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ABSTRACT -- Two bacterial species were isolated from a salt marsh located on privately owned land in Russell County, Kansas. Water samples from the salt marsh were streaked for isolation on tryptic soy agar supplemented with 12 % NaCl. Visual scanning of the plates revealed two prominent colony types. The two colony types were subcultured repeatedly until axenic cultures were obtained. Both of these organisms were shown to be moderately halophilic. The organisms were characterized partially by fatty acid methyl ester analysis, 16S rRNA sequencing, and scanning electron microscopy. These studies revealed that the bacteria previously were unreported members of genera *Marinococcus* and *Halomonas*.

Key words: Electron microscopy, fatty acid methyl ester analysis, *Halomonas*, *Marinococcus*, moderate halophiles, salt marsh, 16S rRNA sequencing.

Halophiles have a worldwide distribution and have been isolated from a wide variety of habitats, including areas of both low and high salt concentrations (Ramos-Cormenzana 1993). Typical sites of halophile isolation have included unpurified salt crystals, saline soils, saltern ponds, saline lakes, deserts, oceans, and salted hides or foods (Ventosa et al. 1998). In one report, *Halomonas muralis* was found colonizing paintings and murals in a castle in Austria (Heyrman et al. 2002). Halophilic bacteria can be either Gram negative or positive, and can exhibit either aerobic or facultatively anaerobic metabolism. They have been shown to grow well in a variety of salt concentrations ranging from 0.2 to 5.2 M (Kushner 1993). Many halophiles have demonstrated the ability to maintain cellular integrity

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in even hypersaline environments, which are those in which the salt concentration is higher than the 3.5 % commonly found in seawater (DasSarma and Arora 2002). The salt required by halophiles need not be sodium chloride, but might be a number of other ions, as has been recently shown for the moderate halophile *Chromohalobacter salexigens* (O'Connor and Csonka 2003). The hypothesis that halophiles might play a role in the bioremediation of selenium-contaminated agricultural soils has been posited (de Souza et al. 2001). The optimum growth temperature of moderate halophiles is influenced by salt concentration, with optimum growth at 4°C occurring in 3.5 %, optimum growth at 15 to 45°C occurring in 20 %, and optimum growth at 23 to 30°C occurring in 32 % NaCl (Vreeland et al. 1980). Due to their diversity and resilience, halophiles also have been of interest to astrobiologists when considering possible characteristics of extraterrestrial micro-organisms (Dundas 1998, Landis 2001, DasSarma 2006).

A common genus of halophilic bacteria isolated from saline habitats is *Marinococcus*. It is Gram-positive, non-sporulating cocci having diameters ranging from 1.0 to 1.2 μm. It can exhibit various cell groupings including singles and pairs, tetrads, or clumped clusters (Novitsky and Kushner 1976, Hao et al. 1984). It is motile, possessing either one or two flagella. Colonies are circular, smooth, and non-pigmented or might be yellow to orange in color. The mol % G+C of DNA ranges from 43.9 to 46.6. Most species grow well in Moderate Halophilic medium, as well as in nutrient agar supplemented with 5 to 20 % sodium chloride, however will not grow in media without salt (Hao et al. 1984). In an extensive study by Marquez et al. (1992), 55 moderately halophilic Gram-positive cocci were isolated from various locations in eastern and southern Spain. When these organisms were subjected to phenotypic and chemotaxonomic characterization, nine of the isolates bore a striking resemblance to *Marinococcus halophilus*, which suggests this organism is relatively common in saline environments (Marquez et al. 1992). Members of genus *Marinococcus* possess metabolic abilities that might be factors in the ecology of saline habitats. For instance, both *Marinococcus halophilus* and *Marinococcus albus* have been shown to have the ability to precipitate carbonates from culture medium to produce bioliths (Rivadenyera et al. 1999).

Halomonas is another genus of moderately halophilic bacteria routinely isolated from saline environments. These organisms generally exhibit a bacillus morphology, but can be pleomorphic under certain physiological conditions. Elongated flexuous filaments of cells occasionally are formed. They are Gram-negative, non-sporulating, and motile with unsheathed polar or lateral flagella. In the presence of nitrate they are either aerobic or facultatively anaerobic. Colonies are white to yellow, unlike halophilic Archaea, which commonly display a red pigment. The mol % G+C of DNA is 60.5 ± 0.5 (Vreeland et al. 1980). While it generally is not considered to be a human pathogen, an instance of a human infection by *Halomonas venusta* from a fish bite has been reported (von Graevenitz et al. 2000).

Members of *Halomonas* have proven difficult to classify based on phenotypic and chemotaxonomic characteristics (Dobson et al. 1993). Although moderate halophiles can be distinguished by morphological features, physiological characteristics, and biochemical assays (Vreeland 1993), they also can be classified by 16S rRNA gene sequencing (Dobson et al 1993). Even with the acceptance of 16S rRNA sequence analysis as a means of *Halomonas* identification, discrepancies occasionally arise (Baumgarte et al. 2001).

The objective of our study was not to generate an exhaustive list of all halophiles and their biochemical characteristics at our study site, but rather to determine whether moderately halophilic bacteria could be isolated and identified from the site. Previous work has addressed the seed bank at this location (Burr 1998), however to our knowledge, no study of the microbial flora has ever been conducted.

METHODS

Surface water grab samples were collected (50 ml in a sterile capped centrifuge tube) from the edge of a salt marsh on privately owned land in Russell County, Kansas. The marsh was located 3.6 km south and 6.8 km east of Fairport, Kansas and was included in the following land description: T12S, R15W, E 1/2 of the NW 1/4 of Section 14. It was adjacent to the north side of the Saline River and occupied approximately 20.7 ha. It was situated in the bottom of a small drainage valley located in the Blue Hills Upland section of the Great Plains Province (Burr 1998). The samples were returned to Fort Hays State University and stored at 4°C for one day prior to analysis. We used a sterile glass L-rod to plate 500 µl of each sample on trypticase soy agar (TSA) (Becton Dickinson Microbiology Systems, Cockeysville, Maryland) supplemented with 12 % NaCl. Visual examination of the plates revealed two prominent colony types. These colonies were labeled Halophile A and Halophile B and were sub-cultured numerous times on TSA containing 12 % NaCl (12 % NaCl TSA) until axenic cultures were obtained.

We submitted axenic cultures of both organisms, on 12 % NaCl TSA, to MIDI Labs (Newark, Delaware) for fatty acid methyl ester (FAME) analysis. MIDI Labs used a standard FAME analysis protocol, which was as follows: Colonies were re-streaked on 12 % NaCl TSA and incubated at 28° C for 24 hours. Approximately 30 mg of an isolated colony was harvested and subjected to fatty acid saponification with Reagent 1 (45 g sodium hydroxide, 150 mL methanol, and 150 mL distilled water). Next, Reagent 2 (325 mL 6.0N hydrochloric acid and 275 mL methyl alcohol) was used to methylate the saponified fatty acids. The methylated fatty acids were then extracted by using Reagent 3 (200 mL hexane and 200 mL methyl-tert-butyl ether). The organic layer was cleaned-up by using Reagent 4 (10.8 g sodium hydroxide dissolved in

900 mL distilled water). The resulting fatty acid methyl esters subsequently were resolved by gas chromatography on an ultra 2 column (Sasser 2001).

For partial 16S rRNA sequencing analysis, axenic cultures of both Halophile A and B were submitted on 12 % NaCl TSA to MIDI Labs. MIDI Labs used the Applied Biosystems MicroSeq 500 gene kit (Applied Biosystems, Foster City, California) to determine the DNA sequence of the first 500 base pairs of the 16S rRNA gene. The resulting DNA sequences were analyzed by using the commercial MicroSeq Analysis Software and Sequence Database package, which is based on phylogenetic trees and pair wise alignment algorithms. In addition, the derived sequences were aligned with sequences in GenBank.

We prepared, mounted, and examined both Halophile A and B samples by scanning electron microscopy and followed standard methods (Postek et al. 1980). We fixed colonies growing on 12 % NaCl TSA overnight by flooding the agar plate with 1 % glutaraldehyde in 0.15 M cacodylate buffer. We collected fixed cells with a Pasteur pipette and centrifuged them and decanted off the fixing solution. Next, we dehydrated the cells with the series of cacodylate buffer/ethanol baths at the following ratios: 90/10, 75/25, 50/50, 25/75, and 0/100. After the 25/75 wash step, we filtered the bacteria by using 0.45 μm pore filter membranes (Millipore, Bedford, Massachusetts). The membrane filters, containing the fixed bacteria, were stored in 100 % ethanol at 4°C for a minimum of 24 hours. We subsequently dried the membranes by using hexamethyldisalizane (HMDS) in a fume hood and stored them in a desiccator. The fixed and dried filter membranes were mounted directly to an aluminum stub with silver cement, sputter coated with gold palladium in a Pelco sputter coater for one minute and observed by using an ISI SX-30 scanning electron microscope (Topcon America Corporation, Paramus, New Jersey).

RESULTS

For identification of bacteria based upon FAME profiles, MIDI Labs employs the Sherlock Microbial Identification System (MIS). In this system, a similarity index is assigned to an unknown organism, based upon how closely its fatty acid composition compares with the mean fatty acid composition of known organisms in the MIS database. A similarity index of 1.00 indicates an exact match of the unknown organism with an organism in the MIS database. The similarity index will decrease as each fatty acid varies from the mean percentage. The similarity indices assigned by the MIS to Halophiles A and B are shown in Table 1.

Fatty acid methyl ester analysis conducted by MIDI Labs suggested that Halophile A was an atypical *Bacillus* organism, and was as yet unclassified. The fatty acid used as a reference peak was 16:1ω7c alcohol, and was indicative of a member of genus *Bacillus*. The FAME analysis indicated that Halophile A most closely resembled either *Bacillus coagulans* or *Bacillus atrophaeus*. Other organisms that

exhibited a similar FAME profile were *Clavibacter michiganesis* and *Nesterenkonia halobia*.

FAME analysis of Halophile B also indicated an atypical *Bacillus* organism, as yet unclassified. Again, the fatty acid used as a reference peak was 16:1ω7c alcohol. Based on the similarity index assigned to Halophile B, it most closely resembled *Bacillus coagulans* and *Clavibacter michiganensis*. Other organisms in the MIS database that have FAME profiles similar to Halophile B were *Bacillus atropheus* and *Curtobacterium flaccumfaciens*.

The DNA sequences of the first 500 base pairs of the 16S rRNA gene from both Halophiles A and B were determined by MIDI Labs. These sequences were then compared to known sequences in GenBank and in the MIDI Labs MicroSeq database. Microorganisms showing the closest matches from both databases are indicated in Table 2.

Table 1. Similarity (SIM) indices of the fatty acids of Halophile A and B resolved by fatty acid methyl ester analysis.

Halophile A		Halophile B	
SIM Index	Organism	SIM Index	Organism
0.455	*Bacillus coagulans*	0.489	*Bacillus coagulans*
0.414	*Bacillus atropheus*	0.462	*Clavibacter michiganensis*
0.411	*Clavibacter michiganensis*	0.408	*Bacillus atropheus*
0.376	*Nesterenkonia halobia*	0.400	*Curtobacterium flaccumfaciens*

Table 2. Summary of the results of the 16S rRNA sequence analysis. The closest matches in first 500 base pairs of the 16S rRNA gene from the GenBank and MicroSeq databases are shown. The column designated as % diff (difference) represents the percentage by which each organism listed differs from Halophile A or B in the first 500 base pairs of the 16S rRNA gene.

Halophile A			Halophile B		
Database	% diff	Organism	Database	% diff	Organism
GenBank	1.0	*Marinococcus halophilus*	GenBank	3.0	*Halomonas variabilis*
MicroSeq	12.86	*Bacillus clarkii*	MicroSeq	2.12	*Halomonas aquamarina*

When the first 500 bases of the 16S rRNA gene from Halophile A were compared to sequences in GenBank, they most closely resembled *Marinococcus halophilus* with a difference of 1.0 %. However, when it was aligned with sequences in the MicroSeq the closest match was *Bacillus clarkii*, with a difference of 12.86 %.

The partial 16S rRNA gene sequence of Halophile B also was aligned with sequences in the GenBank and MicroSeq. The closest match with sequences in GenBank was to *Halomonas variabilis*, with a difference of 3.0 %. The organism in MicroSeq that most closely matched the sequence of Halophile B was *Halomonas aquamarina* with a difference of 2.12 %.

The scanning electron micrographs obtained for Halophiles A and B are shown in Figure 1. Scanning electron microscopy of Halophile A revealed cocci with diameters of approximately 1.0 μm, with cells arranged in clusters or tetrads. (Fig. 1a). Scanning electron microscopy also showed that Halophile B exhibited coccus morphology, with cells having a diameter of approximately 1.0 μm arranged primarily in clusters (Fig. 1b). The coccus morphology revealed by scanning electron microscopy also was seen when the organisms were initially observed by Gram staining (data not shown).

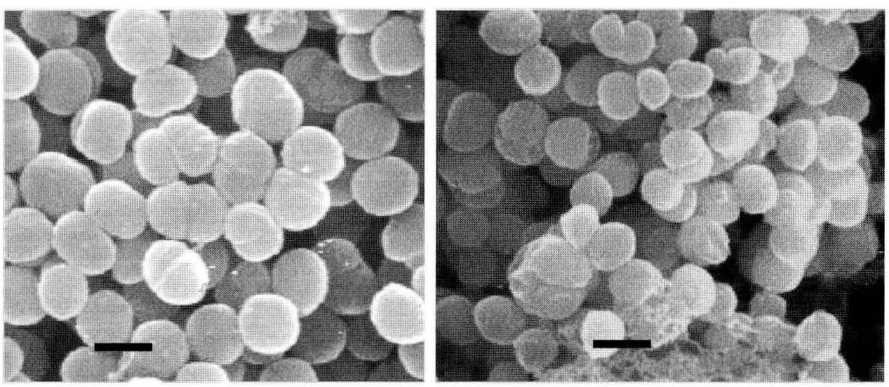

Figure 1. Scanning electron micrographs of Halophile A (A) and B (B). Both organisms exhibit distinct coccus morphology with cells arranged in clusters. Scale bar = 1μm.

DISCUSSION

The FAME analysis of Halophile A suggested that it should be placed within the genus *Bacillus*. This was not initially surprising, as a number of moderately

halophilic *Bacillus* species have been isolated from hypersaline environments (Garabito et al. 1997, Arahal et al. 1999, Caton et al. 2004). Based upon data obtained from partial 16S rRNA sequencing analysis and scanning electron microscopy, the placement of Halophile A within *Bacillus* seems unlikely.

Part of the difficulty in using FAME analysis for bacterial identification at this time could stem from the fact that relatively small numbers of FAME profiles have been obtained from known organisms from which to base a comparison. According to MIDI Labs general guidelines, strains with at least a 0.600 similarity index and with more than a 0.100 distance from the second choice are considered good matches. A similarity index between 0.400 and 0.600 with good separation from other organisms might be a species match, indicating an atypical strain. A value of 0.400 or less on the similarity index indicates that the sample species is not in the MIS database. The organisms chosen as matches for Halophile A (Table 1) have similarity indices ranging from 0.376 to 0.455, however they are not separated by at least 0.100. Thus, there was not a match for Halophile A in the FAME profile database.

When the sequence of the first 500 base pairs from the 16S rRNA gene from Halophile A was compared with sequences in the MicroSeq, the closest match was *Bacillus clarkii*, which showed a 12.86 % difference from Halophile A. When GenBank was searched, the closest match was *Marinococcus halophilus*, which showed only a 1.0 % difference from Halophile A (Table 2). According to previous work, a sequence similarity greater than or equal to 97 % is considered a genus level match. A species level match is based on a similarity greater than or equal to 99 % (Drancourt et al. 2000). Based on this criterion, it seems more likely that Halophile A should be placed within the genus *Marinococcus*, rather than *Bacillus*. Members of genus *Bacillus* exhibit a distinct rod-like morphology, with many members showing evidence of sporulation, neither of which was seen in the scanning electron micrographs of this organism (Fig. 1). The presence of cocci in clusters in the scanning electron micrographs of Halophile A also supported the placement of this organism within *Marinococcus* rather than *Bacillus*.

The FAME analysis of Halophile B also suggested that it is a member of genus *Bacillus*. As with Halophile A, however, this conclusion also seemed unlikely in light of the 16S rRNA sequencing and scanning electron microscopy data. The similarity indices derived for Halophile B ranged from 0.400 to 0.489 (Table 1). Using the FAME criteria discussed for Halophile A, MIS database did not contain a match for Halophile B.

The partial 16S rRNA gene sequence analysis of Halophile B did not support its placement within the genus *Bacillus*. When the first 500 base pairs of the 16S rRNA gene from Halophile B were aligned with MicroSeq, the closest match was *Halomonas aquamarina*, with a difference of 2.12 %. When the sequence was compared with GenBank, the closest match was *Halomonas variabilis*, with a difference of 3.0 %. Based upon the work of Drancourt et al. (2000), where a 1 %

difference is required for a species level match and 3 % is required for a genus level match, it seemed likely that Halophile B should be placed within the genus *Halomonas*, but was not a definitive match with any previously reported species of that genus.

Scanning electron microscopy clearly indicated that Halophile B cells were cocci arranged in clusters (Fig. 1b). This would initially seem to rule out the placement of Halophile B among *Halomonas*, which are normally rod-like in appearance. However, under certain physiological conditions members of *Halomonas* assume a pleomorphic appearance, which might be the case with Halophile B (Vreeland et al. 1980). Gram stains of Halophile B also revealed cocci in clusters.

Our study revealed some of the difficulties that can arise when attempting to identify bacteria from environmental samples. In many instances, classical phenotypic and chemotaxonomic characteristics are not helpful in identifying these organisms (Dobson et al. 1993).

Sequence analysis of the 16S rRNA gene is one of the most reliable methods to delineate phylogenetic relationships among bacteria. Even though the sequence of the 16S rRNA gene is conserved highly among bacteria, it still contains variable regions and is thought to be only weakly affected by horizontal gene transfer (Acinas et al. 2004). Using automated DNA sequencing technology, the entire 16S rRNA gene can be sequenced relatively rapidly, which makes it a popular technique in bacterial classification (Vandamme et al. 1996, Thurlow and Gillock 2005).

Fatty acid methyl ester analysis (FAME) also is becoming a readily accepted tool for delineation of phylogenetic relationship, especially among pathogens (Haack et al. 1994). The use of FAME analysis for the identification of environmental bacteria is relatively recent when compared to 16S rRNA sequencing and might not be entirely reliable, at least for some organisms. This might change in the future as a wider variety of FAME profiles are added to the databases. Scanning electron microscopy, when used alone, is not very useful in identifying unknown bacteria. However, it does provide a powerful means to verify morphological features initially revealed in standard light microscopy. In the identification and characterization of bacteria from environmental samples, more than one analysis technique is often required. When two techniques give contradictory results, often a third method must be used. In our case, the bacteria we isolated seemed to be members of genera *Marinococcus* and *Halomonas*.

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