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ORIGINAL PAPER

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Identification of archaea and some extremophilic bacteria using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

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Abstract Archaea and a number of groups of environmentally important bacteria, e.g., sulfate-reducing bacteria, anoxygenic phototrophs, and some thermophiles, are difficult to characterize using current methods developed for phenotypically differentiating heterotrophic bacteria. We have evaluated matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-MS) as a rapid method for identifying different groups of extremophilic prokaryotes using a linear mass spectrometer (Micromass, UK). The instrument is designed to acquire mass-spectral patterns from prokaryotic cell-wall components between masses of 500 and 10,000 Da in a statistically robust manner and create a database that can be used for identification. We have tested 28 archaea (10 genera, 20 spp.) and 42 bacteria (25 genera, 37 spp.) and found that all species yield reproducible, unique mass-spectral profiles. As a whole, the profiles for the archaea had fewer peaks and showed less differentiation compared to the bacteria, perhaps reflecting fundamental differences in cell-wall structure. The halophilic archaea all had consistent patterns that showed little differentiation; however, the software was able to consistently distinguish *Halobacterium salinarium*, *Halococcus dombrowski*, and *Haloarcula marismortui* from one another, although it could not always correctly distinguish four strains of *Hb. salinarium* from one another. The method was able to reliably identify 10^5 cells of either *Albidovulum inexpectatum* or *Thermococcus litoralis* and could detect as low as 10^3cells. We found that the matrix, alpha-cyano-4-hydroxy-cinnamic acid yielded better spectra for archaea than 5-chloro-2-mercapto-benzothiazole. Overall, the method was rapid, required a minimum of sample processing, and was capable of distinguishing and identifying a very diverse group of prokaryotes.

Keywords Archaea · Bacteria · Extremophiles · Identification · Intact cell · MALDI-TOF

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Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) shows promise as a technique for the identification of intact microorganisms by providing a unique mass-spectral fingerprint characteristic of low (≥500 Da)- to intermediate (< 30,000 Da)- mass cellular components. In intact cell MALDI-TOF-MS or MALDI, cells are mixed together with an energy-adsorbing matrix and irradiated with a UV-laser; the matrix promotes laser energy transfer to the cells that results in ionization of cell-surface macromolecules, principally proteins. The ions are ejected under high vacuum and travel down a flight tube to a mass detector, and the result is a mass-spectral profile that should be cell-specific.

To date, much of the work using MALDI for microbial identification has focused on demonstrating that reproducible mass spectra can be obtained using intact cells and developing algorithms for interpretation and comparison of these spectra (Claydon et al. 1996; Holland et al. 1996; Krishnamurthy and Ross 1996; Demirev et al. 1999; Jarman et al. 2000; Bright et al. 2002; Wahl et al 2002). Several recent reviews provide excellent perspectives on whole-cell MALDI (Krishnamurthy et al. 2000; Lay 2000, 2001; Fenselau et al. 2001). The potential advantages that MALDI offers over other techniques for microbial characterization (such as genotyping, fatty acid methyl-ester analysis (FAME), or biochemical methods) include minimal sample preparation, rapid results, and negligible reagent costs. Perhaps most importantly, this technique is dependent on a trait common to all prokaryotes, namely, a complex macromolecular cell wall.

To our knowledge, no one has evaluated the effectiveness of using MALDI to characterize archaea, and with a few exceptions (Fastner et al. 2001), environmentally important bacteria have also received little attention. MALDI could be especially effective for these organisms, since, for the most part, standardized biochemical tests have not been developed. In addition, routine genotypic methods of comparison are not readily available beyond the sequencing of the SSU rRNA gene, which might not provide strain-level differentiation. The purpose of this work was to determine if characteristic and reproducible mass spectra could be obtained from a variety of archaea and bacteria. In addition, we evaluated other important factors that might affect the results, such as growth time, analyte concentration, matrix, and media type (agar vs broth).

We report here results from analyses of 74 different microorganisms, representing 29 different prokaryotic genera and 57 species. The microorganisms include thermophiles, halophiles, methanogens, sulfate reducers, phototrophs and cyanobacteria, *Streptomyces*, and acidophilic iron oxidizers. Each organism displayed a unique pattern that was reproducible, suggesting this technology has promise for the authentication of all prokaryotes.

Methods and materials

Instrumentation

We used a Micromass-Waters MALDI-TOF-MS (MALDI-L) operated in linear mode. The instrument utilized a 337-nm nitrogen laser to excite matrix/specimen compounds and generate ionized molecules that are launched into a flight tube between a 15-kV potential and registered at the detector operated in positive-ion mode. The entire system operates under a high vacuum. This machine comes with Masslynx software for instrument control and acquisition of spectra and Microbelynx software designed for establishing a database of bacterial profiles and subsequent comparative analysis.

For data acquisition, the laser fires in a random pattern (spot size approximately 2 μm) at different points within a sample well until 15 spectra per well of sufficient intensity for data analysis are acquired. The instrument then moves to a calibration well and performs a lock mass calibration on a set of standard polypeptides of known molecular weight (MW) before moving to the next sample well. When the laser is operated at a firing rate of 20 MHz, it takes between 60 and 90 min to acquire the data from a 96-well plate. The data is then processed with the Microbelynx software. This software integrates all the spectra that are collected for a given organism (typically 12 replicates of 15 spectra each) and performs a statistical analysis to determine if the spectra are acceptable, i.e., the standard deviation for all the analyte peaks from different spectra are within a threshold value. All the acceptable data are integrated into a reference pattern that is stored in the database and used for comparisons. The software differentiates spectra based on both peak placement and intensity. For comparisons of samples within the database, a root mean square (RMS) value is reported for the comparison of different profiles; the closer this value is to zero, the better the match.

Matrices and standards

Two different matrix solutions were used: α-cyano-4-hydroxycinnamic acid (CHCA) for Gram-negative bacteria and archaea, and a saturated solution of 5-choloro-2-mercaptobenzothiazole (CMBT) for Gram-positive bacteria. They were both made up in a 1:1:1 water:acetonitrile:methanol mixture that contained 0.1% (v/v) formic acid and 0.01 M 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6). Crown ether is useful for removing metal-ion adducts, and formic acid promotes positive ion formation in the sample material (Evason 2001). Matrix solutions were made fresh daily by adding 3.0 mg CMBT or 14.0 mg CHCA to 1.0 ml acetonitrile/methanol solution and mixing for 15 min in a sonicating water bath.

A calibration solution of seven polypeptides of known MW was made using a set of standards. A stock solution (1 mg/ml) of each standard was made up in HPLC-grade water, and stored at −20°C. The calibration mix was made as follows: 2.00 μl bradykinin (1,060 Da), 2.60 μl angiotensin (1,296.5 Da), 3.14 μl glu-fibrino peptide B (1,570.6 Da), 3.52 μl renin substrate tetra decapetide (1,759 Da), 4.94 μl ACTH (18-39, 2,465.7 Da), 22.93 μl insulin (bovine, 5,733.5 Da), and 171.20 μl ubiquitin (bovine, 8,564.9 Da) were added to 789.67 μl 0.1% TFA solution. The calibration mix could also be stored at –20°C. All chemicals were from Sigma/Aldrich Chemical (St. Louis, Mo.).

Culture preparation

All cultures used in this study were from the American Type Culture Collection (ATCC) and are listed in Table 1; growth media and conditions are available online (http://www.atcc.org: choose "Bacteria" under "Search a collection", then click on "Media formulations"). Most of the previous studies that have analyzed intact bacterial cells using MALDI have focused on common laboratory bacteria that were grown on agar plates using standard heterotrophic media. The microbes used in this study were metabolically diverse; many of them grew best in liquid medium. For this reason, most of the organisms tested here were grown in tubes of broth, each containing 5 or 10 ml of the appropriate medium. The cells were grown to the late-log phase of growth, and then harvested by centrifugation (14,000 rpm for 2 min), and the cell pellet was washed two times with HPLC-grade water or a salt solution depending on the salt tolerance of the organism. After the second wash, the cell pellet was resuspended in 30 μl of HPLC-grade water. A 1-μl subsample of the cell

Table 1 Organisms and media used in this study. *ATCC* American Type Culture Collection

Organism name	ATCC number	ATCC medium[a]
Archaea		
Aeropyrum pernix	700893	2183
Haloarcula marismortui	43049	1218
Halobacterium salinarium	17051	217
H. salinarium	17052	217
H. salinarium	29341	217
H. salinarium	33170	217
H. salinarium	33171	217
Halococcus dombrowskii	BAA-364	2301
Methanocaldococcus jannaschii	43067	2121
Methanococcus maripalidus	43000	1439
Methanothermococcus thermolithotrophicus	35097	1439
Methanococcus vannielii	35089	1439
Methanocaldococcus vulcanius	700851	2121
Methanothermobacter thermautotrophicus	29096	2133
M. thermautotrophicus	35610	2133
M. thermautotrophicus	43846	2133
M. thermautotrophicus	700791	2133
Pyrococcus furiosus	43587	1915
Thermococcus celer	35543	1915
T. gorgonarius	700654	1922
T. litoralis	51850	1922
T. litoralis	55233	1922
T. pacificus	700653	1922
T. peptonophilus	700098	1922
T. profundus	51592	1922
T. siculi	BAA-270	1922
Thermococcus sp.	55659	1922
T. sulfurophilus	BAA-394	1922
Bacteria		
Acidithiobacillus ferrooxidans	53985	2039
A. ferrooxidans	23270	2039
A. thiooxidans	8085	125
A. thiooxidans	15494	125
Amycolatopsis mediterranei	27643	196
Anoxybacillus kamchatkensis	BAA-549	2359
Caloramator uzoniensis	BAA-503	2107
Chlorohenpeton thalassium	35110	2198
Dechlorosoma sp.	BAA-592	2361
Dechlorosoma sp.	BAA-593	2361
Desulfitobacterium hafniense	BAA-391	Lactate
Desulfonatronum paiuteum	BAA-395	2319
Leucovolum marinus	BAA-387	2308
L. marinus	BAA-388	2308
Nostoc sp.	29133	819
Oxalophagus oxalicus	49686	2309
Rubrobacter taiwanensis	BAA-406	2322
R. taiwanensis	BAA-407	2322
Sphaerotilus natans	13338	1103
Spirocheta americana	BAA-392	2311
Spirulina platensis	53844	1679
Streptomyces capensis	BAA-411	196
Streptomyces coelicolor	BAA-471	196
Streptomyces toxytricini	19813	196
Tetragenococcus halophilus	33315	1148
Thermotoga lettingae	BAA-301	2299
Thermus aguaticus	25105	461
Thermus antranikianii	700961	461
Thermus filiformis	43280	461
Thermus igniterrae	700962	461
Thermus lacteus	31557	461
Thermus oshimai	700435	461
Thermus scotoductus	51532	461
Thermus sp.	31674	461
Thermus sp.	700910	461
Thermus sp.	BAA-302	461
Thermus taiwanensis	BAA-405	2322
Thermus thermophilus	33923	461
T. thermophilus	BAA-163	461
Tindallia californica	BAA-393	2310
Truepera sp.	BAA-389	461
Victivallis vadense	BAA-548	2362

[a]Medium recipes are available at http://www.atcc.org under "Bacteria," then "Media formulations"

suspension was added to each of 12 wells of a MALDI plate using a 10-µl pipette. Alternatively, in a few cases, cultures grown on agar plates were used; then a single colony was picked and placed in the well with a sterile loop. Each stainless steel MALDI plate holds 96 samples plus wells for calibration or lock mass standards. After the sample had dried for approximately 1 h, 1 µl of the appropriate matrix solution was added to the same wells, and the plate dried for an additional 15 min. A standard peptide mixture was mixed 1:1 with CHCA and 1 µl of this

standard solution was added to each lock mass well on the plate. After the plate had dried, it was ready for analysis either immediately, or it could be stored in a desiccator at room temperature for several days before analysis.

To study the effects of culture age on the mass-spectral profiles, the experimental strains (*Amycolatopsis mediterranei*, *Streptomyces coelicolor*, and *Thermus thermophilus*) were grown in 30-ml volumes of the appropriate medium. At the prescribed time periods, 1.5 ml of culture media was removed from the flask and processed for MALDI as described above.

Plate cleaning

The standard plate used for the MALDI-TOF has 96 etched sample wells and 24 lock mass-correction wells. The plates could be reused. To clean them, they were soaked in HPLC-grade methanol for 30 min, then rinsed in distilled water and scrubbed using a soft-bristle toothbrush. They were then submerged in a beaker containing 30% (v/v) nitric acid solution, and sonicated in a water bath (Fisher Scientific FS20) for 30 min. They were then rinsed three times with distilled water, sonicated in HPLC-grade water (30 min) and sonicated again in HPLC-grade methanol (30 min). Finally, they were air dried and stored in a closed container until use.

Results

Spectral variation

In every case, the organisms listed in Table 1 yielded reproducible mass spectra. Figure 1 shows the mass-spectral fingerprints obtained from a representative group of environmental bacteria. As is evident, the spectra differ significantly between the different organisms, with patterns ranging from quite simple, e.g., *Acidithiobacillus ferrooxidans* to complex, e.g., *Streptomyces coelicolor*. As yet, we have not found an organism that does not produce a spectral fingerprint. A common feature of the spectral profiles from most of the bacteria and the archaea is that the mass range of the spectral peaks is normally below 3,000 Da, with the majority of peaks between 500 and 2,000 Da. Compared to CMBT, the matrix CHCA consistently gave better peak intensities and more reproducible spectral profiles for the archaea (comparisons not shown) as well as for the Gram-negative bacteria. The work of others has shown that CHCA works better with Gram-negative organisms, and CMBT works better with Gram-positives (Evason 2001); thus, our results are consistent with these findings and extend the use of CHCA to archaea.

Sensitivity

Two unrelated organisms, an archaeon *Thermococcus litoralis* ATCC 55233, and a marine, heterotrophic, moderately thermophilic bacterium *Albidovulum inexpectatum* BAA-387 (Albuquerque 2002) were used to determine the sensitivity of the method to cell concentrations. *T. litoralis* cells were concentrated to 5.2×10^9 cells/ml, and this cell suspension was diluted 1:5, 1:10, 1:50, 1:100, and 1:1000. Analysis was done on 1-µl aliquots from the undiluted suspension (1:1) and each of the dilutions, which yielded the following cell concentrations: 5.2×10^6, 1.04×10^6, 5.2×10^5, 1.04×10^5, 5.2×10^4, and 5.2×10^3. The same procedure was

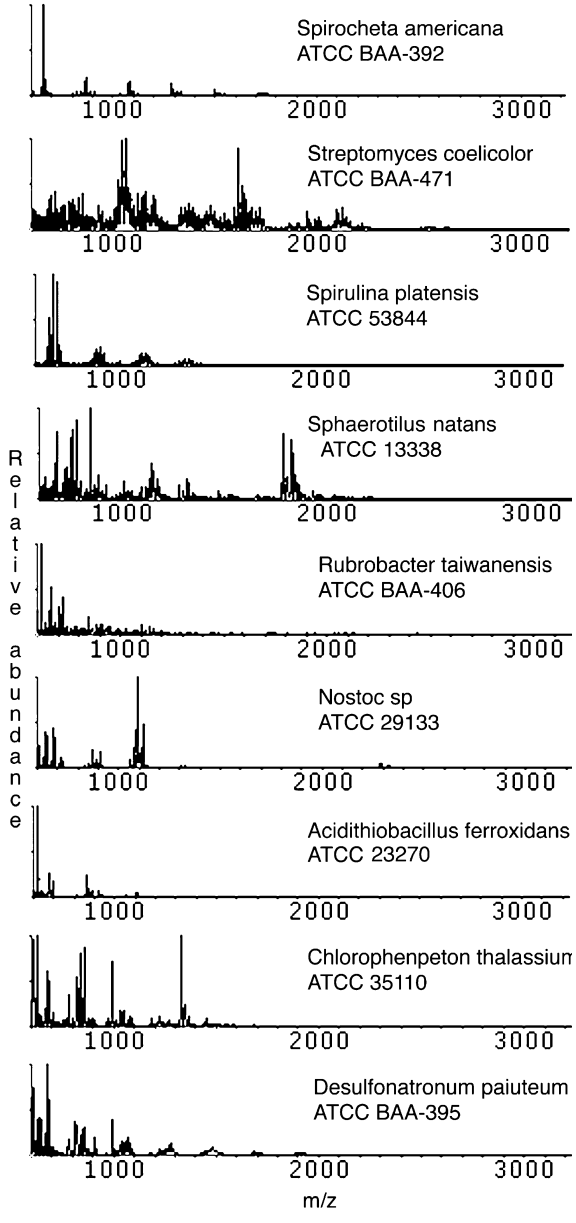
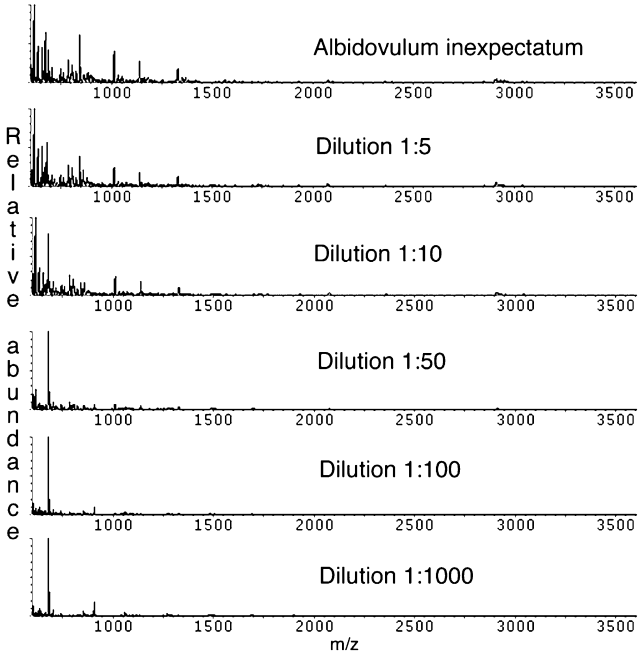


Fig. 1 Mass-spectral variation among diverse bacteria. *Spirocheta americana* is an alkaliphilic free-living spirochete; *Spirulina platensis* and *Nostoc* sp. are cyanobacteria; *Sphaerotilus natans* is a sheath-forming bacterium; *Acidithiobacillus ferrooxidans* is an acidophilic iron oxidizer; *Desulfonatronum paiuteum* is an alkaliphilic sulfate-reducing bacterium; *Rubrobactor taiwanensis* is a themophilic aerobe that grows at 60°C; and *Chlorophenpeton thalassium* is a photosynthetic green sulfur bacterium. The *m*/*z* on the *x-axis* gives the mass:charge ratio



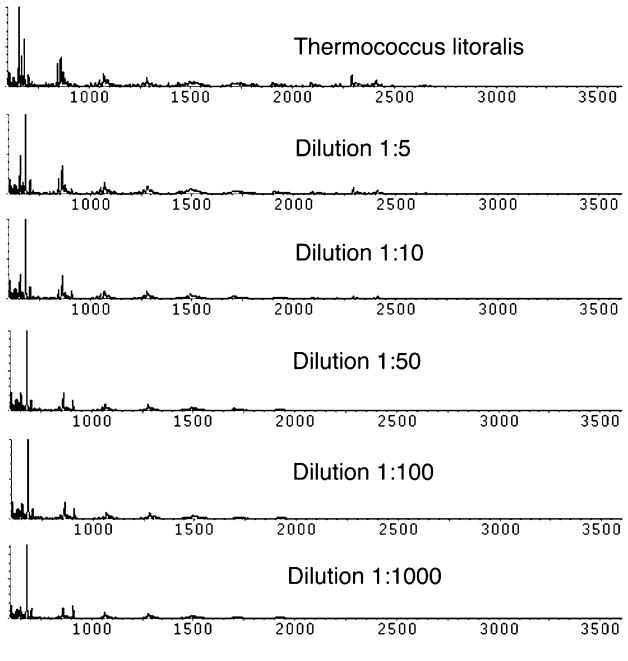


Fig. 2 Sensitivity test for a bacterium, *Albidovulum inexpectatum*, and an archaeum, *Thermococcus litoralis*. *A. inexpectatum* was able to be reliably identified at a dilution of 1:100, which corresponded to 3.4×10^5 cells. *T. litoralis* was identified reliably up to a dilution of 1:10, which corresponded to 5.2×10^5 (see text for details). As is evident, spectral peaks were still present at dilutions of 1:1,000, but reliable identifications could not be made

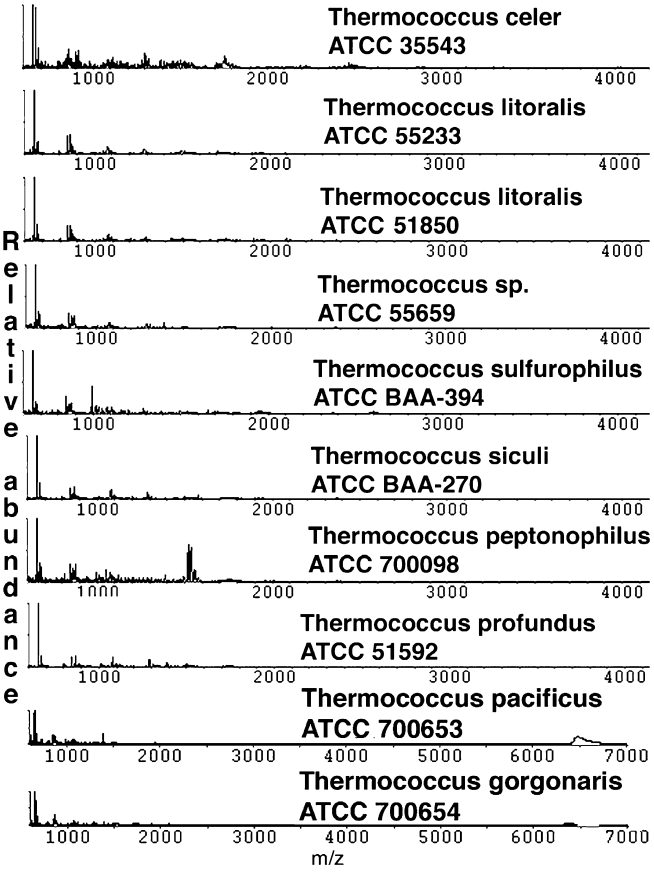


Fig. 3 Mass-spectral profiles for *Thermococcus* spp. The type strain of *T. litoralis* is 51580

used for *A. inexpectatum*, starting with a concentrated cell suspension of 3.4×10^{10} cells/ml, and the following dilutions: 3.4×10^7 (1:1), 6.8×10^6 (1:5), 3.4×10^6(1:10), 6.8×10^5 (1:50), 3.4×10^5 (1:100), and 3.4×10^4 (1:1,000). As expected, for both organisms the peak height and the number of peaks of each spectrum decreased with increasing dilution (Fig. 2). For *A. inexpectatum*, the reliability of an identification as reflected in the RMS values was 1.83 (1:1), 0.87 (1:5), 0.25 (1:10), 1.54 (1:50), 2.42 (1:100), and there was no identification at 1:1,000. For *T. litoralis*, the RMS values were 2.41 (1:1), 2.26 (1:5), 2.85 (1:10), and Microbelynx was unable to identify the organism and calculate an RMS value at higher dilutions. These results suggest that 10^5 to 10^6 cells are best for yielding a reliable identification, although prominent peaks for a given organism can be detected with as few as 10^3 cells. These results are similar to those of Evason et al. (2001) who showed that they could identify 10^4*Escherichia coli* cells. A report on using MALDI to identify *Bacillus* spores showed that as few as 5,000 spores could be detected (Hathout et al. 1999).

Identification of *Thermococcus* and *Thermus*

To test the ability of the MALDI-TOF to differentiate between species within the genus *Thermococcus*, ten different strains of *Thermococcus* spp. were selected that represent nine different species. Nine of these strains were grown in a sulfur-containing marine medium (Neuner 1990), except for *T. celer*, which was

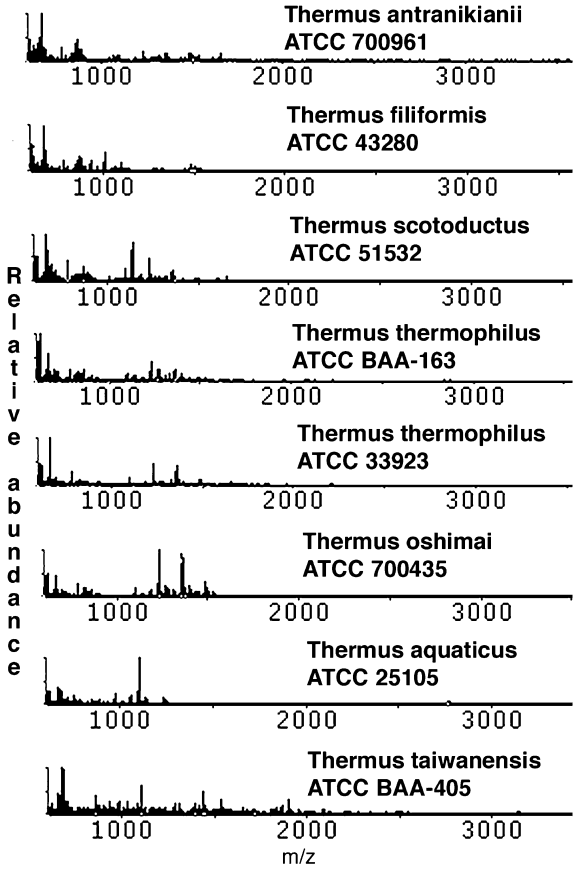


Fig. 4 Mass-spectral profiles for *Thermus* spp. Compare the overall diversity of the patterns for these thermophilic bacteria to those of the thermophilic archaea in the *Thermococcus* group

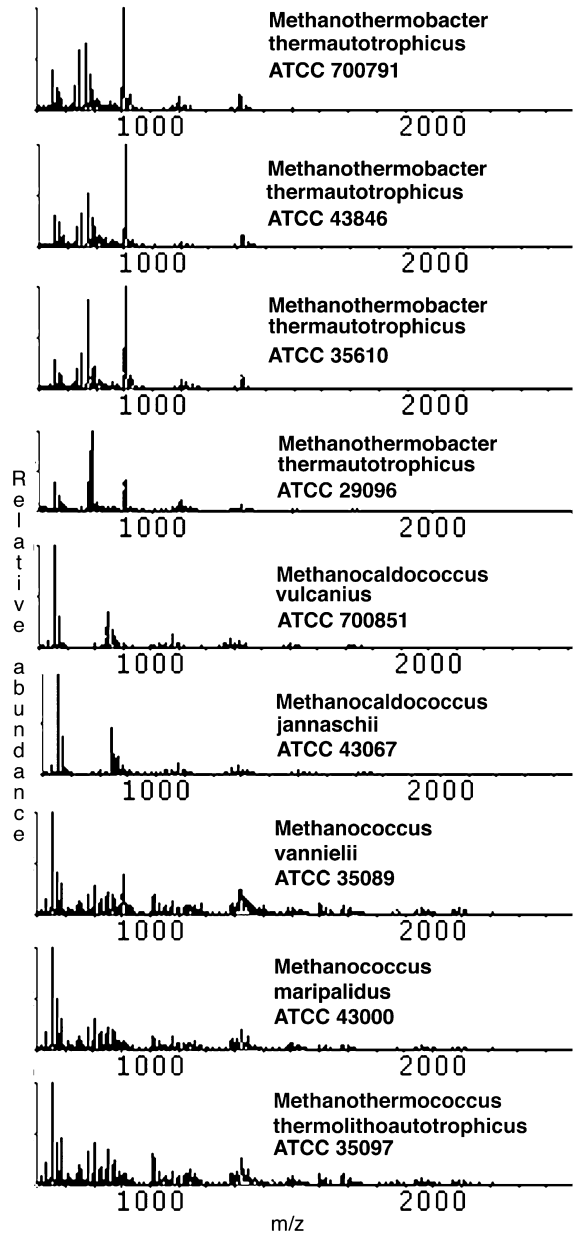


Fig. 5 Mass-spectral profiles for a group of methanogens. The type strain of *Methanothermobacter thermautotrophicus* is 29096. *Methanococcus vannielii* and *Mc. maripaludis* are mesophiles; the others are all thermophiles

grown in a sulfur-containing *Pyrococcus* medium (Zillig 1983). A representative composite spectrum from each strain is shown in Fig. 3. As visual inspection shows, there was less variation between the spectra of these related species than there was in patterns from the widely divergent bacteria shown in Fig. 1. All strains shared a dominant peak with a mass around 655 Da and then had a varying number of peaks of less intensity. The two *T. litoralis* strains had patterns that are very similar. *T. pacificus* and *T. gorgonarius* were both unusual in having small but consistent peaks at around 6,500 Da. Despite the visual similarities in the spectral profiles for the *Thermococcus* strains, the Microbelynx software was able to correctly identify each strain based on the database comparison. This was based on a minimum of three tests for each strain.

For comparison between thermophilic archaea and thermophilic bacteria, spectra from eight strains representing seven different species in the bacterial genus *Thermus* are shown in Fig. 4. The *Thermus* strains were all grown in the same media at a temperature of 65°C. The spectra of these organisms showed greater diversity both in the size range and intensity of the peaks compared to *Thermococcus*. The two strains of *Thermus thermophilus* showed the most similarity of all the *Thermus* strains tested, but were readily distinguished by the software.

Identification of methanogens and halophiles

We tested nine strains of methanogens, including four strains of *Methanobacter thermautotrophicus*, as well as other thermophilic and mesophilic isolates that represented seven different species within four genera of methanogens. A representative composite spectrum for each strain is shown in Fig. 5. Once again, the patterns

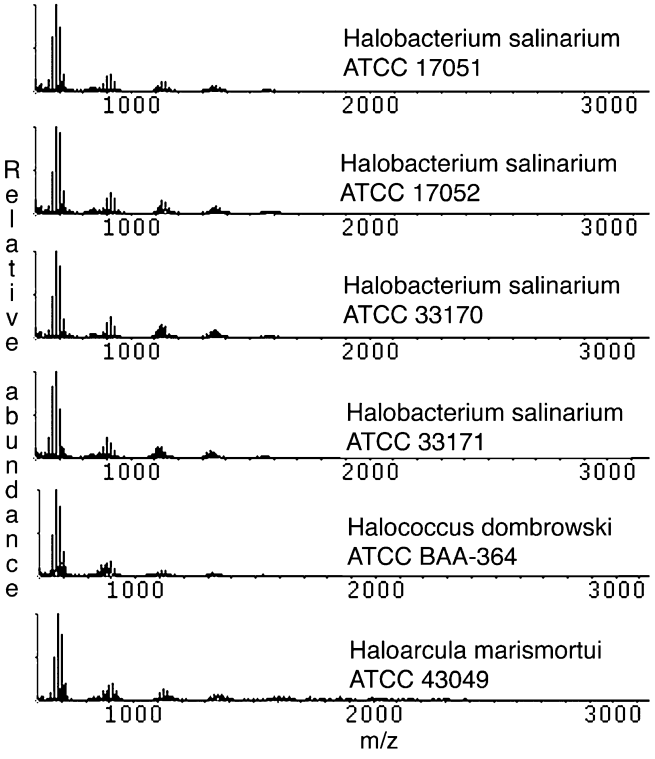


Fig. 6 Mass-spectral profiles for some extreme halophiles. This group had the most uniform patterns of any of the organisms that were tested. The type strain of *Halobacterium salinarium* is 33171

within a given genus appear relatively uniform; however, there are significant differences between genera. The four *Mtb. thermautotrophicus* strains all yielded profiles with only subtle differences in peak placement and intensity. The Microbelynx software was able to consistently identify all these strains as *Mtb. thermautotrophicus*, although it did not always resolve the strains correctly.

We also tested a group of halophiles representing three different genera and including four strains of *Halobacterium salinarum*. Representative spectra of these strains are illustrated in Fig. 6. Visually, it is clear that the patterns for the halophiles are the most uniform of all the archaea, regardless of the genus or species. The four *Halobacterium salinarium* strains differ from one another primarily by peak intensity. They differ from the other two genera by peak intensity and small shifts in the masses of some peaks.

Because of the high overall similarity of the halophiles, the RMS values were compared for all of these organisms and the results are shown in Table 2. In this case, each organism was searched against the Microbelynx database and the RMS match was recorded. The closer to zero the RMS value was, the better the identification. As the results indicate, the four *Hb. salinarium* strains all share RMS values close to or below 1, and each strain identified with itself with the lowest RMS value (Table 2). The *Hb. salinarium* strains do not match as well with the *Halococcus dombrowski*, and *Haloarcula marismortui*. *Methanococcus jannaschii* was included as an unrelated strain. These results are consistent with the database search results, which consistently match *Hb. salinarium* with the correct species, but did not always identify the correct strain.

Effect of culture age

We conducted a limited study on how the growth phase of an organism effected the mass-spectral profiles. Results from two organisms, the thermophilic bacterium *Thermus thermophilus* (ATCC BAA-163) and a streptomycete, *Amycolatopsis mediterranei* (ATCC 27643), are shown in Fig. 7. *T. thermophilus* reached the late log phase of growth by 25 h, and as the profiles show, there was little change through 65 h. All profiles taken after 10 h were identified as *T. thermophilus* by the database. For *A. mediterranei*, the case was different. This organism reached late log phase at 5 days, subsequent profiles (9 and 14 days are shown) all showed significant changes and the database could not reliably identify *A. mediterranei* at these subsequent time points. Another streptomycete, *Streptomyces coelicolor* (ATCC BAA-411), yielded similar results (not shown). These latter findings are not surprising, given that streptomycetes undergo significant morphological differentiation during growth as well as begin producing secondary metabolites in the stationary phase (Korn-Wendisch 2003).

Table 2 Root mean square matrix comparing halophilic archaea

	33170 *Halobacterium salinarium*	33171 *H. salinarium*	17051 *H. salinarium*	17052 *H. salinarium*	BAA-364 *Halococcus dombrowskii*	43049 *Haloarcula marismortu*	43067 *Methanocaldococcus jannaschii*
33170 *Halobacterium salinarium*	**0.25**						
33171 *H. salinarium*	1.60	**0.28**					
17051 *H. salinarium*	1.19	1.31	**0.19**				
17052 *H. salinarium*	0.84	1.13	0.62	**0.26**			
BAA-364 *Halococcus dombrowskii*	2.15	1.36	1.68	2.04	**0.14**		
43049 *Haloarcula marismortu*	3.01	3.66	3.56	2.59	3.33	**0.37**	
43067 *Methanocaldococcus jannaschii*	11.49	8.26	10.34	11.35	5.55	9.56	**0.2**

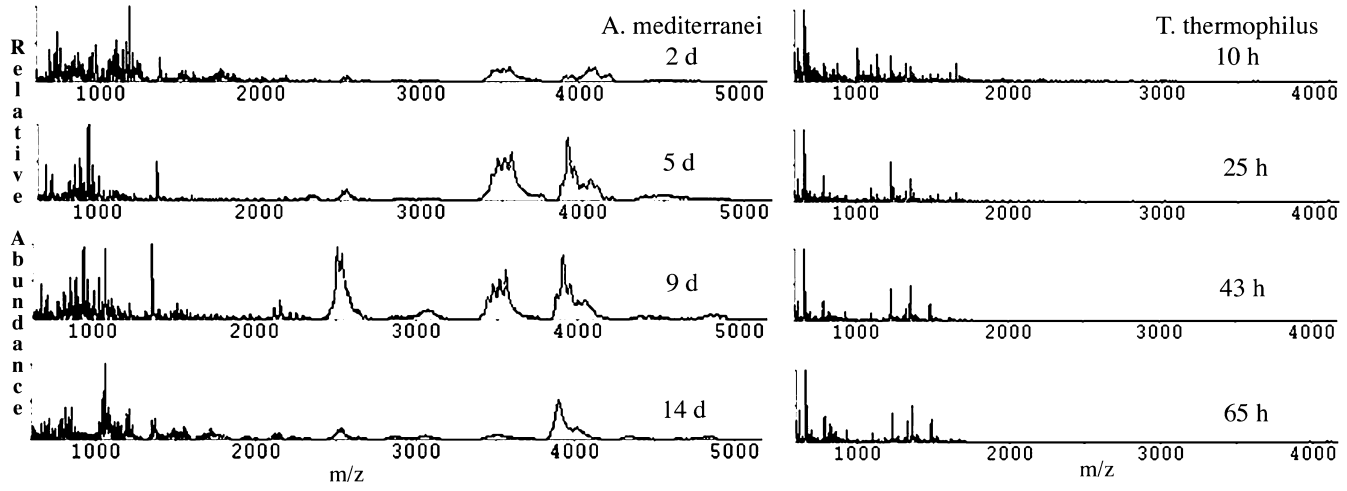


Fig. 7 Effect of culture age on mass-spectral profiles. The *left panel* shows the results for the streptomycete *Amycolatopsis mediterranei* with times in days (note the appearance and disapperance of peaks especially of higher mass). The *right panel* shows the thermophile *Thermus thermophilus* (note that by 25 h, late log phase, the pattern becomes invariant)

Discussion

Our findings are consistent with those of others who have successfully used MALDI to characterize and type strains of bacteria; however, we have used a commercially available MALDI and software package to significantly expand the range of prokaryotes that can be authenticated using this technique. To our knowledge, this is the first time that members of the archaea have been analyzed by MALDI. It is encouraging that all these organisms yield spectra and can be reliably identified.

The mass-spectral profiles were predominated by relatively low-MW ions. The detection cutoff for the instrument was set at 500 Da, and most of the ions that were detected were between 500 and 2,000 Da. It has been pointed out that a potential advantage of using lower laser energies (15 kV, in this case) for intact cell analysis is that it reduces cellular damage by the laser; that is, only molecules on the cell surface are ionized (Evason 2001). Higher laser energies can rupture the cell surface and result in ionization of cytoplasmic molecules. In addition, washing the cells in water or a salt solution rather than treating them with TFA, as used by many investigators, may reduce cell lysis and result in more specificity for cell-wall components. While this cell-wall-biased analysis limits the amount of information available in comparison to cell-lysis methods, it might also increase the reproducibility of the signal. Cell-surface macromolecules are thought to vary less as a function of growth rate or stage of a given cell culture. The precise cellular constituents that make up the mass profiles are not known. It is thought the ions consist of smaller polypeptides, oligosaccharides, and higher-MW lipid fragments (Bright et al. 2002).

As noted above, the mass-spectral profiles for the archaea, as a whole, appear more uniform with a more limited number and distribution of peaks than for the bacteria. In general, the signal intensity of peaks for the archaea is less than that of the bacteria. Since we do not know the identities of the individual mass peaks, we are unsure of what fundamental cellular properties may underlie this difference; however, there are very obvious differences in the cell-wall structure of bacteria and archaea. The cell walls of archaea often contain glycoprotein surface layers or S-layers that are composed of repeating protein subunits (Kandler 1998). This is especially true for members of the *Thermococcaceae* and the extreme halophiles (Boone 2001). The cell walls of *Methanothermobacter* spp., on the other hand, consist of pseudomurein, which is analogous to the thick murein or peptidoglycan layers of the Gram-positive bacterial cell wall, but is composed of different aminosugars (Kandler 1998). Given the similarity between pseudomurein and murein, we do not understand why better spectra were obtained using CHCA as a matrix than CMBT, since the latter generally works better with Gram-positives (Evason 2001). Understanding matrix interactions with diverse macromolecules is a continuing challenge in MALDI-TOF and will require further analysis.

The extremely halophilic archaea present a special problem because they must be maintained in a high-salt solution, including washing in a 20% NaCl solution to prevent the cells from lysing. Because Na^+ ions are known to cause adducts upon ionization (Watson 1997), this in part may explain the quite uniform patterns for all the halophiles. While the addition of crown ether (see "Materials and methods") may reduce the formation of adducts, the high concentration of Na^+ ions used with the halophiles may have saturated the crown ether. When 20% salt solutions are analyzed alone by MALDI, they yield spectra between 500 and 2,000 Da, although these are significantly different from the organismal spectra and much reduced in intensity (data not shown).

Reproducibility is, of course, a key component of any prokaryotic identification scheme. Under the conditions described here, the Microbelynx software

was consistently able to identify a microbe by comparison of its composite spectrum with the reference spectra in the database. When the organisms used in this study were compared against the "extremophile" database that was created as a part of this study and that had 65 entries, in ≥90% of the cases the correct match at the species level was made. In the cases of *Halobacterium salinarium* and *Mtb. thermautotrophicus,* where multiple strains of the same species were compared, the correct species was identified, but the correct strain was not always called. Since our extremophile database is limited, we compared *Thermococcus* spp., halophiles, and methanogens against a much larger database of 389 strains, and again, in ≥90% of the cases they were correctly identified to the species level (results not shown). This latter database consists mostly of strains of Gram-negative and Gram-positive bacteria that are primarily of clinical interest.

At this point, it is not clear the degree to which relationships can be made between organisms based on mass-spectral profiles. The Microbelynx software was used to create a dendrogram comparing the spectral profiles for the halophiles and the methanogens (Fig. 8). The two species of *Methanocaldococcus* cluster together and are distinct from the other *Methanococcales*. The two mesophilic *Methanococcus* species also show a high degree of similarity. The dendrogram shows that the four strains of *Mtb. thermautotrophicus* also cluster together as would be expected based on their conserved spectral profiles (Fig. 5). This analysis clusters most of the halophiles together in a closely related group, with the exception of *Haloarcula marismortui*, which clusters with the methanogens. The sulfate-reducing bacterium *Desulfonatronum paiuteum* (Pikuta et al. 2003) was included in this analysis as a phylogenetic outgroup; however, it clustered with the halophile group.

This latter finding that a bacterium clustered in the midst of a group of archaea would suggest it is difficult to infer anything beyond close identities based on dendrogram comparison. On the other hand, this method did resolve two species of *Methanocaldococcus* from the other *Methanoccocus* spp. (Fig. 7). Until recently *Mc. jannaschii* and *Mc. vulcanius* were members of the genus *Methanococcus*; however, a new systematic analysis showed they warranted their own genus (Whitman 2001). It will require substantially more data and a more refined analysis of specific prokaryotic groups before judgments can be made about the capability of MALDI for higher-taxa-level discrimination. It is unlikely that MALDI will provide the phylogenetic discrimination for identifying microbes afforded by standard methods like sequence comparison of SSU rRNA genes.

In terms of higher-order taxonomic comparisons, it must be remembered that intact-cell MALDI is characterizing a phenotypic state of the cell that can be sensitive to growth conditions (Fenselau 2001; Lay 2001). Composition of the growth medium, time of growth, gas composition of the headspace, as well as temperature and pH all have the potential to effect the results. In this study, we attempted to minimize the types of media that were used; nevertheless, given the extreme metabolic diversity of the organisms tested, by necessity most were grown on different media. There are relatively few MALDI studies that have looked at the effects of media

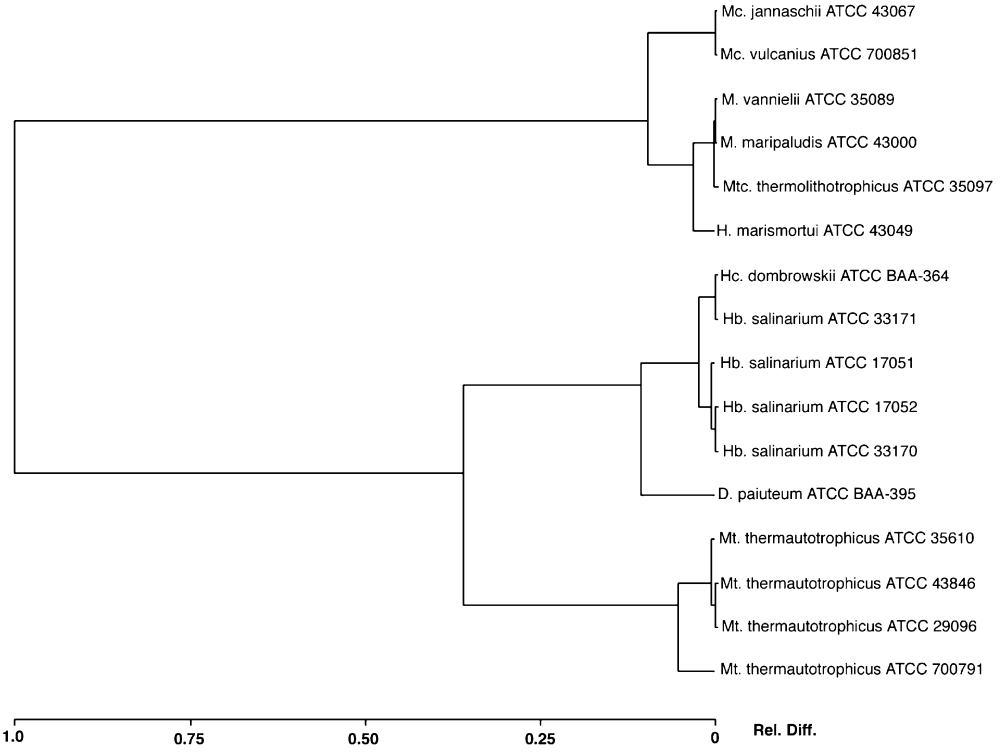


Fig. 8 A dendrogram created using the Microblynx software showing the relative differences between the methanogens and the extreme halophiles. *Desulfonatronum paiuteum* is a mesophilic, alkaliphilic bacterium that was included as an outgroup to these Crenarchaeota

composition on mass-spectral profiles. One study did show that the spectra for *Staphylococcus aureus* were consistent when it was grown on the same media, but the spectra varied with media composition (Lay 2000; Walker et al. 2002). This is not surprising, and it is presumed that so long as growth media and conditions are kept consistent, the profiles should be comparable.

Several studies have looked at the effect of culture age on the reproducibility of MALDI patterns in enteric bacteria and shown there can be significant changes in the overall patterns, although some ion peaks may remain quite constant with time (Arnold 1999; Saenz 1999; Lay 2000). The results shown here suggest that for organisms that are recognized for undergoing differentiation during growth, there will be substantial changes in their mass-spectral profiles (Fig. 7). However, *T. thermophilus* showed little change in its MALDI profiles well into the stationary phase of growth. Again, further studies will be required to establish the extent to which the effects of culture age can be generalized for different groups of prokaryotes.

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