaches and the application of methods to characterize the microbial physiological and metabolic properties can be employed to better understand the ecology of these microbial communities and the role they play in both health and disease processes [8–11]. In our continuing studies of traditional communities in Peru, a novel, Gram-stain positive, strictly anaerobic bacterium was isolated from a fecal sample. Based on the phylogenetic, phenotypic and chemotaxonomic evidence, strain NE08V^T represents a novel species within the genus *Clostridium* for which the name *Clostridium amazonense* sp. nov. is proposed. The type strain is NE08V^T (DSM 23598^T = CCUG 59712^T).

2. Materials and methods

2.1. Cultures and cultivation

Strain NE08V^T (DSM 23598^T = CCUG 59712^T) was isolated from a freshly voided fecal sample of an individual residing within a small, remote traditional community, from the Loreto region, Peru. The sample was collected, processed and stored anaerobically, and transported on ice to the Lawson Microbial Systematics Laboratory (University of Oklahoma, Norman, Oklahoma) for further processing. Multiple enrichments using an array of substrates were constructed and inoculated with 1 ml of fecal slurry. Strain NE08V^T was isolated from an enrichment (modified MRS broth) that contained (per Liter): casein peptone, tryptic digest 10.00 g, meat extract 10.00 g, yeast extract 5.00 g, glucose 20.00 g, Tween 80 1.00 g, potassium phosphate 2.00 g, sodium acetate 5.00 g, ammonium citrate 2.00 g, magnesium sulfate 0.20 g, manganese sulfate 0.05 g with 20% ethanol and 0.1 % hemin solution, pH 6.5. Enrichment cultures were incubated under an anaerobic gas mix of 85 % nitrogen, 10 % carbon dioxide, and 5 % hydrogen at 37°C. After incubation for 7 days, serial dilution and repeated sub-culturing onto fresh modified MRS agar was performed until pure colonies were obtained. The strain was then transferred to and maintained on Brucella (BD/BBL, NJ) agar supplemented with 5 % defibrinated sheep's blood.

2.2. Phenotypic and biochemical characterization

For morphological observations, strain NE08V^T was grown on Brucella (BD/BBL) agar supplemented with 5 % defibrinated sheep's blood at 37°C for 48 h. Cell morphological characteristics were characterized with an Olympus CX41 light microscope. Phenotypic tests to determine standard characteristics were performed at the Center for Microbial Identification and Taxonomy (University of Oklahoma, Norman, Oklahoma). The pH range, temperature range and salinity range were determined using peptone-yeast extract (PY) broth (DSMZ 104 medium). The pH range for growth was assessed over the range of pH 5.0–9.0 (increments of 0.5 pH units) using sodium citrate buffer for pH 5.0–6.0, potassium phosphate buffer for pH 6.0–8.0, and Tris buffer for pH 8.0–9.0. The salinity range for growth was tested with NaCl concentrations of 0 %, 0.5 %, 1 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 %, 8 %, and 9 % (w/v). The temperature range was determined by incubating strain NE08V^T at 4, 10, 15, 20, 25, 30, 37, 43, 47, and 60 °C. Optimum growth conditions were determined by monitoring the optical density using a spectrophotometer at 600 nm (Spectronic 20D,

ThermoFisher Scientific, MA). Additional biochemical characterization was determined using the Rapid ID32A system (bioMeriux, Marcy l'Etoile, France) and all reactions were performed in duplicate. Further, metabolic characterization was performed by the BIOLOG system (BIOLOG, Hayward, CA) following the supplied manufacturer's instructions. Briefly, strain NE08V^T was cultivated on BUATM agar supplemented with 5 % defibrinated sheep's blood for 48 hrs and used to inoculate an MicroPlateTM. The AN MicroPlateTM was incubated at 37 °C under anaerobic conditions for 72 hr. All tests were performed in duplicate.

Metabolic end products were determined from cultures grown under anaerobic conditions in PYG broth. Sample analyses were carried out in duplicate on an Aminex HPX-87H organic acid analysis column (Bio-Rad), using ion-exclusion HPLC with 0.015 HCl running buffer at a flow rate of 0.9 mL/min. Retention times and peak areas of fermentation products were compared to standards of acetate, butyrate, lactate, succinate, formate, and propionate.

2.3. DNA isolation and 16S rRNA gene sequencing and phylogenetic analysis

For phylogenetic analysis, DNA of strain NE08V^T was extracted using the UltraClean[®] Microbial DNA Isolation Kit (MoBio Laboratories, Inc., CA) following manufacturer's instructions. 16S rRNA gene fragments were generated by PCR using universal primers 8f (positions 8 to 28, *Escherichia coli* numbering) and pH* (1542 to 1522) [12]. The amplicon was purified using Exo-SapIt (USB Corporation, OH) and the sequence determined using the Big Dye terminator cycle sequencing kit (ver. 3.1), with an automatic DNA sequencer (model 3100 Avant, Applied Biosystems, Life Technologies, Grand Island, NY). The closest known relatives of the new isolate based on the 16S rRNA gene sequence were determined by performing database searches using the program EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>)[13]. These sequences and those of other related strains were aligned with the sequence derived from NE08V^T using the program ClustalW. Phylogenetic reconstructions were performed in MEGA (version 4)[14] using the neighbour-joining method [15], applying evolutionary genetic distances that had been calculated by the Kimura two-parameter model[16].

2.4. GenBank accession numbers

The 16S rRNA gene sequence for strain NE08V^T was deposited with the GenBank Sequence Database under the following accession number KP281434.

2.5. Chemotaxonomic methods

Analysis of fatty acids and mol% G+C content was performed at the Center for Microbial Identification and Taxonomy (CMIT) (University of Oklahoma, Norman, Oklahoma) under conditions chosen to directly compare strain NE08V^T with its closest relative, *Clostridium vulturis*. Biomass for fatty acid analysis was collected from Brucella (BD/BBL) agar supplemented with 5 % defibrinated sheep's blood at 37°C for 48 h. Cells were harvested from the fourth quadrant of the streaked plate. Fatty acid methyl esters were extracted using the Sherlock Microbial Identification System (MIDI Labs Inc, Newark, DE) version 6.1 as described previously [17,18]. Analysis was performed using an Agilent Technologies 6890N gas chromatograph equipped with a phenyl methyl silicone fused silica capillary column

(HP-2 25m × 0.2 mm × 0.33 μm film thickness) and a flame ionization detector with hydrogen used as the carrier gas. The temperature program was initiated at 170°C and increased at 5 °C min⁻¹ reaching a final temperature of 270°C. Fatty acids were identified and expressed in the form of percentages using the QBA1 peak naming database. The mol% G+C was determined according to the method of Mesbah et al. [19], the value obtained was taken from an average of five experimental replications.

3. Results and discussion

3.1. Phenotypic and biochemical characterization

NE08V^T was determined to be a Grain-stain positive, strictly anaerobic, spore-forming and non-hemolytic rod-shaped bacterium. Cells occurred as single cells or in pairs. Subterminal to terminal spores were observed. Colonies grown on Brucella (BD/BBL) agar supplemented with 5 % defibrinated sheep's blood at 37°C for 48 h were observed to be yellow-brown in color, irregular 1 mm in diameter with undulate margins. Oxidase and catalase activities were not observed. The pH and temperature ranges for growth were pH 5.0–9.0 and 30–47 °C with optimal growth at pH 7.0 and temperature 37 °C. The strain grew well in NaCl concentrations between 0–3 % (w/v). The main fermentation end-products detected from PYG media were acetate and butyrate. Positive reactions of the AN MicroPlate™ indicate the organism's ability to utilize L-fucose, palatinose, L-rhamnose, L-glutamine, and L-serine as sole carbon sources.

Using the API Rapid ID 32A system, positive reactions were obtained with arginine dehydrogenase, alkaline phosphatase, arginine arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase, mannose, and pyroglutamic acid arylamidase. No activity was observed for urease, α-galactosidase, β-galactosidase, β-galactosidase-6 phosphate, α-glucosidase, α-arabinosidase- β-glucuronidase, N-acetyl- β-glucosidase, raffinose, proline arylamidase, phenyl arylmidase, tyrosine arylamidase, alanine arylamidase, glutamic acid decarboxylase, α-fucosidase, glutamyl glutamic acid and serine arylamidase. Morphological, physiological and biochemical properties are provided in the species description and Table 2.

3.2. Phylogenetic analysis

Phylogenetic analysis demonstrated that the organism was a member of the *Firmicutes* sharing a relationship with members of the genus *Clostridium sensus stricto*, also recognized as *Clostridium* rRNA group I [20,21] (Fig. 1). The pairwise comparisons showed that strain NE08V^T was most closely related to *Clostridium vulturis* (97.4% 16S rRNA sequence similarity) with the two organisms forming a cluster in the phylogenetic tree. A number of organisms demonstrated lower sequence similarity values with the tree topology revealing a more loose relationship; these included *Clostridium bowmanii* (94.0% 16S rRNA sequence similarity), *Clostridium huakuii* (95.0% 16S rRNA sequence similarity), *Clostridium schirmacherense* (95.1% 16S rRNA sequence similarity), *Clostridium subterminale* (95.5% 16S rRNA sequence similarity), *Clostridium sulfidigenes* (95.2% 16S rRNA sequence similarity) and *Clostridium thiosulfatireducens* (95.2% 16S rRNA sequence similarity). All the major groupings in the neighbor-joining tree were confirmed using the maximum

parsimony algorithm (data not shown). Although no precise correlation exists between % 16S rRNA sequence divergence and species delineation, for many years 3% divergence values were generally considered adequate for the separation of species [22]. However, studies by Stackebrandt and Ebers [23] demonstrated that this value could be lowered to 1.3%. Their exhaustive investigation determined that where 16S sequence similarities values of 98.7 % and below were obtained between strains, DNA-DNA hybridizations could be omitted without a risk for incorrect species differentiation and these values were confirmed by Meier-Kolthoff et al. (2013) [24].

3.3. Chemotaxonomic analysis

The cellular fatty acid profile of strain NE08V^T determined that the predominant fatty (10 % of total fatty acids) were C_{13:0} anteiso (19.7%), and C_{16:0} (26.4%) with lower amounts of C_{16:1} ω₉c (5.6%), C_{14:0} (6.0%), and C_{18:1} ω₉c (8.2%) (Table 1). The profile differs significantly from *Clostridium vulturis* in that this organism does not produce C_{13:0} anteiso but produces major amounts of C_{18:1} ω₉c in comparison to strain NE08V^T. *Clostridium vulturis* and also produces C_{18:1} ω₉c DMA which strain NE08V^T does not. The lack of C_{18:1} ω₉c DMA in strain NE08V^T appears to be an important differential characteristic with other members of this loose group of organisms that includes *Clostridium vulturis*, *Clostridium schirmacherense*, *Clostridium subterminale*, *Clostridium sulfidigenes* and *Clostridium thiosulfatireducens* all of which synthesis this particular fatty acid (Table 2). The DNA G+C content of the strain was determined to be 31.6 mol%.

In addition to its unique 16S rRNA gene sequence, strain NE08V^T was found to possess biochemical and chemotaxonomic traits consistent with organisms belonging to members of the genus *Clostridium* but could clearly be distinguished from its nearest phylogenetic relatives using characteristics shown in Tables 1 and 2. Although there is much discussion on the validity of describing novel genera and species on a single strain, this continues to be a common practice; in our opinion this is warranted and allows species that would previously have gone unnoticed to be described and additional strains or related species to be identified in the future. The increasing number of cultured strains recovered from individuals from indigenous communities will augment the insights gained from the molecular tools commonly used in these studies. Thus, a clearer picture of the interrelationships between organisms that constitute this ecosystem and the overall ecology of these environments will emerge.

Phenotypic, phylogenetic and chemotaxonomic characteristics support the novel species status for strain NE08V^T within the genus *Clostridium*, for which we propose the name *Clostridium amazonense* sp. nov.

4.0. Description of *Clostridium amazonense* sp. nov

Clostridium amazonense (am.a.zon,en'se. N.L. neut. adj. *amazonense* pertaining to the community from where the organism was first isolated). Cells are Gram-stain positive, obligately anaerobic rods occurring as single cells or in pairs. Spores are formed and located either subterminally or terminally. When grown anaerobically on Brucella blood agar for 48 h colonies are irregular, 1 mm in diameter with undulate margins and have a yellow-brown

color. Catalase, oxidase and urease are negative. Optimal growth temperature is 37°C, and occurs at 30–47°C. Growth occurs at pH 5.5–9.0, and is optimal at pH 7.0. Growth occurs in the presence of 0–3% (w/v) NaCl. Using the ID 32A system, positive reactions are obtained for arginine dehydrogenase, alkaline phosphatase, arginine arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase, mannose, and pyroglutamic acid arylamidase. Negative reactions are obtained for α -galactosidase, β -galactosidase, β -galactosidase-6 phosphate, α -glucosidase, α -arabinosidase- β -glucuronidase, N-acetyl- β -glucosidase, raffinose, proline arylamidase, phenyl arylamidase, tyrosine arylamidase, alanine arylamidase, glutamic acid decarboxylase, α -fucosidase, glutamyl glutamic acid and serine arylamidase. Can utilize L-fucose, palatinose, L-rhamnose, L-glutamine, and L-serine as sole carbon sources. The main fermentation end products from PYG medium were acetate and butyrate. The major fatty acids (>10%) are C_{13:0} anteiso and C_{16:0}. The DNA G + C content of the type strain is 31.6 mol%.

The type strain is NE08V^T (=DSM 23598^T = CCUG 59712^T) is isolated from a fecal sample of an individual from Nuevo Eden in the province of Loreto, Peru.

Acknowledgments

This study was supported by the U.S. National Institutes of Health: RO1 GM089886.

Abbreviations

BD	Becton, Dickinson and Company
CCUG	Culture Collection-University of Goteborg
HMP	Human Microbiome Project
PY	peptone-yeast extract

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Research Highlights

A novel *Clostridium* species was isolated from a remote Amazonian community in Peru.

Novel organisms will provide insights into differences between remote and westernized communities.

Interrelationships between taxa will provide functional aspects of such ecosystems.

Figure 1.

Phylogenetic tree showing the relationship of NE08V^T to the most closely related species and other representative members of the genus *Clostridium*. The tree was constructed using the neighbor-joining method based on the pairwise comparison of approximately 1,340 nucleotides. Bootstrap values (>90%), expressed as a percentage of 1000 replications. The scale bar indicates 1% sequence divergence.

Table 1Cellular fatty acid composition of NE08VT and its closest relative *C. vulturis*

Fatty Acids	NE08VT ^T	<i>C. vulturis</i> YMB-57 ^T
C _{12:0}	1.9	
C _{13:0} anteiso	19.7	
C _{14:0}	6.0	2.4
C _{15:0} anteiso	1.3	
C _{15:0} iso		
C _{15:1} ω8c	4.6	
C _{16:0}	26.4	31.9
C _{16:0} aldehyde		
C _{16:0} DMA		
C _{16:0} iso	1.9	
C _{16:1} ω7c		
C _{16:1} ω9c	5.6	
C _{16:1} ω5c	1.2	
C _{16:1} ω11c		
C _{16:1} ω9c DMA		
C _{17:0}		1.4
C _{17:1} ω8c	1.7	
C _{17:1} ω9c	1.7	
C _{18:0}		8.7
C _{18:1} ω9c	8.2	27.0
C _{18:1} t 11?		2.5
C _{18:2} ω9,12c		3.1
C _{18:0} DMA		1.1
C _{18:1} ω9c DMA		10.3
C _{18:1} ω11c DMA		
C _{19:0} cyclo 9, 10/1		1.6
C _{19:0} cyclo 9, 10 DMA		1.2
Summed feature 3	3.9	
Summed feature 4		
Summed feature 6		
Summed feature 7		
Summed feature 10		
Summed feature 11		
Unknown 14.969	2.4	
Unknown 15.301	2.5	

Predominant products are shown in bold, products that represented <1.0 % in all strains were omitted; cyclo, cyclopropane; DMA, dimethylacetal
Summed feature 3 consists of C_{16:1} ω7c or C_{16:1} ω6c

Summed feature 4 contains UN14.762 15:2 ? FA or C15:2.
Summed feature 6 contains C15:0 anteiso 3-OH or C16:1 ω7c
DMA. Summed feature 7 contains C17:2@ 16.760 or C17:1 ω8c.
Summed feature 10 contains C18:0/ C17:0 cycl0.
Summed feature 11 contains C17:0 iso 3-OH or C18:2 DMA

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Table 2

Comparison of characteristics of strain NE08V^T and closely related *Clostridium* species. Data: Strains: 1, strain NE08V^T (data from this study); 2, *C. vulturis* [25]; 3, *C. subterminale* [25]; 4, *C. thiosulfatireducens* [25,26]; 5, *C. sulfidigenes* [25,27].

Characteristic	1	2	3	4	5
Temperature range °C	30–47	30–45	25–45	18–45	18–48
Optimal temperature °C	37	37	37	37	34
NaCl range (% w/v)	0–3%	0–3%	0–80	0–20	0–60
Optimum NaCl	0	ND	0–10	0	0
pH range	5.5–9	5–10	5.4–9.3	6–9.8	5.5–9.0
Optimal pH	7.0	7.5	7.5	6.0	6.6
β-hemolysis	–	–	–	+	+
Enzyme activity					
Alanine arylamidase	–	–	+	+	+
Arginine arylamidase	+	–	+	+	+
α-Fucosidase	–	–	+	+	–
Glutamic acid arylamidase	–	–	+	–	–
Glutamic acid decarboxylase	–	+	–	–	–
Glycine arylamidase	+	–	+	+	+
Leucyl glycine arylamidase	+	–	+	+	+
Mannose fermentation	+	–	+	–	+
Pyroglutamic acid arylamidase	+	–	+	–	–
Tyrosine arylamidase	–	–	–	+	–
Major fatty acids	C _{13:0} anteiso, C _{16:0}	C _{16:0} , C _{18:1} o9c, C _{18:1} o9c DMA	C _{14:0} , C _{16:0} , C _{18:1} o9c, C _{18:1} o9c DMA	C _{14:0} , C _{16:0} , C _{18:1} o9c, C _{18:1} o9c DMA	C _{14:0} , C _{16:0} , C _{18:1} o9c, C _{18:1} o9c DMA
DNA G+C content (mol%)	31.6	34.0	28–30.8	31.4	32.3
Source	Human fecal sample	Vulture intestine	Human infection	Cheese factory wastewater	Pond sediment