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FT-IR for Rapid Discrimination of Halophilic Archaea and Bacteria

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Abstract

Fourier transform infrared spectroscopy (FT-IR) was applied for the first time for the discrimination of halophilic prokaryotes. In the current study, halophilic Bacteria and Archaea species were investigated according to their FT-IR profiles. Some of the halophilic isolates collected from Çamaltı Saltern in Turkey were also analyzed and their FT-IR profiles were compared with reference strains. Hierarchical cluster analysis was used to indicate discrimination. This study implies that FT-IR can be applied to discriminate species of Archae and Bacteria.

Keywords: Halophilic; Bacteria; Archaea; Fourier transform infrared spectroscopy

Introduction

Microorganisms requiring salt for growth and specialized for living in hypersaline environments are referred as halophiles. Kushner (1985) defined several categories of microorganisms according to the salt concentration that was optimal for growth. These organisms are phylogenetically (on the basis of comparative ribosomal RNA gene sequencing) found in all three domains of life: Archaea, Bacteria and Eukarya, the domain being the highest taxon. Two of these domains, the Archaea and Bacteria, are exclusively microbial and are composed of single cells that lack a membrane-enclosed nucleus (i.e., prokaryotic cells) [1].

Halophilic microorganisms able to live in saline environments have garnered a great deal of attention as they have the potential in various biotechnological applications such as the production of biopolymers, compatible solutes, cancer detection, drug screening, biodegradation of toxic compounds, fermentation of foods and most importantly the production of enzymes [2-4].

Most of the halophilic Archaea are found in hypersaline lakes and saltern crystallizer ponds, giving a red coloration due to C-50 carotenoid pigments. However, the domain Bacteria contains many types of halophilic and halotolerant microorganisms in many phylogenetic subgroups [5].

Although the domains Archaea and Bacteria are founded on phylogenetic criteria, these two domains can also be characterized by phenotypic properties, e.g. membrane lipids [6]. In contrast to the lipids of Bacteria in which ester linkages bond fatty acids to glycerol, the lipids of Archae contain ether bonds between glycerol and their hydrophobic side chains [7].

The diversity of Archaea and Bacteria in various hypersaline environments worldwide has been characterized by culture-based, pigment and molecular-phylogenetic studies [7-12]. It is known that halophilic Archaea are mostly orange-red pigmented and grow optimally at salt concentrations from above 20% [13]. Archaeal strains grow in the presence of penicillin G in the growth medium, whereas Bacterial strains could not [7]. However, the more recent technique using Archaeal and Bacterial primers in PCR has proved itself as a more reliable way of distinguishing between the two domains [14-15].

Conventional techniques for identifying microorganisms are phenotypic, biochemical or genotypic assays including culturing, immunological methods such as the enzyme-linked immunosorbent assay (ELISA), and molecular biology techniques such as polymerase chain reaction (PCR) and in-situ hybridization. These techniques are accurate, but none of these techniques are readily automated for rapid (<1 min) identification under industrial or field conditions [16]. However, the FT-IR method is rapid and non-invasive, permitting users to collect full spectra in a few seconds per sample. Differences in the structure and quantity of cell wall polysaccharides, lipids and proteins are reflected in the FT-IR spectra. These data provide fingerprint-like patterns which are highly reproducible and typical for different bacteria [17-19]. Compared to conventional methods, FT-IR identification is not only based on cell characteristics, but is achieved by selecting discriminating features from a large amount of spectral information. The technique is applicable to all microorganisms including prokaryotes and eukaryotes (e.g. fungi) that can be grown in culture [20].

Identification with FT-IR has been used for the identification of various members of Bacteria at the genus, species, and even strain level and typical examples are given in Table 1 [21-49]. However, there has been no such study of Archaea except for one study by Hedrick et al. [6] who used diffuse reflectance FT-IR spectroscopic analysis for the rapid differentiation of Archaea from Bacteria.

Therefore, in this study, we aimed to investigate the similarities and differences between some representatives of halophilic Archaea and Bacteria domains, and if possible to differentiate between genera and species using FT-IR spectroscopy. To our knowledge, this is the first study to investigate FT-IR differentiation of halophilic prokaryotes.

Materials and Methods

Organisms

A total of twelve halophilic reference strains representing Archaea and Bacteria domains were kindly provided by Josefa Anton (Spain), Michael Dyall-Smith (Australia), Helga Stan-Lotter (Austria) and Dr. Mehmet Burçin Mutlu (Anadolu University, Turkey). The strains used and their sources are given in Table 2.

Microorganisms	Year	Reference
Staphylococcus, Streptococcus, Clostridium, Legionella species and Escherichia coli	1990	Helm et al. [21]
Bacillus cereus, Bacillus mycoides, Bacillus thrungiensis	1998	Beattie et al. [22]
Candida albicans	2000	Orsini et al. [18]
Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, and Pseudomonas stutzeri	2001	Filip et al. [23]
	2003	Guibet et al. [24]
Listeria monocytogenes ATCC 19114, L. innocua ATCC 51742, L. innocua ATCC 33090, and L. monocytogenes ATCC 7644	2004	Lin et al. [25]
Streptococcus crista, Strep. Salivarius and *Strep. Rattus*	2004	van der Mei et al. [26]
Bacillus subtilis	2004	Filip et al. [27]
Lactobacillus, L. sakei, L. plantarum, L. curvatus and L. paracasei	2004	Oust et al. [28]
Acinetobacter species	2004	Winder et al. [29]
Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis, Candida krusei, and Candida kefyr	2005	Essendoubi et al. [30]
Bacillus cereus, Salmonella enterica, Escherichia coli and *Listeria spp.*	2006	Al-Holy et al. [31]
Campylobacter coli and *Campylobacter jejuni.*	2006	Mouwen et al. [32]
S. arlettae, S. capitis, S. caprae, S. carnosus, S. epidermis, S. equorum, S. felis, S. gallinarum, S. lentus, S. intermedius, S. pasteuri, S. pulvereri	2006	Lamprell et al. [33]
Enterobacter aerogenes, Enterobacter cloacae, Klebsiella spp., Citrobacter koseri; Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, and *Enterococcus faecium*	2006	Sandt et al. [34]
Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and *Streptococcus* strains	2007	Dziuba et al. [35]
Listeria monocytogenes samples	2006	Oust et al. [36]
Staphylococcus aureus	2007	Amiali et al. [37]
Mesophilic and termophilic bacteria	2007	Garip et al. [38]
Listeria innocua FH, Lactococcus lactis, Pseudomonas fluorescens, Pseudomonas mendocina and Pseudomonas putida	2008	Alexandrakis et al. [39]
Acinetobacter baumanni, Enterococcus faecium and Staphylococcus aureus / epidermidis	2008	Preisner et al. [40]
Bacillus cereus, B. megaterium, B. subtilis, B. thrungiensis	2009	Forrester et al. [41]
Bacillus cereus	2010	Mietke et al. [42]
Filamentous fungi and yeasts	2010	Santos et al. [43]
Lactic acid bacteria	2011	Samelis et al. [44]
E. coli O157:H7	2012	Davis et al. [45]
Salmonella enterica	2012	Preisner et al. [46]
Trichophyton	2013	Ergin et al. [47]
Staphylococcus aureus	2014	
Filamentous Fungi	2015	

Table 1: Examples of application of FT-IR for microbial identification.

Reference strains			
Archaea	**Source**	**Bacteria**	**Source**
Haloferax mediterranei	Josefa Anton, Spain	*Halomonas elongata*	Josefa Anton, Spain
Haloferax alexandrinus	M. Burcin Mutlu, Turkey	*Pseudomonas halophila* DSM 3050	Josefa Anton, Spain
Haloarcula hispanica	Michael Dyall-Smith, Australia	*Chromahalobacter salexigens*	Josefa Anton, Spain
Haloarcula mukohatei	M. Burcin Mutlu, Turkey	*Salinibacter ruber*	Josefa Anton, Spain
Haloarcula marismortui	M. Burcin Mutlu, Turkey	s	
Haloarcula argentinensis	M. Burcin Mutlu, Turkey		
Halococcus dombrowskii	Helga Stan-Lotter, Austria		
Halorubrum xinjiangense	M. Burcin Mutlu, Turkey		
Çamalti strains			
Archaea		**Bacteria**	
C23		C15	
C27		C18	
C29		C19	
C37		C21	
C43		C22	
C46			
C50			
C51			

C52	

Table 2: Halophilic Archaea and Bacteria strains used in the study.

Nine of the Archeal and five of the Bacterial strains isolated from the Çamaltı solar saltern in Turkey for a previous study were also included in the assay (Table 2). Domain tests of these isolates were carried out by PCR amplification of 16S rRNA genes using universal primers for Bacteria and Archaea, as indicated by Mutlu et al [12].

Growth Media. Halophilic microorganisms were grown on sea water agar (25% SW) containing (g L^{-1}): NaBr 0.65, $NaHCO_3$ 0.17, KCl 5, $CaCl_2$ 0.72, $MgSO_4$•$7H_2O$ 49.5, $MgCl_2$ •$6H_2O$ 34.6, NaCl 195, 1 g yeast extract (YE), and 20 g of agar, for one week at 37°C. Two loops of massive growth were then placed in Eppendorf tubes.

Sample Preparation. Cells were collected and dried in a vacuum evaporator at room temperature and then were ground into fine particles using a mortar and pestle. About one milligram of biomass was encapsulated in 100 milligrams KBr with a pressure of 100 kg/cm^2 (2000 psi) in order to prepare the sample pellet. Three sample pellets were prepared for each microorganism tested.

Fourier transform infrared spectroscopy and data analysis

Infrared spectra were obtained by scanning the pellets with a Perkin Elmer Spectrum 100. The spectrum of air was recorded as the background and subtracted automatically using the spectrum software program. Atmospheric vapour was also automatically subtracted. The FT-IR spectra of bacterial samples were recorded in the 4000-450 cm^{-1} region at room temperature. Three spectra were acquired for each bacterial sample.

The averages of the spectra belonging to the same strain, baseline correction, normalization and the bands were obtained by the software. The average spectra and normalization process were applied only for visual representation of the differences; however, for the determination of the spectral parameters and calculation of mean values and statistical analyses, each baseline-corrected original spectrum was taken into consideration.

Statistical analysis

The features that reflect the important characteristics of different biochemical components was taken into account while assessing the significant values in spectra. Hierarchical cluster analysis was applied to classify the differences in samples by using Clustan 8.0 software. For calculating the spectral pair-wise distance, the Euclidian distance was used in the 4000-450 cm^{-1} region. Average Linkage (UPGMA) method was used to join the clusters together.

Results

In this study, FT-IR spectroscopy was carried out on halophilic members of the Archaea and Bacterial domains and spectral differences and similarities were compared between the domains and species.

The FT-IR spectra of reference strains of Archaea are shown in Figure 1. As can be seen from the figure, there were differences mainly in the 450-1700 cm^{-1} region. Table 3 shows the regions numbered from 1 to 6 to discriminate Archaeal members. All of the Archaeal strains showed ether bonds (C-O) in the 1000-1300 cm^{-1} region. Also, CH_2 asymmetric stretching of methylene groups in fatty acids was observed at 2930 cm^{-1} as broadened peaks (peak no. 1).

The FT-IR spectra of Bacteria strains are given in Figure 2. As can be seen in the figure, there were differences mainly in the 1000-1700 cm^{-1} region, and typically strong ester bonds in the 1735-1750 cm^{-1} region.

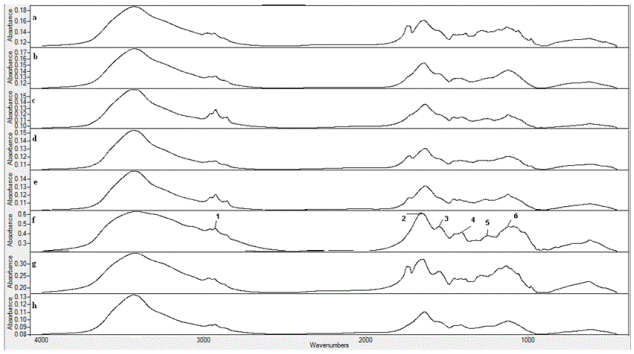

Figure 1: FT-IR spectra of strains belonged to Archaea domain. a: *Haloferax mediterranei*, b. *Haloferax alexandrinus*, c. *Haloarcula hispanica*, d. *Haloarcula mukohatei*, e. *Haloarcula morismortii*, f. *Haloarcula argentinensis*, g. *Halococcus dombrowski*, h. *Halorubrum xinjiangense*.

Peak number	Wavenumbers (cm^{-1})	Functional group assignments and remarks
1	2929.25	CH_2 asymmetric stretch: methylene groups in fatty acids
2	1659.46	Amide I (protein CO stretching): α helices
3	1549.2	Amide II (protein N–H bend, C–N stretch): α helices
4	1410.48	Symmetric CO_2 streching
5	1243.31	Aromatic ether band
6	1102	C-O stretch, ether band

Table 3: Comporison of halophilic reference *Archaea* species.

A strong peak indicating amide group N–H stretching at 3200 cm^{-1} (peak no. 1) was observed (Figure 2). CH_2 asymmetric stretching of methylene groups in fatty acids were observed at 2960 cm^{-1} as narrow peaks (peak no. 2-3). Table 4 shows regions 1 to 7 in Figure 2 used to discriminate Archaeal members. Hierarchical cluster analyses on FT-IR spectra from the reference Archaea and Bacteria strains resulted in the dendrogram shown in Figure 3.

Comparison of strains isolated from the camaltı saltern

The bands centered at 1000-1300 cm^{-1} corresponds to the C-O stretching of ether groups (Figure 4) for Archaeal strains obtained from the Çamaltı solar saltern. A strong band occurred at 1735-1750 cm^{-1} corresponding to C=O stretching of the esters (Figure 5) in Bacterial strains obtained from the Çamaltı solar saltern. Hierarchical

cluster analyses of the FT-IR spectra from Çamaltı strains of Archaea and Bacteria resulted in the dendrogram shown in Figure 6.

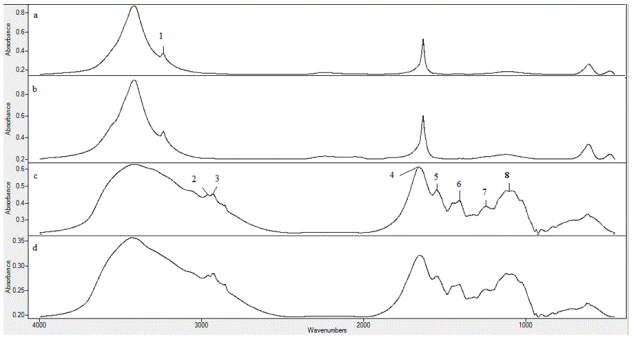

Figure 2: Discrimination between reference bacteria species: a: *Chromahalobacter salexigens*, b: *Salinibacter ruber*, c: *Halomonas elongata*, d: *Pseudomonas halophila*.

The spectra were analyzed for the differentiation of each genus/species. The FT-IR spectra for known examples of each class of bacteria were collected. These were used to generate a discrimination method based on the assignment of spectra to classes. As with any biological sample, there was considerable variation within each class, so region selection and spectral processing were critical.

Clear segregations between different domains, genera and species of bacteria were observed by hierarchical cluster analyses by using Clustan 8.0. Euclidean distances were taken into account.

Discussion

In this study, we used a spectroscopic approach for the rapid discrimination and identification of halophilic microorganisms. These extreme microorganisms are important for their newly discovered biotechnological purposes.

Peak number	Wavenumber	Functional group assignments and remarks
1	3235,74	Amide group N–H streching
2	2965,51	Aromatic C–H
3	2930,41	CH_2 asymmetric stretch: methylene groups in fatty acids
4	1735	C=O stretch, ester group
5	1547,67	Amide II (protein N–H bend, C–N
6	1410,79	Symmetric CO_2 streching
7	1249,36	ring C–H in- plane bending vibrations
8	1101,96	C–O streching, ester group

Table 4: Comporison of halophilic reference Bacteria species.

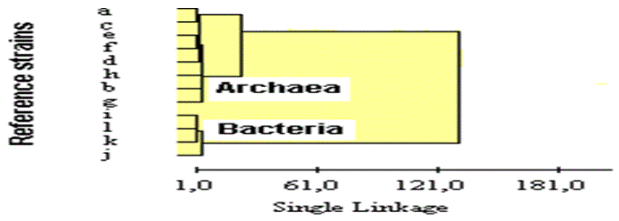

Figure 3: Classification of FT-IR spectra obtained from different strains of halophilic Archaea and Bacteria domains. Dendogram shows the dissimilarity level of strains tested by Clustan 8. Abbreviation: a: *Haloarcula argentinensis*, b: *Haloarcula mukohatei*, c: *Haloarcula hispanica*, d: *Haloferax alexandrines*, e: *Haloarcula marismortui*, f: *Halorubrum xinjiangense*, g: *Halococcus dombrowskii*, h: *Haloferax mediterranei*, i: *Chromahalobacter salexigens*, j: *Halomonas elongata*, k: *Pseudomonas halophila*, l: *Salinibacter ruber*.

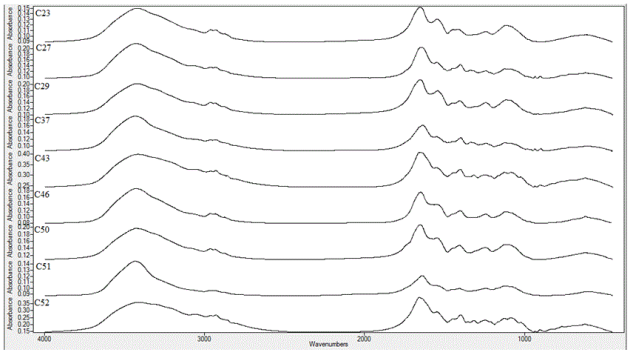

Figure 4: FT-IR Spectra of Archaeal strains obtained from Çamaltı saltern.

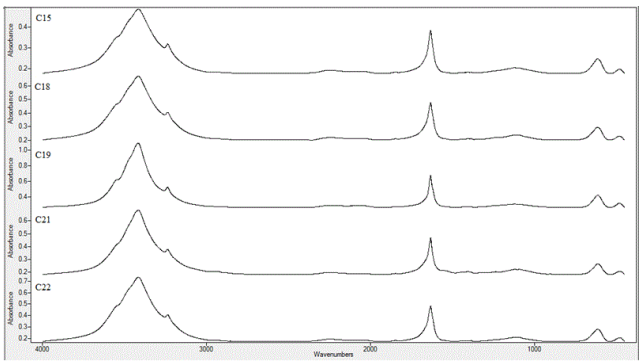

Figure 5: FT-IR Spectra of Bacterial strains obtained from Çamaltı saltern.

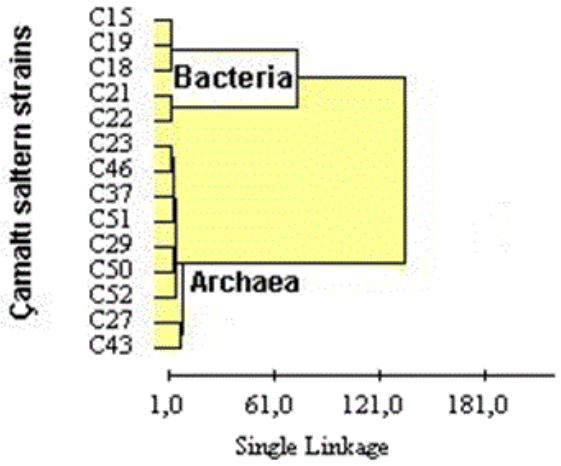

Figure 6: Classification of FT-IR spectra obtained from Çamaltı strains of halophilic *Archaea* and *Bacteria* domains. Dendogram shows the dissimilarity level of strains tested by Clustan 8. Strains numbered from C15 to C-52 were isolated from Çamaltı saltern.

Traditional methods used for the identification of various microorganisms are based on morphological characteristics, biochemical reactions, serological reactions, sensitivity to bacteriophages and 16s rDNA sequencing. However, these methods can often be tedious, time consuming and occasionally inconclusive [38].

The only study on the rapid differentiation of archaebacteria from eubacteria by diffuse reflectance FT-IR spectroscopic analysis of lipid preparations was carried out by Hedrick et al. [6]; the authors concluded that archaebacterial ether lipids had broadened peaks in the alkyl region between 2800-3000 cm^{-1}; however, eubacterial spectra were narrower and clearly separate [39]. It is known that ether bonds (C-O) appear in the 1000-1300 cm^{-1} region (http://www2ups.edu/faculty/hanson/spectroscopy/IR/IRfrequencies.html). We also observed that archaeal strains gave ether bands in this region; however, both reference and Çamaltı saltern bacterial strains had typically a strong ester bonds in the 1735-1750 cm^{-1} region.

The application of various vibrational spectroscopic techniques to identify and characterize microorganisms has been studied with Fourier transform infrared (FT-IR) spectroscopy are the most promising. FT-IR spectroscopy is a rapid and non-destructive technique that has been demonstrated to provide a highly sensitive and reproducible means for the identification of microorganisms without the use of reagents. Furthermore, it can provide results faster than virtually any other conventional method in microbiology, which are generally time consuming and labour intensive. Moreover, FT-IR identification methods have easy and simple protocols together with low running costs [50]. Infrared spectroscopy is based on the measurement of molecular vibrations [51]. Fourier transform infrared spectroscopy with high-resolution power is able to distinguish microbial cells at different taxonomic levels [52]. This technique is referred to as whole-organism fingerprinting because it measures the vibrations of chemical bonds within the biochemical constituents of cells [6]. The chemical composition of the cell contributes to the FT-IR spectrum [22]. As microorganisms consist of pure biochemicals, the IR spectra of these microorganisms provide few features and peaks since the bonds and functional groups of a particular molecular structure are often give a simple, unique, and reliable spectral fingerprint. The IR vibrations of microorganisms are related to thousands of other molecular components in microorganisms. With the use of different statistical methods like principal component analysis (PCA), hierarchical cluster analysis (HCA), partial least square regression (PLSR), soft independent modelling of class analogy (SIMCA), K-nearest neighbor (KNN), artificial neural networks (ANNs) and canonical variate analysis (CVA), extracting subtle details from the IR spectra of whole microbial samples has become possible. This method has been successfully applied to correlate the nearest neighbor along appropriate taxa [53-54]. In this study, clear segregations between different domain, genera and species of bacteria were observed by hierarchical cluster analyses.

In conclusion, identification of halophilic microorganisms using FT-IR seems to have great value, confirming that FT-IR can detect biochemical differences between halophilic prokaryotic cells in this preliminary study. However, future studies should be carried out to optimize the results by including many reference strains and environmental isolates.

This study has contributed basic data to the FT-IR studies of halophilic prokaryotes and the method could become a potentially useful tool for rapid differentiation of domains of Archaea and Bacteria at the strain level.

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