Determining the Effects of Nutrient Sources on Halophile Membranes through Analysis of Fatty Acid Methyl Ester (FAME) Profiles

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Abstract - This project attempts to demonstrate two applications of fatty acid methyl ester (FAME) analysis in the study of bacterial cell membranes. Gas chromatography/mass spectrometry was utilized to analyze membrane fatty acids following conversion to their respective FAMEs. The data collected, in the form of "FAME profiles" are used for two purposes. The first is to test the procedure as a viable method in differentiating strains of bacteria, specifically halophiles, based on their FAME profiles. The second is to investigate the effects of specific carbon and nitrogen sources on halophile membrane composition. The data show that the procedure can differentiate species of halophiles, though this particular study was unable to distinguish strains of the same species. They also demonstrate that membrane composition does change when nutrient sources are defined.

I. Introduction

Halophiles make up a group of bacteria specialized to tolerate or to thrive in extraordinarily saline environments. The salt concentration in these environments is often too high to allow any other kinds of life. To cope with osmotic stress, halophiles utilize a variety of strategies.[1] Specifically of interest to this study is the synthesis of particular fatty acids to create the plasma membrane.

Using fatty acid methyl ester (FAME) analysis, previous studies have demonstrated that genera of bacteria can be distinguished based solely on their relative proportions of specific fatty acids.[2] Similarly, halophiles have been shown to alter the fatty acid composition of their membranes in order to cope with varying concentrations of salt in media.[3]

With the exception of one, the bacterial strains in this study belong to a single species, *Salinivibrio* costicola. They are thus far differentiated based solely on their susceptibility to specific bacteriophages, i.e. each strain is killed by a phage that targets that strain specifically.

With this knowledge, it is believed these strains must differ at least slightly from one another on a molecular level. Therefore, this study not only attempts to further the conclusion that genera of halophiles can be differentiated based on FAME profiles, but also tests the method's ability to compile unique profiles for strains within a single species. The study also tries to track changes in membrane composition in response to limiting and changing specific nutrients.

II. Experimental

Materials

Strains - All strains were provided by Dr. Matt Domek of Weber State University. Strains 36, 39, 40, and 50 were isolated from the Great Salt Lake by Dr. Domek. Strain ATCC came originally from the American Type Culture Collection. Strain B1 was isolated originally from a saltern in Alicante, Spain[4] and G3 from a saline pond near Saskatchewan, Canada.[5] Apart from G3, all strains are thus far classified as *Salinivibrio* costicola. Cells were cultured on plates of "halophile medium" as described below, with the addition of 15g agar per liter. Cultures were re-plated every 2 weeks, incubated at 30°C for 48 hours after streaking, and stored at room temperature thereafter.

Media - 10mL Halophile Medium (HM) served as the control condition. It consisted of 80g NaCl, 5.24g anhydrous $MgSO_4$, 5g casamino acids, 5g KCl, 3g sodium citrate, 1g KNO_3, 1g yeast extract, and 0.1g $CaCl_2$ in 1 liter dH_2O. Test samples for limited conditions consisted of 4mL twice concentrated solution of salts (NaCl, $MgSO_4$, KCl, and $CaCl_2$), 3mL carbon source at 0.14M, and 2mL nitrogen source at 0.15M. An additional 1mL of 2x concentrated salt solution inoculated with cells was added after all other components. All media were pH adjusted to 7.45 ± 0.14. All cultures were placed in a 30°C shaking incubator at 200 rpm, and grown until turbid enough to yield a viable pellet after centrifugation.

FAME Standard – The FAME standard for GCMS analysis was Supelco Analytical's Bacterial Acid Methyl Ester Mix (47080-U) in 10mL toluene.

Methods:

Preparation of pellet - Once turbid, the samples were spun on a Centra CL2 tabletop centrifuge at 4000 rpm for 10 minutes. The supernatant was poured out, and the pellets were resuspended in dH_2O. This was done a total of three times. The pellets were then suspended in methanol and centrifuged as before.

FAME preparation – The method for deriving FAMEs followed the protocol of Schultz and Pugh.[6] Pellets were resuspended in 2-3mL 4:1 methanol toluene and transferred to a conical vial with a triangular stir bar. The open cap was fitted with a Teflon-lined silicon septum. After stirring for 3 minutes, 200µL acetyl chloride was added from a syringe through the silicon septum. The open cap was replaced with a closed silicon-lined cap. The vials were placed in 100°C heating block and stirred for 1 hour. After reacting, the vials were removed and placed in a cool water bath. The mixture was poured into 30mL glass Corex centrifuge tubes containing 5mL 6% Na_2CO_3 and 2mL toluene. The tubes were vortexed for 15 seconds and spun on a Sorvall Superspeed RC2-B centrifuge at s3000 rpm for 20 minutes to separate the phases. The organic phase was removed by pipette, dried on $MgSO_4$, and filtered through glass wool into a 2.5mL screw cap vial.

GCMS Analysis of Samples – Samples were injected into a Varian 450-GC Gas Chromatogram/Varian 240-MS IT Mass Spectrometer and analyzed using Varian Inc. MS Workstation MS Data Review Software (v. 6.9.3). The initial column oven temperature was set to 150°C for 5 minutes, then increased at a rate of 5°C for another 22 minutes for a final temperature of 260°C. Following this, the temperature increased by 0.2°C per second for another 5 minutes. Helium was used as the carrier gas at a rate of 1mL per second.

III. Results

Growth Success

The following table indicates whether or not each strain was able to grow in the test media.

Table 1: Growth Success by Strain in Defined Media

	36	39	40	50	ATCC	B1	G3
Citrate + Nitrate	-	+/-	-	-	-	-	+
Glucose + Nitrate	-	-	-	-	-	-	+
Glucose + Alanine	+	+	+	-	+	+	+
Glucose + Glucosamine	+	+	+	+	+	n/a	+
Glucose + Ammonium	+	+	+	+	+	+	+
Acetate + Ammonium	+	+	-	+	+	+	+
Glucosamine + Ammonium	-	-	-	-	-	-	-
Glucosamine only	-	-	-	-	-	-	-
Alanine only	-	-	-	-	-	-	+

Media listed as carbon source + nitrogen source.
+ indicates that the turbidity of the culture increased after several days in the respective medium.
– indicates that no growth was observed after one week.
+/-Strain 39 showed very minor growth in one trial, and none in a second trial.

Qualitative Analysis of Chromatograms

The following figures give a qualitative overview of chromatograms. Figure 1 demonstrates a sample chromatogram from each strain. Figure 2 shows the chromatograms of three trials of the same strain (36) following the decision to limit the study to focus on only two strains. In both figures, the chromatograms are labeled by strain (36, 39, 40, 50, B1, ATCC, or G3) before the "HB" in the top right string of characters.

Figure 1: Example Chromatograms of all Strains In Halophile Medium.

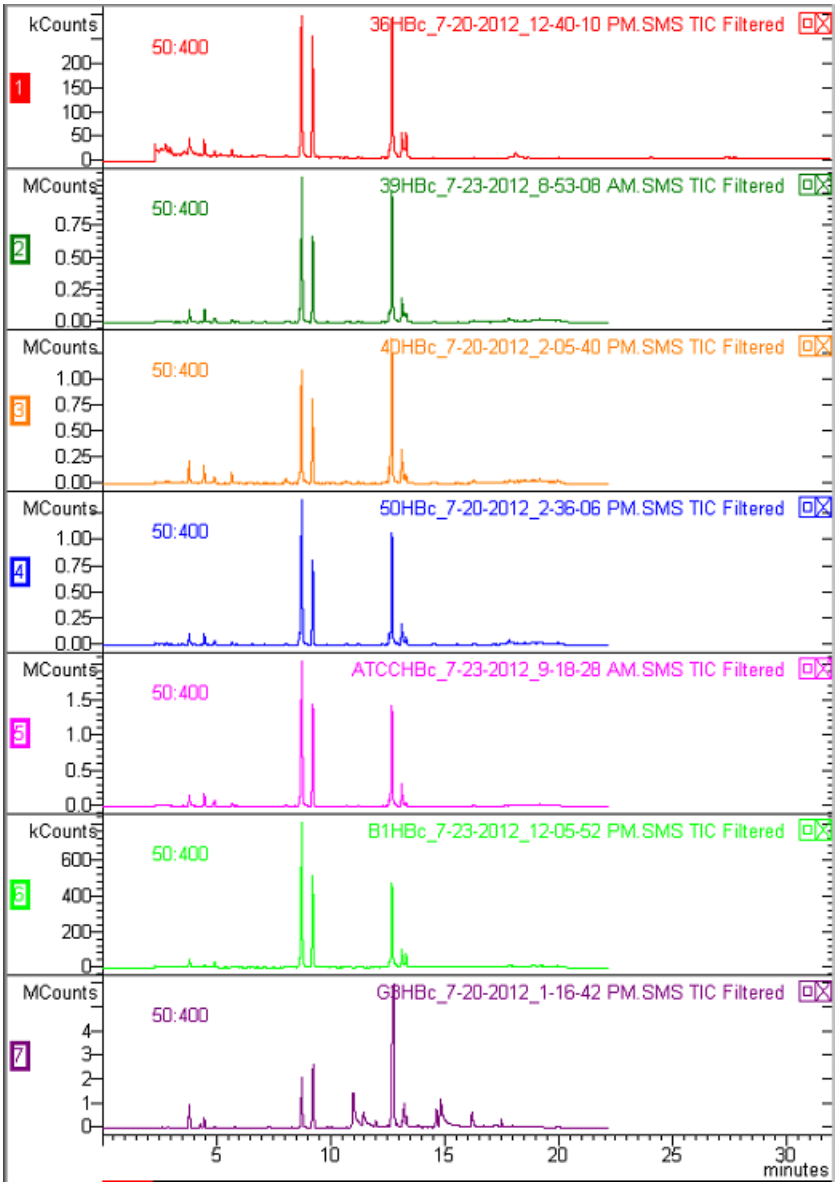
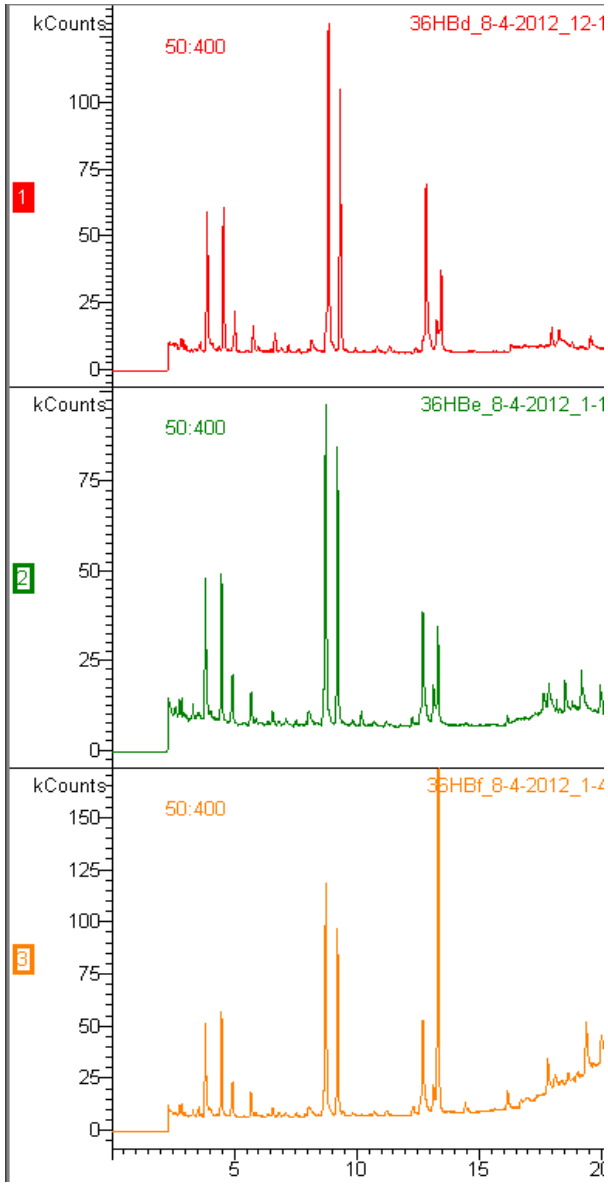


Figure 2: Chromatograms of Triplicate Samples Of Strain 36 Grown in Halophile Medium



FAME Profiles Across Conditions

The following data were obtained through integration of chromatogram peaks. The identity of each peak was compared to standard by retention time. Values were calculated by adding only values of FAMEs (confirmed by mass spectrometry) and finding each FAME's percentage of that total.

Table 2a: FAME Identities of Strain 36 in Varying Media

FAME	Acetate	Glucose	HM
I	0	0	0
II	0	0	11.53 ± 0.88
III	0	0	11.58 ± 0.15
IV	24.22 ± 9.77	18.85 ± 6.55	32.23 ± 1.32
V	39.17 ± 4.52	30.57 ± 1.12	23.08 ± 0.59
VI	36.60 ± 13.62	42.36 ± 8.76	14.11 ± 2.63
VII	0	8.224 ± 2.110	3.889 ± 0.688
VIII	0	0	0
IX	0	0	3.584 ± 0.426

Table 2b: FAME Identities of Strain G3 in Varying Media

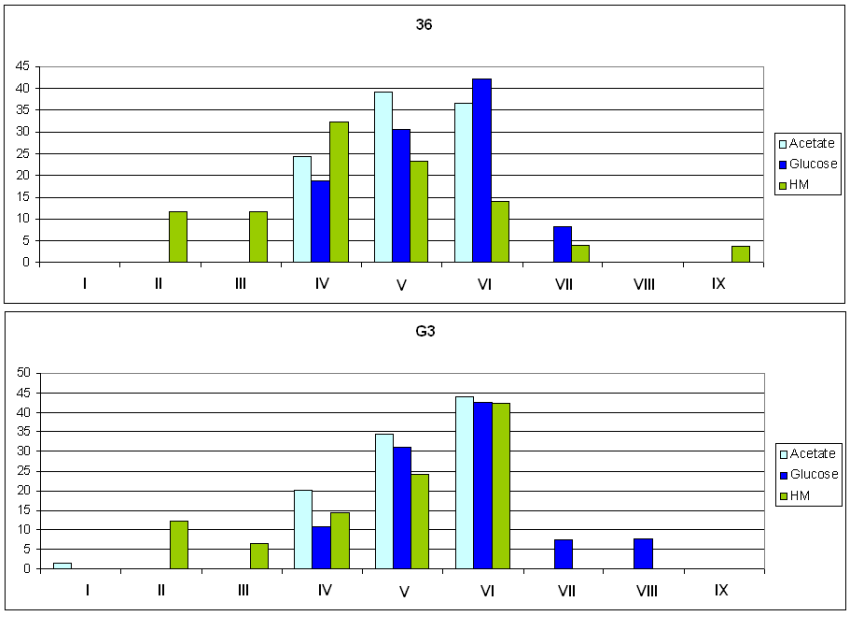
FAME	Acetate	Glucose	HM
I	1.422 ± 0.812	0	0
II	0	0	12.41 ± 2.93
III	0	0	6.682 ± 1.486
IV	20.11 ± 11.53	10.93 ± 0.68	14.37 ± 0.68
V	34.24 ± 2.56	31.41 ± 5.32	24.21 ± 0.93
VI	43.92 ± 11.63	42.06 ± 4.78	42.33 ± 3.54
VII	0	7.706 ± 0.653	0
VIII	0	7.895 ± 3.120	0
IX	0	0	0

Values expressed as percentages of total FAMEs in sample (averages and standard deviations of triplicate cultures). Acetate and Glucose cultures were grown with Ammonium as the nitrogen source.

Table 3: FAME Identification Based on Retention Time Comparison With Standard Mix

Peak ID	Standard Match
I	Unknown FAME 1
II	Methyl ± 3-hydroxydodecanoate
III	Unknown FAME 2
IV	Methyl cis-9-hexadecanoate
V	Methyl hexadecanoate
VI	Unknown FAME 3
VII	Methyl octadecanoate
VIII	Unknown FAME 4
IX	Methyl tetradecanoate

Figure 3: Graphic Representation of Data in **Tables 2a** and **2b.**



IV. Discussion

Growth Success

Table 1 - No strains were able to utilize glucosamine for carbon, but grew quite well when supplemented with glucose. The most notable trend in Table 1 is G3's ability to utilize nutrients that strains of *S.* costicola cannot. The table, however, does not show the turbidity differences in the cultures. G3 consistently grew faster and more vigorously in all media. Given these data, it would be viable to construct tests to differentiate G3 from *S.* costicola based on metabolism alone. The small differences in other growth patterns among *S.* costicola strains, such as 50's lack of growth in glucose and alanine, could be attributed to improper inoculation of cultures, i.e., the culture received an insufficient number of living cells to produce a viable population within the tube. With the exception of citrate + nitrate and glucose + nitrate, all tests were done in single trials. Repeated trials would be required to confirm growth success and to construct tests to differentiate *S.* costicola strains by metabolism.

Qualitative Analysis of Chromatograms

Figure 1 – The FAME profiles of *Salinivibrio* costicola strains (1-6) are highly consistent within the species when grown in Halophile Medium. Strain G3 (bottom), however, deviates from the rest. The consistency within the *S.* costicola strains demonstrates the reproducibility of the method, and furthers the hypothesis that species (or at least genera) of halophiles can be distinguished based on FAME analysis. While the method does not produce noticeable differences within a species, the possibility still remains that individual strains could be distinguished when responding to different media conditions. Further experiments would be required to determine this.

Figure 2 – In the interest of time, the study was narrowed to focus only on two strains, 36 and G3, to see how their FAME profiles would differ when grown in defined media. As shown previously, a high level of consistency existed among *S.* costicola strains. For this reason, strain 36 was chosen as a representative to be compared with G3. Figure 2 is an example of the reproducibility of three identical cultures of strain 36 grown in HM, as the relative height of peaks to one another remains consistent across the three trials. The final peak is a contaminant found in all samples in varying concentrations, and was ignored with all other contaminants in calculating relative amounts of FAMEs in each sample.

FAME Profiles across Conditions

Tables 2a and 2b – The data show that under different conditions, halophiles do change their membrane composition. Both the identities and relative amounts of FAMEs differ across media conditions. Of interest are the values collected for cells grown with acetate as the carbon source. The consistently large standard deviations show that the relative amounts of certain fatty acids, mainly IV, V, and VI, vary significantly from culture to culture. This indicates that, among the major fatty acids present for that species, one particular pattern of fatty acids in the membrane is no more successful than another.

Table 3 - The FAMEs of most interest are IV, V, and VI, as they are the most abundant in all cultures, and appear to change most readily across media conditions and across species. Methyl hexadecanoate (V) is the saturated form of methyl cis-9-hexadecanoate (IV). According to library values recorded within the software, Unknown 3 (VI) appears to be a saturated FAME with 17 carbons.

Figure 3 – When grown in an enriched medium (HM), strains 36 and G3 yield quite different profiles. Both have FAMEs IV, V, and VI as their major components, but the relative amounts of are reversed. The profile of Strain 36 is comprised mostly of IV, followed by V, followed by VI. Conversely, G3's dominant FAME is VI, followed by V, followed by IV. For the purpose of this study, these are considered the default profiles for these strains, as they are not subjected to the stress of limited nutrients.

Looking at the bottom half of Figure 3, it is apparent that, regardless of growth conditions, the values of IV, V, and VI in G3's profiles remain roughly in the same concentration relative to one another. This is consistent with the aforementioned observations of G3's ability to grow quickly and abundantly

in limited conditions. It appears that its default arrangement of membrane fatty acids is already conducive to surviving in stressful conditions. This conclusion is further supported by the profiles of 36. While its default arrangement of IV, V, and VI are very different from G3's, the relative amounts of those FAMEs converge with G3 in the two limited conditions. This indicates that that particular arrangement, VI > V > IV, allows for better growth when limited to one source of carbon. Since the limited media both used ammonium as the nitrogen source, it is possible that the ammonium is the reason for the convergence. More experiments will need to be performed to test the effects of other nitrogen sources. Furthermore, tests wills also be done to test the effects of other carbon sources, to either support or refute the hypothesis that the FAME profiles of *S.* costicola strains will converge with G3's under limited conditions.

V. Acknowledgements

I would like to thank Dr. Matt Domek for providing the bacterial strains, and for instruction on culturing them properly. My deepest gratitude goes to Dr. Brown, for agreeing to oversee the project, and for his patient guidance throughout the summer.

VI. References

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