AN ABSTRACT OF THE THESIS OF

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in Food Science and Technology presented on June 26, 1978

Title: NUTRITIONAL AND BIOCHEMICAL CHARACTERIZATION OF MORAXELLA SPECIES FROM FISHERY SOURCES

Abstract approved: Redacted for privacy

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One hundred and fourteen strains of Moraxella species isolated from marine fishery sources were investigated to determine the cause of this organism's predominance in seafood. The nutritional requirements, effect of pH, NaCl concentration, and temperature on growth, utilization of carbohydrates, protein, and lipid, resistance to 12 antibiotics, sensitivity to phenethyl alcohol (PEA), production of PEA, and a study of the deoxyribonucleic acids (DNA) of the Moraxella strains were performed.

Two strains of Moraxella had a requirement for biotin; most other strains, however, had no requirement for growth factors. The majority of strains had a multiple amino acid requirement which was satisfied by seven amino acids: leucine, isoleucine, valine, alanine, glycine, serine, and hydroxyproline.

All strains grew in broth in the pH range of 6.9 to 8.8, with pH

limits for growth by any strain being 4.9 to 10.4. All strains grew in the presence of 6% NaCl, with most strains tolerating up to 10%. Ninety five percent of strains tested grew at 5 C and no strain grew at 37 C or above.

Thirty percent of all Moraxella strains were able to produce acid from glucose. Xylose and arabinose were oxidized by 17.5 and 1.7% of strains, respectively. Sucrose, fructose, lactose, and galactose were not oxidized. Seven percent of strains were weakly proteolytic while lipolytic activity was not observed in any strain.

All Moraxella strains were sensitive to tetracycline (Tc), chlorotetracycline (Ct), oxytetracycline (Ot), streptomycin (St), neomycin (Nm), kanamycin (Km), and nalidixic acid (Na). Half of all strains were resistant to chloramphenicol (Ch) and nitrofurazone (Nf), 36% were resistant to ampicillin (Am), and 70% were resistant to sulfathiazole (Su). Thirty one strains (27.2%) were resistant to 3 IU of penicillin G (Pe), thus, being classified as atypical Moraxella. Atypical Moraxella strains were significantly more resistant to Ch and Am.

Moraxella strains were more resistant to PEA than were other gram negative bacteria of fishery origin. At a concentration of 0.82 mmoles PEA/liter, Moraxella strains were not affected; however, an Arthrobacter strain of marine origin was totally inhibited and marine Pseudomonas strains showed an extended lag period.

Moraxella strains produced PEA in concentrations ranging from undetectable to 3.25 mmoles per liter. PEA production was strain

dependent and substrate dependent and was not the direct function of the cell concentration.

Moraxella strains tested showed a guanine plus cytosine mole percent (G+C mole %) between 42.8 to 44.4. No relationship was observed between G+C mole % and other parameters: penicillin resistance, proteolytic ability, PEA production, or acid production from glucose oxidation.

Four factors appear to allow Moraxella to predominate in seafood: NaCl tolerance, the ability to grow at low temperatures, a simple nutritional requirement, and production of PEA.

# Nutritional and Biochemical Characterization of *Moraxella* Species Isolated from Fishery Sources

by

Stephen M. Vernon

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Commencement June 1979

APPROVED:

Professor of Food Science and Technology
in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented June 26, 1978

Typed by Christi Blodgett for Stephen Michael Vernon.

## ACKNOWLEDGEMENTS

I would like to thank Dr. Jong S. Lee, my major professor, for his guidance and help throughout my entire stay at Oregon State University. Also, to Don Pfeifer, for his assistance with my laboratory work.

I would like to thank Dr. Paul Kifer, the department head, Dr. Allen Anglemier, head of the graduate students, and all the fine faculty and staff of the Department of Food Science for the time and effort. A special thank you is extended to Dr. Dan Selivonchick and Mr. Jim Barbour for their assistance with the gas chromatography work.

I would like to thank Dr. Ramon J. Seidler of the Department of Microbiology for his help with the GC analysis of my bacteria.

Finally, a special thank you to my fiance Christi, for her love and moral support throughout my thesis work.

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## INTRODUCTION

The spoilage of cold-stored proteinaceous foodstuffs, such as fish, poultry, and meat, is accompanied by the large increase in the numbers of bacteria of the *Pseudomonas-Moraxella* type. *Pseudomonas* has long been recognized as a spoilage organism, producing off odors and off flavors in refrigerated seafoods. The role of *Moraxella* in seafood spoilage, however, is not clear. *Moraxella* seem to have a low spoilage potential, as judged from pure culture studies on sterile fish; yet this organism's persistence and predominance on seafoods raises questions as to its natural role.

*Moraxella* are also isolated from clinical sources as an opportunistic pathogen of debilitated hosts. This group of well-defined *Moraxella* species are morphologically, biochemically, and genetically similar to their fishery counterparts, except for their growth temperature. Clinical isolates are strict mesophiles, while the fishery strains are psychrotrophic.

The morphological, biochemical, and genetic studies of clinical and fishery *Moraxella* are described in the literature review. The criteria of seafood spoilage, description of spoilage organisms, and the role of protein in seafood spoilage are also reviewed. This study was initiated to determine the nutritional requirements and biochemical characteristics of fishery *Moraxella* isolates. Antibiotic resistance, resistance

to and production of phenethyl alcohol, and deoxyribonucleic acid studies of fishery Moraxella strains are also presented. Finally, a hypothesis was presented to explain the possible role of Moraxella in seafoods.

# LITERATURE REVIEW

## General Description

*Moraxella* are gram negative to gram variable, short, plump rods, often coccus shaped, which exist singly, in pairs, or in chains. Spores are never formed, nor are flagella found. All species are strict aerobes. *Moraxella* are chemoorganotrophic with an oxidative metabolism. Carbohydrates are usually not oxidized, though some strains do oxidize glucose and other carbohydrates. *Moraxella* are cytochrome oxidase positive and most strains are quite sensitive to penicillin G. The guanine plus cytosine mole percent (G+C mole %) of *Moraxella* ranges from 40 to 46 (10).

*Moraxella* has been isolated from mucous membranes of man and other warm-blooded animals and from marine fishery sources. Five species are recognized and other "*Moraxella*-like" strains have been reported. Fishery isolates fall under this latter group.

*Moraxella* is differentiated from *Acinetobacter* by their cytochrome oxidase positive reaction and penicillin sensitivity. *Neisseria* differ in that they divide on two planes while *Moraxella* divide on only one and *Neisseria* have a G+C mole % of 50 to 55. The separation between *Moraxella* and *Branhamella* is not so clearly defined. It has been proposed that the two genera be merged (28); however, *Branhamella* are always coccus shaped, while *Moraxella*, though often pleomorphic, are more often rod shaped.

The literature regarding *Moraxella* and related bacteria is

quite confusing. Different organisms have been incorrectly grouped together and seemingly identical organisms have been classified differently. Genera associated with Moraxella in the past were: Morax-Axenfeld bacillus, Mimeae, Herellea, Bacterium, Hemophilus, Pseudomonas, Acinetobacter, Achromobacter, and Branhamella. Several review articles have helped to clarify our knowledge of clinical and fishery Moraxella species (3,24,27,28,29,33).

## Comparison of Clinical and Fishery Moraxellas

Similarities and differences exist between clinical isolates of Moraxella and those of fishery origin. The clinical Moraxella are opportunistic parasites that reside on mucous membranes of man and animals and often are isolated from the respiratory and genitourinary tracts of man (40,58,59,63). Animals found to harbor Moraxella include swine (38), sheep and cattle (58,59). Moraxella species have been implicated in infectious keratoconjunctivitis of cattle (59,59) and catarrhal pneumonia, arthritis, and abscesses in swine (39). In humans, Moraxella has been isolated from clinical cases of bacterial endocarditis (73), conjunctivitis (16,79), septicemia (21), arthritis (22), urethritis (37,63), meningitis (47), and vaginitis (16).

The pathogenic potential of Moraxella appears to be low (28). Predisposing conditions often are necessary for infection to occur. The highest rate of Moraxella infections in swine occurred during the winter months among those less than one week of age (39).

Clinical Moraxella species have varying growth requirements (28). Moraxella osloensis and M. urethralis had simple growth requirements. Both grew well in a simple medium that contained ammonia as the nitrogen source and acetate as the carbon source (3,9,28). M. nonliquifaciens and M. bovis exhibited fair growth on nutrient agar. Improved growth was noted when media were supplemented with blood or serum (28). M. lacunata, M. phenylpyrouvica, and M. kingii, on the other hand, were quite fastidious and required blood or serum supplementation (28). Moraxella species are relatively inactive biochemically. Most were shown to be asaccharolytic (28). Some of the few exceptions were M. osloensis, which has been reported to produce acid from ribose and xylose (60) and M. kingii, which was reported to produce acid from glucose, maltose, and galactose (28). None of the 48 strains of oxidase-positive Moraxella investigated by Baumann et al., however, were able to utilize sugars or amino acids (3).

All clinical isolates of Moraxella are mesophilic, with an optimum temperature range for growth of 33 to 37°C. Poor growth, if any, was observed at 25°C (28).

Moraxella isolates from bovine keratoconjunctivitis infections showed the spreading-corroding (SC) colony morphology on agar (59). Under the electron microscope, these cells appeared fimbriated. It was reported that only fimbriated strains of M. bovis, which produced the SC type of colony morphology, were able to colonize the mucous membranes of the bovine eye (8). Fimbriation is thus

a possible adherence-promoting factor. Fimbriae of bacteria have been found to confer adhesion to many types of tissue cells (19).

Shewan (70) described Achromobacter species from fishery sources as being nonmotile, nonpigmented, short, stout rods that were often coccus shaped. They occurred singly, in pairs, or in short chains. Colonies were grey to off-white and were opaque on agar. They were relatively inactive biochemically and were sensitive to 3 IU of penicillin G. In Hugh and Leifson media (32), most strains of Achromobacter produced an alkaline reaction, others, no reaction, and a few strains showed an acid reaction, indicating the oxidative utilization of glucose. In contrast to the clinical isolates, the fishery isolates grew at $0^{o}C$ and were not able to grow at $35^{o}C$.

Strains of Achromobacter or Moraxella from fishery sources are usually classified only to the genus level. Shewan (68) compared many of his fishery isolates to known species of clinical Moraxella in regard to cellular and colony morphology, biochemical reactions, and growth requirements and found these strains to be quite similar to M. lacunata and M. osloensis.

Thornley (77) compared 120 strains of gram negative short rods previously identified as Achromobacter. She divided the strains into five phenotypic groups (phenoms), based on similarities of phenotypic characteristics. Phenoms 1 and 5 consisted of motile strains, not characteristic of Moraxella. Phenom 2 strains were cytochrome oxidase negative and were resistant to 2.5 IU of penicillin. These bacteria thus fit into the latest Bergy's Manual

description of the genus Acinetobacter. Strains from phenom 4 varied both in biochemical reactions and G+C mole %. It is difficult to determine which, if any, strains from phenom 4 fit into the present genus Moraxella. Strains from phenom 3, isolated from cold stored poultry and fish, were oxidase positive, sensitive to 2.5 IU of penicillin, and produced acid from glucose, arabinose, xylose, lactose, and galactose. Their G+C mole % was 44 to 45. Thornley (77) felt that phenom 3 was closely related to M. saccharolytica. She also believed phenom 3 was very similar to the strains of Shewan (67) and Hendrie et al. (27). Also bearing similarities to phenom 3 were four oxidase positive, penicillin sensitive Achromobacter strains isolated from fresh meat by Gardner (23).

Thornley (77), in characterizing the strains of phenom 3, noted the presence of capsules and fimbriae in some strains. Phenom 3 consisted of seven strains of poultry origin and one strain of fishery origin. No reference was made of the presence of fimbria in the strain of fishery origin. Thornley did note, however, the very high degree of relatedness among the strains of phenom 3.

## Seafood Spoilage and Moraxella

Moraxella species often predominate in freshly landed seafoods (42,43,45,49,50,66,68,71). Upon storage, Moraxella and Pseudomonas species become the predominant flora of refrigerated fish and shellfish (42,43,45,49,66,69). Due to their resistance to irradiation, Moraxella species become the predominant flora of

irradiated Dover sole (15), Dungeness crab (72), and chicken (78). Lee and Pfeifer (42) noted the predominance by Moraxella in Dungeness crab was due to a high initial population and rapid growth while Pseudomonas predominated due only to its initial high numbers. They reported a similar phenomenon with Pacific shrimp (43). The environment is the major factor that determines the microbial flora of seafoods (43,67). Shewan (67) noted that different species of fish, such as cod, haddock, sole, skate, and herring, caught during the same season, from the same area, had very similar microbial floras. The presence of Moraxella species in high proportions in freshly caught seafoods reflects their environmental origin.

The role of autolytic enzymes in fish spoilage appears to be minor and microorganisms are primarily responsible for spoilage of seafoods (20,31,50,62). Spoilage microorganisms are restricted to the Pseudomonas-Achromobacter group (30,45,69,80). Lerke et al. (45) tested 213 Achromobacter strains and found 29% of them spoiled fish press juice. Spoilage organisms were found to increase considerably in numbers during cold storage of fish but never accounted for more than 20% of the total microbial flora (30). This observation has been substantiated by others (1,65).

Much work has been done on determining the best method to objectively measure fish spoilage and to determine which organisms were responsible for it. Heat sterilized fish substrate was used (11), though it did not duplicate the natural substrate. Sterile fish muscle press juice (44), and later, press juice separated into

protein and nonprotein fractions have also been employed with some success (46). Finally, sterile fish muscle, obtained by aseptic technique, has been used as a substrate in pure culture studies (50). From such studies, it was shown that total volatile acids (TVA) and total volatile bases (TVB), produced in fish muscle, were due to microbial action (50). Further, it was found that Achromobacter isolates produced low levels of TVB though TVA was produced in amounts comparable to that produced by Pseudomonas. However, TVA levels were reduced during storage due to further degradation. This indicated that TVA measurements were not a suitable indicator of fish spoilage (50).

Lerke et al. (46) showed that the protein fraction of sterile fish muscle press juice supported microbial growth yet did not give off the characteristic spoilage odor. The protein-free fraction, containing small molecular weight nitrogenous compounds, however, spoiled normally, according to the organoleptic and chemical criteria of spoilage, measured by the levels of volatile reducing substances (VRS), trimethyl amine nitrogen (TMA-N), and total volatile nitrogen (TVN). Thus, the authors reported that off-odors and off-flavors were produced prior to degradation of large protein molecules (46).

The proteolytic ability of microorganisms has not been correlated to their spoilage potential. Proteolysis, it was reported, represented the advanced state of spoilage, happening long after the seafood had passed the spoilage stage (4). The ability to liquify gelatin or to digest egg albumin were not the characteristics found

in all spoilers and some nonspoilers were able to liquify gelatin and digest egg albumin (45). Pseudomonas fragi, which produced strong off-odors from fish muscle extracts, was unable to hydrolyze protein (11). It has been shown that lower amines, such as dimethyl amine (DMA), trimethyl amine (TMA), and ammonia, which are used as indications of spoilage, were not always produced by the microorganisms producing the off-odors (30).

Shewan (66) stated that fish spoilage by marine bacteria was the result of degradation of small molecular weight nitrogenous compounds: TMA oxide, simple peptides, amino acids, and nucleotides. In beef muscle with a high microbial load ($10^7/cm^2$), the free amino acid content decreased with the onset of spoilage (36). When a medium containing amino acids was inoculated with a strain of Pseudomonas and a strain of Achromobacter, the amino acid levels decreased with the growth of bacteria and the production of spoilage odors (36). The nucleotide level was also decreased by 43% when equal numbers of a fluorescent Pseudomonas species and an Achromobacter strain were inoculated in a semimembranous muscle (36).

The slime on fish is a major source of the microbial flora of fish. The composition of fresh cod slime showed that the amino acids alanine, tyrosine, leucine, and taurine comprised 13% of the total solids (68). Amino sugars were also detected. The glycoprotein and amino acid constituents of a fish body mucin contained glucoseamine, N-acetylneuraminic acid, galactoseamine, and the amino acids: aspartic acid, alanine, glutamic acid, glycine, threonine, leucine, valine,

lysine, serine, histidine, proline, isoleucine, phenylalanine, arginine, tyrosine, and methionine (26). In one sample, the free amino acids found in the highest quantities were: aspartic acid, threonine, serine, glutamic acid, glycine, and alanine (24). Seventeen free amino acids have been detected in organic matters in sea water (57). Found, in order of abundance, were: glutamic acid, lysine, glycine, aspartic acid, serine, alanine, leucine, valine plus cystine, isoleucine, ornithine, methionine sulfoxide, tyrosine plus phenylalanine, histidine, arginine, proline, and methionine.

DNA Determination and Antibiotic Resistance of Moraxella

Most work on DNA guanine plus cytosine (G+C) mole % studies, DNA homology, and genetic transformation has been with the clinical isolates of Moraxella (3,6,7,9,12,28). A comprehensive survey of the G+C mole % of the recognized Moraxella species is to be found in a review by Henriksen (28). G+C mole % values ranged from 40.0 to 44.5. Thornley (77) determined the G+C values for phenom 3, which contained isolates of poultry and fishery origin, to be between 44 to 46 mole %.

The 8th edition of Bergey's Manual of Determinative Bacteriology (10) differentiated Moraxella from Acinetobacter on the basis of the cytochrome oxidase test. Moraxella species are cytochrome oxidase positive, while Acinetobacter species are cytochrome oxidase negative. Moraxella species are also quite sensitive to 3 IU of penicillin G, while Acinetobacter species are quite resistant. The antibiotic

resistance patterns of Moraxella have been reviewed (12, 58). Catlin and Cunningham (12) found most strains of Moraxella were sensitive to a large number of antibiotics: penicillin, streptomycin, tetracycline, neomycin, chloramphenicol, erythromycin, novobiocin, kanamycin, and vancomycin. M. bovis was found to be sensitive to penicillin, streptomycin, chloramphenicol, tetracycline, sulfathiazole, neomycin, and polymyxin (58).

The effects of penicillin G and chloramphenicol on M. osloensis were quite different (17). Penicillin G, at a concentration of 0.5 IU, was bacteriostatic to the culture. Increasing the concentration of penicillin G, up to 5000 IU, only weakly increased its bactericidal effect, with an inactivation rate independant of the antibiotic concentration. There was no evidence of cell lysis. Cells in media containing penicillin G were nearly exclusively filamentous. Chloramphenicol was bactericidal to M. osloensis at concentrations as low as 50 μg/ml.

## Phenethyl Alcohol

Phenethyl alcohol (PEA) and phenol, two high boiling volatiles, were detected in refrigerated haddock fillets by gas chromatography-mass spectroscopy (GC-MS) (14). PEA was produced by Achromobacter strains of fishery origin from L-phenylalanine and ethanol (13). This was significant taxonomically as the fishery Achromobacter strains produced detectable amounts of PEA while no Acinetobacter strains and only one of nine strains of clinical Moraxella produced detectable

amounts (13). This discrepancy, however, may be due to the inability of clinical Moraxella strains to grow in vitamin-free casein hydrolysate (Nutritional Biochem. Corp.), which was used to grow the bacteria to reduce the size and number of background peaks in GC-MS analysis, a problem encountered with other media.

The effects of PEA on bacteria DNA, especially to gram negative bacteria, is well documented (5,18,48,54,56,75,76). PEA was shown to inhibit repair of gamma-ray induced single-strand breaks in the DNA of Escherichia coli (54). However, synthesis of DNA and protein was only partially inhibited. This suggested that PEA action on the cellular metabolism of E. coli was selective and limited.

PEA reduced the rate of incorporation of fatty acids into the phospholipids of a fatty acid auxotroph E. coli (56). Phospholipid synthesis was shown to be more sensitive to inhibition by low concentrations of PEA than were protein, DNA, and RNA syntheses (56).

The outgrowth of spores of Mucor rouxii was inhibited by PEA concentrations of 0.05 to 0.3% (75). Production of PEA by Candida albicans was shown to produce autoinhibition (48).

PEA was found to selectively inhibit gram negative bacteria in a mixed culture (5). It was proposed that reversible inhibition of DNA synthesis in gram negative bacteria was the cause of this bacteriostatic action. In contrast, it was reported that gram positive and gram negative non-sporulating anaerobes were somewhat resistant to PEA in both liquid and solid media (18). Both gram negative and gram positive cultures grew in thioglycollate broth containing 0.25%

PEA.

PEA inhibited the growth of a marine Pseudomonas strain at a concentration of 0.15% (76). In this study, PEA was found to interfere with the uptake of an amino acid analog. Thus, PEA appears to have broader inhibitory effects on gram negative bacteria beyond that of its well known effect on DNA synthesis.

# MATERIALS AND METHODS

## Moraxella Cultures

Most strains of Moraxella used in this investigation were isolated by us from various marine fishery sources. Table 1 lists the origins of these strains. A total of 114 strains, from ground beef, from shrimp processing plants, and from fishing vessels were employed in this study. All cultures conformed to the description of the genus Moraxella of the 8th edition of Bergey's Manual of Determinative Bacteriology (10). The cells were oval to coccobacillary in morphology, did not produce pigments, gram negative to variable, cytochrome oxidase positive, and non-motile.

## Nutrient Requirements of Moraxella

Thirty one randomly selected Moraxella strains were examined for their growth factor and amino acid requirements (Table 1). Cultures were grown on the initial isolation medium (TPE) that contained tryptone, peptone, and yeast extract as major ingredients (Table 2). The colonies on TPE mother plates were then replicated onto series of daughter plates that contained the test nutrients in various combinations. After incubation, the growth responses were recorded as: +++: excellent growth, ++: good growth, +: some growth, -: no growth.

## Growth Factors

Table 1. List of Moraxella strains investigated.

| SOURCE | STRAIN DESIGNATION | | | | |
|---|---|---|---|---|---|
| Ground Beef | A-41* | A-42* | A-43* | A-44*** | A-45* |
| Crab | A-101* | A-102*** | A-103* | A-151* | A-171* |
| | A-401* | A-402* | A-404* | A-491*** | A-4211* |
| | A-4213* | A-4214* | A-2-11* | | |
| Shrimp processing Plant | C-2-15* | D-1-12*** | D-2-29* | D-4-10* | |
| Shrimp Boat | DD-2-4-2* | DD-2-4-3 | DD-2-4-5 | DD-2-4-6** | DD-2-4-10* |
| | DD-2-4-14* | DD-2-4-18 | DD-4-5** | S-1-4* | S-1-5* |
| | S-1-19* | S-1-20 | S-1-23*** | A-1-1 | A-1-2 |
| | A-1-3 | A-1-4** | A-1-24 | A-1-25 | A-1-27 |
| | A-1-28 | A-2-4 | A-2-5 | A-2-12 | A-2-30 |
| | B-1-3 | B-1-4 | B-1-10 | B-1-11 | B-1-14 |
| | B-1-15 | B-1-16 | B-1-19 | B-1-21 | B-1-23 |
| | B-1-25 | B-1-30 | C-1-2 | C-1-3 | C-1-10 |
| | C-1-15 | C-1-16 | C-1-17 | C-1-20 | C-1-25 |
| | C-1-30 | C-2-2 | C-2-6 | C-2-10 | D-1-1 |
| | D-1-5 | D-1-6 | D-1-7 | D-1-10 | D-1-14 |
| | D-1-15 | D-1-22 | D-1-27 | D-1-30 | D-2-1 |
| | D-2-10 | E-1-1** | E-1-4 | E-1-8 | E-1-9 |
| | E-1-10 | E-1-11 | E-1-20 | E-1-23 | E-1-26 |
| | E-1-27 | E-1-29 | E-1-30 | F-1-2 | F-1-6 |
| | F-1-8 | F-1-9 | F-2-1 | F-2-2 | F-2-6 |
| | F-2-8 | F-2-9 | F-2-10 | F-2-15 | AC-1-11 |
| | AC-1-12** | 4-1-7 | 5-1-1 | 5-1-2** | 5-1-27** |
| | 5-2-2** | 5-2-13** | | | |

*Strains used for nutritional study.

**Strains used for PEA production study.

***Strains used for both studies.

Table 2. Tryptone-peptone-extract agar[a] (TPE).

| INGREDIENT | QUANTITY |
|---|---|
| Bacto-tryptone[b] | 5 g |
| Bacto-peptone[b] | 5 g |
| Sodium chloride, analytical reagent[c] | 5 g |
| Yeast extract[b] | 2.5 g |
| Dextrose, reagent[d] | 1 g |
| Bacto-agar[b] | 15 g |
| Distilled water | 1000 ml |

[a]Agar was sterilized at 15 psi for 20 minutes.

[b]Difco Laboratories

[c]Mallinckrodt Chemical Works.

[d]J. T. Baker Chemical Co.

Davis Minimal Agar (DMA) (Difco) was selected as the basal medium to test the growth factor requirements of Moraxella strains. DMA was developed for testing the nutrient requirements of Escherichia coli (41). The growth factors and their respective concentrations we examined have also been previously tested with E. coli (41), with the exception of biotin, which was added to a final concentration of 0.02 mg/liter instead of 0.0001 mg/liter. Five growth factors: biotin, nicotinic acid, pyridoxine, riboflavin, and thiamine were tested. All growth factors were filtered through a sterile 0.22 μm cellulose nitrate filter (Millipore Corp.) and stored as stock solutions at 4°C.

Moraxella strains were replica plated onto DMA, DMA plus the five growth factors, DMA plus the five growth factors plus 0.5% vitamin free casamino acids (VFCAA) (Difco), 0.5% VFCAA alone , and DMA plus 0.5% VFCAA. The composition of VFCAA is listed in Table 3. The growth factors were added to molten media, previously autoclaved at 15 psi for 20 minutes and cooled to 45°C, prior to pouring. All plates were incubated at 25°C and the growth response recorded after 48 hours.

After the preliminary growth factor requirement study, a second study was undertaken to determine which growth factor or combination of growth factors was stimulatory or essential for growth. The basal medium was DMA plus 0.5% VFCAA. All 32 possible combinations of the growth factors were examined. Again, growth response was recorded after 48 hours incubation at 25°C.

Table 3. Composition of casamino acids (VFCAA)[a]

| MACRO INGREDIENT | % |
|---|---|
| Total nitrogen | 11.15 |
| Arginine | 3.8 |
| Aspartic acid | 0.49 |
| Cystine | - |
| Glutamic acid | 5.1 |
| Glycine | 1.1 |
| Histidine | 2.3 |
| Isoleucine | 4.6 |
| Leucine | 9.9 |
| Lysine | 6.7 |
| Methionine | 2.2 |
| Phenylalanine | 4.0 |
| Threonine | 3.9 |
| Tryptophan | 0.8 |
| Tyrosine | 1.9 |
| Valine | 7.2 |
| **GROWTH FACTORS** | **µgram/gram** |
| Biotin | 0.102 |
| Nicotinic acid | 2.7 |
| Pyridoxine | 0.073 |
| Riboflavin | 0.03 |
| Thiamine | 0.12 |

[a]Difco Laboratories.

## Amino Acids

Table 4 lists the amino acids and their respective concentrations used in this experiment. The tests were again modeled after that with E. coli (40). Amino acids were divided into five groups. The basal medium was DMA plus the five growth factors. All 32 possible combinations of the five amino acid groups, along with the growth factors, were added to molten DMA prior to pouring. After replica plating, all plates were incubated at 25°C for 48 hours and the growth response recorded.

Once nonessential amino acid groups had been determined, remaining amino acids were added singly to the basal medium (single addition) and all amino acids, except one (single deletion), were added to the basal medium. Again, all plates were incubated at 25°C for 48 hours and the growth response recorded.

## Effect of pH

Twenty Moraxella strains were tested for growth at various pH levels in 10 ml TPE broth. The broth was made acidic by dropwise addition of 5N HCl and made alkaline by dropwise addition of 5N NaOH. The pH was measured by a Beckman Zeromatic II pH meter. The pH levels tested were: 3.0, 3.9, 4.9, 5.9, 6.9, 8.0, 8.8, 9.4, and 10.4. Culture tubes were inoculated, in duplicate, and incubated at 25°C. Visible turbidity after 48 hours was recorded as growth. All negative tubes were reincubated for 48 hours to check for any delayed growth.

Table 4. Amino acids and the concentrations tested.

| AMINO ACID[a] | ABBREVIATION | FINAL CONC.(mg/l) |
|---|---|---|
| Group 1 | | |
| L-lysine | lys | 10 |
| L-arginine | arg | 10 |
| L-methionine | met | 10 |
| L-cystine | cys | 50 |
| Group 2 | | |
| L-leucine | leu | 10 |
| L-isoleucine | ile | 10 |
| L-valine | val | 10 |
| Group 3 | | |
| L-phenylalanine | phe | 10 |
| L-tyrosine | tyr | 10 |
| L-tryptophan | trp | 10 |
| Group 4 | | |
| L-histidine | his | 10 |
| L-threonine | thr | 20 |
| L-glutamic acid | glu | 10 |
| L-proline | pro | 10 |
| L-aspartic acid | asp | 10 |
| Group 5 | | |
| L-alanine | ala | 10 |
| glycine | gly | 10 |
| L-serine | ser | 10 |
| L-hydroxyproline | h-pro | 10 |

[a]All amino acids from Calbiochem.

The pH optimum for growth was determined by inoculating, in duplicate, 20 mm optically matched culture tubes with TPE broth adjusted to various values: 6.7, 7.0, 7.4, 7.7, 8.1, and 8.5. Spectrophotometric measurements were recorded at four hour intervals with a Bausch and Lomb Spectronic 20 spectrophotometer at 425 nm. Change in absorbance was plotted against time to determine the generation time. The pH level at which the generation time was the shortest was recorded as the optimum pH for that strain.

## Effect of NaCl

The twenty cultures of *Moraxella* were grown, in duplicate, in culture tubes with 10 ml of TPE broth (minus NaCl) with various concentrations of NaCl added. NaCl concentrations tested were: 0, 1.5, 3.0, 4.5, 6.0, 7.5, 10, 15, and 20% (w/v). The pH of all tubes was standardized to 7.9. Tubes were incubated at 25°C for 48 hours and visible turbidity was recorded as growth.

The optimum concentration of NaCl for each strain was determined by inoculating 20 mm optically matched culture tubes, in duplicate, with TPE broth (minus NaCl), standardized at pH 7.9, supplemented with various salt concentrations: 0.5, 1.0, 2.0, and 3.0% (w/v). Spectrophotometric measurements were recorded at four hour intervals with a Bausch and Lomb Spectronic 20 spectrophotometer at 425 nm. The optimum salt concentration for each strain was determined as in the pH study.

## Utilization of Carbohydrates, Protein, and Lipid

All strains of Moraxella were tested for carbohydrate utilization and for proteolytic and lipolytic capabilities.

Hugh and Leifson media (32), solidified with 1.5% Bacto-agar (Difco), was used to test for oxidative utilization of glucose (42). All strains able to produce acid from glucose were further tested on Hugh and Leifson plates with sucrose, fructose, galactose, xylose, arabinose, or lactose, replacing glucose, as the test carbohydrate. All plates were incubated aerobically at 25°C and results recorded after 24 hours.

Proteolytic ability of Moraxella was tested on Standard Methods Agar with Caseinate (53). Strains were streaked onto the plates and incubated at 25°C. A clear zone around colonies was indicative of proteolysis. Results were recorded daily for up to two weeks. Pseudomonas strains PB-1-10 and 4-1-11 were used as positive controls.

Lipolytic ability was determined on double layer lipolytic media (2). Strains were spread plated onto the lipid-containing lower layer, then molten nutrient agar (45°C) was overlayed. Plates were incubated at 25°C for up to two weeks. Zones of clearing around colonies were indicative of lipolysis.

## Antibiotic Resistance

All Moraxella strains were tested for their resistance to 12 antibiotics. The antibiotics were added to molten TPE agar by a sterile hypodermic syringe. Table 5 lists the antibiotics and their respective concentrations tested. Cultures were replica plated from

Table 5. Antibiotics and concentrations used in sensitivity testing.

| ANTIBIOTIC (symbol) | TRADE NAME | MANUFACTURER | CONCENTRATION (micrograms/ml) |
|---|---|---|---|
| Chloramphenicol (Cm) | Chloromycetin kapseals | Parke-Davis | 2.5 |
| Streptomycin (St) | Streptomycin sulfate injectable | Eli Lilly | 10 |
| Ampicillin (Am) | Polycillin-N injectable | Bristol | 25 |
| Tetracycline (Tc) | Achromycin injectable | Lederle | 25 |
| Chlorotetracycline (Ct) | Auromycin injectable | Lederle | 25 |
| Oxytetracycline (Ot) | Terramycin injectable | Lederle | 25 |
| Neomycin (Nm) | Mycifradin injectable | Upjohn | 50 |
| Nitrofurazone (Nf) | Furacin soluble powder | Eaton | 25 |
| Nalidixic acid (Na) | Nalidixic acid powder, grade B | Calbiochem | 25 |
| Sulfathiazole (Su) | Sulfathiazole powder | Merck | 500 |
| Kanamycin (Ka) | Kantrex injectable | Bristol | 25 |
| Procaine Penicillin G (Pe) | Crysticillin injectable | Squibb | 3 IU and 75 IU |

TPE mother plates onto the antibiotic plates. A plate of antibiotic free TPE agar was inoculated as the last plate to verify that all preceding plates had been inoculated. All plates were incubated at 25°C and results determined after 48 hours.

## Phenethyl Alcohol Sensitivity

A preliminary test to determine the sensitivity of various bacteria to phenethyl alcohol (PEA) was performed as follows. Cultures were spread plated onto Antibiotic Medium 3 (Difco). Four sterile filter paper disks of approximately 50 mm in diameter were aseptically placed onto each plate. Filter sterilized PEA (Eastman Kodak Co.) solutions were pipetted on the disks in concentrations of: 200, 20, 2, and 0.2 μmoles PEA/ml. PEA was diluted with 100% ethanol to yield the desired concentration of PEA in 0.1 ml of solution. The ethanol used as the solvent had no effect on microbial growth. The seeded plates were incubated at 25°C for 24 hours and the zones of inhibition around the disks were measured.

Five cultures from the disk sensitivity test were judged to be the most sensitive to PEA. These organisms were further tested by growing them in 50 ml of TPE broth in 250 ml side-arm flasks (Bellco) in the presence of three different concentrations of PEA: 80, 8.0, and 0.8 mmoles PEA/liter. The flasks were incubated in a Psycrotherm incubator shaker (New Brunswick Scient. Co.), with shaking, at 25°C. Growth was measured spectrophotometrically in a Spectronic 20 at 520 nm. Absorbance was plotted against time to determine the degree of

inhibition.

## Phenethyl Alcohol Production

A modification of the method employed by Chen and Levin (13) was used to determine the PEA levels in the culture media. Fourteen strains of Moraxella were grown in 0.5% VFCAA plus 0.1% yeast extract. Yeast extract supplementation was necessary because the VFCAA did not support the growth of many Moraxella strains. This supplementation did not greatly interfere with the detection of PEA. Strains were inoculated into 10 ml of media and incubated at 25°C for 72 hours. Absorbance was recorded in a Bausch and Lomb Spectronic 20 spectrophotometer at 520 nm. The cells were removed by centrifugation at 8000xG for ten minutes. The supernatant was decanted off and added to an equal volume of isooctane (J. T. Baker). PEA and other solvent-soluble compounds from the spent media were extracted into the isooctane layer by shaking. The solvent layer was decanted and the solvent evaporated from approximately 10 ml to 0.2 ml under a gentle stream of nitrogen gas prior to gas chromatographic analysis. The injection size for all samples was 1.0 µliters.

A Varian Aerograph model 1200 gas chromatograph, equipped with a flame ionization detector, was used for PEA detection. The recorder was a Barber-Coleman model 8000. Column temperature was maintained at 145°C. Nitrogen (Airco), the carrier gas, was measured at 25 ml per minute across the detector. Hydrogen (Airco) and air (Airco) were operated at 25 ml per minute and 250 ml per minute, respectively.

The gas chromatograph was operated at 150 milliamps and at an attenuation of 1. Chart speed was 16 inches per hour. The ¼ inch I.D., 12 foot aluminum column was packed with 5% Carbowax 20 M on Chromosorb G. PEA (Eastman Kodak Co.) had a retention time of 22 minutes in this experiment.

Known amounts of PEA were injected into the chromatograph and the peak areas calculated. A standard curve relating the quantity of PEA to peak area was constructed. The PEA content of the Moraxella samples was calculated by relating peak area to an equivalent peak area of the PEA standard curve.

## DNA Base Composition

Six strains of Moraxella, listed in Table 6, were studied for DNA base composition. Cultures were grown in two liters of TPE broth at 25°C, with shaking, in a Psycrotherm incubator shaker (New Brunswick Scient. Co.). Cultures were incubated for 48 hours before harvesting.

Cells were centrifuged at 2000xG for 15 minutes and washed in saline-EDTA. Lysozyme (Calbiochem) was added to a final concentration of 100 μgrams/ml and the suspension was incubated with gentle shaking at 37°C for two hours. The suspension was then frozen at 10°C, thawed, and again incubated with shaking at 37°C for two hours. This freeze-thaw lysozyme treatment was repeated three to four times prior to lysis. Lysis was accomplished by addition of sodium lauryl sulfate to a final concentration of 2% (v/v). Lysis resulted in a partial clearing of the suspension and increased viscosity.

Table 6. Phenotypic characteristics of Moraxella strains used in DNA base composition study.

| MORAXELLA STRAINS | CHARACTERISTICS | | | |
|---|---|---|---|---|
| | RESISTANT TO 3 IU Pe | HUGH AND LEIFSON OXIDATIVE | PROTEOLYTIC | PEA PRODUCTION |
| A-44 | - | - | - | + |
| D-1-12 | - | + | - | - |
| DD-4-5 | - | - | - | - |
| C-1-10 | - | - | + | ? |
| C-2-10 | + | - | + | ? |
| 5-1-2 | + | + | - | + |

DNA extraction and purification was carried out by the modified procedure of Marmur and Doty (52). Equilibrated saline-EDTA phenol (64) was used for deproteinization following cell lysis and treatment with ribonuclease. Ribonuclease (Sigma Chemical) was added at 100 μgrams/ml and the samples were incubated with gentle shaking for two to three hours at 37°C. The final DNA precipitation was with isopropanol at a final concentration of 60% (v/v), after which, the DNA was spooled on glass rods and dissolved in 0.1 strength sodium chloride and sodium citrate solution (SSC) (SSC = 0.15M NaCl plus 0.015M sodium citrate, pH 7.0) and stored at 4°C over a drop of chloroform. The purity of each sample was ascertained by absorbance measurements of ultraviolet light at 230, 260, and 280 nm. A ratio between 1.9 to 2.0 for 260/280 nm and a ratio between 2.25 to 2.4 for 260/230 nm indicated sufficient DNA purity from protein.

DNA in 0.1xSSC was pipetted into quartz cuvettes (Beckman) and diluted to approximately 25 μgrams per ml. The samples were heat denatured in a Gilford model 2000 automatic recording spectrophotometer. Relative absorbance versus temperature was plotted, corrected for the thermal expansion of water. The midpoint of the thermal denaturation curve (Tm) was calculated. *E. coli* BOSE26 was used as a reference (Tm = 73.6, G+C mole % = 51.0). The relationship of Mandel *et al.* (51), whereby one degree difference in the Tm of a strain in comparison to the reference strain is equal to a G+C mole % difference of two, was used. Thus, with a reference strain having a Tm and a G+C ratio of 73.6 and 51.0, respectively, a different

strain with a Tm of 71.6 would have a corresponding G+C mole % of 47.0.

# RESULTS AND DISCUSSION

## Nutritional Requirements of Moraxella

### Growth Factors

The results of the initial growth factor requirement screening test of the marine fishery Moraxella strains are presented in Table 7. None of the 31 strains tested grew on Davis Minimal Agar (DMA) or DMA plus the five growth factors: biotin, nicotinic acid, pyridoxine, riboflavin, and thiamine. Growth was noted on 0.5% vitamin free casamino acids (VFCAA) by 87% of the strains. Growth performance was further improved on the more complex media, i.e., VFCAA plus the five growth factors and DMA plus VFCAA plus the five growth factors. DMA contains glucose, sodium citrate, and various salts but contains no organic nitrogen sources. Most clinical Moraxella species: M. bovis, M. nonliquifaciens, M. lacunata, M. phenylpyruvica, and M. kingii require nutrient agar or media supplemented with blood or serum for growth. M. osloensis and M. urethralis have simple nutritional requirements, though ammonia was required as a nitrogen source (3,9,28). Two strains, DD-2-4-2 and ME-1-11 required additional substances not present in even the most complex media prepared and failed to grow. Strain S-1-4 required the presence of both amino acids and growth factors for growth.

Results of the single addition and single deletion of one growth factor analysis are shown in Table 8. Biotin was required by strains

Table 7. Screening test results for growth factor[a] (GF) requirements of Moraxella strains.

| MORAXELLA CULTURE | MEDIA | | | | | |
|---|---|---|---|---|---|---|
| | DMA[b] | VFCAA[c] | DMA + GF | DMA + VFCAA | VFCAA GF | DMA + VFCAA + GF |
| A-41 | - | + | - | + | ++ | ++ |
| A-42 | - | ++ | - | + | ++ | ++ |
| A-43 | - | ++ | - | ++ | ++ | +++ |
| A-44 | - | + | - | + | + | ++ |
| A-45 | - | + | - | + | ++ | +++ |
| A-404 | - | ++ | - | + | + | +++ |
| A-491 | - | + | - | + | + | + |
| A-2-11 | - | + | - | ++ | + | ++ |
| C-2-15 | - | - | - | + | + | + |
| D-1-3 | - | +++ | - | +++ | +++ | +++ |
| D-2-29 | - | - | - | + | + | ++ |
| D-4-10 | - | + | - | + | ++ | ++ |
| DD-2-4-2 | - | + | - | - | + | - |
| ME-1-11 | - | - | - | - | - | - |
| S-1-4 | - | - | - | - | ++ | ++ |
| S-1-23 | - | ++ | - | + | ++ | + |
| A-101 | - | ++ | - | ++ | +++ | +++ |
| A-102 | - | ++ | - | ++ | +++ | +++ |
| A-103 | - | ++ | - | ++ | +++ | +++ |
| A-151 | - | ++ | - | ++ | +++ | +++ |
| A-171 | - | ++ | - | ++ | +++ | +++ |
| A-401 | - | ++ | - | ++ | +++ | +++ |
| A-402 | - | ++ | - | ++ | +++ | +++ |
| A-4211 | - | ++ | - | ++ | +++ | +++ |
| A-4213 | - | ++ | - | ++ | +++ | +++ |
| A-4214 | - | ++ | - | ++ | +++ | +++ |
| D-1-12 | - | +++ | - | +++ | +++ | +++ |
| DD-2-4-10 | - | +++ | - | +++ | +++ | +++ |
| DD-2-4-14 | - | + | - | + | +++ | +++ |
| S-1-5 | - | ++ | - | ++ | +++ | +++ |
| S-1-19 | - | ++ | - | +++ | +++ | +++ |

[a]Biotin, nicotinic acid, pyridoxine, riboflavin, and thiamine.

[b]Davis Minimal Agar (Difco).

[c]Vitamin free casamino acids (Difco).

Table 8. Single addition and single deletion tests to determine growth factor requirements of Moraxella strains.

| MORAXELLA | GROWTH FACTOR[a] ADDED | | | | | GROWTH FACTOR DELETED | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CULTURE | Bi[b] | Ni[c] | Py[d] | Ri[e] | Th[f] | Bi | Ni | Py | Ri | Th |
| A-41 | ++ | - | - | - | - | - | ++ | ++ | ++ | ++ |
| A-42 | ++ | - | + | + | + | + | ++ | ++ | ++ | ++ |
| A-43 | ++ | + | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ |
| A-44 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-45 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-404 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-491 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-2-11 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| C-2-15 | + | + | + | + | + | ++ | + | ++ | + | + |
| D-1-3 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| D-2-29 | ++ | + | ++ | + | + | ++ | ++ | ++ | ++ | ++ |
| D-4-10 | ++ | + | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ |
| DD-2-4-2 | - | - | - | - | - | - | + | - | - | - |
| ME-1-11 | - | - | - | - | - | - | - | - | - | - |
| S-1-4 | ++ | - | - | - | - | - | ++ | ++ | ++ | ++ |
| S-1-23 | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ |
| A-101 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-102 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-103 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-151 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-171 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-401 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-402 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-4211 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-4213 | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| A-4214 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| D-1-12 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| DD-2-4-10 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| DD-2-4-14 | ++ | + | + | + | ++ | + | ++ | ++ | ++ | ++ |
| S-1-5 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| S-1-19 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

[a]Basal medium consisted of Davis Minimal Agar, vitamin free casamino acids, and either the indicated growth factor or all growth factors except one.

[b]Biotin. [c]Nicotinic acid. [d]Pyridoxine. [e]Riboflavin.

[f]Thiamine.

A-41 and S-1-4. Although not required, biotin stimulated the growth of strains A-42 and DD-2-4-14. Again strains DD-2-4-2 and ME-1-11 failed to grow. The majority of the strains tested did not have a growth factor requirement. Failure to grow on DMA and DMA plus growth factors, however, indicated that the Moraxella strains had a requirement for non-growth factor organic nutrients.

## Amino Acids

The results of the single addition of five amino acid groups is shown in Table 9. Group 5 (alanine, glycine, serine, and hydroxyproline) was able to support the growth of 13 strains of Moraxella (42%). Group 3 (phenylalanine, tyrosine, and tryptophan) and group 2 (leucine, isoleucine, and valine) supported the growth of six (19%) and four (13%) strains, respectively. Group 1 (lysine, arginine, methionine, and cystine) and group 4 (histidine, threonine, glutamic acid, proline, and aspartic acid) were unable to support the growth of Moraxella (Table 9).

All 19 tested amino acids were able to support the growth of 28 strains (90.7%) when present simultaneously (Table 10). Strains DD-2-4-2 and ME-1-11, unable to grow on DMA plus VFCAA plus the five growth factors (Table 7), again failed to grow, along with strain S-1-4. S-1-4, however, showed growth on media containing VFCAA plus the five growth factors (Table 7). Moraxella strains A-41 and A-151 required all five amino acid groups for growth (Table 10).

It appeared that amino acid groups 2 and 3 were antagonistic

Table 9. Growth of Moraxella strains in amino acid supplemented media[a].

| MORAXELLA STRAIN | AMINO ACID GROUP[b] | | | | |
|---|---|---|---|---|---|
| | LYS, ARG, MET, CYS | LEU, ILE, VAL | PHE, TYR, TRP | HIS, THR, GLU, PRO, AASP | ALA, GLY, SER, H-PRO |
| A-41 | - | - | - | - | - |
| A-42 | - | - | - | - | + |
| A-43 | - | - | - | - | + |
| A-44 | - | + | + | - | + |
| A-45 | - | + | + | - | + |
| A-404 | - | - | - | - | - |
| A-491 | - | - | - | - | - |
| A-2-11 | - | - | - | - | + |
| C-2-15 | - | - | + | - | + |
| D-1-3 | - | - | - | - | + |
| D-2-29 | - | - | - | - | + |
| D-4-10 | - | - | + | - | - |
| DD-2-4-2 | - | - | - | - | - |
| ME-1-11 | - | - | - | - | - |
| S-1-4 | - | - | - | - | - |
| S-1-23 | - | - | - | - | + |
| A-101 | - | - | - | - | - |
| A-102 | - | - | - | - | + |
| A-103 | - | - | - | - | - |
| A-151 | - | - | - | - | - |
| A-171 | - | - | - | - | - |
| A-401 | - | - | - | - | - |
| A-402 | - | - | - | - | - |
| A-4211 | - | - | - | - | - |
| A-4213 | - | - | - | - | - |
| A-4214 | - | - | - | - | - |
| D-1-12 | - | - | - | - | + |
| DD-2-4-10 | - | + | + | - | + |
| DD-2-4-14 | - | - | + | - | + |
| S-1-5 | - | - | - | - | - |
| S-1-19 | - | + | - | - | - |

[a]Davis Minimal Agar plus: biotin, nicotinic acid, pyridoxine, riboflavin, and thiamine.

[b]Amino acid abbreviations as in Table 4.

Table 10. Growth of Moraxella strains in media[a] supplemented with various groups of amino acids.

| MORAXELLA STRAIN | AMINO ACID GROUPS[b] | | | | |
|---|---|---|---|---|---|
| | 2 PLUS 3 | 2 PLUS 5 | 3 PLUS 5 | 2 PLUS 3 PLUS 5 | GROUPS 1 PLUS 5 |
| A-41 | - | - | - | - | + |
| A-42 | - | + | - | - | + |
| A-43 | - | + | + | - | + |
| A-44 | + | + | + | + | + |
| A-45 | + | ++ | + | + | + |
| A-404 | - | + | + | - | + |
| A-491 | + | - | - | + | + |
| A-2-11 | - | + | + | - | + |
| C-2-15 | ++ | ++ | + | ++ | + |
| D-1-3 | - | ++ | - | - | + |
| D-2-29 | - | + | + | + | + |
| D-4-10 | - | - | - | - | + |
| DD-2-4-2 | - | - | - | - | - |
| ME-1-11 | - | - | - | - | - |
| S-1-4 | - | + | - | - | - |
| S-1-23 | - | + | - | - | + |
| A-101 | - | + | + | + | + |
| A-102 | - | + | + | - | + |
| A-103 | - | ++ | + | - | + |
| A-151 | - | - | - | - | + |
| A-171 | + | + | + | + | + |
| A-401 | - | + | + | - | + |
| A-402 | - | + | - | - | + |
| A-4211 | - | + | + | - | + |
| A-4213 | - | + | + | - | + |
| A-4214 | - | + | - | - | + |
| D-1-12 | - | + | + | - | + |
| DD-2-4-10 | + | + | + | + | + |
| DD-2-4-14 | - | + | + | + | + |
| S-1-5 | - | + | + | - | + |
| S-1-19 | - | + | + | + | + |

[a]Davis Minimal Agar plus biotin, nicotinic acid, pyridoxine, riboflavin, and thiamine.

[b]Group 1: lysine, arginine, methionine, and cystine. Group 2: leucine, isoleucine, and valine. Group 3: phenylalanine, tyrosine, and tryptophan. Group 4: histidine, threonine, glutamic acid, proline, and aspartic acid. Group 5: alanine, glycine, serine, and hydroxyproline.

towards Moraxella when present simultaneously. Strains A-404, A-401, A-4211, D-1-12, and S-1-5 showed growth on plates containing amino acid groups 2 plus 5 and group 3 plus 5 but not on groups 2 plus 3 plus 5 (Table 10). This inhibition was lacking when all five amino acid groups were present (Table 10). Further, only six strains of Moraxella showed growth on plates containing amino acid groups 2 plus 3 and ten strains grew with amino acid groups 2 plus 3 plus 5. However, 19 strains (61%) grew on plates containing amino acid groups 3 plus 5 and 25 strains (81%) showed growth with amino acid groups 2 plus 5 (Table 10).

Individual amino acids from all five amino acid groups were further tested for their ability to support growth of Moraxella (Table 11). The amino acids from group 5, previously shown in Table 9 to best support the growth of Moraxella, again, gave the best results. Alanine supported the growth of ten strains (32%) while serine supported three strains (9.7%). Leucine, from amino acid group 2 and tyrosine, from group 3, supported the growth of two strains and one strain, respectively. Hydroxyproline supported the growth of strain A-4213. All other amino acids from groups 2,3, and 5, along with all amino acids from groups 1 and 4, were unable to support the growth of Moraxella strains.

The majority of Moraxella strains have a multiple amino acid requirement. This multiple amino acid requirement was met for 81% of Moraxella strains by seven amino acids: leucine, isoleucine, valine, alanine, glycine, serine, and hydroxyproline. A summary of the

Table 11. Utilization of amino acids from groups[a] 2, 3, and 5 by *Moraxella* strains isolated from fishery sources.

| MORAXELLA | AMINO ACID TESTED[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| STRAIN | LEU | ILE | VAL | PHE | TYR | TRP | ALA | GLY | SER | H-PRO |
| A-41 | - | - | - | - | - | - | - | - | - | - |
| A-42 | - | - | - | - | - | - | - | - | - | - |
| A-43 | - | - | - | - | - | - | + | - | - | - |
| A-44 | - | - | - | - | - | - | + | - | - | - |
| A-45 | + | - | - | - | - | - | + | - | + | - |
| A-404 | - | - | - | - | - | - | - | - | - | - |
| A-491 | - | - | - | - | - | - | - | - | - | - |
| A-2-11 | - | - | - | - | - | - | - | - | - | - |
| C-2-15 | - | - | - | - | - | - | + | - | - | - |
| D-1-3 | - | - | - | - | - | - | + | - | - | - |
| D-2-29 | - | - | - | - | - | - | + | - | - | - |
| D-4-10 | - | - | - | - | - | - | - | - | - | - |
| DD-2-4-2 | - | - | - | - | - | - | - | - | - | - |
| ME-1-11 | - | - | - | - | - | - | - | - | - | - |
| S-1-4 | - | - | - | - | - | - | + | - | - | - |
| S-1-23 | - | - | - | - | - | - | + | - | - | - |
| A-101 | - | - | - | - | - | - | - | - | + | - |
| A-102 | - | - | - | - | + | - | + | - | + | - |
| A-103 | - | - | - | - | - | - | + | - | - | - |
| A-151 | - | - | - | - | - | - | - | - | - | - |
| A-171 | - | - | - | - | - | - | - | - | - | - |
| A-401 | - | - | - | - | - | - | - | - | - | - |
| A-402 | - | - | - | - | - | - | - | - | - | - |
| A-4211 | - | - | - | - | - | - | - | - | - | - |
| A-4213 | - | - | - | - | - | - | - | - | - | + |
| A-4214 | - | - | - | - | - | - | - | - | - | - |
| D-1-12 | - | - | - | - | - | - | - | - | - | - |
| DD-2-4-10 | + | - | - | - | - | - | - | - | - | - |
| DD-2-4-14 | - | - | - | - | - | - | - | - | - | - |
| S-1-5 | - | - | - | - | - | - | - | - | - | - |
| S-1-19 | - | - | - | - | - | - | - | - | - | - |

[a]Amino acid grouping as in Table 4.

[b]Amino acid abbreviations as in Table 4.

nutritional requirements of Moraxella is presented in Table 12.

Baumann et al. (3) reported that oxidase positive Moraxella were not able to utilize 19 amino acids, 17 of which were tested in this experiment: glycine, alanine, serine, leucine, isoleucine, valine, aspartic acid, glutamic acid, histidine, proline, lysine, arginine, cystine, methionine, phenylalanine, tyrosine, and tryptophan. These strains, however, were from clinical sources. Chen and Levin (13) noted that clinical Moraxella strains were unable to utilize a vitamin free casein hydrolysate which was utilized by Achromobacter strains of fishery origin.

The amino acids utilized by Moraxella in this experiment were found in high quantities in the slime of fish. Shewan (68) reported that alanine and leucine were found in high quantities in cod slime. Serine has been detected in fish slime (26). Eighteen amino acids tested in this experiment, including alanine, serine, and leucine, have been detected in organic matters in sea water (57). Thus, it appears that Moraxella strains require a combination of amino acids for growth and utilize amino acids that are found in their natural habitat more efficiently.

## Effect of pH

All strains of Moraxella tested were able to grow in the pH range of 6.9 to 8.8 (Table 13). The lowest and highest pH values where none of the strains grew were 4.9 and 10.4, respectively. The pH optima of selected Moraxella strains, measured by the rate of growth at various

Table 12. Nutritional requirements of Moraxella species of fishery origin.

| REQUIRED NUTRIENTS[a] | NO. (%) | STRAIN DESIGNATION |
|---|---|---|
| Ala | 10 (32.3) | A-43, A-44, A-45, C-2-15, D-1-3, D-2-29, S-1-4, S-1-23, A-102, A-103 |
| Ser | 3 (9.7) | A-45, A-101, A-102 |
| Leu | 2 (6.5) | A-45, DD-2-4-10 |
| Tyr | 1 (3.2) | A-102 |
| H-pro | 1 (3.2) | A-4213 |
| Leu, ile, val | 1 (3.2) | S-1-19 |
| Phe, tyr, trp | 2 (6.5) | D-4-10, DD-2-4-14 |
| Ala, gly, ser, h-pro | 3 (9.7) | A-42, A-2-11, D-1-12 |
| Leu, ile, val, phe, tyr, trp | 2 (6.5) | A-491, A-171 |
| Leu, ile, val, ala, ser, gly, h-pro | 7 (22.6) | A-404, A-171, A-401, A-402, A-4211, A-4214, S-1-5 |
| Phe, tyr, trp, ala, gly, ser, h-pro | 5 (16.1) | A-404, A-171, A-401, A-4211, S-1-5 |
| All 19 amino acids | 2 (6.5) | A-41, A-151 |
| No growth | 2 (6.5) | DD-2-4-2, ME-1-11 |

[a]Amino acid abbreviations as in Table 4.

Strains A-41 and S-1-4 required biotin.

Table 13. Effect of pH on the growth of *Moraxella* strains isolated from fishery sources.

| pH RANGE FOR GROWTH | *MORAXELLA* STRAIN | | |
|---|---|---|---|
| 5.9 to 8.4 | A-101 | A-103 | A-402 |
| | A-4213 | D-1-12 | S-1-23 |
| 5.9 to 9.4 | A-41 | A-42 | A-44 |
| | A-45 | A-171 | D-2-29 |
| | S-1-4 | | |
| 6.9 to 8.8 | A-491 | DD-2-4-14 | S-1-19 |
| 6.9 to 9.4 | A-43 | A-404 | A-2-11 |

pH levels, ranged from 7.4 to 8.5, with the pH values of 7.7 and 8.1 predominating.

## Effect of NaCl

All strains grew in TPE broth with up to 6% NaCl. Ninety percent were able to grow at 7.5% NaCl and 60% grew at 10% NaCl. Moraxella and Acinetobacter species, isolated from radurized sausages, were able to grow in media with 7.5% NaCl (34). These Moraxella, however, were not typical, due to their resistance to penicillin (34). Nevertheless, NaCl tolerance appears to be one of the distinctive characteristics of the gram variable coccobacilli grouped either under Moraxella or Acinetobacter species.

The optimum NaCl concentrations of the Moraxella strains ranged from 0.5% to 3.0%. Most strains (80%) grew best at 1.0% NaCl or less.

## Effect of Temperature

As expected, the marine fishery Moraxella strains grew well at 5°C but were unable to grow at 37°C or above. All strains grew at 25°C and 10°C. Nineteen strains (95%) grew at 5 C. One strain, D-2-29, grew slowly at 10°C, turbidity was noted only after seven days of incubation. At 5°C, no growth was observed after 14 days.

The temperature range for growth is a major difference between Moraxella species of fishery and clinical origin. Clinical strains of M. urethralis were able to grow at temperatures from 25°C to 44°C (63). The temperature optimum of M. urethralis was reported to be

between 33°C to 37°C (9). Moraxella species from seafood, on the other hand, are psychrotrophic and grow best between temperatures of 10°C to 25°C (70).

## Utilization of Carbohydrates, Protein, and Lipid

Thirty five strains (30.2%) of Moraxella were Hugh and Leifson oxidative with glucose as the carbohydrate. All glucose oxidative strains are listed in Table 14. An interesting observation was that often the size of the inoculum determined the test result. Strains, unable to produce sufficient acid to change the color of the indicator when inoculated as a stab into the agar, were able to produce sufficient acid, indicative of oxidative utilization of the carbohydrate, when streaked over a larger area of the plate.

All strains which utilized glucose were tested further for their ability to oxidize other carbohydrates (Table 14). Xylose was oxidized by 19 of the glucose-positive strains (54.3%) and arabinose was weakly oxidized by two strains (5.7%). Sucrose, fructose, lactose, and galactose were not oxidized. Some strains slowly oxidized the carbohydrates, requiring 48 to 72 hours of incubation to fully develop the indicator color change.

The ability of Moraxella species to oxidize carbohydrates has been a continual source of controversy and confusion. Thornley (77), in her description of phenom 3, reported that strains of Moraxella were able to oxidize five sugars: glucose, galactose, arabinose, xylose, and lactose. Henriksen (28), in his extensive report on

Table 14. Hugh and Leifson carbohydrate oxidation test with various sugars by Moraxella strains isolated from fishery sources.

| CARBOHYDRATES OXIDIZED | MORAXELLA STRAIN | | |
|---|---|---|---|
| Glucose | A-102 | A-103 | A-151 |
| | A-401 | A-4211 | D-1-12 |
| | DD-1-12 | S-1-5 | S-1-19 |
| | A-2-12 | D-1-4 | D-1-30 |
| | E-1-1 | E-1-8 | E-1-10 |
| | E-1-30 | | |
| Glucose and xylose | A-101 | A-402 | A-4213 |
| | A-4214 | DD-2-4-3 | DD-2-4-5 |
| | DD-2-4-10 | DD-2-4-14 | DD-2-4-18 |
| | S-1-20 | D-1-1 | E-1-20 |
| | AC-1-11 | 5-1-1 | 5-1-2 |
| | 5-2-2 | 5-2-13 | |
| Glucose, xylose, and arabinose | A-171 | DD-2-4-6 | |

clinical Moraxella species, stated that M. osloensis was able to oxidize ribose and xylose and M. kingii produced acid from glucose, maltose, and galactose. M. urethralis was found to be unable to produce acid from glucose, xylose, lactose, sucrose, or maltose (63). Other studies have reported on the inability of Moraxella species to oxidize carbohydrates (3,12). Our strains appear to be in an intermediate position in regard to carbohydrate utilization. Glucose was oxidized by 30%, 17.5% oxidized xylose, and 1.7% oxidized arabinose.

During the Moraxella growth factor study, it was noted that Hugh and Leifson oxidative strains grew faster than Hugh and Leifson negative strains (Table 7). The last 15 strains listed in Table 7 were Hugh and Leifson oxidative. All produced excellent growth on plates within 48 hours. These strains not only grew faster on plates containing DMA, which contains glucose, but also grew faster on VFCAA and on VFCAA plus the five growth factors, both of which contained no added glucose. Glucose is utilized by Hugh and Leifson oxidative strains as an energy source. Faster growth would be expected with oxidative strains on minimal media containing glucose as compared to nonoxidative strains. The faster growth rate of Hugh and Leifson oxidative strains on VFCAA was probably due to gluconogenesis from the amino acids and subsequent oxidation of this glucose.

No difference was observed between Hugh and Leifson oxidative strains and negative strains in regard to pH limits or pH optima for growth or NaCl limits or NaCl optimum concentrations for growth.

## Protein

Eight strains (7%) of Moraxella were proteolytic on Standard Methods Agar with Caseinate (53): B-1-30, C-1-10, C-1-17, C-1-20, C-1-30, C-2-2, C-2-6, and C-2-10. These eight strains were isolated from one shrimp boat and seven (C-1-10 through C-2-10) were from the same area in the boat. All eight strains were weakly proteolytic, six to nine days of incubation were required before clearing of the agar, indicative of proteolysis, was apparent. The Pseudomonas strains used as positive controls showed visible proteolysis after three days of incubation.

Microorganisms are the primary agent of fish spoilage (20,30,31, 50,62). The Pseudomonas-Achromobacter group has been implicated as the causative agents of fish spoilage at refrigeration temperatures (30,45,69,80). In one report, 29% of tested Achromobacter strains were found to spoil fish juice (45). The role of proteolytic microorganisms in fish spoilage has been questioned (4,11,30,45,46,66,69). Beatty and Collins (4) claimed that proteolysis became evident only long after seafood had passed the spoilage limit. The degradation of small molecular weight nitrogenous compounds: nucleotides, amino acids, small peptides, and trimethylamine oxide (TMAO) have been implicated as the primary substrates of seafood spoilage (30,36). The hypothesis that spoilage can occur with little or no proteolysis has been disputed (25,61,74). However, the ability to hydrolyze casein within six days is not as significant as the ability to grow in media containing

one amino acid as the sole carbon and nitrogen source within 48 hours (Table 11) or the ability to show growth on VFCAA within 48 hours (Table 7).

Statistical analysis (55) revealed that proteolytic strains of Moraxella were significantly more Hugh and Leifson negative than non-proteolytic strains (Table 15). All eight proteolytic strains listed above were Hugh and Leifson negative. It appears that the proteolytic Moraxella strains need not depend on carbohydrates for their energy source. Amino acids, derived from protein hydrolysis, could act as the energy source.

## Lipid

No lipolytic activity was observed with any Moraxella strain when tested on double layer lipolytic media (2). All strains produced heavy growth but did not form clear zones around colonies, indicative of lipolysis.

## Antibiotic Resistance

The antibiotic resistance pattern of all Moraxella strains is shown in Table 16. All strains were sensitive to tetracycline, chlorotetracycline, oxytetracycline, streptomycin, neomycin, and kanamycin. All of these antibiotics are inhibitors of protein synthesis (35). Nalidixic acid, a urinary antiseptic, also inhibited all strains. Fifty nine strains (51.8%) were resistant to nitrofurazone and chloramphenicol. Ampicillin resistant strains comprised 36% of the total. Sulfa-

Table 15. Significant variables[a] determined by chi-squared analysis for phenotypically grouped strains of Moraxella isolated from fishery sources.

| PHENOTYPIC STRAINS | VARIABLE ANALYZED | CHI-SQUARED VALUE | P |
|---|---|---|---|
| Typical vs Atypical | Cm resistance[b] | 11.24 | .005 |
| Typical vs Atypical | Am resistance[c] | 19.7 | .005 |
| Proteolytic vs Nonproteolytic | Am resistance[d] | 15.3 | .005 |
| Proteolytic vs Nonproteolytic | Hugh and Leifson test[e] | 4.65 | $.025<\chi 2<.05$ |

[a]At alpha = .05, the critical chi-squared value is 3.84.

[b]Atypical Moraxella strains were significantly more resistant.

[c]Atypical Moraxella strains were significantly more resistant.

[d]Proteolytic Moraxella strains were significantly more resistant.

[e]Nonproteolytic Moraxella strains were significantly more Hugh and Leifson oxidative.

Table 16. Antibiotic resistance patterns of phenotypically differentiated strains of Moraxella isolated from fishery sources.

| ANTIBIOTIC[2] | MORAXELLA STRAINS RESISTANT (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALL STRAINS | TYPICAL[1] | ATYPICAL | PROTEOLYTIC | NON PROTEOLYTIC | HUGH AND LEIFSON OXIDATIVE | HUGH AND LEIFSON NEGATIVE |
| Pe, 3 IU | 31 (0) | 0 (0) | 31 (100) | 1 (12.5) | 30 (28.3) | 14 (35) | 17 (27.2) |
| Pe, 75 IU | 7 (6.1) | 0 (0) | 7 (22.6) | 0 (0) | 7 (6.6) | 2 (5) | 5 (8.3) |
| Cm | 59 (51.3) | 35 (42.2) | 24 (77.4) | 5 (62.5) | 54 (50.9) | 25 (62.5) | 34 (45.9) |
| Sm | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Km | 41 (40) | 16 (19.3) | 25 (80.6) | 3 (100) | 33 (31.1) | 14 (35) | 27 (36.5) |
| Tc | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Ct | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Ot | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nm | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Ni | 55 (51.3) | 43 (51.3) | 16 (51.6) | 3 (37.5) | 55 (52.8) | 20 (50) | 39 (52.7) |
| Na | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Su | 79 (69.3) | 56 (67.5) | 23 (74.2) | 7 (87.5) | 72 (67.9) | 25 (62.5) | 54 (73) |
| Km | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

[1]Typical Moraxella species are sensitive to 3 IU of penicillin G. Atypical Moraxella species are resistant to 3 IU of penicillin G.

[2]Antibiotic abbreviations as in Table 5.

thiazole resistance was highest, as nearly 70% of Moraxella were not affected. Ampicillin, like penicillin, iihibits cell wall synthesis in gram positive bacteria. Ampicillin is also effective against some gram negative organisms (35). Thirty one strains (27.2%) of Moraxella were characterized as atypical, due to their resistance to 3 IU of penicillin G (10,30,34,42). Thus, Moraxella are susceptible to the action of penicillin and related antibiotics that inhibit cell wall synthesis of gram positive bacteria.

Antibiotic resistance patterns of Moraxella strains arranged as typical or atypical, proteolytic or nonproteolytic, and Hugh and Leifson oxidative or Hugh and Leifson negative are listed in Table 16. Statistical analysis (chi-squared) revealed significant variations in antibiotic resistance patterns in two groupings (Table 15). Atypical strains of Moraxella were significantly more resistant to chloramphenicol and ampicillin at a level of significance of alpha = 0.05. Proteolytic Moraxella strains were significantly more resistant to ampicillin than nonproteolytic strains. No significant difference in antibiotic resistance was observed when strains were grouped according to their reaction on Hugh and Leifson media. As previously mentioned, proteolytic strains of Moraxella were significantly more Hugh and Leifson negative than nonproteolytic strains.

The antibiotic resistance patterns observed in this experiment agreed with the findings of other workers (12,28,58,77). Thornley (77) reported that her Moraxella food isolates (phenom 3) were sensitive to penicillin and oxytetracycline. Moraxella species have also been

Table 17. Phenethyl alcohol (PEA) sensitivity of various bacteria.

| BACTERIAL STRAIN | 0.2 mmole/ml | 0.02 mmole/ml | $2x10^{-3}$ mmole/ml | $2x10^{-4}$ mmole/ml |
|---|---|---|---|---|
| | ZONE OF INHIBITION, (mm) | | | |
| Moraxella | | | | |
| A-1-1 | 11 | 4 | 0 | 0 |
| A-1-3 | 14 | 4 | 0.5 | 0 |
| A-1-2 | 11 | 1.5 | 1 | 0 |
| A-1-4 | 13 | 2 | 0 | 0 |
| D-1-10 | 11 | 2.5 | 0 | 0 |
| S-1-19 | 8 | 2 | 0 | 0 |
| SD-1-2 | 7 | 0 | 0 | 0 |
| SD-3-14 | 11 | 4 | 0 | 0 |
| Arthrobacter | | | | |
| SD-1-6 | 13 | 2.5 | 0 | 0 |
| SD-3-30 | 7 | 0.5 | 0 | 0 |
| Flavobacter-Cytophaga | | | | |
| BCC-19 | 5 | 1 | 0 | 0 |
| 1-1-4 | 7 | 3 | 0 | 0 |
| Acinetobacter BCC-1-27 | 3 | 1.5 | 0 | 0 |
| Micrococcus | | | | |
| SD-1-1 | 1.5 | 0 | 0 | 0 |
| BCC-1-14 | 1 | 0 | 0 | 0 |
| Ent. aerogenes strain | 6 | 0.5 | 0 | 0 |
| Pseudomonas II | | | | |
| $H_2S$-15 | 10 | 2 | 0 | 0 |
| PB-1-3 | 9 | 4 | 0 | 0 |
| PB-1-10 | 9 | 2.5 | 0.5 | 0 |
| P. putrefaciens BCC-1-11 | 3.5 | 1.5 | 0 | 0 |
| Pseudomonas III | | | | |
| SD-1-1 | 9 | 2.5 | 1 | 0 |
| SD-1-29 | 6 | 0.5 | 0 | 0 |
| SD-3-11 | 14 | 5 | 1.5 | 0 |
| SD-3-21 | 16 | 5.5 | 0.5 | 0 |
| 1-1-11 | 10 | 1 | 0.5 | 0 |

found to be sensitive to streptomycin, tetracycline, neomycin, chloramphenicol, kanamycin (12), nalidixic acid, and ampicillin (28). M. bovis was sensitive to above antibiotics and to sulfathiazole (58).

The varying pattern of resistance to penicillin exhibited by Moraxella raises questions as to the structure of its cell wall. Wiebe and Chapman (81) examined the fine structure of Pseudomonas strains and Achromobacter strains of marine origin and reported that Pseudomonas possessed the typical gram negative morphology, having an irregularly undulant cell wall and a cytoplasmic membrane which was nearly planar. Achromobacter strains, however, possessed a regularly undulant outer cell wall in addition to a planar inner wall. The cytoplasmic membrane was thin and hardly observable. This inner wall, not present in Pseudomonas, was very predominant in Achromobacter and resembled the cell wall structure of gram positive bacteria.

## Phenethyl Alcohol Sensitivity

Table 17 shows the phenethyl alcohol (PEA) sensitivity of various bacteria. All test strains were sensitive to 0.2 mmoles/ml (200 mmoles/liter) of PEA. Micrococcus, a gram positive bacteria, was the least sensitive to PEA. PEA was reported to selectively inhibit gram negative bacteria while not inhibiting the gram positive ones in a mixed culture (5). The Flavobacter-Cytophaga strains were also relatively resistant to PEA.

The growth kinetics of five strains: Moraxella A-103 and A-404, Arthrobacter SD-1-6, and Pseudomonas III SD-3-11 and SD-3-21 were

tested in side-arm flasks (Bellco) in the presence of PEA. Absorbance readings of the five strains at various time intervals at three concentrations of PEA are presented in Tables 18, 19, and 20. Absorbance readings for the five strains grown in TPE broth without PEA are also presented. At a PEA concentration of 82 mmoles/liter, all five strains were completely inhibited (Table 18). This was expected, as previously, the five strains were found to be sensitive to 20 mmoles/liter of PEA (Table 17). At a concentration of 8.2 mmoles of PEA/liter, Moraxella A-103 and A-404 had an increase in onset of logarithmic growth and a reduced growth rate. Arthrobacter SD-1-6 and Pseudomonas III strains SD-3-11 and SD-3-21, however, were totally inhibited at this concentration of PEA (Table 19). At a concentration of 0.82 mmoles of PEA/liter, the onset of logarithmic growth and the growth rate during log phase of Moraxella strains A-103 and A-404 were not affected. Arthrobacter SD-1-6 was still completely inhibited while the two Pseudomonas strains had an increase in onset of logarithmic growth though their growth rate during log phase was not affected (Table 20).

## Phenethyl Alcohol Production

The amounts of phenethyl alcohol (PEA) produced by the fishery strains of Moraxella are presented in Table 21. Chen and Levin (13) reported that the amount of PEA produced by their fishery strains of Achromobacter was strain dependent; this was substantiated by our study. The concentrations of PEA produced by our strains ranged from undetectable in two strains to 3.25 mmoles/liter by strain A-44

Table 18. Growth of bacteria in TPE broth and TPE broth with 82 mmoles phenethyl alcohol (PEA)/liter.

| BACTERIAL STRAIN | ABSORBANCE READINGS AT VARIOUS TIMES (HOURS) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HOURS | | | | | | | | | |
| | 6 | | 8 | | 12 | | 16 | | 24 | |
| | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL |
| Moraxella | | | | | | | | | | |
| A-103 | .021 | .040 | .022 | .082 | .020 | .360 | .019 | THTR[a] | .021 | THTR |
| A-404 | .029 | .045 | .027 | .118 | .027 | .470 | .029 | THTR | .028 | THTR |
| Arthrobacter | | | | | | | | | | |
| SD-1-6 | .027 | .008 | .024 | .007 | .025 | .012 | .027 | .040 | .025 | .195 |
| Pseudomonas | | | | | | | | | | |
| SD-3-11 | .019 | .003 | .019 | .003 | .018 | .009 | .018 | .028 | .018 | .180 |
| SD-3-21 | .021 | .004 | .022 | .004 | .023 | .010 | .020 | .045 | .019 | .195 |

[a]Too high to record.

Table 19. Growth of bacteria in TPE broth and TPE broth with 8.2 mmoles phenethyl alcohol (PEA)/liter.

| BACTERIAL STRAIN | ABSORBANCE READINGS AT VARIOUS TIMES (HOURS) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HOURS | | | | | | | | | |
| | 10 | | 16 | | 24 | | 30 | | 40 | |
| | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL |
| Moraxella | | | | | | | | | | |
| A-103 | .012 | .048 | .023 | 1.20 | .068 | 1.70 | .142 | THTR[a] | .290 | THTR |
| A-404 | .005 | .053 | .011 | 1.10 | .014 | 1.60 | .014 | THTR | .125 | THTR |
| Arthrobacter | | | | | | | | | | |
| SD-1-6 | .004 | .001 | .005 | .004 | .006 | .004 | .005 | .011 | .003 | .147 |
| Pseudomonas | | | | | | | | | | |
| SD-3-11 | .007 | .002 | .008 | .009 | .009 | .070 | .008 | .250 | .008 | THTR |
| SD-3-21 | .008 | .004 | .011 | .014 | .013 | .023 | .010 | .110 | .010 | THTR |

[a]Too high to record.

Table 20. Growth of bacteria in TPE broth and TPE broth with 0.82 mmoles phenethyl alcohol (PEA)/liter.

| BACTERIAL STRAIN | ABSORBANCE READINGS AT VARIOUS TIMES (HOURS) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HOURS | | | | | | | | | |
| | 8 | | 12 | | 24 | | 27 | | 64 | |
| | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL | PEA | CONTROL |
| Moraxella | | | | | | | | | | |
| A-103 | .032 | .034 | .340 | .490 | THTR[a] | THTR | THTR | THTR | THTR | THTR |
| A-404 | .059 | .074 | .87 | 1.00 | THTR | THTR | THTR | THTR | THTR | THTR |
| Arthrobacter | | | | | | | | | | |
| SD-1-6 | .003 | .002 | .002 | .002 | .001 | .007 | .003 | .007 | .002 | .102 |
| Pseudomonas | | | | | | | | | | |
| SD-3-11 | .002 | .002 | .001 | .005 | .077 | .204 | .183 | .580 | THTR | THTR |
| SD-3-11 | .012 | .018 | .008 | .012 | .010 | .009 | .008 | .011 | .048 | .120 |

[a]Too high to record.

Table 21. Production of phenethyl alcohol (PEA) by Moraxella strains isolated from fishery sources in 0.5% VFCAA[a] plus 0.1% yeast extract[b].

| MORAXELLA STRAIN | ABSORBANCE | PEA mmoles/liter |
|---|---|---|
| A-44 | .415 | 3.25 |
| A-102 | .144 | 1.67 |
| A-491 | .438 | 0.23 |
| D-1-12 | .100 | - |
| DD-2-4-6 | .585 | 0.08 |
| DD-4-5 | .100 | - |
| S-1-23 | .200 | 0.50 |
| A-1-4 | .383 | 1.72 |
| E-1-1 | .277 | 0.81 |
| 5-1-2 | .203 | 2.66 |
| 5-1-27 | .179 | 3.00 |
| 5-2-2 | .500 | 2.82 |
| 5-2-13 | .508 | 2.80 |
| AC-1-12 | .240 | 0.79 |

[a]Difco Laboratories.

[b]Difco Laboratories.

(Table 21).

The amount of PEA produced by Moraxella strains in this experiment was approximately 1000 fold higher than that reported by Chen and Levin (13). Their Achromobacter strains produced PEA in the range of 1.5 to 4.0 μmoles/liter. Our strains which produced PEA produced it in the range of 0.075 to 3.25 mmoles/liter. Eight of the strains they tested were from this laboratory. One such strain, A-102, produced 1.67 mmoles/liter as compared to their reported value of 3.3 μmoles of PEA/liter (13). This large increase in PEA production was perhaps due to the media employed. Chen and Levin (13) used, as the growth medium, 0.5% vitamin free casein hydrolysate (VFCH) (Nutritional Biochemicals Corp.), a substrate similar to VFCAA (Difco). The 0.5% VFCAA plus 0.1% yeast extract (Difco) broth we used supported growth better and although it produced larger background peaks than VFCAA alone, PEA was still readily detectable.

PEA production appears to be substrate dependent, since the production of PEA in our growth media was approximately 1000 fold higher than in VFCH broth. PEA production is not proportional to cell growth. PEA was detected in refrigerated haddock fillets (14) though the amount was not quantitated. In that experiment, PEA was detected on the fifth day of incubation at $0^{\circ}C$, was highest after nine days, afterwhich, the level declined. The total bacterial count, stabilized at approximately $10^7$/gram after five days of incubation, rose to $10^9$ bacteria/gram at the time the level of PEA began declining.

The role of metabolites and natural inhibitors produced by

bacteria in the spoilage of seafood is unknown. PEA was produced by Moraxella strains of fishery origin to the level of 3.25 mmoles/liter. Previously, it was shown that at the level of 0.82 mmoles PEA/liter, fishery Moraxella strains were not inhibited while fishery strains of Pseudomonas had a lengthened onset of logarithmic growth and a fishery strain of Arthrobacter was completely inhibited (Table 20). Thus, PEA produced by fishery Moraxella species might be a factor in its predominance over other bacteria on refrigerated seafoods. Kwan and Lee (38) characterized an inhibitor of Clostridium botulinum type E produced by a Moraxella species. This antibiotic-like substance was only inhibitory to the outgrowth of spores, not the germination of them. The role of this substance, produced by a Moraxella, in competition with C. botulinum, in nature, is not known (38).

## Guanine plus Cytosine Mole Percent

All six strains of Moraxella possessed a guanine plus cytosine mole percent (G+C mole %) between 42.8 to 44.4 (Table 22). This range is in accordance with the values reported elsewhere for Moraxella (3,12,28,77). No apparent relationship was observed between strains in regard to G+C mole % and other parameters: penicillin resistance, proteolytic ability, Hugh and Leifson reaction, and PEA production.

Table 22. Guanine plus cytosine mole percent (G+C mole%) of Moraxella strains isolated from fishery sources.

| STRAIN | PENICILLIN RESISTANT | PROTEOLYTIC | HUGH AND LEIFSON OXIDATIVE | PEA[a] PRODUCED | Tm[b], C | G+C MOLE% |
|---|---|---|---|---|---|---|
| A-44 | - | - | - | + | 70.1 | 44.0 |
| D-1-12 | - | - | + | - | 70.3 | 44.4 |
| DD-4-5 | - | - | - | - | 70.3 | 44.4 |
| C-1-10 | - | + | - | ? | 69.3 | 42.4 |
| C-2-10 | + | + | - | ? | 69.5 | 42.8 |
| 5-1-2 | + | - | + | + | 70.1 | 44.0 |

[a]Phenethyl alcohol.

[b]Tm in 0.1xSSC (SSC = 0.15 M NaCl plus 0.015 M TSC, pH 7.0).

# CONCLUSION

*Moraxella* species of marine fishery origin are a group of bacteria which predominate on cold-stored seafood and other proteinaceous foodstuffs. Their spoilage potential is low, as indicated from pure culture studies using sterile fish muscle. Due to their relative resistance to irradiation, however, *Moraxella* are the primary spoilage organisms of irradiated seafoods.

What are the properties of *Moraxella* which select for its predominance in cold-stored seafoods? First, *Moraxella* are a natural member of the marine environment, often isolated from freshly landed fish and shellfish. Tolerance to NaCl is a key factor in its predominance. The majority of our strains grew at 10% NaCl (w/v). NaCl tolerance appears to be an innate characteristic of gram negative to gram variable coccobacilli, as *Acinetobacter* has also been shown to be NaCl tolerant. Fishery *Moraxella* strains are psychrotrophic, able to grow at temperatures ranging from $0^{\circ}C$ to $30^{\circ}C$. This temperature factor selects for organisms naturally present on foodstuffs harvested from marine environments of temperate regions. Spoilage and growth are a surface phenomenon, anaerobic microorganisms are selected against. The strict aerobic *Pseudomonas-Moraxella* group thus becomes the predominant microflora of seafoods.

Our experiments demonstrated *Moraxella* species to be relatively similar metabolically and typical in their biochemical reactions to other reported strains of fishery *Moraxella*. The majority of strains

were asaccharolytic, only 30% oxidized glucose. Xylose and arabinose were seldom oxidized. Most strains grew in the same pH range, NaCl concentrations, and temperature limits. Eighty one percent of our Moraxella strains grew on a glucose-salt medium supplemented with five growth factors and seven amino acids: leucine, isoleucine, valine, alanine, glycine, serine, and hydroxyproline. Leucine, serine, and alanine alone were able to support the growth of 6, 10, and 32% of all strains, respectively. These three amino acids have been found in large quantities in the slime of fish. The ability of Moraxella species to utilize these amino acids presents another selective advantage over nutritionally more demanding microorganisms.

The production of a metabolite, phenethyl alcohol (PEA), by marine fishery Moraxella strains, which was shown to selectively inhibit gram negative bacteria, also appears to select for Moraxella. Moraxella strains were more resistant to PEA at concentrations that could have inhibited other bacteria found in seafoods. Thus, PEA could have been responsible for Moraxella domination in seafoods and would also explain the poor microbial growth in some seafood items that are occasionally observed. Another Moraxella strain used in our laboratory, A-43, was shown to produce a non-dialyzable protein inhibitor against Clostridium botulinum type E.

Thus, four factors: NaCl tolerance, ability to grow at low temperatures, a simple nutritional requirement, and production of PEA would allow Moraxella predominance in seafood.

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