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Identification of Seafood Bacteria from Cellular Fatty Acid Analysis via the Sherlock® Microbial Identification System

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Abstract

The current taxonomic positions of psychrotrophic bacterial strains isolated from seafood and housed in the Kodiak Seafood Culture Collection (KSCC) were determined based on membrane fatty acid profiles and compared with profiles for representative American Type

Culture Collection (ATCC) strains. Gram-positive organisms had a high percentage (64–96%) of branched-chain fatty acids, except for *Streptococcus faecium*. Gram-negative, oxidase-negative isolates were composed of 38–69% of saturated fatty acids and 19–50% of unsaturated fatty acids. Hydroxyl fatty acids were present in all the gram-negative, oxidase-positive isolates and the branched-chain fatty acids were <1–74%. Of the 33 strains tested, 55% were identified to species with a similarity index (SI) >0.5 and <0.1 SI in range of the first rank. Strains of *Pseudomonas fluorescens* and those belonging to the Enterobacteriaceae were responsible for this lower percentage. The modern taxonomic positions of three KSCC strains were found to be *Shewanella putrefaciens*, *Psychrobacter immobilis* and *Myroides odoratus*. Sherlock MIS proved to be an effective and rapid technique in the identification of bacteria associated with seafood.

Keywords: Fatty acids, Seafood bacteria, Identification

1. Introduction

Lipids compose almost 50% of the cytoplasmic membrane of bacterial cells and are composed of unique fatty acids of straight chains (saturated, unsaturated, cyclopropane and hydroxylated) and branched chains (saturated, unsaturated or hydroxylated iso-, anteiso- and ω-alicyclic) (Suutari and Laakso, 1994). The diversity of fatty acids and their species specific distribution among bacteria can be used as important chemotaxonomic biomarkers in bacterial identification (Thornabene, 1985; Komagata and Suzuki, 1987; Kaneda, 1991).

The only automated system available for the bacterial identification based on fatty acid profile analysis is by Microbial Identification Inc. (MIDI) and introduced in 1985. The computerized Sherlock® Microbial Identification System (MIS) identifies fatty acids (9:0 to 20:0) and compares individual fatty acid percentages with its databases which are fragmented into libraries. The output is a list of possible microbial identities and corresponding similarity index (SI) which is calculated using multivariate statistical analysis (Welch, 1991; O'Hara, 2005).

Some microbiology researchers conclude the MIS is accurate, efficient, reproducible, and rapid and correctly identifies clinical bacteria (Kunitsky et al., 2005; Himelbloom et al., 2011). Others report that this system cannot be used for sub-grouping of bacteria since the typing capability of MIS is genus- and/or species-dependent (Osterhout et al., 1991; Birnbaum et al., 1994; Steele et al., 1997; Odumeru et al., 1999). Lower specificity of MIS and other commercial systems to correctly identify non-clinical bacterial isolates could be due to the databases being mainly composed of membrane fatty acid profiles derived from clinical isolates (Nedoluha and Westhoff, 1995). Upon investigating the applicability of the MIS to bacteria isolated from fish, they concluded this system is not suitable for such application. Initial studies to determine the ability of the MIS to identify bacteria other than those of clinical importance are recommended (Nedoluha and Westhoff, 1995; Brown and Leff, 1996). The fatty acid signatures of microorganisms can be used successfully as a complementary tool for taxonomic identification of novel bacterial species isolated from food, clinical specimens, and environmental sources (Welch, 1991; Bertone et al., 1996; Neyts et al., 2000; Hinton et al., 2004; Hoffmann et al., 2010). A particular interest to us is the use of these biomarkers to distinguish marine bacteria isolated from sea ice of polar regions (Hoffmann et al., 2010; Zhang et al., 2008). It may be possible to apply the MIS to bacterial isolates from cold-water seafood. Bacteria isolated from Alaska fishery products are mostly psychrotrophic organisms, in which membrane fatty acids profiles have not been determined. The objective of this research was to establish the fatty acid profiles of bacteria previously detected in Alaska seafood products and to assess the ability of the MIS to accurately identify this select group. A

variety of genera, with an emphasis on known seafood spoilage bacteria, were selected for a system challenge test (Miller, 1991) and to determine the effect of repeated analysis on the variation in the fatty acid profiles.

2. Materials and Methods

2.1 Bacterial Isolates

Thirty-three selected isolates from the Kodiak Seafood Culture Collection (KSCC; Table 1) and from the American Type Culture Collection (ATCC, Manassas, VA; Table 2) were used in this study. The KSCC bacteria were identified previously to the genus or species level by classical taxonomic techniques and via the API system of bacterial identification (bioMérieux, Hazelwood, MO). Bacterial sample vials (Wheaton, 4 ml with rubber-lined screw cap; VWR) contained 2 ml of cell concentrate from: ~1 ml of sterile brain heart infusion(BHI; Difco; Becton, Dickinson and Co., Sparks, MD) added to freshly-grown pure cultures in individual Petri plates (VWR International, Inc., Brisbane, CA) for pipetting the colony slurry, 1 ml of sterile 50% glycerol (w/v; Sigma Chemical Co., St. Louis, MO) in sterile reverse osmosis water (roH_2O) and 40 µl dimethyl sulfoxide (Sigma). These vials had been frozen (-80 ℃) long-term for archival purposes. Selected vials were thawed for less than 30 minutes at room temperature. Psychrotrophic bacterial cells (~0.1 ml of cell concentrate) was inoculated in 2 ml of BHI (Difco) and incubated at 25 ℃ for 24 h, while mesophilic bacterial cells were incubated at 35 ℃ for 24 h. Culture purity was determined by streaking on plate count agar (Difco) at the psychrotrophic and mesophilic incubation temperatures for 72 h and 48 h, respectively, before confirming basic taxonomic characterizations. Isolated, representative colonies of each test strain were transferred using sterile toothpicks to clean plastic Petri plate lids to observe the cell wall reaction (Powers, 1995) with a drop of 3% KOH (VWR) in roH_2O (w/v), instead of the standard Gram staining protocol. The catalase test (Harrigan, 1998) used was to add a drop of 3% hydrogen peroxide (Fisher Scientific Co., Fair Lawn, NJ) in roH_2O to colonies transferred to a second lid. The cytochrome oxidase test (Kovacs, 1956) for colonies was performed in a third lid containing a filter paper (Whatman 1 circle, 11 cm diam.; VWR) wetted with ~1 ml of the fresh 1% N, N, N´, N´-tetramethyl-p-phenylenediamine dihydrochloride (Sigma) in roH_2O. Morphological characteristics regarding cell shape and motility of the selected bacterial cells were observed under a microscope (Montagesatz T-UL, Zeiss, Germany) by using the 40X high-dry and 100X oil-immersion lenses.

Table 1. Confirmation of identities of KSCC bacterial strains isolated primarily from raw and processed Alaska fish to genus and species by MIS

Strain	Source – year isolated	Genus no. ID	Species	
			no. ID	Mean SI ±s.d.[a]
Alteromonas sp. 8C2	Pacific flatfish – 1990	5	5	0.689 ±0.031
Arthrobacter sp. 5B6	Pacific pollock – 1989	5	5	0.659 ±0.071
Bacillus subtilis 5D1	Kodiak College stock – 1990	5	5	0.907 ±0.05
Citrobacter freundii 4E5	Pacific sole – 1991	4	4	0.653 ±0.088
Enterobacter cloacae 12D5	dried dill weed – 1992	5	3	0.777 ±0.001
Flavobacterium sp. 5C6	Pacific pollock – 1989	5	5	0.620 ±0.034

Hafnia alvei 2F1	pollock surimi – 1989	3	3	0.716 ±0.057
H. alvei 12E1	commercial salmon pate – 1992	4	4	0.725 ±0.171
Micrococcus varians 7F1	smoked salmon – 1996	5	0	0.767 ±0.058[b]
Morganella morganii 2E5	pollock surimi – 1989	5	5	0.535 ±0.134
Moraxella sp. 5E6	Pacific pollock – 1989	6	6	0.533 ±0.082
Providencia alcalifaciens 7F2	Pacific herring – 1996	4	0	0.751 ±0.05[c]
Pseudomonas fluorescens 2A1	pollock surimi – 1989	5	5	0.740 ±0.180
P. fluorescens 11A1	fillet processing line – 1992	5	4	0.535 ±0.057
P. fluorescens 11B3	surimi stuffing equipment-1992	7	3	0.375 ±0.022
P. fluorescens 12A2	commercial salmon pate – 1992	5	1	0.422[d]
P. putida 7E4	smoked salmon – 1996	6	6	0.765 ±0.115
P. putida 12A3	commercial salmon pate – 1992	5	5	0.579 ±0.184
Serratia fonticola 2D3	pollock surimi – 1989	5	5	0.414 ±0.221
Staphylococcus xylosus 13A4	experimental fish food - 1992	5	0	0.387 ±0.017[e]
Streptococcus faecium 6A4	Stabisil starter culture[f] - 1990	5	5	0.777 ±0.051
				Overall SI for 21 KSCC strains = 0.659

[a]Similarity index and standard deviation

[b]SI for strain but identified by MIS as *Micrococcus luteus* GC subgroup B

[c]SI for strain but identified by MIS as *Erwinia chrysanthemi* biotype III

[d]SI of one species level identification

[e]SI for strain but identified by MIS as *Staphylococcus gallinarum*

[f]manufactured commercially in Iowa

Table 2. Confirmation of identities of ATCC bacterial strains to genus and species by MIS

Strain	Source-country of origin	Genus no. ID	Species no. ID	Mean SI±s.d.[a]
Aeromonas hydrophila 35654 (quality control strain)	unknown	5	5	0.889 ±0.023
Escherichia coli 11303 biotype B	unknown	5	5	0.744 ±0.068
Pseudoalteromonas nigrifaciens 19375 (type strain)	butter – UK	5	5	0.851 ±0.046
Pseudomonas fluorescens 13525 biotype A (type strain)	pre-filter tanks – UK	5	5	0.874 ±0.112
P. fluorescens 17574 biotype C or 49	polluted seawater-USA	5	5	0.709 ±0.123
P. fluorescens 31419 biotype G	unknown – Japan	5	3	0.624 ±0.110
P. putida 12633 biotype A (type strain)	unknown – USA	5	5	0.716 ±0.047
Psychrobacter immobilis 43116 (type strain)	poultry – UK	5	5	0.900 ±0.012
Serratia marcescens 13880 (type strain)	pond water – unknown	4	3	0.619 ±0.063
Shewanella putrefaciens 49138 (quality control strain)	clinical isolate – USA	5	5	0.955 ±0.012

S. putrefaciens 8071(type strain)	unknown	5	5	0.771 ± 0.043
Staphylococcus epidermidis 14990 (type strain)	nose – USA	5	4	0.679 ± 0.079
Overall SI for 12 ATCC strains = 0.758				

[a]Similarity index and standard deviation

2.2 Identification of Bacteria by Fatty Acid Methyl Ester Composition

Pure cultures were streaked on Trypticase soy broth agar (TSBA; 30 g of Trypticase soy broth [Difco], 15 g granulated agar [Difco] per L of roH_2O) and incubated at 28 ± 1 ℃ for 24 ± 2 h. Fatty acid methyl esters (FAME) were produced in five or more replicates using the standard MIDI procedures (Paisley, 2004). A loopful (20-40 mg) of cells, from the 3rd streaked quadrant after the 24 ± 2 h culture, was harvested and placed into a clean screw-capped glass tube (16x125 mm, VWR). For slow-growing bacteria, two to three plates were cultured simultaneously to allow sufficient cells to be collected (Paisley, 2004). Cellular fatty acids were saponified by boiling at 100 ℃ with 1 mL of 3.75N certified ACS-grade NaOH (Fisher) in 1:1 (v/v) HPLC-grade methanol (Burdick & Jackson, Muskegon, MI): roH_2O for 30 minutes. Methylation occurred after 2 mL of 1.18:1 (v/v), 6N HCl (VWR): HPLC-grade methanol was added and the mixture heated at 80 ℃ for 10 minutes. The FAME were extracted into the organic phase by adding 1.25 mL of 1:1 (v/v), HPLC-grade hexane (Burdick & Jackson): HPLC-grade methyl tert-butyl ether (J.T. Baker, Phillipsburg, NJ) followed by a base wash (0.3N NaOH in roH_2O) to remove any free fatty acids and residual reagents. The purified FAME were analyzed immediately using a gas chromatograph (GC model 6850, Agilent Technologies, Wilmington, DE) coupled to a flame ionization detector using the protocol recommended by MIDI (Paisley, 2004). The GC Chemstation enhanced integrator program (rev. A. 10.02, 1757, Agilent Technologies) was used to integrate the chromatogram peaks and to electronically transfer results to the MIS for library comparison with the TSBA method 5.0 (TSBA50) aerobe database. The output contained a list of fatty acids corresponding to percent composition and identifications with similarity indexes (SI) ranging from 0-1 in which the higher number being the closest match to the library of fatty acids for aerobic bacteria (RTSB50). The GC was calibrated twice before the start of each analysis sequence and after every 11th sample injection (Paisley, 2004) using rapid calibration standards (No. 1300-AA, MIDI). During all the calibration analyses, the peak percent named for the standard was >99% with the root mean square error <0.003. Genus-level and species-level identification were designated if the possible list from the MIS consisted of only a genus match to the known isolate or a species match, irrespective of its ranking. If the replicate of a known organism did not show a correct identification to genus or species, irrespective of the SI, it was categorized as unidentified. The isolates which were consistently identified had a SI >0.5 and were <0.1 SI within the range of the first rank. Only fatty acids >5% of the total were shown, otherwise the amounts were designated as trace.

3. Results

3.1 Gram-Positive Isolates

Arthrobacter sp. 5B6 was identified as *A. aurescens* based on its fatty acid profile (Table 3). Alteration in *anteiso* 15:0 fatty acid percentage led two of five replicates to be identified as *Brevibacillus choshinensis* as the first rank with <0.1 SI difference for *A. aurescens*. However, the MIDI indicated that a good match is considered when a SI≥0.1 occurs between the first and second rank. Three replicates identified as *A. aurescens* at the first rank showed *A. agilis* at the

second rank with a SI>0.1. *Bacillus subtilis* 5D1 was identified correctly to the species level due to a very high SI (Table 1). For all replicates, *B. subtilis* was the first rank on the possible match list indicating that the fatty acid profile (Table 3) matched the RTSB50 entry. *Micrococcus varians* 7F1 was identified previously using API Staph Trac (good ID) but the MIS identified the isolate as *M. luteus*-GC subgroup B (Table 3). Saturated straight- and branched-chain fatty acids were prominent in *Staphylococcus epidermidis* and *S. xylosus* but differed quantitatively and qualitatively (Table 3). Among the five replicates for *S. epidermidis* ATCC 14990, four were correct (Table 2) while one replicate was identified with low confidence (SI = 0.322) as *S. simulans*. All five replicates of *S. xylosus* 13A4 were identified as *S. gallinarum* which was the only option in the MIS list. Most of the fatty acids of strain 13A4 (Table 3) were within the RTSB50 range for *S. gallinarum* but *iso* 13:0, *anteiso* 13:0, *anteiso* 15:0, and *iso* 17:0 concentrations differed by approximately 14%, 4%, 14%, and 5%, respectively, from the library means. *Streptococcus faecium* 6A4 exhibited a narrow fatty acid composition (Table 3) and a high SI (Table 1). Although the total response was below the recommended limit of 50 000 for all replicates, these were identified as *Enterococcus faecium*. All possible identifications in the listing consisted of *E. faecium* GC subgroups A and B.

Table 3. Fatty acid profiles[a] of Gram-positive bacteria from the KSCC and ATCC

Fatty Acids	*Arthrobacter* sp. 5B6	*Bacillus subtilis* 5D1	*Micrococcus varians* 7F1	*Staphylococcus epidermidis* ATCC 14990	*Staphylococcus xylosus* 13A4	*Streptococcus faecium* 6A4
14:0	T	T	T	T	T	7.21 ±0.37
16:0	T	T	T	6.33 ±1.77	T	16.68 ±0.56
18:0	T	T	T	14.67 ±3.49	T	T
20:0	A	A	A	11.44 ±3.36	T	A
i13:0	T	T	T	T	27.29 ±0.94	A
i14:0	6.33 ±1.23	T	T	5.16 ±0.75	T	A
i15:0	6.52 ±3.10	23.68 ±3.27	13.81 ±0.33	14.44 ±1.66	13.81 ±0.44	A
i16:0	T	T	8.48 ±0.49	T	T	A
i17:0	T	10.27 ±0.47	T	T	7.74 ±0.24	A
ai13:0	T	T	T	T	9.20 ±0.58	A
ai15:0	77.94 ±4.03	38.72 ±1.34	57.09 ±0.18	31.45 ±4.64	16.89 ±0.47	A
ai17:0	T	11.47 ±1.42	13.09 ±0.20	T	T	A
18:1 ω7c	A	T	A	T	A	47.79 ±2.07
19:0 cy8c	A	A	A	A	A	8.74 ±1.45
SF3	A	A	A	A	T	17.37 ±0.52
Total[b]	91	84	92	83	75	98

[a]Each value is an average ± standard deviation of five replicates. T denotes trace amounts <5% ; A denotes absent/below detection; *iso* (i); *anteiso* (ai); cyclopropane (cy); *cis* (c); summed feature 3 (SF3) indicates fatty acids are 16:1 ω6c or 16:1 ω7c

[b]Total is the sum % of the fatty acid values >5%

3.2 Gram-Negative, Oxidase-Negative Isolates

Fatty acid profiles of *Citrobacter freundii* 4E5 indicated that 16:0, SF3 and 18:1 ω7*cis* were the major fatty acids, while branched-chain fatty acids formed a minor component (Table 4). Previously identified using API 20E, strain 4E5 was identified correctly in four of five replicates (Table 1) irrespective of its position in the MIS list. In two of the replicates, the first match was *Erwinia chrysanthemi* and separated from the *C. freundii* match by a SI<0.1. Strain 4E5 was not identified for one replicate from any choices in the RSTB50 list. Most of the fatty acids of all replicates were near the mean or within the prescribed percentage range. The replicate not identified as *C. freundii* had lower 16:0 (21%) and 18:1 ω7*cis* (14%) and higher SF2 (13%) and SF3 (34%) than the percentage ranges of the corresponding library entries. In three of five replicates, *E. cloacae* 12D5 was identified to species but it was incorrectly identified as *E. coli* at the first rank in the RSTB50 list. The difference between the first rank and *E. cloacae* had a SI>0.1. All identifications as *E. cloacae* had a narrow SI range (Table 1) and the differences between fatty acids quantities were subtle. Major fatty acids comprising strain 12D5 were SF3, 17:0 cyclopropane (cy), and 18:1 ω7*cis* (Table 4). The possible explanation for *E. cloacae* not being the first rank was the difference between the mean fatty acid percentage of the library entry and the replicates. Similarly, the identifications of the other two replicates identified only to genus may be explained by the higher percentage of 17:0 cyclopropane (11%) and lower percentages of SF3 (14%) and 18:1 ω7*cis* (21%). The differences between these fatty acids and the RSTB50 entry means were approximately 10% for SF3, 6% for 17:0 cy, and 4% for 18:1 ω7*cis*. The identification capability of strain 12D5 was challenged by its incorrect identification as *E. coli*. The five replicates of *E. coli* ATCC 11303 were identified correctly to species at the first or second rank with a SI<0.1. Two replicates had *Salmonella enteritidis* as the first rank, followed closely (SI<0.1) by *E. coli* as the second possible candidate. Similarly, in three correct replicates, the first rank was followed by *S. enteritidis* and a SI<0.1. The two *H. alvei* strains were identified previously (Table 1) using API 20E (excellent ID). Among the five replicates of strains 12E1 and 2F1, two replicates were identified as the first match. Major fatty acids comprising *H. alvei* included SF2, an unknown fatty acid (equivalent chain length of 14.502), SF3, and 17:0 cy (Table 4). *Serratia fonticola* 2D3 and *S. marcescens* ATCC 13880 were identified to species in all replicates and three of five replicates, respectively (Table 1 and 2). Strain 2D3 was identified previously using API RapidE (excellent ID) but showed ambiguous MIS results (SI<0.5) in the five replicates and might be due to the major fatty acids such as 15:0, 16:0 and SF3 (Table 4) falling outside the percentage range for the library entry. For the remaining two replicates, *Yersinia aldovae* (SI = 0.703) and *S. fonticola* (SI = 0.614) were the first ranks. Of the three correct replicates, *S. marcescens* was the first rank once while *Salmonella choleraesuis* subsp. *houtenae* and *Klebsiella pneumoniae* were the first ranks for the other two replicates separated by a SI>0.1. This variation in identifications in replicate analysis might be due to absence of minor fatty acids such as *anteiso* 16:0, SF6 and *iso* 19:0 (Table 4). Chromatograms showed the presence of these fatty acid peaks, but were not integrated by the software and subsequently not considered during comparison with the library. Fatty acid profile of the *S. marcescens* replicate, not being identified, was missing *anteiso* 16:0 and *iso* 19:0 but had a higher percentage of SF2 (11%), 13:0 was present, and a lower percentage of 16:0 (27%) compared to the RSTB50 entry.

Table 4. Fatty acid profiles[a] of Gram-negative, oxidase-negative bacteria from the KSCC and ATCC

Fatty Acids	*Citrobacter freundii* 4E5	*Enterobacter cloacae* 12D5	*Escherichia coli* 1D3	*Hafnia alvei* 12E1	*Hafnia alvei* 2F1	*Serratia fonticola* 2D3	*Serratia marcescens* ATCC 13880
14:0	6.70 ±0.42	7.28 ±0.21	5.64 ±0.26	7.45±0.46	6.28 ±0.41	5.79 ±0.18	6.15 ±0.38
15:0	T	T	T	T	T	5.53 ±2.92	T
16:0	24.02±1.58	26.57 ±0.75	27.32 ±0.88	33.36 ±0.68	33.07±0.56	28.15 ±0.92	28.24 ±0.81
18:1 ω7c	16.56±1.73	21.28 ±1.03	28.61 ±3.01	7.47 ±1.11	7.44 ±0.89	8.12 ±0.59	17.01 ±1.18
17:0 cy	T	8.64 ±1.71	9.84 ±3.06	17.63 ±5.31	21.57±5.11	12.25 ±2.78	7.77 ±3.00
SF2	9.82 ±1.45	8.52 ±0.55	8.37 ±0.54	8.96 ±0.69	9.21 ±1.25	8.74 ±0.56	9.86 ±0.69
SF3	33.25 ±0.87	16.18 ±3.01	8.96±2.73	14.88 ±5.07	10.63±2.99	21.79 ±6.38	19.11 ±2.82
Total[b]	90	88	89	90	88	90	88

[a] Each value is an average ± standard deviation of five replicates. T denotes trace amounts <5%; A denotes absent/below detection; *cis* (c); cyclopropane (cy); summed feature 2 (SF2) indicates peak tentatively designated as 12:0 aldehyde or unknown at retention time 10.947 min; SF3 indicates fatty acids are 16:1 ω6c or 16:1 ω7c

[b]Total is the sum % of the fatty acid values >5%

3.3 Gram-Negative, Oxidase-Positive Isolates

The MIS identified all replicates of *A. hydrophila* ATCC 35654 correctly and with a high SI (Table 2). The first rank was the *A. ichthiosmia-A. hydrophila* complex followed closely by other *Aeromonas* species in the RTSB50. Only two fatty acids were prominent in the profile for *A. hydrophila* (Table 5) but it was a repeatable identification. The fatty acid profiles of *Alteromonas* sp. 8C2 and *S. putrefaciens* ATCC 8071 and 49138 were very similar. Strain 8C2 was identified previously only to genus using classical taxonomic techniques plus it was proteolytic, lipolytic and hydrogen sulfide positive (data not shown). Strain 8C2 was identified as the *S. putrefaciens-S. alga* complex, the only entry for *Shewanella*. Although the *Alteromonas* name exists, there was no separate entry for any bacterium belonging to this genus in the RTSB50. Similarly, *Shewanella putrefaciens* ATCC 8071 and ATCC 49318 were identified as the *S. alga-S. putrefaciens* complex. *Pseudoalteromonas nigrifaciens* ATCC 19375 was correctly identified to species and had a high SI (Table 2). There are six species in the RTSB50 for *Pseudoalteromonas* separated by qualitative and quantitative differences in their fatty acids profiles (Table 5). *Flavobacterium* sp. 5C6 was the only isolate that belonged to the Gram-negative, oxidase-positive bacteria and was comprised of hydroxyl fatty acids (Table 5). In five replicates, strain 5C6 was identified as *Myroides odoratus*. The RTSB50 has five entries for *Flavobacterium* with only one entry for *Myroides*. Fatty acid profiles of *Moraxella* sp. 5E6 indicated a higher percentage of SF3 (16%) compared to 4% in *Psychrobacter immobilis* ATCC 43116 (Table 5). All replicates of strain 5E6 and *P. immobilis* ATCC 43116 were identified as *P. immobilis* even though the RTSB50 entries for *Moraxella* include several species. For *Psychrobacter*, library entries included only *P. phenylpyruvicus* and *P. immobilis*. The fatty acid profiles of strain 5E6 were different quantitatively from any of the *Moraxella* entries but matched well with the entry for *P. immobilis*. The SI for *P. immobilis* ATCC 43116 was high (Table 2) indicating that its fatty acid profile was very similar to the fatty acid profile of the RSTB50 entry. The lower SI determined for strain 5E6 might be due to higher percentages of 16:0 and SF3 (Table 5) which were close to the upper limit in the RSTB50. Low 18:1 ω9*cis* content, absence of SF6 (*anteiso* 18:0 or 18:2 ω6,9*cis*), and the low percentage of SF7 (19:1 ω11c or 19:1 ω9c), likely contributed to the lower SI (data not shown).

Providencia alcalifaciens 7F2 was identified previously from API 20E (excellent ID). The fatty acid profile indicated the presence of 14:0 which was not in any other isolate in the Gram-negative, oxidase-positive group (Table 5). All replicates were identified as *Erwinia chrysanthemi* biotype III as the first rank. The possible identification list of three replicates matched *P. rustigianii* which had a SI<0.1 from the first rank; in one replicate, the second rank with a SI<0.1 to *E. chrysanthemi*. Since strain 7F2 was identified only to genus, the average fatty acid profile was superimposed on the RSTB50 comparison charts of *P. alcalifaciens* and *P. rustigianii* to determine the closeness of the observed fatty acid profiles and the library entries for these bacteria. Most of the fatty acid quantities were within the range for *P. rustigianii* library entry rather than those for *P. alcalifaciens* (data not shown). Although the API 20E (excellent ID) was determined for strain 7F2 (Table 1), the MIS identified the same strain as *E. chrysanthemi*. Among the five replicates *Morganella morganii* 2E5, three were identified correctly as the first rank (Table 1). Although, the fatty acid profile of strain 2E5 was similar to *P. alcalifaciens*, the presence of 17:0 cy separated the two isolates (Table 5). Comparison charts of these replicates showed the SI increased when SF2 and 18:1 ω7*cis* were closer to the average percentage of these fatty acids in the RSTB50 entry (data not shown). The other two replicates were identified as *Salmonella typhimurium* GC subgroup B (SI = 0.68) and *Ewingella americana* (SI = 0.738) and separated from *M. morganii* by SI = 0.23–0.32 . Ten fluorescent *Pseudomonas* species comprising *P. fluorescens* and *P. putida* strains (Table 1 and 2) resulted in *P. putida* listed as the first rank while *P. fluorescens* had either a SI<0.1 or >0.1. Additionally, fatty acids such as 10:0 3-OH, 12:0 2-OH/3-OH, SF3, 17:0 cy, and 18:1 ω7*cis* were found to deviate from the mean fatty acid profiles (Table 6) for the RSTB50 entries of *P. fluorescens* biotypes A, B, C, F and G.

Table 5. Fatty acid profiles[a] of Gram-negative, oxidase-positive bacteria from the KSCC and ATCC

Fatty Acids	*Aeromonas hydrophila* ATCC 35654	*Alteromonas* sp. 8C2	*Shewanella putrefaciens* ATCC 49138	*Shewanella putrefaciens* ATCC 8071	*Pseudoalteromonas nigrifaciens* ATCC 19375	*Flavobacterium* sp. 5C6	*Moraxella* sp. 5E6	*Psychrobacter immobilis* ATCC 43116	*Providencia alcalifaciens* 7F2	*Morganella morganii* 2E5
14:0	T	T	T	T	T	T	T	T	5.69±0.08	6.56±0.33
15:0	T	7.25±1.15	9.15±1.44	6.39±0.67	8.76 ±0.19	T	T	T	T	T
16:0	17.29±0.50	9.11 ±0.93	T	6.89 ±0.65	16.13 ±0.68	T	T	T	28.56 ±0.48	32.21 ±1.11
i13:0	T	6.52 ±0.64	5.48 ±0.25	7.91 ±0.70	T	T	A	A	A	A
i15:0	T	10.17 ±0.64	23.41 ±0.88	15.69 ±1.46	T	52.57 ±2.77	T	T	A	A
15:1 ω8c	T	T	T	T	5.69 ±0.22	A	T	T	T	A
17:1 ω8c	T	13.02 ±1.84	20.14 ±2.00	14.21 ±1.19	11.43 ±0.72	A	9.91 ±1.53	8.64 ±1.70	T	T
18:1 ω7c	18.77±0.53	T	T	T	A	A	A	A	11.99 ±0.26	8.69 ±1.59
18:1 ω9c	A	T	T	T	T	T	48.37±1.74	63.04 ±2.25	A	A
i15:0 3-OH	T	T	T	T	A	6.41 ±0.72	A	A	A	A
i17:0 3-OH	A	A	A	A	A	10.92 ±1.64	A	A	A	A
17:0 cy	A	A	A	A	A	A	A	A	A	18.26 ±3.05
SF2	6.67 ±0.97	T	T	T	T	T	T	T	8.07 ±0.51	9.62 ±0.92
SF3	36.35±1.59	20.51±1.31	10.03±1.25	18.60±0.80	30.33±0.99	2.20±0.30	16.13±1.08	3.99±0.15	37.39±0.81	8.97±1.56
SF4	T	A	T	A	A	16.84±1.30	T	A	A	A
Total[b]	79	67	68	70	72	89	74	76	92	84

[a]Each value is an average ± standard deviation of five replicates except for *Moraxella* 5E6 where six replicates were used. T denotes trace amounts <5%; A denotes absent/below detection; *iso* (i); *cis* (c); cyclopropane (cy); summed feature 2 (SF2) indicates peak tentatively designated as 12:0 aldehyde or unknown at retention time 10.947 min; SF3 indicates fatty acids are 16:1 ω6c or 16:1 ω7c; SF4 indicates fatty acids are 10-methyl 16:0 or iso 17:1 ω9

[b] Total is the sum % of the fatty acid values >5%

Table 6. Fatty acid profiles[a] of Gram-negative, oxidase-positive bacteria belonging to *Pseudomonas* from the KSCC and ATCC

Fatty Acids	*Pseudomonas fluorescens* 11A1	*Pseudomonas fluorescens* 11B3	*Pseudomonas fluorescens* 12A2	*Pseudomonas fluorescens* 2A1	*Pseudomonas fluorescens* ATCC 17574	*Pseudomonas fluorescens* ATCC 31419	*Pseudomonas fluorescens* ATCC 13525	*Pseudomonas putida* ATCC 12633	*Pseudomonas putida* 7E4	*Pseudomonas putida* 12A3
16:0	30.11 ±0.39	24.29 ±0.82	32.14 ±1.51	27.37 ±0.82	26.83 ±1.40	29.20 ±1.49	28.19 ±1.26	25.73 ±0.72	29.62 ±0.67	27.66 ±1.70
17:1 ω7c	A	A	A	A	A	A	A	A	17.67 ± 0.6	A
18:1 ω7c	11.80 ±0.47	20.23 ±1.91	8.51 ±0.77	14.97 ±0.47	15.37 ±1.33	15.29 ±0.83	18.92 ±0.79	18.04 ±0.71	A	10.68 ±1.23
12:0 2-OH	6.45 ±0.29	5.93 ±1.09	5.54 ±0.68	T	5.11 ±0.87	6.12 ±0.96	T	5.20 ±0.44	5.85 ±0.30	T
13:0 3-OH	5.37 ±0.58	5.79 ±0.96	T	5.08 ±0.31	5.16 ±0.85	5.14 ±0.77	T	5.04 ±0.37	T	5.19 ±0.21
17:0 cy	6.89 ±2.63	T	8.78 ±1.54	T	T	T	T	7.51 ±2.88	10.75 ±2.52	7.50 ±3.68
SF3	31.03 ±2.61	30.08 ±1.71	30.45 ±0.97	36.38 ±0.63	36.54 ±0.72	29.69 ±1.93	33.16 ±0.72	28.21 ±3.07	22.57 ±2.45	32.23 ±3.62
Total[b]	92	86	85	84	89	85	80	90	86	83

[a]Each value is an average ±standard deviation of 5 replicates except for *P. putida* 7E4 and *P. fluorescens* 11B3 where six and seven replicates were used, respectively. T denotes trace amounts <5%; A denotes absent/below detection; cyclopropane (cy); summed feature 3 (SF3) indicates fatty acids are 16:1 ω6c or 16:1 ω7c

[b]Total is the sum % of the fatty acid values >5%

4. Discussion

The 33 bacterial strains belonged to 18 genera comprising Gram-negative and Gram-positive strains. The majority of the isolates tested were Gram-negative since these have been determined as dominant bacteria in spoiling raw seafood (Himelbloom *et al.*, 1991; Himelbloom *et al.*, 1994; Gram and Huss, 2000). Overall, 57% of the KSCC isolates and 75% of the ATCC strains were identified correctly to species in all replications with a SI>0.5 and a SI range<0.1 of the first rank in the MIS list. Isolates belonging to Enterobacteriaceae and *Pseudomonas* species formed the remainder of the KSCC isolates. Gram-positive organisms had a high percentage of branched chain fatty acids (64–96%) with one exception. Gram-negative, oxidase-negative isolates were composed of 38–69% of saturated fatty acids and 19–50% of unsaturated fatty acids. Hydroxyl fatty acids were present in all Gram-negative, oxidase-positive isolates with only one exception, the branched chain fatty acids varied considerably (<1–74%) .

Pseudomonas species misidentification at the first rank may be attributed to the closeness in the fatty acid profiles of *P. fluorescens* and *P. putida* to form a tight taxonomic cluster (Osterhout *et al.*, 1991; Moss and Dees, 1976; Oyaizu and Komagata, 1983; Vancanneyt *et al.*, 1996). Variation in fatty acid and subsequent identification between the replicates can be due to harvesting cells from the stationary phase along with the log phase of the culture (Osterhout *et al.*, 1991). Contrary to our findings, cellular fatty acid analysis effectively distinguishes clinical specimen strains of *P. fluorescens* and *P. putida* (Hsueh *et al.*, 1998). *Pseudomonas* strains analyzed in the present study were isolated from the psychrotrophic environment and may have changed in fatty acid profile when grown at 28 ℃ (Zachariah and Liston, 1973; Morita, 1975). This alteration in fatty acids by psychrotrophs from the same genus can be variable and strain-dependent (Gill, 1975; Herbert, 1981). The identity of the KSCC isolates belong in the *P. fluorescens-P. putida* complex designated by MIDI (Osterhout *et al.*, 1991) but the reproducibility of bacterial identification is strain-dependent.

The MIS reduced the microbial analysis time to ~1 h after a 24 h pure culture of isolates compared to classical techniques requiring several days. The system correctly identified the ATCC strains with higher SI compared to the KSCC strains (Table 1 and 2). These observations concur with those that show bacterial identifications using MIS are genus- or species-specific (Osterhout *et al.*, 1991; Birnbaum *et al.*, 1994; Steele *et al.*, 1997; Odumeru *et al.*, 1999). Challenges in the identification of certain isolates or low SI during repeated analysis can be attributed to: (1) difficulty in ascertaining the growth phase of the bacterial isolates. The MIDI protocol states that a bacterial mass of 40 mg should be collected from the 3rd quadrant which corresponds to the late log-phase. This phenomenon might not be always true since different organisms have different growth rates. Hence, the cells harvested from 3rd quadrant can belong to a different growth phase resulting in altered fatty acid profiles (Kaneda, 1991); (2) pinpoint colonies. For *S. epidermidis* ATCC 14990, it was observed that the required weight of 40 mg could not be procured from one plate and hence cells were collected from seven different plates. This resulted in the differences in percentages of *iso* 13:0, 14:0, 16:0, lower 20:0, and absence of 19:0 and *anteiso* 19:0, as compared to the RTSB50 entry and ultimately lower SI; (3) inclusion or omission of minor fatty acids. Replicate analysis of *S. xylosus* indicated a presence of minor fatty acids *iso* 17:1 ω10*cis* (<1%) and *anteiso* 19:0 led to its identification as *S. gallinarum*; (4) increase or decrease in percentages of certain fatty acids. Deviation in the fatty acids such as cyclopropane fatty acid, SF3, and 18:1 ω7*cis* lead to either low SI as in case of *Pseudomonas fluorescens* (KSCC strains) or change in ID as for *Enterobacter cloacae* 12E5. Comparison charts should be used to understand the variations in replicate analysis and to

increase the number of replications for specific bacterial isolates. Correct identification for some bacteria was not listed within the top ranked candidates and indicated that prior knowledge of the bacteria source is crucial to help eliminate misidentifications.

Therefore, the MIS must include accurate fatty acid profiles for targeting unique non-clinical bacteria by using well-identified, laboratory-isolated strains and the ATCC or other national repository strains. Library entries could be created prior to utilizing the MIS as a rapid screening method or for complementary identification. There exists a need to develop a customized database of cell membrane fatty acid profiles of spoilage bacteria isolated from cold-water fishery products. Then, the MIS may become a rapid method for identifying these bacteria in seafood and for tracking the microflora changes occurring during chilled storage.

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