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Title: Characterization of Arthrobacter Species Isolated From Marine and Seafood Sources.

Abstract approved: Dr. Jong S. Lee

Forty Arthrobacter species isolated from Pacific and Atlantic fishery sources were examined to determine their significance in seafood. All strains were able to utilize inorganic nitrogen as the sole nitrogen source and grew in the absence of vitamins or growth factors. In addition, 78% grew on a medium containing only agar although no agar digestion was observed. When grown in 0.01 strength trypticase soy broth that simulated the nutrient level in seawater, Arthrobacter species attained a greater cell density than that of Pseudomonas or Moraxella, reaching the maximum absorbance at 580 nm of 0.113 for Arthrobacter, 0.094 for Pseudomonas and 0.044 for Moraxella. Ninety-seven percent of Arthrobacter isolates utilized glucose, but only 8% showed an oxidative reaction on Hugh-Leifson medium and none showed a fermentative reaction. None of the isolates hydrolyzed cellulose, but 60% degraded starch. Hydrolysis of gelatine and casein were observed in 72% and 5% of the isolates, respectively and lipolytic activity was present in 92%.

The isolates tested showed a tolerance to a broad range of pH levels and 85% of them grew at pH 6.3 to 9.5. Thirteen percent could grow at a pH of 5.0 and 28% at a pH of 10.5. Salt tolerance was high, with 43% able to grow in the presence of 10% NaCl.

All isolates grew at 2 and 25°C; 33% also grew at 37°C. Low temperature growth was slow for the four representative strains studied; generation times were 14, 16, 28 and 32 hours at 2°C. The optimal growth temperature for these strains averaged 28.1°C. Heat resistance varied from a D_{50} of 1.2 to 100 minutes and the average was 31 minutes. Potassium sorbate at an average level of 2.3% completely inhibited the growth of the same four isolates. The Arthrobacter isolates were inhibited by an average of six ppm Quaternary Ammonium Compound (QAC) compared to seven ppm and greater than 17 ppm for Moraxella and Pseudomonas species. Forty-two ppm of iodophor was sufficient to inhibit Arthrobacter, whereas 41 and greater than 50 ppm were necessary to prevent the growth of Moraxella and Pseudomonas species.

Arthrobacter species were sensitive to antibiotics. Chloramphenicol (2.5 µg/ml), streptomycin (10 µg/ml), ampicillin (25 µg/ml), tetracycline (25 µg/ml), chlortetracycline (25 µg/ml), oxytetracycline (25 µg/ml), neomycin (50 µg/ml), kanamycin (25 µg/ml), and penicillin G (3 and 75 IU/ml) inhibited 89% of the isolates. The least effective antibiotics against Arthrobacter species were nitrofurazone (25 µg/ml), nalidixic acid (25 µg/ml) and sulfathiazole (500 µg/ml), to which 93, 90 and 50% of the strains were resistant, respectively.

The ability to store carbohydrate was observed for the four isolates tested at an average level of 63% by dry weight.

Marine Arthrobacter species appear to possess characteristics that are well suited for growth and survival in harsh environments.

Characterization of *Arthrobacter* Species Isolated From Marine and Seafood Sources

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INTRODUCTION

Arthrobacter comprise a diverse group of gram positive, non-spore forming bacteria which have a distinct morphological cycle, changing from cocci or coccobacilli to rods and back to cocci or coccobacilli. Members of the genus *Arthrobacter* were originally isolated from soil but cheese, activated sludge, poultry litter, and seafoods have also yielded *Arthrobacter*.

Arthrobacter species generally are found in low numbers in the marine environment but under certain circumstances have been shown to dominate the microbial flora of fresh caught seafoods. Their growth in seafood appears to be limited, as *Arthrobacter* species are invariably overgrown by such bacteria as *Pseudomonas* and *Moraxella*. Pathogenicity has not been demonstrated among the members of this genus.

Most taxonomic, morphological, nutritional, and biochemical characteristics of *Arthrobacter* described in the literature deal with *Arthrobacter* species isolated from soil, sludge, or cheese, and relatively little work has been done on those Arthrobacters isolated from a marine environment. Available information does not explain how *Arthrobacter* species occasionally dominate the microbial flora of seafood or the significance of this occurrence.

This study was initiated to determine the conditions under which *Arthrobacter* could play a significant role in seafoods. Growth potential of *Arthrobacter* species were determined by studying their nutritional requirements, energy sources, biodegradative abilities,

and temperature characteristics. Their resistance to extremes of temperature, pH, salinity, and ability to grow on minimum substrate levels, formation of endogenous carbohydrate reserves, and resistance to antimicrobial agents were also examined. Finally, a hypothesis is presented to explain the occasional predominance of Arthrobacter species in seafood and its significance.

LITERATURE REVIEW

Description of Genus Arthrobacter and Its Distinctive Characteristics

Morphology

The genus Arthrobacter was first described by Conn and Dimmick (1947, 1948) as pleomorphic soil bacteria exhibiting a morphology similar to Mycobacterium and Corynebacterium. During the rod phase, the gram reaction was said to change from predominantly positive to mostly negative. The description of the genus Arthrobacter in the latest issue of Bergey's Manual (1974) includes strictly aerobic, non-spore forming, catalase-positive, pleomorphic cells having cell walls conforming to the gram positive structure, which may contain either but not both meso-diaminopimelic acid (m-DAP) or arabinose. Members of this group of bacteria are often referred to as coryneforms; however, only those isolates specifically identified as Arthrobacter, Arthrobacter-like, and those coryneforms conforming to the aforementioned description of Arthrobacter are included in present discussion.

Pleomorphism is a major distinctive characteristic of Arthrobacter and it was originally described by Conn (1947, 1948) and others (Kuhn, 1960; Mulder, 1963; Veldkamp, 1963; Ensign and Wolfe, 1964). They showed a sequence involving a complete transformation from coccus to rod to coccus in a rich medium. The cells, however, remained in coccus form throughout an entire growth cycle in a poor or nutritionally deficient medium. Larger (two to four times diameter) spherical cells (cystites) are often seen in older cultures of Arthrobacter and they

give rise to rods upon transfer to fresh media via "germination tubes" (Keddie, 1974; Mulder, 1963). Duxbury (1977b) demonstrated that cystites may be formed from either cocci or rods by exposure to a nutritionally unbalanced, but not carbon-limiting, medium. Ensign and Wolfe (1963) also found that A. crystallopoietes would readily reproduce in a glucose plus mineral salts medium but would remain in coccus form. Addition of L-lysine, L-aspartic acid, L-arginine, L-phenylalanine, butyrate, succinate, malate, fumarate or lactate would induce transformation of coccus form to rod form. Lucas and Clark (1975) found most of the Arthrobacter strains they studied showed the morphological change during growth when subjected to 38 chemicals. These included all of the 20 common amino acids except cysteine; hydroxyproline, glycerol, ribose, arabinose, glucose, galactose, lactose, fructose, sucrose, mannose, lactate, citrate, malate, succinate, pyruvate, gluconate, propionate, butyrate, and valerate also induced morphological change. Luscombe and Gray (1971) utilized a chemostat to determine if the morphological transformation was related solely to growth rate or to specific inducers. They showed that coccus to rod transformation could be induced simply by raising the dilution rate. Hamilton (1977) observed that at the onset of morphological change in A. crystallopoietes the cyclic AMP (c-AMP) level increased dramatically to 30 times the level found in the coccus form. The c-AMP level subsequently dropped and stabilized at levels four to five times that of the coccus form in glucose + salts media during the exponential growth phase and the cells remained in the rod form. The same authors recently investigated the relationship between c-AMP levels and morphogenesis in

a morphological mutant of A. crystallopoietes. They found a similar production of c-AMP by the mutant as evidenced by measurement of extracellular c-AMP but much lower levels of intracellular c-AMP compared to the parent strain, during growth on a medium which induced morphological change in normal strains (Hamilton, 1978). Kimberlin-Hariri (1977) treated A. crystallopoietes cells with TRIS/EDTA and the permeable cells were then exposed to extracellular c-AMP; further breakdown of c-AMP was prevented by exposing the treated cells to theophylline. This treatment, however, failed to stimulate initiation of the morphological cycle when cocci were transferred to fresh glucose + salts medium and actually retarded morphogenesis at concentrations of 1mM c-AMP or above in media that would normally induce rod formation. Thus, the role of c-AMP in the morphogenesis of Arthrobacter has yet to be fully resolved. The possible reasons for the morphological cycle in Arthrobacter are discussed at length in a review by Clark (1972) and they will be discussed later under "Relationship of Arthrobacter to Its Environment."

Relationship of Marine Coryneforms to Arthrobacter and Corynebacterium

As more Arthrobacter-like microorganisms were isolated from nature it became apparent that the diversity found in this group of bacteria had not been represented by the reference strains. This has prompted a number of numerical taxonomy studies wherein a multitude of tests are applied to a group of isolates and their relatedness determined (Skyring, 1968, 1969a, 1969b; Bousfield, 1972; Vanderzant, 1972; Hagedorn, 1975a,

1975b, 1975c; Jones, 1975). Bousfield (1972) studied 158 named and unnamed coryneform bacteria from a variety of origins and found that all aerobic, catalase positive, non-sporulating coryneforms or marine origin clustered together with all named Arthrobacter and Brevibacterium species at approximately 50% similarity (S-level), but did not merge at all with Corynebacterium species even at the 30% S-level. That 50% similarity is a considerable degree of relatedness is evidenced by his finding that most (7/9) of the named Arthrobacter species clustered only at the 55% S-level. Shewan (1971) stated that no true marine Corynebacterium has ever been found. Most Brevibacterium species are currently listed as "species incertae sedis" in Bergey's Manual (1974) and are likely to be consolidated with Arthrobacter; B. linens has already been renamed A. linens largely as a result of Mulder's work (Mulder, 1966). It appears, therefore, that the marine species are related to the terrestrial Arthrobacter closely enough to be included in the same genus.

Gram Reaction and Cell Wall Composition

The relationship of the morphological cycle to the gram reaction of Arthrobacter was found to vary. In general, all were predominantly gram positive or gram variable at one or more stages in the morphological cycle but this depends on whether the coccoid or rod form withstood decolorization (Mulder, 1964; Antheunisse, 1974; Clark, 1972). In Yamada's (1972) study of 112 strains of coryneform bacteria that included Arthrobacter, he noted that the intensity of the gram reaction was inversely related to the degree of pleomorphism. All 19 Arthrobacter

species exhibited a "weak" gram reaction and 84% (16/19) exhibited "distinct" pleomorphism.

The structure of the Arthrobacter cell wall remains typical of gram positive organisms throughout the morphological cycle. The constituent amino acids of A. globiformis were found in constant molar ratios throughout the life-cycle by Duxbury (1977a). Although only glucose, galactose, and rhamnose were found in the peptidoglycan regardless of morphological form, their molar ratios varied from rods to cocci to cystites, yielding ratios of 1.0:3.1:2.0, 1.0:3.7:3.0, and 1.0:1.1:0.9, respectively. Previc and Lowell (1975), however, found a definite increase in the meso-diaminopimelic acid (m-DAP) to lysine ratio as A. crystallopoietes transformed from cocci to rods. Their data tends to support Gillespie's (1963a, 1963b) observation that the lysine:aspartic acid ratio of A. globiformis strain 425 changed appreciably with changes in morphology from coccus to rod form. Although the ratios of cell wall constituents apparently change with changes in cell morphology, no study indicated any loss or gain of new sugars or amino acids during morphogenesis. Indeed, since the early work of Cummins and Harris (1956) on cell wall composition, the identity of the constituent sugars and amino acids has been shown to be constant and distinctive for a number of bacteria (Boone and Pine, 1968; Gillespie, 1963a, 1963b; Keddie, 1966, 1977; Bowie, 1972; Cummins, 1971; Yamada, 1970; Bergey's Manual, 1974). This uniqueness has allowed the use of cell wall composition as a taxonomic characteristic for a number of genera including Arthrobacter; Arthrobacter species vary in the sugars and amino acids which constitute their cell wall

peptidoglycan but no species combines both arabinose and m-DAP (Keddie, 1974). In recent years cell wall composition has become even more significant to the identification and classification of coryneforms since the morphological cycle, once thought to be an exclusive attribute of the genus Arthrobacter, is now known to exist within Brevibacterium, Cellulomonas, Kurthia, Corynebacterium, Microbacterium, Micrococcus, and possibly other genera (Bergey's Manual, 1974; Keddie, 1977, Yamada, 1970; Veldkamp, 1970).

Motility

Motility is occasionally observed in Arthrobacter species but is definitely the exception rather than the rule. Antheunisse (1974) studied the motility and type of flagellation of 120 Arthrobacter strains isolated from soil. Among them, 79% were non-motile. Even the motile rods developed into flagellated but non-motile cocci. In a study published the following year, he found 80% of coryneform bacteria from poultry litter to be non-motile. In Yamada's (1972) study which included 19 Arthrobacter strains 68% (13) of the Arthrobacter species and 75% of all coryneforms studied were non-motile. Stanlake and Clark (1976) studied the relationship of flagellation and motility to the morphological cycle and found that the extent of flagellation correlated well with the degree of cell elongation. As cells elongated, the percent of flagellated and motile cells increased; as cells reverted to cocci, de-flagellation occurred.

Relationship of Arthrobacter to Its Environment

Carbohydrate Storage and Resistance to Environmental Stress

One of the common characteristics of soil Arthrobacter is the formation of up to 60 to 70% in dry weight of carbohydrates (CHO) (Mulder, 1962, 1963, 1966, 1967; Zevenhuizen, 1966). Zevenhuizen (1966) found that this occurred under conditions of growth inhibition such as nitrogen, sulfur or phosphorus deficiency, or at a low pH in a CHO-containing medium. The structure of the storage CHO was a highly branched glycogen (Mulder, 1967) and it contained only glucose. It is the accumulation of large amounts of this material that accounts for the increased size of the cystites (Mulder, 1967; Duxbury, 1977a). Boylen (1970) found rods to store approximately 10% of this CHO whereas cocci contained approximately 40% CHO. Presumably the higher values (60 to 70% CHO) were from cultures containing a high number of cystites (Mulder, 1966; Duxbury, 1977a). Unlike other bacteria which rapidly deplete their stored glycogen, Arthrobacter metabolizes it relatively slowly (Zevenhuizen, 1966). Indeed, during dessication Boylen (1973) found the endogenous respiration rate was such that half-life of self-consumption was estimated to be 12 years. No one has yet performed a dessication-survival experiment of such duration, but resistance to dessication has been clearly demonstrated for this genus. Robinson (1965) compared the resistance of Arthrobacter and Pseudomonas soil isolates to drying in sterilized soil. The freshly-isolated Arthrobacter species fared somewhat better than those isolates maintained on laboratory media for several years. Among older isolates, the Arthrobacter

species suffered a ten-fold initial population loss during drying but no further loss of viability was observed after one month. Recent Arthrobacter isolates were essentially unaffected by either the drying or one month's storage. The older laboratory cultures of Pseudomonas species, however, suffered a 10^4- to 10^5-fold population loss at the time of drying and a further 10^2-fold decline after one month. Newly isolated Pseudomonas species only suffered a 10^2-fold decrease at time of drying and 10^1- to 10^2-fold decline after 30 days. Mulder (1964) found Arthrobacter species to be very dessication-resistant and reported that Arthrobacter survived in air-dried soil for more than 10 months. Cameron (1971) recovered Arthrobacter species from Antarctic soils kept four years under laboratory dessication. Chen and Alexander (1970) reported drought-resistant as well as drought-susceptable strains of Arthrobacter; drought-resistance was said to be increased by osmotic acclimatization in high salt media, thus suggesting a natural selection for bacteria with high internal osmotic tension during periods of dessication.

The advantages of storage CHO as an endogenous energy source during periods of starvation not accompanied by dessication are obvious. Boylen (1970) found no difference in survival potential of either rods of spheres in phosphate buffer; viabilities were 100%, 65%, and 60% after 30, 60, and 80 days starvation, respectively. This was in spite of the fact that he found the rods to store only about 10% (dry weight) of CHO versus approximately 40% CHO for the cocci. In the very long term, one would expect the cells having greater endogenous reserves to outlast those containing lesser amounts, but the duration of Boylen's

experiments evidently were not sufficiently long enough to deplete the intracellular reserves of the cells in either morphological form. Since the coccus or cocco-bacillus form is the preferred form during the resting stage, it must confer some advantage over the rod form. Clark (1972) explored various possibilities and suggested that this might be merely related to the larger amounts of stored CHO and reduced surface area to volume ratio which would give greater dessication resistance. There may be additional undetected physiological changes. Likewise, the rod form has the obvious advantage of greater surface area per unit volume for nutrient exchange during times of rapid growth. The precise reason for the existance of the morphological cycle in Arthrobacter and other related bacteria remains to be resolved.

Salt Tolerance

Arthrobacter species are also able to withstand relatively high concentrations of NaCl and other salts. Mulder's (1966) study compared soil Arthrobacter species to Brevibacterium linens (now A. linens) and other coryneforms isolated from cheese and found that none of the soil isolates grew in 8% NaCL but all of the cheese isolates were able to grow in the presence of 8% NaCl. Crombach (1974) found that the soil Arthrobacter species were considerably less salt-tolerant than the orange-pigmented sea fish and cheese coryneforms and the "non-orange" cheese coryneforms. One-third of the soil isolates were able to grow in 5% NaCl and all were able to grow in 3% NaCl. Seven of eight (88%) orange-pigmented cheese isolates tolerated at least 10% NaCl, with 3/8 (38%) able to grow in the presence of 15% NaCl. Nine of 13 (88%) of the non-orange cheese isolates grew in 10% NaCl and 14/16 (88%) of

the orange-pigmented sea fish isolates tolerated 10% NaCl. The average concentration of NaCl tolerated was approximately 4, 12, 10, and 12%, respectively. In Adamse's (1970) study of Arthrobacter from dairy waste activated sludge, 59 (83%) of 71 strains grew in 5% NaCl and only 5 (7%) could grow in 8% NaCl. Yamada (1972) noted that NaCl tolerance was not a significant feature of the 19 soil Arthrobacter strains tested; 17/19 (89%) grew in 5% NaCl but only one (5%) grew in 10% NaCl. In Jones' (1975) study of 60 Arthrobacter strains, 52 (87%) grew in 5% NaCl and 10 (17%) grew in 10% NaCl. Thus, it appears that Arthrobacter species isolated from saline environments such as cheese and sea fish tolerate approximately two to three times as much NaCl in their growth media as that tolerated by Arthrobacter species isolated from soil.

Temperature Relation

Keddie (1974) lists the growth temperature optimum for the genus Arthrobacter at 20 to 30°C, with growth usually occurring at 10°C but not at 37°C. In her study of 60 Arthrobacter strains from a variety of sources, Jones (1974) found 22% would grow at 37°C but only 2% could grow at 45°C. Two isolates, or only 3%, survived 30 minutes at 60°C. Antheunisse (1975) tested 30 coryneforms isolated from poultry litter and found that all grew at 10°C but none grew at 37°C. Lee and Pfeifer (1977) compared the heat resistance of marine Arthrobacter isolates and found a D_{52} value of 6.3 minutes. This was equal to Micrococcus and greater than that of the Staphylococcus, Pseudomonas, Moraxella, Vibrio parahemolyticus and Flavobacterium-Cytophaga species tested. Their respective D_{52} values were 6.3, 1.5, 1.2, 0.7, 0.7, and 0.4 minutes.

The only species showing greater heat resistance was Acinetobacter with a D_{52} of 8.0 minutes.

Antibiotic Resistance

Antibiotic sensitivity of Arthrobacter species have not been studied extensively as would be the case with bacteria of proven pathogenicity. Skyring (1969a, 1969b, 1970) subjected at least 400 named and unnamed Arthrobacter isolates from soil to seven antimicrobial agents and found the resistance of streptomycin (0.5 µg/ml) to be a distinctive characteristic of some groups of Arthrobacter species. Similarly, Vanderzant (1975) subjected 66 coryneforms isolated from pond shrimp to seven antibiotics but could not find any distinct resistance pattern. Jones (1975) provides detailed information on the effectiveness of antibiotics against Arthrobacter. The overwhelming majority of the 60 Arthrobacter species isolated from various sources were sensitive to most of the 23 antibiotics tested. Ten of the antibiotics inhibited 100% of the isolates with 19/23 antibiotics effective against at least 85% of the isolates. The least effective antibiotics, nitrofurantoin, colomycin, celbenin, and sulphonamide, still inhibited 78, 75, 62, and 62% of the test strains, respectively. It appears that Arthrobacter species are relatively more sensitive to a variety of antibiotics than most other bacteria, in spite of a well known ability of this group of bacteria to metabolize exotic compounds (see "Biodegradative Activities: Degradation of Exotic Compounds").

Growth Requirements

Nitrogen Nutrition and Growth Factor Requirements

The genus Arthrobacter encompasses a metabolically diverse group of bacteria. Nearly all soil and sludge isolates of Arthrobacter were capable of utilizing inorganic nitrogen as sole nitrogen source when growth factors and vitamins were supplied (Mulder, 1963, 1966; Owens, 1969, Adamse, 1970; Bousfield, 1972; Jones, 1975). Only 11% (3/27) of Arthrobacter isolated from poultry litter were reported to be able to grow in an ammonium salts + citrate medium (Antheunnisse, 1975). The ability to utilize inorganic nitrogen is variously reported to exist in 52% (24/46) and 33% (17/51) of non-pigmented cheese isolates (Mulder, 1966; Crombach, 1974). Crombach (1974) found only 13% (2/16) of his orange-pigmented sea fish isolates capable of growth with inorganic nitrogen, but in Bousfield's (1972) study, all 30 marine coryneform isolates were found to have this capacity.

Vitamins or vitamin-like growth factors are required by a majority of soil, sludge, and cheese isolates. Mulder (1963, 1966) found 62% (69/112) of soil isolates tested to require one or more growth factors or vitamins, while Keddie (1966) and Owens (1968, 1969) found this to be true of 87% (58/67) and 85% (47/55) of their Arthrobacter species of soil origin, respectively. Adamse (1970) found 55% (39/71) of Arthrobacter species isolated from sludge required vitamins for growth on inorganic nitrogen. Mulder's (1963) earlier study of cheese isolates indicated 46% (21/46) needed vitamins but this figure was later revised to 63% (32/51) for the non-pigmented isolates and 70% (19/27) for the

orange-pigmented isolates (Mulder, 1966). Mulder in this latter study also examined five orange-pigmented sea fish isolates and found all of them to have a vitamin requirement. In addition, Crombach (1974) reported that 16 orange-pigmented sea fish isolates he studied showed a requirement for organic nitrogen.

Carbon/Energy Sources and Carbohydrate Utilization

Most Arthrobacter species are known to seldom produce acid from glucose or any of the other common hexoses or pentoses (Keddie, 1974; Bousfield, 1972; Yamada, 1972; Jones, 1975). The ability to hydrolyze starch varies with the source of the isolates and appears to be present in about one-half of the members of this genus. Mulder (1963) and Crombach (1974) found 49 and 58% of Arthrobacter species isolated from soil could hydrolyze starch as compared to Antheunisse's (1975) report that 96% of coryneforms isolated from poultry litter could do the same. Eighty percent of sludge isolates were reported by Mulder (1963) to hydrolyze starch; Adamse (1970) confirmed this finding in his report and found that 89% of his Arthrobacter species isolated from sludge could degrade starch. Crombach (1974) found only 15% of non-pigmented cheese isolates and none of his orange isolates from cheese and sea fish to have this ability. Similarly, Mulder (1963) found 22% of his test isolates from cheese able to break down starch. Vanderzant (1975) indicated a minority (less than 40%) of his pond isolates capable of starch hydrolysis, as compared to Jones' (1975) report that 52% of Arthrobacter species isolated from various sources could degrade starch.

Proteolytic Activity

With the exception of non-pigmented strains isolated from cheese the majority of Arthrobacter species have been shown to be proteolytic when tested against gelatine and casein. Mulder (1963) found 65% of soil Arthrobacter to be proteolytic, and 90% of sludge isolates but only 33% of cheese isolates had this ability. In a later study, however, he found all strains isolated from soil to be proteolytic as were 93% of orange-pigmented cheese isolates and 22% of non-pigmented cheese isolates (Mulder, 1966). Crombach (1974) also reported all soil isolates tested to be proteolytic as were all orange-pigmented strains isolated from cheese. He also concurred with Mulder's (1966) finding that the proteolytic activity of non-pigmented cheese isolates was limited, and reported only 23% of them demonstrated proteolysis. Eighty-eight percent of orange-pigmented strains isolated from sea fish were also reported to be proteolytic. Mulder (1963) and Adamse (1970) are in close agreement with regards to proteolysis among Arthrobacter isolated from sludge; they reported 90 and 92%, respectively, of their test isolates were able to degrade protein. As an estimate of the capabilities of the genus as a whole, Jones' (1975) finding that 65% of Arthrobacter species isolated from various sources were proteolytic seems reasonable; of five published reports describing a total of 439 individual tests on Arthrobacter species isolated from soil, sludge, cheese and sea fish, 66% (287/439) were found to be proteolytic (Mulder, 1963, 1966; Adamse, 1970; Crombach, 1974; Jones, 1975).

Lipolytic Activity

Lipolytic activity is apparently widespread among Arthrobacter species of diverse origins. In his earlier article describing the characteristics of Arthrobacter species isolated from soil, sludge, and cheese, Mulder (1963) found 11, 0, and 7%, respectively, were able to utilize Tween-80 (polyoxyethylene sorbitan monooleate) as a carbon + energy source. Adamse (1970) found 8% of his sludge isolates capable of hydrolyzing Tween-80. In his articles reporting numerical taxonomy studies of Arthrobacter species of soil origin, Skyring (1969a, 1969b, 1970) tested for clearing in palmitic, oleic, and tributyrin agar, but failed to give specific data. Jones (1975) found 75% of 60 Arthrobacter species from various origins capable of Tween-80 hydrolysis; this figure increased to 83% when Tween-20 was substituted but only 40% showed lipolysis on egg yolk agar.

Degradation of Exotic Compounds

Arthrobacter as a group is known to possess an ability to degrade exotic chemicals. They have been found capable of decomposing such substances as nicotine; amino acids; mono-, di- and tri-carboxylic acids; alantoin; lignin; 2-hydroxypyridine; phenol; ethanol; certain pesticides such as 4,6 dinitroorthocresol; 2,4 dichorophenoxyacetate; endoxohexahydrophthalic acid; and aromatic and aliphatic hydrocarbons (Mulder, 1963, 1964; Sguros, 1955; Morris, 1960; Vogel, 1963; Ensign, 1963; Jensen, 1963, 1964; Loos, 1967a, 1967b; Stevenson, 1967; Jones, 1968; Veldkamp, 1970). Indeed, the ability to utilize unusual compounds has been used in enrichment techniques wherein substances such

as pyridines, hydrocarbons, puromycin, amyl alcohol, toluene or cyclohexanol were incorporated in the isolation media (Foster, 1962; Mulder, 1963; Engisn, 1963; Veldkamp, 1970). Silicate decomposing Arthrobacter species capable of fixing molecular nitrogen have been isolated from rocks (Smyk, 1963).

Arthrobacter and Seafood Spoilage

Arthrobacter species are readily isolated from freshly caught seafood taken in Atlantic, Pacific and Gulf Coast waters. Shewan (1971) listed Arthrobacter as representing 18% of the microbial flora of North Sea fish, thus putting them below Moraxella but about even with Pseudomonas species in predominance. In Boeye's (1975) study of microbial flora from North Sea sediments, he found pleomorphic rods to comprise the most numerous group after Bacillus species, and accounted for 23% of the total. In Lee and Pfeifer's (1975) study of Dungeness crab microbial flora, Arthrobacter species were the second most numerous in raw crabs, comprising 14% of total counts versus 40% for Moraxella. In a later study of Pacific shrimp, Lee and Pfeifer (1977) found Arthrobacter to account for 13% of the landed shrimp counts, vying for third place in predominance with Acinetobacter but behind Moraxella and Pseudomonas in predominance. Vanderzant (1970) examined the microbial flora of Gulf of Mexico and Texas pond-reared shrimp and found coryneforms to predominate, respectively, accounting for 40 and 43% of the counts of freshly harvested shrimp. Water samples from earthen ponds yielded even high proportions of coryneforms which often exceeded 90% of total counts.

Without exception, however, Arthrobacter species are gradually replaced during refrigerated storage of seafoods, giving way to such bacterial species as Pseudomonas and the Moraxella group (Shewan, 1971; Vanderzant, 1970). By the time Dungeness crab had reached the retail outlets, the proportion for Arthrobacter had dropped from 14 to 7% while Pseudomonas had risen from 6 to 14% (Lee, 1975). Vanderzant (1970) showed that after seven days storage at 1°C the Pseudomonas population in Gulf shrimp increased from 22 to 40% and at the same time the Arthrobacter population decreased from 40 to 22%. This may have been due to the more rapid growth of Pseudomonas species at low temperatures. Lee and Pfeifer (1977) examined the growth rate of five genera of bacteria most common to seafood and found that the generation times at 10°C of Pseudomonas, Moraxella, Arthrobacter, Flavobacterium-Cytophoga, Acinetobacter, and Vibrio parahemolyticus were 3.9, 4.2, 8.0, 8.0, 8.1, and 14 hours, respectively. During processing steps involving heating such as cooking crab or blanching/peeling shrimp, Arthrobacter may increase in proportion probably due to its higher heat resistance (Lee, 1975, 1977). Lee and Pfeifer (1977) found Arthrobacter isolated from shrimp to have a D_{52} value of 6.3 minutes as compared to D_{52} values of 8.0, 6.3, 1.5, 1.2, 0.7, 0.7, and 0.4 minutes for Acinetobacter, Micrococcus, Staphylococcus, Pseudomonas, Moraxella, Vibrio parahemolyticus and Flavobacterium-Cytophaga, respectively.

The association between Arthrobacter and mud or soil appears to be a close and continuing one. Bousfield (1972) showed the taxonomical relatedness of marine Arthrobacter isolates to those from soil. Vanderzant (1970) found the predominance of Arthrobacter species to be

noticeably greater in Gulf of Mexico and pond-reared shrimp than that found by Lee (1977) in Pacific shrimp. In comparing shrimp taken from the same area of the Pacific but processed at different plants, Lee (1977) found consistantly higher Arthrobacter counts associated with those processing operations which utilized minimal washing of the shrimp prior to processing.

MATERIALS AND METHODS

Arthrobacter Isolates

All Arthrobacter cultures were isolated by this laboratory in our previous study. Nineteen of the 40 isolates studied had originated from frozen Atlantic scallops. Of the remaining 21 isolates, eight were from refrigerated seawater collected from the Pacific shrimp boats that utilized the refrigerated seawater spray (RSWS) system for cooling the catch. The remaining 13 cultures were isolated from the shrimp hold surfaces of various Pacific shrimp boats which used traditional ice refrigeration (Table 1). All test cultures were streaked three successive times to ascertain their purity and were periodically restreaked throughout this study. All isolates included in this study conformed to the description of Arthrobacter. They were gram positive, non-spore forming, strictly aerobic, catalase positive, pleomorphic rods showing spherical or coccobacillary cells in older cultures (Keddie, 1966, 1974; Clark, 1972; Veldkamp, 1970). Cultures were maintained on TPE (5 g Bacto-tryptone, Difco; 5 g Bacto-peptone, Difco; 2.5 g yeast estract, Difco; 1 g glucose; 15 g Bacto-agar, Difco per liter). All media were sterilized at 15 psi for 20 minutes; heat-labile constituents were filter sterilized (0.45 μ Millipore filter, HAWG-047-SO; Millipore Corp., Bedford, MA) and added aseptically to cooled media. Unless otherwise stated, all tests were made on agar media via replica plates and were incubated at 25°C. Motility was checked under phase contrast in wet mount preparations, and with semisolid Motility-Nitrate medium (1 g KNO_3, 3 g Bacto-beef extract, 5 g Bacto-peptone, 1 g glucose,

Table 1. Origin of Arthrobacter isolates.

Pigmentation	Strain Number		
	Refrigerated Seawater[1]	Shrimp Boat Hold[1]	Atlantic Scallops
White	1, 2	15, 16, 20[2]	24, 31, 35, 38, 39
Pink	--	8	25, 34[2]
Orange	4, 5[2], 6	--	--
Yellow	3, 7, 41	9, 10, 11[2], 12, 13, 14, 17, 19, 21	22, 23, 26, 27, 28, 29, 30, 32, 33, 26, 37, 40

[1]Pacific Coast shrimp vessels.

[2]Representative strains used for optimum temperature, sorbate, disinfectant, and CHO storage tests.

2.5 g Bacto-agar per liter). Gram staining was by the Kopeloff modification.

Nitrogen Nutrition

Ability to utilize inorganic nitrogen as sole nitrogen source was tested with EG (Mineral Base E plus 1% glucose) agar and broth. Mineral Base E contained 1.10 g K_2HPO_4, 0.72 g KH_2PO_4, 0.025 g $CaCl_2$, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.5 g $(NH_4)_2SO_4$, and 3 ml of EDTA Trace Metals Mix (5 g EDTA, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.57 $MnSO_4 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.161 g $CoCl_2 \cdot 6H_2O$, 0.157 g $CuSO_4 \cdot 5H_2O$, 0.151 g $Na_2MoO_4 \cdot 2H_2O$ per liter, adjusted to pH 6.0 with KOH) per liter. Nitrogen preference was determined by comparison of growth on ZN agar (1.9 g K_2HPO_4, 0.025 g $CaCl_2$, 0.2 g $MgSO_4$, 0.1 g NaCl, 10 g glucose, 15 g agar per liter) plus one of the following: 3 g $(NH_4)_2SO_4$, 1.82 g NH_4NO_3, 3.86 g $NaNO_3$, 6.09 g Vitamin-Free Casamino Acids (VFCAA, Difco), 6.09 g tryptone, 6.09 g peptone, or no added nitrogen sources (ie. ZN1-ZN7, respectively). Colony size was measured daily for five days and then scored as -, ±, +, ++, +++, or ++++ according to the degree of growth. Nitrate reduction was tested with sulfanilic acid and alpha napthylamine as outlined in Manual of Microbiological Methods (1957) using three day cultures in Motility-Nitrate medium.

Growth factor requirement/stimulation was tested by comparing growth on EG (see above) to growth on Z66C (4 g yeast extract, 10 g glucose, 15 g agar per liter).

Carbon/Energy Sources and Carbohydrate Utilization

Cellulose hydrolysis was tested using strips of Whatman Number 1 (W. and R. Balston, Ltd.) filter paper half submerged in a liquid medium [2.5 g peptone, 2.5 g yeast extract, 0.05 g Tween-80 (polyoxyethylene sorbitan monooleate; Baker), 20 µg B_{12} per liter of Mineral Base E] inside culture tubes. Cultures were incubated for one month and checked periodically for disintegration of the filter paper and for turbidity.

Oxidative/fermentative utilization of glucose, fructose, rhamnose, galactose and lactose was tested using the Hugh-Leifson medium (10 g tryptone, 1 g yeast extract, 0.04 g bromcresol purple) containing 10 g of the sugar to be tested and 15 g agar per liter; anaerobic incubation was via GasPak Anaerobic Jar (BBL). Presence of cytochrome oxidase was determined by means of Pathotec-CO test strip (General Diagnostics, Morris Plains, NJ). Glucose utilization was determined by comparing growth on Z66E (1 g K_2HPO_4, 0.2 g $MgCl_2$, 0.025 g $CaCl_2$, 3 g $(NM_4)_2SO_4$, 2 µg B_{12}, 0.05 g Tween-80, 3 ml EDTA Trace Metals Mix, 15 g agar per liter) with and without 1% glucose. Utilization of xylose, lactose, cellobiose, sucrose, and mannitol was tested by comparing the growth on TPE3 (1 g tryptone, 1 g peptone, 1 g yeast extract, 15 g agar per liter of Mineral Base E), which contained 1% of the carbohydrate to the growth on TPE3 alone and TPE3 plus 1% glucose.

Starch hydrolysis was tested on a medium containing 10 g potato starch, 1 g glucose, 2.5 g yeast extract, 2.5 g peptone, and 15 g agar per liter. After 5 days incubation, colonies were gently washed from agar surface with distilled water and the Petri plates flooded with 1%

iodine solution. Absence of blue color where colony had been indicated starch hydrolysis.

Proteolytic Activity

Hydrolysis of casein was tested on sodium caseinate agar that contained 5 g tryptone, 2.5 g yeast extract, 1 g glucose, 10 g Casein-Sodium (U.S. Biochemical Corp.) and 15 g agar per liter. Plates were checked daily for zones of clearing for eight days. Gelatine hydrolysis was determined on a gelatine medium that contained 0.05 g Tween-80, 2 μg B_{12}, 2.5 g peptone, 2.5 g yeast extract, 2.5 g glucose, and 150 g gelatine per liter of Mineral Base E. Plates were examined daily for eight days for gelatine liquifaction.

Lipolytic Activity

Esterase activity on Tween-80 was determined by formation of opaque precipitate zones around colonies in a modification of the lipolysis medium used by Adamse in 1970. This medium contained 2.5 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0.1 g $CaCl_2 \cdot 2H_2O$, 10 g Tween-80, and 15 g agar per liter. Hydrolysis of tributyrin was tested in L-3 agar (3 g beef extract, 5 g peptone, 50 g tributyrin, 1 g glucose, 15 g agar per liter) and was evidenced by zones of clearing in the resultant emulsion.

pH Range

The pH range for growth was tested in M-2 broth (2.5 g peptone, 0.1 g Tween-80, 5 g VFCAA, 5 g yeast extract per liter of Mineral Base

E). After autoclaving, media were adjusted to pH 5.0, 6.3, 7.0, 8.5, 9.5, and 10.5 with sterile 0.1 M KOH and 0.1 M H_3PO_4 solutions and aseptically transferred in 10 ml amounts to sterile culture tubes. Inoculated tubes were incubated at least five days on a shaker and periodically checked over the next 21 days for turbidity.

NaCl Tolerance

Salt tolerance was tested on M-2 agar containing 0, 1, 4 and 10% of added NaCl. Duplicate plates were incubated at 2°C and 25°C. The growth of isolates representing the four pigment groups in the presence of 0, 1, 4, 8, and 12% NaCl was monitored with Spectronic 20 spectrophotometer (Bausch & Lomb) and absorbance read at 580 nm. The medium used for this, and in a subsequent experiment wherein acclimatization to salt was attempted, was TPE5 (2 g peptone, 2 g tryptone, 1 g yeast extract, 1 g Tween-80, 2 µg B_{12}, 10 g glucose, and 3 ml EDTA Trace Metals Mix per liter). The cultures monitored spectrophotometrically were grown on a shaker in 300 ml side arm flasks (Bellco) containing 50 ml of the medium to be tested. In the salt acclimitization experiment, these same representative Arthrobacter cultures were inoculated into a series of culture tubes containing TPE5 with 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% added NaCl. As each tube in a series became turbid, it was used to inoculate the tube containing the next higher salt concentration in the series. Incubation and reinoculation continued for one month.

Temperature Effects

Growth Range and Optimum Growth

Growth on solid media at 2, 10, 20, 25, and 37°C was observed using TPE agar. Low temperature plates were incubated for two weeks, 20 and 25°C plates for one week, and 37°C plates for two days. Optimum growth temperatures for representative isolates were determined in TPE5 broth in a Temperature Gradient Incubator (Scientific Industries, Bohemia, NY). The gradient was from approximately 2.8°C to 41°C in approximately 2.6°C increments. Growth was monitored by reading absorbance at 580 nm in a Spectronic 20 spectrophotometer.

Low Temperature Growth

Representative strains of Arthrobacter, Moraxella and Pseudomonas were grown in TSB at 2°C in 300 ml side arm flasks (Bellco). Again growth was monitored by reading absorbance at 580 nm in a Spectronic 20 spectrophotometer.

Heat Inactivation Kinetics

Heat inactivation kinetics of representative strains were determined in a manner similar to that of Chung (1979) and involved heating of cell suspensions for 1, 3 and 5 minutes in a 50°C water bath. Subsequent dilution and spread-plating was on TSA. Come-up time was predetermined using distilled water blanks of equal volume to that used in actual treatments, and after treatment, culture tubes were cooled by agitation in an ice bath. Nonheated controls were placed in the ice

bath simultaneously with the samples of shortest heat exposure and were diluted and plated first. All dilutions were plated in duplicate, incubated at 25°C, and counted at 1, 2, 3, and 5 days. In a separate experiment, heat treated cell suspensions were inoculated into 300 ml Bellco side arm flasks containing 50 ml of sterile TSB and incubated in a Psychrothern Incubator Shaker (New Brunswich Scientific Co.) at 26°C. Growth was monitored with a Spectronic 20 spectrophotometer and absorbance read at 580 nm. Generation times were calculated and the length of lag period was adjusted to compensate for the population decrease due to heating.

Storage Carbohydrate

Storage carbohydrate was determined for representative Arthrobacter isolates by the method of Dubois (1956) in which concentrated sulfuric acid is added to a suspension of cells containing aqueous phenol. Cells were grown for five days in TPE5, inactivated with 1% formaldehyde, washed three times in distilled water, and dried to a constant weight (stored one week under vacuum over P_2O_5) prior to CHO determination. Absorbance was read at 480 nm in a Spectronic 20 spectrophotometer and compared to glucose standards; results were expressed as glucose equivalents.

Growth in Low-Nutrient Environments

Growth in low-nutrient environments was tested on solid and liquid media. Isolates were tested for ability to grow on agar alone, as well as on special agar-Noble (Difco) alone prepared in acid-washed glassware.

Four strains of Arthrobacter, representing each of the pigment groups, along with two species each of Pseudomonas and Moraxella were tested first for ability to grow in 0.3, 0.1, and 0.01 strength TSB using culture tubes and observing turbidity after five days. These same eight isolates were then inoculated into 300 ml side arm flasks containing 50 ml 0.01 strength TSB and their growth was monitored spectrophotometrically at 580 nm with a Spectronic 20 spectrophotometer.

Resistance to Potassium Sorbate

Resistance to potassium sorbate was tested after the manner of Chung (1979) and involved the inoculation of culture tubes containing TPE plus 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, or 3.0% potassium sorbate (Monsanto Co.). After six days incubation at 26°C, turbidity was noted and compared to inoculated sorbate-free controls. The lowest sorbate concentration producing an easily detected reduction in growth compared to positive controls was determined to be the minimum inhibitory concentration. Maximum inhibitory concentration was the lowest concentration of sorbate that completely inhibited growth as evidenced by the absence of turbidity when compared to uninoculated control tubes.

Resistance to Quaternary Ammonium Compound (QAC) and Iodophor Disinfectants

Four representative Arthrobacter isolates plus two species each of Pseudomonas and Moraxella were tested according to A.O.A.C. procedures 4.001-4.003 (1970). After five-minute exposures, test cultures were

subcultured in TSB. After initial range finding tests, these isolates were tested by exposure to 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 ppm QAC (Airchem A-3, Airwick Industries, Carlstadt, NJ) and to 0, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, and 50 ppm iodophor (Mikroklene DF, Klenzade Products, Beloit, WI). Concentrations tested were prepared according to label directions; sterility of disinfectant solutions was ensured by Millipore filtration (0.45 μm). Elapsed time from first dilution of disinfectant to subculture of the last exposure was no more than 2.5 hours.

Resistance to Antibiotics

Antibiotic sensitivity of the 40 Arthrobacter isolates was tested on TPE agar according to the method of Kelch (1978). The antibiotics tested, concentrations used, and their manufacturers are presented in Table 2. Exposure plates were incubated for seven days and inspected daily after the second day for growth.

Table 2. Antibiotics and concentrations used in resistance tests.

Antibiotic (symbol)	Trade Name	Manufacturer	Concentration (µg/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
Chlortetracycline (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

RESULTS AND DISCUSSION

Microorganism

Morphology

Pleomorphism is a characteristic of the genus Arthrobacter. All isolates we tested conformed to a varying degree to the coccus-to-rod-to-coccus morphological cycle. Not all isolates could be induced to form perfect cocci; many rods merely shortened and plumped to a coccobacillus form. All isolates, however, formed slender rods, V-forms, and club-shaped rods upon transfer to fresh media. Gram staining was usually positive in the elongated rod forms and gram-variable in the coccobacillus or coccus form. A few isolates did closely follow Conn and Dimmick's (1947) original description for the genus, staining gram variable in the rod and pleomorphic stages and becoming increasingly positive as the coccus shape returned. Cell size varied with the age of the culture but was largest in older cultures grown in rich media and smallest in media affording slower growth, such as glucose plus salts. Filaments were occasionally seen in rapidly growing cultures but reduced to well-defined strings of four to six cells or less in mature cultures. During the lag and early log phases upon transfer to fresh media, one to three "germination tubes" were often seen growing from large plump rods or the larger cocci (cystites). These larger cells increased in number as the cultures aged. This is not surprising in view of the fact that their increased (two to four times) size has been shown to result from an accumulation of large amounts of glycogen, a development that follows when growth conditions become unfavorable

but glucose is still available (Zevenhuizen, 1966; Mulder, 1967; Boylen, 1970; Duxbury, 1977a, 1977b).

Pigmentation

Thirty of the 40 isolates showed distinct pigmentation (Table 1). The intensity and hue varied somewhat with the media used. Since very similar colors were produced when cultures were grown at 2, 10, 20, 25, and 30°C, and whether exposed to light or grown in darkness, no attempt was made to associate color variations with the growth conditions. The "non-pigmented" (white) isolates eventually turned the growth medium dark in a couple of weeks. While testing to see whether any of the isolates could hydrolyze cellulose (and thus potentially be Cellulomonas rather than Arthrobacter) it was found that the pigments migrated along the filter paper strips. There was no pigment separation and at the tip of most was a spot corresponding to the normal pigment of that isolate (e.g., yellow for yellow isolates, orange-brown for orange isolates). Fifteen of 24 yellow isolates produced this diffusible pigment as did two-thirds orange and one-third pink isolates. Four of the ten non-pigmented isolates also demonstrated a pigment-like spot identical to that produced by the orange-pigmented isolates. Presumably this substance was the one responsible for darkening the media in aged cultures of white isolates.

Motility

Although motility is sometimes encountered in this genus, and is not uncommon among marine microorganisms, it was never observed among

the strains examined. All isolates were checked for motility using Motility-Nitrate Medium (Manual of Microbiological Methods, 1957) but results were inconclusive. Most of the cultures had been repeatedly tested in wet mount preparations during the course of this investigation but again no motility was seen. The rapidly growing and spreading cultures which were the most likely candidates for motility have been checked repeatedly for motility but the results were always negative. Although it cannot be stated unequivocally that motility is completely absent from this group of Arthrobacter, it can be safely said that none are predominantly motile and that motility, if it exists, is an uncommon characteristic. In the literature, motility has been reported primarily for Arthrobacter of soil origin, is usually present in only a minority of cultures, as well as in only a minority of cells within a given culture, and is apparently related to the morphological cycle, with virtually all motile cells being rods (Yamada, 1972; Antheunisse, 1974; Stanlake and Clark, 1976). In this respect, it would seem that the marine Arthrobacter species we tested deviate slightly from the soil forms.

Nutrition and Biodegradative Activities

Nitrogen Nutrition

All isolates grew slowly on a mineral salts + glucose medium (EG) which supplied nitrogen in the form of $(NH_4)_2SO_4$. In a test of two media similar to EG [ZN + $(NH_4)_2SO_4$ and ZN + $NaNO_3$], all isolates again showed at least a weak growth response on ammonium sulfate, but 13% (5/40) failed to do so on sodium nitrate (Table 3). This is in agreement with that observed with soil and sludge Arthrobacter species by Mulder (1963,

Table 3. Growth responses of Arthrobacter species in various nitrogenous media.

	EG (13d)	EG (5d)	YE+G (Z66C) (2d)	ZN $(NH_4)_2SO_4$	ZN NH_4NO_3	ZN $NaNO_3$	$NO_3 \rightarrow NO_2$	ZN VFCAA	ZN Tryptone	ZN Peptone	ZN No Added Nitrogen	Casein Hydrolysis	Gelatine Hydrolysis
White													
1	+++	++	+++	++	+++	+++	+	++++	++++	+++	++	-	-
2	+	-	++	+	+	-	+	++	++	++	-	-	-
15	+	-	++	+	±	-	-	+	++	-	-	ND[1]	ND
16	+	+	++	++	++	++	+	+	+	++	+	-	-
*20	+	-	+	+	++	++	-	+++	+++	++	-	-	-
24	+	-	++	+	+	+	-	+	++	+	-	-	-
31	+	-	++	+	+	±	-	+	+	+	LA	-	-
35	++	-	+	+	+	±	-	+	++	++	LA	-	+
38	++	±	++	+	±	-	-	+	+	+	LA	-	+
39	++	-	++	+	+	-	-	+	+	+	LA	-	+
Orange													
4	++	-	++	+	+	±	+	++	+	+	-	-	-
* 5	++	+	++	+	+	±	+	++	+	++	-	-	++
6	++	+	++	+	+	+	+	+	++	++	-	-	++
Pink													
8	++	+	+++	+	+	-	±	++	++	++	-	+	++
25	+++	+++	+++	+	+	+	-	+	++	+++	++	-	-
*34	+++	+++	+++	+++	+++	+++	-	+++	+++	+++	LA	-	-
Yellow													
3	++	+	++	++	++	++	+	+++	+++	+++	+	-	++
7	+	-	++	+	+	+	+	++	+	+	-	-	++
9	++	+	+++	++	++	++	+	+++	+++	+++	+	-	++
10	++	++	+++	++	++	+	+	+++	+++	+++	+	-	++

Table 3. Continued

	EG (13d)	EG (5d)	YE+G (Z66C) (2d)	ZN $(NH_4)_2SO_4$	ZN NH_4NO_3	ZN $NaNO_3$	$NO_3 \rightarrow NO_2$	ZN VFCAA	ZN Tryptone	ZN Peptone	ZN No Added Nitrogen	Casein Hydrolysis	Gelatine Hydrolysis
*11	++	+	+++	+	+	+	+	++	+++	+++	+	-	++
12	++	-	+++	+	+	+	+	+	+++	+++	+	-	++
13	++	+	+++	++	++	+	+	+++	+++	+++	+	-	++
14	++	++	+++	++	++	++	+	+++	+++	+++	+	-	++
17	++	++	+++	++	++	+	+	++	+++	+++	+	-	++
19	++	++	+++	++	++	+	+	+++	+++	+++	+	-	++
21	+++	+++	+++	++	++	++	+	+++	+++	+++	++	-	++
22	++	+	++	+	+	+	+	+	+++	+++	++	-	-
23	+	-	+	+	+	±	-	+	+	+	-	-	-
26	++	++	++	++	++	++	+	+++	+++	+++	+	-	++
27	++	++	++	++	+	+	-	++	+++	+++	+	-	++
28	+	±	++	+	+	±	-	+	+	+	-	-	+
29	++	+	++	+	+	+	-	+	++	++	+	+	++
30	++	+	++	+	+	+	-	+	++	++	+	-	++
32	+	±	++	±	+	±	-	+	++	++	LA	-	+
33	+	-	++	±	±	±	-	+	+	+	LA	-	+
36	++	±	++	+	±	±	-	+	++	++	LA	-	+
37	++	±	+	+	±	±	-	+	+	++	LA	-	+
40	+++	+	+++	+	+	+	+	++	+++	+++	LA	-	+
41	+++	++	+++	+	+	+	+	++	+++	+++	LA	-	++

1966), Owens (1969), Adamse (1970), and Hagedorn (1975). Table 4 compares the growth characteristics of Arthroacter species isolated from various sources on inorganic nitrogen media. It shows that nearly all soil and sludge Arthrobacter could utilize ammonium nitrogen and a lesser proportion of them could utilize nitrate as well. Among investigators who have studied marine Arthrobacter there is no agreement as to the proportion of isolates possessing the ability to utilize inorganic nitrogen (Mulder, 1966; Bousfield, 1972; Crombach, 1974). Bousfield tested a large number of marine Arthrobacter-like coryneforms and stated that all could grow on inorganic nitrogen. This is in agreement with our present study. Mulder, however, did not find this ability among five isolates from sea fish, and Crombach found only 13% (2/16) of sea fish Arthrobacter able to grow on inorganic nitrogen. The reason for this disagreement may lie in the duration and conditions of testing procedures, sample size, chance selection of a given population, or some combination of these factors. In view of the diversity within the genus Arthrobacter, all four observations including our present study could very well represent some subgroups within the larger spectrum of all marine Arthrobacter species. Jones' (1975) finding that 90% (54/60) of assorted Arthrobacter species from various sources utilized inorganic nitrogen may more closely reflect our present status of knowledge on this genus. At any rate, the independence from organic nitrogen sources for growth affords these microorganisms a greater capacity to grow and survive in a wider range of environmental conditions.

In spite of their ability to utilize inorganic nitrogen, 83% (33/40)

Table 4. Utilization of inorganic nitrogen by Arthrobacter.

Source	Number	Pigmentation	Nitrogen	Growth (%)	Reference
Marine	10	White	$-NO_3$	4 (40)	Present study
"	3	Pink	"	2 (66)	"
"	3	Orange	"	1 (33)	"
"	24	Yellow	"	18 (75)	"
"	40	All	"	25[1] (63)	"
"	40	All	$-NH_4$	40 (100)	"
Marine & Soil	30		Inorganic	30 (100)	Bousfield, 1972
Seafish	5	Orange	$-NH_4$	0 (0)	Mulder, 1966
"	16	Orange	$-NH_4$ or NO_3	2 (13)	Crombach, 1974
Soil	112		NH_4NO_3	116 (95)	Mulder, 1963
"	55		$-NH_4$	50 (90)	Owens, 1969
"	55		$-NO_3$	44 (80)	Owens, 1969
"	177		$-NO_3$	158 (90)	Hagedorn, 1975
Sludge	15		NH_4NO_3	15 (100)	Mulder, 1963
"	71		NH_4NO_3	71 (100)	Adamse, 1970
Poultry Litter	27		$-NH_4$	3 (11)	Antheunisse, 1975
Cheese	46		NH_4NO_3	24 (52)	Mulder, 1963
"	51		$-NH_4$	17 (33)	Mulder, 1966
"	27	Orange	$-NH_4$	2 (7)	Mulder, 1966
Assorted	60		$-NH_4$	47 (78)	Jones, 1975
"	60		$-NO_3$	54 (90)	Jones, 1975

[1]Five day incubation; does not include ± growth responses (see Table 3).

of the isolates tested demonstrated a preference for peptides and/or amino acids as nitrogen sources (Table 3). Growth was more rapid and more luxuriant on media containing tryptone, peptone, or vitamin-free casamino acids than that on media where the organic nitrogen compounds were replaced with $(NH_4)_2SO_4$, $NaNO_3$, or NH_4NO_3. Most (4/7) of the isolates that did not show improved growth responses on organic nitrogen media were from the non-pigmented (white) group with one pink and two yellow isolates sharing this characteristic. Only one of these seven strains was positive for nitrate reduction in Nitrate-Motility Medium, whereas 61% (20/33) of the strains which showed a pronounced preference for organic nitrogen sources were nitrate reductase-positive. Jones (1975) found 90% (54/60) of Arthrobacter species isolated from various sources able to utilize nitrate as a sole nitrogen source, but only 30% (18/60) tested positive for nitrate reduction. Based on the present data and that of Jones, the ability to use nitrate as a nitrogen source does not guarantee that it will be used equally or preferentially in the presence of amino acids by members of this genus. The fact that although all of our isolates could utilize inorganic nitrogen the majority (83%) grew more rapidly and luxuriantly on media providing organic nitrogen, perhaps reflected their preference for amino acids as energy sources. All 40 isolates grew well on glucose-free media that contained peptides. In low-nitrogen media, 62% (18/29) of our cultures grew on the nitrogen-free mineral salts plus glucose medium (ZN + No N; Table 3). Little can be said of nitrogen preference based on these experimental findings other than that the strains involved were evidently capable of growth in very dilute sources of nutrients,

such as are found in seawater (Zobell, 1962). All 18 strains also grew on agar alone.

Growth Factors

None of the 40 isolates tested showed a requirement for growth factors. Our findings did not agree with those reported previously for Arthrobacter species (Table 5). Fifty-three percent (21/40) of the strains tested, however, grew better on yeast extract plus glucose medium (Z66C) than on a mineral salts plus glucose plus vitamin-free casaminoacids medium (ZN + VFCAA). This growth stimulation might still have been due to vitamins plus other growth factors present. Nevertheless, their absence did not prevent growth. The lack of growth factor (vitamins, amino acids, etc.) requirements could be an added survival attribute for Arthrobacter in an environment such as the ocean wherein nutrient levels can vary and generally are present in low concentrations (Zobell, 1962).

The ability to grow in a minimal nutritional environment may not be an advantage for growth of this group of bacteria in seafoods. In fresh or spoiling seafoods concentrated nutrients are available and the capacity to manufacture all needed vitamins and growth factors from basal substrates would be a redundant capability. This may help to explain why Arthrobacter is not recognized as a significant spoiler of seafood (Vanderzant, 1970; Shewan, 1971; Lee, 1975, 1977).

Table 5. Vitamin requirements of Arthrobacter species from various sources.

Source	Number	No Requirement (%)	Vitamins Required (%)	Specific Requirements (%)						Reference
				Biotin	Thiomine	Bi Thi	Bi Thi B_{12}	Thi B_{12}	Other Factors	
Marine	40	40 (100)	0 (0)							Present study
Seafish[1]	5	0 (0)	5 (100)							Mulder, 1966
Soil	112	43 (38)	69 (62)	51 (46)					18 (16)	Mulder, 1963
Soil	67	9 (13)	58 (87)	17 (25)	6 (9)	18 (27)	3 (4)	7 (10)	7 (10)	Keddie, 1966
Sludge	10	1 (10)	9 (90)							Mulder, 1963
Sludge	71	32 (45)	39 (55)							Adamse, 1970
Cheese[1]	46	24 (52)	22 (48)	3 (7)					19 (41)	Mulder, 1963
Cheese[1]	51	24 (47)	27 (53)							Mulder, 1966
Cheese[2]	27	4 (15)	23 (85)	23 (85)						Mulder, 1966

[1]Non-pigmented isolates.

[2]Orange-pigmented isolates.

Carbon/Energy Sources and Carbohydrate Utilization

Early in the investigation various sugars were tested for acid production; results were generally negative, with only 8% (3/40) producing acid from glucose; all isolates grew on the Hugh-Leifson media containing fructose, rhamnose, galactose and lactose but no acid production was observed (Table 6). That glucose was utilized by the majority of these isolates was evidenced by the variation in growth response on a mineral salts medium (Z66A) with and without glucose; of the 34 isolates growing after seven days at 25°C, 97% grew better in the presence of glucose rather than in its absence. Because no acid was produced from carbohydrates by the vast majority of the 40 isolates tested, and their preference for amino acids as energy plus nitrogen sources, attempts to demonstrate utilization of fructose, rhamnose, galactose, mannitol, cellobiose, sucrose, and lactose were inconclusive. Xylose was somewhat inhibitory to 30% (12/40) of the strains tested.

None of the isolates hydrolyzed cellulose in the form of filter paper strips, in agreement with the accepted description of the genus (Bergey's Manual, 1974).

Sixty percent (24/40) of the isolates tested hydrolyzed starch, including all three orange-pigmented isolates (Table 7). This is in contrast to Crombach's (1974) finding that none of his 16 orange-pigmented isolates from seafish could degrade starch, but in agreement with Norkrans' (1978) report that 60% of 44 psychrotrophs of undetermined species from deep sea sediments could hydrolyze starch. Vanderzant (1975) also found starch hydrolysis to be less widespread among 66 isolates from earthen ponds and pond shrimp, indicating 29 to

Table 6. Growth characteristics of marine Arthrobacter isolates.

	2°C (TPN)	37°C (TPN)	pH 5.0	pH 6.3	pH 7, 8.5	pH 9.5	pH 10.5	No Salt	1% NaCl	4% NaCl	10% NaCl	H-L O[1]
White												
1	+	+	-	+	+	+	-	+++	+++	-	-	-
2	+	-	-	+	+	-	-	++	+++	++	+	-
15	+	-	-	-	+	+	-	++++	+++	++	-	-
16	+	-	-	+	+	-	-	+++	+++	+++	+	±
*20	+	-	-	+	+	-	-	+	++	-	+	-
24	+	±	-	+	+	+	+	+++	+++	++	-	-
31	+	±	-	+	+	+	+	+++	+++	++	-	-
35	+	+	-	+	+	+	+	ND[2]	++	++	-	+
38	+	+	-	+	+	+	+	++	++	+½	-	-
39	+	+	-	+	+	+	+	++	++	1½+	-	-
Orange												
4	+	-	-	+	+	-	-	+++	ND	++	+	-
* 5	+	-	-	+	+	-	-	++	++	+	+	-
6	+	-	-	+	+	+	-	++	++	1½	+	-
Pink												
8	+	+	+	+	+	+	+	++	+++	+++	+	+
25	+	+	+	+	+	+	-	++++	+++	++	-	-
*34	+	+	+	+	+	+	-	+++	+++	++	-	-
Yellow												
3	+	-	-	+	+	+	-	+++	+++	+++	+	-
7	+	-	+	+	+	+	-	++	+++	++	+	-
9	+	-	-	+	+	+	-	+++	+++	2½+	+	-
10	+	-	-	+	+	+	-	+++	+++	2½+	+	-
*11	+	-	-	+	+	+	-	+++	+++	+++	+	-
12	+	-	-	+	+	+	-	+++	+++	2½+	+	-
13	+	-	-	+	+	+	-	+++	+++	2½+	+	-
14	+	-	-	+	+	+	-	+++	+++	+++	-	-
17	+	-	-	+	+	+	-	+++	+++	+++	+	-
19	+	-	-	+	+	+	-	+++	+++	++	+	-
21	+	-	-	+	+	+	-	+++	+++	+++	-	-
22	+	-	-	+	+	+	-	+++	+++	+	+	-
23	+	-	-	+	+	-	-	+	+	-	-	±
26	+	-	-	+	+	+	-	ND	+++	++	-	-
27	+	-	-	+	+	+	-	++	+++	++	-	-
28	+	+	-	+	+	+	+	+++	+++	++	-	-
29	+	+	+	+	+	+	+	+++	+++	++	-	-
30	+	+	-	+	+	+	+	+++	+++	++	-	±
32	+	-	-	+	+	+	-	+++	++	+	-	-
33	+	+	-	+	+	+	+	+++	++	++	-	-
36	+	+	-	+	+	+	+	+++	++	+	-	-
37	+	±	-	+	+	+	-	++	++	+	-	+

Table 6. Continued.

40	+	+	-	+	+	+	-	+++	+++	++	-	-
41	+	ND	-	+	+	+	-	+++	+++	++	-	-

[1]Hugh-Leifson oxidative

[2]No data

*Representative strain used for CHO storage, temperature optimum, disinfectant resistance, etc., tests.

Table 7. Starch hydrolysis by Arthrobacter species.

Source	Number	Pigmentation	Number Positive (%)	Reference
Marine	10	White	2 (20)	Present study
"	3	Pink	1 (33)	"
"	3	Orange	3 (100)	"
"	24	Yellow	18 (75)	"
"	40	All	24 (60)	"
Seafish	16	Orange	0 (0)	Crombach, 1974
Pond	66		19-26 (29-39)	Vanderzant, 1975
Soil	112		55 (49)	Mulder, 1963
"	12		7 (58)	Crombach, 1974
Sludge	10		8 (80)	Mulder, 1963
"	71		63 (89)	Adamse, 1970
Poultry litter	27		26 (96)	Antheunisse, 1975
Cheese	46		10 (22)	Mulder, 1963
"	13	White	2 (15)	Crombach, 1974
"	8	Orange	0 (0)	Crombach, 1974
Various	60		31 (52)	Jones, 1975)

39% able to hydrolyze starch. It was also noteworthy that our yellow- and orange-pigmented isolates of both Atlantic and Pacific origin hydrolyzed starch more readily than Vanderzant's (1975) Texas Gulf Coast pond isolates, with 75% of the yellow and all of the orange isolates positive for starch hydrolysis. When compared to Arthrobacter species of terrestrial origin, our marine isolates demonstrated this ability with a frequency comparable to published reports of soil isolates, and somewhat less frequently than Arthrobacter species isolated from sludge or poultry litter. Mulder (1963) and Crombach (1974) found 49 and 58%, respectively, of their soil isolates able to digest starch. Mulder (1963) and Adamse (1970) found 80 and 89%, respectively, of their isolates from activated sludge to have this capability as compared to 96% of Antheunisse's (1975) strains from poultry litter. If Jones' (1975) finding that 52% of 60 Arthrobacter species from various origins were able to hydrolyze starch were assumed to reflect the tendencies of the genus as a whole, the present data would indicate that our marine Arthrobacter isolates are about average in this regard.

Proteolytic Activity

Seventy-two percent (28/39) of the isolates tested were able to hydrolyze gelatine and 5% (2/39) were also able to hydrolyze casein. These proportions are somewhat lower than those found by Crombach (1974) in tests of 16 orange coryneforms from sea fish (Table 8). He found 88% and 44% able to hydrolyze gelatine and casein, respectively. Compared to the published reports, the proportion of proteolytic isolates from our present study are slightly higher (72% versus an overall

Table 8. Proteolytic activity of Arthrobacter species isolated from various sources.

Source	Number	Pigmentation	Number Gelatine Hydrolysis (%)	Number Casein Hydrolysis (%)	Reference
Marine	9	White	3 (33)	0 (0)	Present study
"	3	Pink	1 (33)	1 (33)	"
"	3	Orange	2 (66)	0 (0)	"
"	24	Yellow	22 (92)	1 (4)	"
"	39	All	28 (72)	2 (5)	"
Seafish	16	Orange	14 (88)	7 (44)	Crombach, 1974
Soil	112		73 (65)		Mulder, 1963
"	11		11 (100)	11 (100)	Mulder, 1966
"	12		12 (100)	12 (100)	Crombach, 1974
Sludge	10		9 (90)		Mulder, 1963
"	71		65 (92)		Adamse, 1970
Cheese	46		15 (33)		Mulder, 1963
"	51	White	11 (22)	11 (22)	Mulder, 1966
"	29	Orange	27 (93)	1 (3)	Mulder, 1966
"	8	Orange	8 (100)	0 (0)	Crombach, 1974
"	13	White	3 (23)	2 (15)	Crombach, 1974
Various	60		39 (65)		Jones, 1975

average of 66%). The inability to degrade casein, a more complete protein than gelatine, probably implies that proteolysis is not one of the major functions or activities of marine Arthrobacter. This is in agreement with Norkrans' (1978) finding that although approximately one-half (48%) of his 44 psychrotrophs of undetermined species from deep marine sediments could hydrolyze gelatine, only 20% could hydrolyze casein. All of our Arthrobacter strains grew well on media containing tryptic or peptic digests of casein. However, 23% (9/40) did not show an equivalent preference for tryptone as they did for peptone in their growth responses, implying that the peptides thus produced are not equally metabolized (Table 3). Proteolysis was more pronounced among the orange- and yellow-pigmented isolates than in the pink- and non-pigmented groups, and was found in 66, 92, 33, and 33% of these isolates, respectively. When regrouped according to geographical origin, the Pacific isolates were similar in proteolytic ability to those from the Atlantic Coast, with 75 and 68% demonstrating proteolysis, respectively.

Lipolytic Activity

Table 9 lists the lipolytic activities of Arthrobacters from several sources. With regards to hydrolysis of Tween-80 (polyoxyethylene sorbitan monooleate) the results of the present study confirm those of Mulder (1963) and Adamse (1970) but are in contrast to the findings of Jones (1975). Her indicated incidence of 75% of 60 isolates able to degrade Tween-80 is several times greater than the 5% (2/39) found in the present study. However, when tested for ability to digest tributyrin suspended in agar, nearly all (92%) of our isolates

Table 9. Lipolytic activity of Arthrobacter species isolated from various sources.

Source	Number	Pigmentation	Number Lipolytic (%) Tween-80	Other Lipids	Reference
Marine	9	White	2 (22)	8 (89) (tributyrin)	Present study
"	3	Pink	0 (0)	3 (100) "	"
"	3	Orange	0 (0)	2 (67) "	"
"	24	Yellow	0 (0)	23 (96) "	"
"	39	All	2 (5)	36 (92) "	"
Soil	95		10 (11)		Mulder, 1963
Sludge	10		0 (0)		Mulder, 1963
"	71		6 (8)		Adamse, 1970
Cheese	46		3 (7)		Mulder, 1963
Various	60		45 (75)	50 (83)[1]	Jones, 1975
"	60			24 (40)[2]	Jones, 1975

[1]Tween-20

[2]Egg yolk

produced a positive reaction. Jones also found an increased incidence of lipolysis when smaller molecular weight lipid was used (Table 9). The reason for the difference in lipolytic activities of our isolates when tested against Tween-80 versus tributyrin could as easily be due to the method of analysis as in an inability to degrade the larger Tween-80 molecule. Because of its miscible nature in aqueous systems, detection of Tween-80 hydrolysis is dependent upon the formation of an insoluble calcium salt resulting from the de-esterification of the oleic acid residues from the larger molecule. If, however, an enzyme capable of decarboxylating fatty acids is also present, the oleic acid may be metabolized as rapidly as it is de-esterified. With no available carboxyl group for form the calcium salt, no precipitate is formed and the results will be interpreted as negative. The ability to metabolize carboxylic acids by Arthrobacter is shown by Hagedorn (1974) who found that fatty acids, dicarboxylic acids and organic acids were readily utilized by 59, 89, and 87%, respectively, of 160 soil isolates. In testing for lipolysis of other fats such as tributyrin or egg yolk, which are less soluble in aqueous systems, the evidence for hydrolysis is a change in the emulsion, evidenced by a zone of clearing. Thus, whether the end products of the hydrolysis are used rapidly or slowly, the reaction remains positive. In view of this, the isolates tested could be characterized as being lipolytic in nearly all cases. This is in agreement with the findings of Norkrans (1978), Morita (1975), and Quigley (1968), all of whom found lipolysis to be widespread among marine bacteria in general and in a greater incidence than proteolytic activity.

In terms of what effect lipolysis may have on seafood spoilage, Hanson (1964) found a definite correlation between the level of free fatty acids (FFAs) in frozen fish muscle and the rate at which the proteins denatured. In fresh seafood, however, Liston (1964) indicated that lipid deterioration brought about by microbial enzymes was of minor importance. In the native environment of sea mud, lipids are known to contribute about 1% of the organic carbon and thought to be an important substrate for the resident microbial community (Norkrans, 1978).

pH

As a group, the marine Arthrobacter species tested were more sensitive to acidic pH than to alkaline environment (Table 6). Ninety-eight percent (39/40) grew at pH 6.3 to 8.5 and 85% (34/40) grew at pH 9.5. Only 28% (11/40) were able to grow at pH 10.5, the highest pH tested, and only 13% (5/40), including all members of the pink pigment group, grew at pH 5.0, the lowest pH tested. No correlation between the oxidative metabolism of sugars (acidic reaction in Hugh-Leifson test) and ability to grow at low pH was found. Of the six cultures giving a + or ± Hugh-Leifson oxidative reaction, only one was capable of growth at pH 5.0.

Salt Tolerance

As Tables 6, 10, 11, and 12 show, increasing concentrations of salt, especially above 4% had a detrimental effect on the growth of the marine isolates studied. Low concentrations were stimulatory to at

Table 10. Salt tolerance of Arthrobacter species of various origins.

Source	Number	Pigmentation	Salt Levels Tested, %	Mean % NaCl Tolerated	Reference
Marine	40	All	1,4,8,10	6.4	Present study
"	4	All[1]	2 → 20	10.0	"
Seafish	16	Orange	3,5,8,10,12,15	12.2	Crombach, 1974
Soil	5		3,5,8	3.8	Mulder, 1966
"	19		5,10	4.7	Yamada, 1972
"	12		3,5,8	3.7	Crombach, 1974
Sludge	71		3,5,8,12	5.1	Adamse, 1970
Poultry litter	30		8 only	30% grew	Antheunisse, 1975
Cheese	3	White	5,8,12	8.0	Mulder, 1966
"	13	White	3,5,8,10,12	9.6	Crombach, 1974
"	8	Orange	5,8,12	10.5	Mulder, 1966
"	8	Orange	3,5,8,10,12	11.9	Crombach, 1974
Various	60		5,10	5.2	Jones, 1975

[1]Strains representing white, pink, orange and yellow pigment groups.

Table 11. Salt tolerance of marine Arthrobacter species on M-2[1] agar (pH 7, 25°C).

Pigmentation	Growth (%)			
	No NaCl	1% NaCl	4% NaCl	10% NaCl[2]
White	9/9 (100)	10/10 (100)	8/10 (80)	3/10 (30)
Pink	3/3 (100)	3/3 (100)	3/3 (100)	1/3 (33)
Orange	3/3 (100)	2/2 (100)	3/3 (100)	3/3 (100)
Yellow	23/23 (100)	24/24 (100)	23/24 (96)	10/24 (42)
All	38/38 (100)	39/39 (100)	37/40 (93)	17/40 (43)

[1]See Materials and Methods for contents of M-2 media.

[2]pH changed to approximately 6.5.

Table 12. Growth of representative Arthrobacter species in TPE5[1] broth in the presence of salt (pH 7.6).

	Generation Time (hours)					Incubation
Pigmentation	No NaCl	1% NaCl	4% NaCl	8% NaCl	12% NaCl	Temperature
White	3.1	0.9	1.4	2.3	5.8	26°C
Pink	4.5	5.3	8.6	>100	∞[2]	26-27°C
Orange	2.0	2.4	8.6	23	∞	27-28°C
Yellow	1.5	1.3	1.7	9.6	72	26°C

[1]See Materials and Methods for contents of TPE5.

[2]Growth too slow to calculate generation time.

least 13% (5/40), giving a better growth response than the basic media without salt. As a group the three orange strains were more tolerant to higher salt concentrations on solid media than the other three pigment groups. This would tend to support Shewan's (1971) contention that orange-pigmented marine isolates were closely related to the more salt-tolerant Arthrobacter linens (formerly Brevibacterium linens) group (Table 11). This group includes the orange-pigmented cheese isolates which Mulder (1966) found to have the highest salt tolerance. Crombach (1974) found his orange strains from seafish and those of like pigmentation from cheese tolerated an average of 12% salt, as compared to 10% salt by one of the representative orange cultures tested in the present study.

NaCl tolerance could not be increased by progressive acclimitization to higher concentrations of NaCl. One of the representative yellow isolates, strain 11, grew very slowly in 12% salt once but this could not be reproduced on repeated tries. All four of the representative isolates tolerated at least 8% NaCl in broth; on solid media 8% (3/40) had not grown after five days incubation when exposed to only 4% NaCl. The discrepancy between the results on solid versus liquid media might have been due to duration of incubation and cultural conditions. The broth cultures were grown on a shaker in flasks wherein nutrient exposure and gas exchange would be more favorable than on solid media. Over the five day incubation period the plates could have become progressively dessicated, thus raising the effective concentration of NaCl above that indicated by the original composition.

The growth responses of the four representative isolates to 0 to

12% NaCl showed that the optimum concentration of NaCl for growth was between 0.0 and 4.0% (Table 12). Perhaps not coincidentally, the optimum NaCl concentrations of these Arthrobacter species were near that found in seawater of 3.0 to 4.0% (Zobell, 1962). Their salt tolerance could afford them a selective advantage over less salt-tolerant organisms in a fish hold where dessication and subsequent brine concentration might take place.

Temperature

All 40 isolates were able to grow at 2, 10, 20, and 25°C and 33% (13/39) showed some growth at 37°C after two days incubation (Table 6). This is somewhat higher than the 22% (13/60) reported by Jones (1975) for Arthrobacter of various origins. In a group by group analysis, the pink-pigmented isolates showed the greatest tendency to grow at elevated temperatures; the representative pink strain also had the highest optimum temperature and greatest resistance to heat. Forty, 100, 0 and 16% of the white-, pink-, orange-, and yellow-pigmented isolates, respectively, were able to grow at 37°C. When grouped according to the geographic origin, the isolates from the Atlantic were noticeably more tolerant of the 37°C incubation than those from Pacific waters with 58 and 10%, respectively, showing growth at this temperature.

One of the procedures we employed for the identification of gram positive bacteria was to subject them to 65°C for 30 minutes (Lee, 1975). This was to detect sporeformers and Arthrobacter, by definition, would not have survived this treatment. None of our isolates showed any growth after this treatment.

If we assume that the natural habitat of marine Arthrobacter is the ocean bottom, they will be constantly subjected to the colder bottom waters, and temperatures below 5°C. If so, this group of bacteria should readily multiply at these temperatures but would not necessarily have an unusually low optimum temperature for growth. That is, they would have to be psychrotrophs but not necessarily psychrophiles (Norkrans, 1978; Quigley, 1968; Boeye, 1975). We tested the four representative strains for optimum temperature and found it to be 27.4, 31.4, 27.4, and 26.1 for white-, pink-, orange-, and yellow-pigmented isolates, respectively.

When we tested the four representative Arthrobacter strains for ability to grow at 2°C, none of our isolates grew any faster than the well-known seafood spoilage bacteria of Pseudomonas-Moraxella groups. The generation times in Table 13 show that Pseudomonas species averaged 7.8 hours, followed by 12 hours for Moraxella species. The average generation time of 22 hours for Arthrobacter species was twice as long as that for Moraxella and three times as long as that of Pseudomonas species. Therefore, when Arthrobacter predominates the microbial flora of seafood, it is not likely due to outgrowth at refrigeration temperatures. In order to determine their optimum growth temperatures these four representative Arthrobacter species were grown in a temperature gradient incubator between 2.8 and 41°C with increments averaging 2.6°C. Generation times of our present isolates at temperatures near 10, 25 and 30°C were noticeably longer than those studied by Lee and Pfeifer (1977). The mean generation time at near 10, 25 and 30°C was 17, 2.6 and 3.9 hours, respectively, in the present study, compared to 8, 1.4 and 0.9

Table 13. Generation times of selected marine microorganisms at 2°C in TSB.

Microorganism	Strain (Pigmentation)	Lag (h)	Generation Time (h)
Arthrobacter	20 (White)	1.3	13.8
"	34 (Pink	13	32
"	5 (Orange)	0	28
"	11 (Yellow)	6.5	16
Pseudomonas I	B-6-1-5	2.8	9.5
P. putrifacians	A-11-1-24	5.5	6.1
Moraxella[1]	A-44	7.5	16
Moraxella[2]	DD-2-4-6	4.5	8.3

[1]High-phenethyl-alcohol-producing strain

[2]Strain producing relatively little phenethyl alcohol

hours at 10, 25 and 30°C, respectively, for Arthrobacter species studied by Lee and Pfeifer (1977) (Table 14). The medium used in the present study was TPE5, a medium similar to, but about one-half as concentrated as TPE, the medium used by Lee and Pfeifer. Although the different media could have contributed to the different growth rates, the most likely explanation is that marine Arthrobacter species comprise a diverse group and such differences could be expected from different isolates. In any event, it appears that low temperature does not afford a competitive advantage to Arthrobacter species.

One aspect of adaptation to deleterious changes in its environment is for an organism to have a high resistance to heat; however, optimal growth temperature and heat resistance are two separate aspects of a microorganism's relationship to its environment. In nearly all species, the growth rate declines very rapidly after the optimum has been exceeded; the relationship of super-optimal temperatures to the rate of cell death is a characteristic that varies from species to species (and sometimes from strain to strain within a given species) and is related to the nature of an organism's enzyme systems, cell wall, membrane structure, etc. (Stanier, 1970). The heat resistance of our isolates were measured by the time it took to inactivate 90% of the viable cells at 50°C or the D-value at 50°C (D_{50}).

The Arthrobacter species tested varied in their response to 50°C heat treatment and demonstrated no apparent correlation between heat resistance (D_{50} value) and generation time or speed of repair of sub-lethal injury. Generation times for the white-, pink-, orange-, and yellow-pigmented isolates (strains 20, 34, 5 and 11) averaged 1.3, 2.7,

Table 14. Generation times of selected marine microorganisms at temperatures near 10, 25 and 30°C.

Microorganism	10°C Temp (°C)	10°C Gen. Time (hr)	25°C Temp (°C)	25°C Gen. Time (hr)	30°C Temp (°C)	30°C Gen. Time (hr)	Opt. Temp	Reference
Arthrobacter sp. (White)	8.5	8.2	26	2.0	30.5	6.5	27.4	Present study
Arthrobacter sp. (Pink)	8.5	25	26	3.1	30.4	2.7	31.4	"
Arthrobacter sp. (Orange)	10	13	25	3.3	29.1	3.8	27.4	"
Arthrobacter sp. (Yellow)	10.1	22	25	1.8	29.4	2.6	26.1	"
Arthrobacter sp. ($\bar{X}$ above)	9.3	17	25.5	2.6	29.9	3.9	28.1	"
Arthrobacter	10	8.0	25	1.4	30	0.9		Lee, 1977
Pseudomonas	10	3.9	25	1.2	30	1.0		"
Moraxella	10	4.2	25	1.3	30	1.2		"
Flavobacterium-Cytophaga	10	8.0	25	1.7	30	1.6		"
Acinetobacter	10	8.1	25	1.5	30	1.2		"
Vibrio parahaemolyticus	10	13.5	25	0.8	30	0.1		"

2.5, and 1.4 hours, respectively (Table 15). Strains 20 and 11 had very similar generation times but showed an eight-fold (1.2 versus 9.6 minutes) difference in D_{50} values. Likewise, strains 5 and 34 had very similar generation times (2.5 and 2.7 hours) but D_{50} values showed a seven-fold difference (14 versus 100 minutes).

Since strain 34 had a very rapid repair period (short lag time), it is tempting to assume that lag period is inversely related to heat resistance; that is, the more easily cells are inactivated, the longer should be the time to repair the sublethal injuries as well, and vice versa. When the lag times of these four isolates were compared (Table 15), this was not the case. After five minutes exposure to 50°C, the most severe heat treatment, the lag times of the most heat-resistant (strain 34, D_{50} = 100 minutes) and heat-sensitive (strain 20, D_{50} = 1.2 minutes) strains were 1.4 and 14 hours, respectively. Strain 11, with a lag time of 13 hours should have shown a D_{50} value close to that of strain 20 (lag time 14 hours); instead, strain 11's D_{50} was eight times greater, as mentioned previously. Likewise, strain 5 with a lag time of 31 hours, should have been much more sensitive to heat than strain 20, but in fact strain 5's D_{50} was approximately 12 times greater (14 minutes). Apparently, different injuries and/or systems of repair have occurred in the different strains of Arthrobacter. This again points to the great diversity within the genus.

In Lee's (1977) study of changes in microbial flora during shrimp processing he noted that the heat resistance of bacterial species commonly isolated from shrimp ranged from a D_{52} of 0.4 to 8.0 minutes. The D_{52} value of Arthrobacter species was 6.3 which was the second most

Table 15. Growth response of representative *Arthrobacter* strains in TPE (26°C) after 50°C heat treatment.

Strain	Pigmentation	Exposure Time (min)	Lag Time[1] (hr)	Generation Time (hr)
20	White	0	0	1.1
		1	4.6	1.2
		3	9.3	1.3
		5	14	1.4
34	Pink	0	0	2.6
		1	0.2	2.7
		3	0.7	2.7
		5	1.4	2.7
5	Orange	0	0	2.2
		1	6.4	3.0
		3	18	2.5
		5	31	2.4
11	Yellow	0	0	1.3
		1	5.1	1.3
		3	10	1.4
		5	13	1.4

[1]Corrected for optical lag due to population reduction as a result of heat exposure.

heat resistant group. The representative isolates of the present study were similarly tested and their heat resistance found to be variable. Within the four isolates the measured D_{50} values ranged from 1.2 to greater than 100 minutes, representing a difference of approximately 80-fold (Table 16). This again may reflect the great heterogeneity known to exist within this genus. Even if isolate 34, the pink-pigmented strain with the greatest heat resistance, were removed from our discussion, the remaining three isolates tested still yielded an average D_{50} value of 8.2 minutes. Arthrobacter species, therefore, appear to be more heat-resistant than most other typical seafood isolates. Steaming or blanching is a part of crab and shrimp processing. Arthrobacter species would survive these treatments better than other marine bacteria and may conceivably become a predominant group in such seafoods. However, other factors such as secondary contamination may play a role and negate the heat-selection. In Lee's (1975) study of Dungeness crab microflora, five of the seven genera listed by heat resistance in Table 16 responded to the heat selection according to their relative resistance to heat. Two genera, Moraxella and Acinetobacter, however, did not react as one would expect with Moraxella increasing in importance after the cooking step and Acinetobacter, the genus with the greatest heat resistance of those tested, assuming a position of lesser importance after cooking (Lee, 1975). A similar occurrance was noted in the monitoring of shrimp processing operations in one of two processing plants; the relatively heat-sensitive Flavobacterium-Cytophaga increased from 13% to 18% of total counts as a result of the blanching step (Lee, 1977). In the other shrimp plant, however, the microbial selection

Table 16. Heat inactivation kinetics of selected microorganisms.

Microorganism	Temp (°C)	D-value	Reference
Arthrobacter (White)	50	1.2	Present study
Arthrobacter (Pink)	50	100	"
Arthrobacter (Orange)	50	14	"
Arthrobacter (Yellow)	50	9.6	"
A. globiformis (Rods)	45	8.0	Mulder, 1963
A. globiformis (Cocci)	45	17	"
A. globiformis (Rods)	50	<2	"
A. globiformis (Cocci)	50	4.7	"
Arthrobacter	52	6.3	Lee, 1977
Acinetobacter	52	8.0	"
Micrococcus	52	6.3	"
Staphylococcus	52	1.5	"
Pseudomonas	52	1.2	"
Moraxella	52	0.7	"
Vibrio parahaemolyticus	52	0.7	"
Flavobacterium-Cytophaga	52	0.4	"

followed that predicted from Table 16.

Whether the heat resistance of Arthrobacter could play a significant role for its selection during seafood processing or not is questionable. With the exception of crab and shrimp, the processing of most fresh seafood does not include a heating step. The overall effect of all processing steps in the abovementioned crab and shrimp studies on Arthrobacter predominance was that of marked increase (3% to 8%), marked decrease (17% to 6%) and virtually no change (12% to 14%), respectively. Heat resistance thus is demonstrated by the present study, in support of Lee's (1977) findings but cannot be relied upon solely to account for Arthrobacter's predominance in fresh-caught seafood. What advantage it afforded these isolates in the environment from which they have originated could not be ascertained from the available data. It may be of some advantage to Arthrobacter species found in processed seafood such as shrimp, crab, or cooked or frozen seafood, but this advantage, if it exists, is a temporary one; as has been previously shown, Arthrobacter species continue to decrease in proportion as storage time increases.

Storage Carbohydrate

The four representative Arthrobacter strains were tested for the presence of storage carbohydrate after five days growth in a medium with a high carbon:nitrogen ratio and were found to contain an average of 63% CHO (calculated as glucose) on a dry weight basis. This is comparable to the report of Zevenhuizen (1966) that his Arthrobacter isolates from soil formed 50 to 70% storage CHO. Mulder (1966) found

an average of 53, 18 and 14% storage CHO in Arthrobacter species isolated from soil and cheese (non-pigmented and orange-pigmented), respectively. Adamse (1970) found his Arthrobacter species isolated from sludge to be similar to those from soil in this respect, forming an average of 51% storage CHO (Table 17). Our marine Arthrobacter isolates appear to be more closely related to soil and sludge isolates in this respect than to Arthrobacter isolated from cheese. Based on the amount of storage CHO present, these marine isolates could survive starvation at least as well as those of soil origins at a given temperature. Taking into account the lower temperature of the marine environment and the probability that metabolic processes proceed more slowly at these temperatures, these marine Arthrobacter species should have a starvation survival potential even greater than the 12 years estimated by Boylen (1973) for soil forms.

Growth in Low-Nutrient Environments

During the course of this investigation, it was found that the majority (78%) of the isolates tested could grow slowly on plain agar, without any added nutrients. No evidence of agar digestion was seen, however. To further examine this unusual ability, the isolates were inoculated on Difco Special Agar-Noble prepared in acid-washed glassware. This medium supported the growth of at least 45% (18/40) of the isolates (Table 18).

The cells of four representative Arthrobacter isolates plus two species each of two typical marine bacteria (Moraxella and Pseudomonas) for comparison, were then inoculated into broth which contained 0.3,

Table 17. Storage carbohydrate of *Arthrobacter* species.

Source	% CHO (dry wt)	Reference
Marine[1]	63	Present study
Soil	50-70	Zevenhuizen, 1966
Soil	53	Mulder, 1966
Sludge	51	Adamse, 1970
Cheese[2]	18	Mulder, 1966
Cheese[3]	14	Mulder, 1966

[1]Four isolates, three determinations

[2]Non-pigmented strains

[3]Orange-pigmented strains

Table 18. Growth[1] of marine Arthrobacter species on "nutrient-free" agar.

Pigmentation	Number	Number Grown on Bacto$_R$ Agar (%)	Number Grown on Noble$_R$ Agar[2] (%)
White	10	7 (70)	2 (20)
Pink	3	2 (67)	2 (67)
Orange	2	3 (100)	3 (100)[3]
Yellow	24	19 (79)	14 (58)
All	40	31 (78)	21 (53)

[1]± growth responses not shown, with exception of 3 below.

[2]Prepared in acid-washed glassware.

[3]All growth responses ±

0.1 and 0.01 strength trypticase soy broth (TSB) in distilled water. After incubation for five days, all tubes showed turbidity, but it appeared to be greater in those tubes inoculated with Arthrobacter isolates than in those tubes containing Moraxella or Pseudomonas species. To confirm and quantitate this, these same eight cultures were again grown in 0.01 strength TSB, but this time in side arm flasks so that growth could be monitored by a spectrophotometer. The growth data from this experiment are presented in Table 19 and the growth curves plotted are shown in Figure 1. As expected, the rapidly growing Pseudonomas species had the lowest generation times; the Arthrobacter, however, reached the highest average optical density, approximately 20% greater than that of the Pseudomonas species. This appears to further confirm our earlier suspicions that Arthrobacter species are capable of concentrating nutrients from a dilute menstrum. Such ability has an obvious advantage in the marine environment where the levels of available organic matter in the water column are often less than 0.01% and usually less than 1% in bottom sediment (Zobell, 1962; Morita, 1955; Boeye, 1975; Norkrans, 1978). Such ability, combined with the ability to store large amounts of carbohydrate reserves, gives Arthrobacter a means to persist in an environment of sea mud or water column, in spite of its relatively slow growth rate.

Resistance to Antimicrobial Agents

Potassium Sorbate

Chung (1979) tested a number of marine isolates from several different genera for resistance to potassium sorbate. She found that

Table 19. Growth of some marine bacteria in 0.01 strength trypticase soy broth at 26°C.

Species	Strain	Pigmentation	Minimum Generation Time (hr)	Maximum Absorbance at 580 nm
Arthrobacter	20	White	4.8	049
Arthrobacter	34	Pink	4.5	121
Arthrobacter	5	Orange	6.3	095
Arthrobacter	11	Yellow	3.0	185
Pseudomonas I	B-6-1-5		1.1	076
P. putrafacians	A-11-1-24		1.1	112
Moraxella	A-44		12	046
Moraxella	DD-2-4-6		15	042

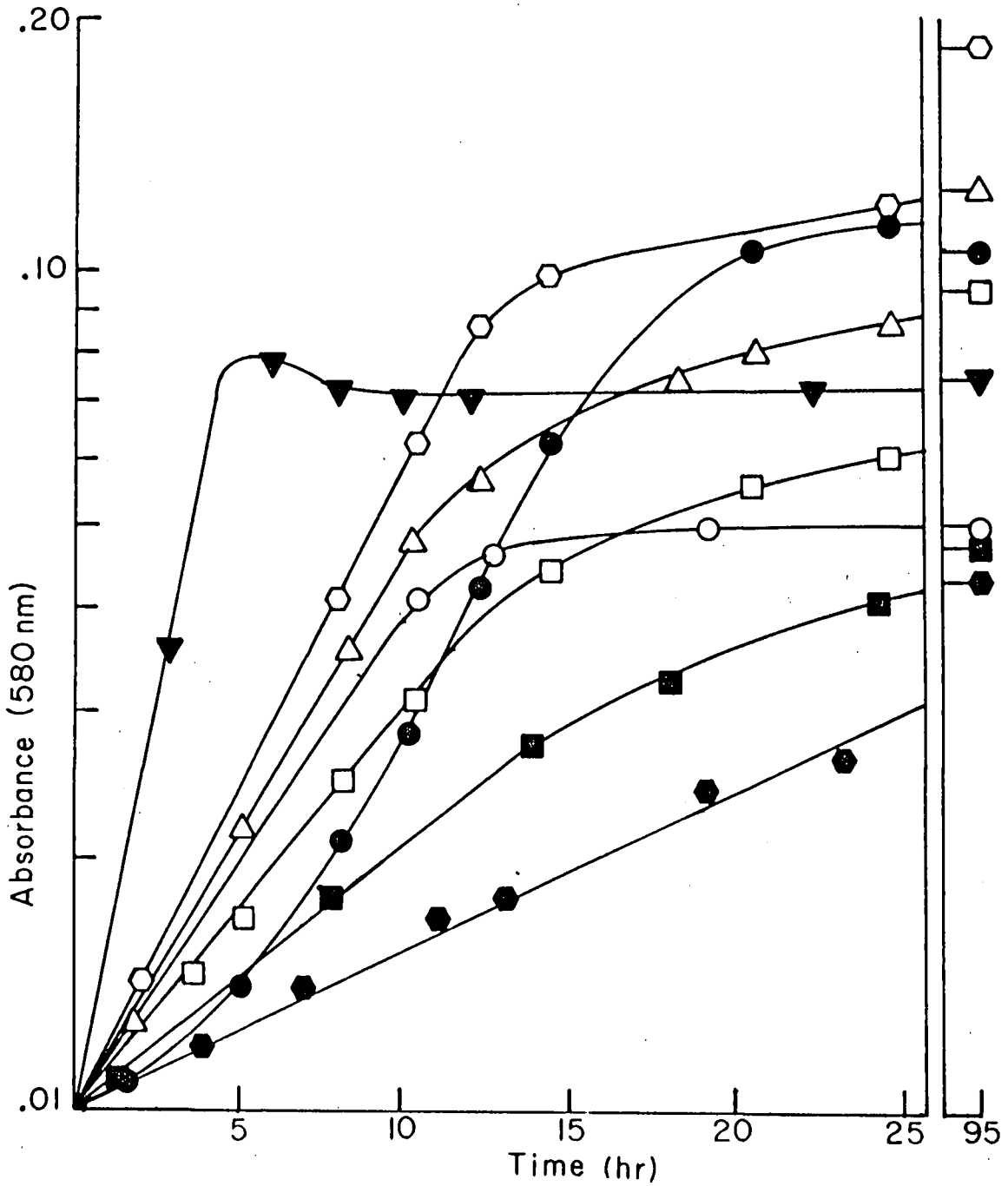

Figure 1. Growth of representative Arthrobacter, Pseudomonas and Moraxella species of marine origin in 0.01 strength TSB at 26°C.

⬡ Arthrobacter strain 11; △ Arthrobacter strain 34; □ Arthrobacter strain 5; ○ Arthrobacter strain 20; ▼ Pseudomonas I strain B-6-1-5; ● Pseudomonas putrafacians strain A-11-1-24; ■ Moraxella strain A-44; ⬢ Moraxella strain DD-2-4-6.

growth of Arthrobacter was influenced by potassium sorbate in concentration as low as 0.08% but a complete inhibition of growth required an average of 2.7% of potassium sorbate. Based on this finding and the published reports of Arthrobacter species' ability to degrade unusual and noxious compounds, it was postulated that Arthrobacter might possess an unusually high resistance to such antibacterial agents as disinfectants and antibiotics. Four isolates, representative of the four pigment groups, were tested first for their sorbate resistance to see if Chung's isolates were similar to those of the present study. The results of this test are shown in Table 20. The present isolates required an average of 0.88% potassium sorbate to cause a noticeable inhibition and were totally inhibited by a sorbate concentration of 2.30%. One isolate, strain 5, grew in the presence of 3.0% sorbate. This confirms Chung's (1979) finding that Arthrobacter were approximately twice as resistant to potassium sorbate as Pseudomonas and Acinetobacter species, and about four times as resistant as Moraxella species. Thus, only Staphylococcus species were more resistant to sorbate than Arthrobacter species (Table 20).

Quaternary Ammonium Compound (QAC) and Iodophor Disinfectants

To determine whether Arthrobacter species are as resistant to the common disinfectants as they were to potassium sorbate these same four representative isolates (strains 20, 34, 5, and 11) were tested against QAC and iodophor along with two strains each of Pseudomonas and Moraxella. The Pseudomonas strains included one of high sorbate resistance (B-6-1-5) and one of low sorbate resistance (A-11-1-24); likewise, the

Table 20. Effect of potassium sorbate on selected marine microorganisms in TPE broth at 25°C.

Microorganism	Number of Isolates	$\bar{X}$ Minimal Inhibitory[1] Concentration (%)	$\bar{X}$ Maximum Inhibitory[2] Concentration (%)	Reference
Arthrobacter sp. (White)	1	0.2	1.2	Present study
Arthrobacter sp. (Pink)	1	0.6	2.8	"
Arthrobacter sp. (Orange)	1	0.9	>3.0	"
Arthrobacter sp. (Yellow)	1	1.8	2.2	"
Arthrobacter sp.	4	0.88	>2.30	"
Arthrobacter	6	0.08	2.73	Chung, 1979
Pseudomonas I	6	0.35	1.62	"
Pseudomonas II	6	0.20	1.37	"
Pseudomonas III	6	0.27	1.48	"
Acinetobacter	6	0.15	1.18	"
Flavobacterium-Cytophaga	6	0.05	0.60	"
Yeast	2	0.08	0.70	"
Staphylococcus	2	0.50	>3.0	"

[1]Lowest concentration showing observable difference from control.

[2]Lowest concentration showing complete inhibition of growth.

Moraxella strains included one of high phenethyl alcohol (PEA) resistance (A-44) and one of low PEA resistance (DD-2-4-6). Arthrobacter species were not particularly resistant to QAC and were completely inactivated by a five-minute exposure to four to eight ppm of the QAC tested (Table 21). This degree of sensitivity was approximately the same as that exhibited by Moraxella species and noticeably less than that of the Pseudomonas species tested, one of which survived a 20 ppm exposure, the highest level tested. The latter finding was not unexpected since QAC is known to be relatively ineffective against Pseudomonas species; the former results show that Arthrobacter species possess no significant resistance to QAC and can easily be inactivated by exposure to QAC.

When exposed to iodophor, Arthrobacter species again showed no significantly greater resistance and were completely inhibited by an exposure to an average of 42 ppm, compared to 41 ppm for Moraxella species. Pseudomonas species survived exposure to 50 ppm, the highest concentration of iodophor tested (Table 22). Iodophor is a non-selective disinfectant whereas QAC is selective. QAC is more effective against gram-positive bacteria than against gram-negative species (Lee, 1973). Our finding that Arthrobacter species have a low resistance to both QAC and iodophor suggests that Arthrobacter species probably have a low resistance to disinfectants of all types. Iodophors are frequently used as sanitizers in Pacific shrimp and crab boats as well as in seafood processing plants. Based on our results, Arthrobacter species could not survive exposure to iodophors to any greater extent. This eliminates the possibility that the Arthrobacter species predominate

Table 21. Quaternary Ammonium Compound (QAC) resistance of some marine bacteria.[1]

Species	Strain	Pigmentation	"No Survival" Exposure Level (ppm)
Arthrobacter	20	White	6
Arthrobacter	34	Pink	4
Arthrobacter	5	Orange	6
Arthrobacter	11	Yellow	8
Pseudomonas I	B-6-1-5		>20
P. putrafacians	A-11-1-24		14
Moraxella	A-44		10
Moraxella	DD-2-4-6		4

[1]After exposure for five minutes as per A.O.A.C. 4.003, 1970.

Table 22. Iodophor resistance of some marine bacteria.[1]

Species	Strain	Pigmentation	"No Survival" Exposure Level (ppm)
Arthrobacter	20	White	38
Arthrobacter	34	Pink	42
Arthrobacter	5	Orange	42
Arthrobacter	11	Yellow	44
Pseudomonas I	B-6-1-5		>50
P. putrafacians	A-11-1-24		>50
Moraxella	A-44		42
Moraxella	DD-2-4-6		40

[1]After exposure of five minutes, as per A.O.A.C. 4.003, 1970.

seafood microbial flora as a result of their survival after disinfection and their presence in large proportion may not reflect an inadequate sanitization.

Although there was no apparent connection between the sorbate resistance of the Arthrobacter species tested and their resistance to either QAC or iodophor, the Pseudomonas and Moraxella species showing the higher resistance to sorbate and phenethyl alcohol (PEA), respectively, also showed the greater resistance to QAC (Table 21).

Resistance to Antibiotics

Most of the 40 Arthrobacter isolates tested were sensitive to most of the 12 antibiotics tested. This is in general agreement with the results reported by Jones (1975). Of the eight antibiotics common to the present study and that of Jones, in all cases a slightly higher percentage of isolates demonstrated resistance in the present study compared to the findings of Jones. None of her 60 isolates were resistant to chloramphenicol, streptomycin, tetracycline, chlortetracycline, oxytetracycline, neomycin, or kanamycin, but 17, 27, 5, 5, 25, 2, and 12% of our 40 Arthrobacter isolates demonstrated resistance to these compounds (Table 23). Jones did report some isolates resistant to penicillin G, a finding confirmed by our results; we found an average of 20% of Arthrobacter species resistant to penicillin G compared to her report of 13%. These minor differences aside, both studies indicated that Arthrobacter species have a low resistance to antibiotics. Out of 520 individual growth responses to 12 antibiotics in the present study, 72% (376) were negative; out of 1,320 individual growth responses to 22

Table 23. Antibiotic resistance of *Arthrobacter* species.

	Growth (%)	
Antibiotic	Marine[1]	Assorted[2]
Chloramphenical	7 (17)	0
Streptomycin	11 (27)	0
Ampicillin	3 (7)	NT[3]
Tetracycline	2 (5)	0
Chlortetracycline	2 (5)	0
Oxytetracycline	10 (25)	0
Neomycin	1 (2)	0
Nitrofurazone	37 (92)	NT
Nalidixic acid	36 (90)	NT
Sulfathiazole	20 (50)	NT
Kanamycin	5 (12)	0
Penicillin	8[4] (20)	8 (13)
Ledermycin	NT	0
Bacitracin	NT	0
Paromomycin	NT	0
Polymyxin	NT	1 (2)
Oleandomycin	NT	1 (2)
Novobiocin	NT	1 (2)
Erythromycin	NT	1 (2)
Spiramycin	NT	2 (3)
Vancomycin	NT	2 (3)
Penbritin	NT	3 (5)
Fucidin	NT	5 (8)
Colomycin	NT	15 (25)
Sulphonomide	NT	23 (38)
Celbenin	NT	23 (38)

[1]Forty isolates from present study. See Table for concentrations used.
[2]Sixty *Arthrobacter* from varied origins; Jones, 1975.
[3]Not tested.
[4]$\bar{X}$ of results for 3 IU and 75 IU/ml.

antibiotics in Jones' (1975) study, 94% (1,235) were also negative. Only nitrofurazone (Nf) and nalidixic acid (Na) appeared to exert no inhibitory effect on the majority of the isolates tested in our present study. Over 90% of the 40 isolates tested were resistant to Nf and Na but sensitive to ampicillin (Am), tetracycline (Tc), chlortetracycline (Ct), neomycin (Nm) and the higher concentration (75 IU/ml) of penicillin G [Pen(H)] (Table 24). This raises the possibility of developing an Arthrobacter-sensitive isolation scheme utilizing some combination of these antibiotics that would reduce the current heavy reliance upon cell morphology and cell wall analysis as criterion for differentiation. One-third or less of the test isolates were resistant to chloramphenicol (Cm), streptomycin (St), oxytetracycline (Ot), kanamycin (Ka) or 3 IU/ml of penicillin G [Pen(L)] (Table 25). Resistance to sulfathiazole (Su) was found in only half (20/40) of the test isolates, but present in all of those possessing pink or orange pigments.

With regards to patterns of resistance, 70% (28/40) conformed to seven resistance patterns with only three resistance patterns shared by three or more isolates (Table 26). The most common resistance pattern was found only among the yellow-pigmented group and consisted of resistance to only two antibiotics, Nf and Na; just under half (11/24) of the yellow isolates belonged to this group. The second most common resistance pattern was similar to the first but added resistance to Su as well; this pattern was shared by 15% (6/40) of the isolates tested and included representatives of the white-, orange-, and yellow-pigmented groups. The only other resistance pattern shared by more than two isolates was resistance to Nf + Na + Pe(L) and was similar to

Table 24. Resistance to specific antibiotics.

Pigmentation	No. Tested	Cm[1]	St	Am	Tc	Ct	Ot
White	10	4 (40)[2]	4 (40)	2 (20)	1 (10)	1 (10)	5 (50)
Pink	3	2 (67)	3 (100)	1 (33)	1 (33)	1 (33)	3 (100)
Orange	3		1 (33)				
Yellow	24	1 (4)	2 (8)				1 (4)
All	40	7 (18)	11 (28)	3 (8)	2 (5)	2 (5)	9 (23)

					Penecillin G[3]	
Nm	Nf	Na	Su	Ka	Low	High
1 (10)	8 (80)	6 (60)	5 (50)	3 (30)	5 (50)	2 (20)
	2 (67)	3 (100)	3 (100)	2 (67)	2 (67)	
	3 (100)	3 (100)	3 (100)			
	24 (100)	24 (100)	9 (38)		7 (29)	
1 (5)	37 (93)	36 (90)	20 (50)	5 (13)	14 (35)	2 (5)

[1]Abreviations listed in Table

[2]Percentages given in parentheses.

[3]Pe(L) = 3 IU/ml; Pe(H) = 75 IU/ml.

Table 25. Growth responses of Arthrobacter species on media containing antibiotics.

	Cm	St	Am	Tc	Ct	Ot	Nm	Nf	Na	Su	Ka	Pe (L)	Pe (H)
White													
1	++	+	++	-	+	±	-	-	-	++	-	++	+
2	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	++	+++	+	-	-	-
16	-	-	-	-	-	-	-	++	-	+	-	+	-
*20	-	-	-	-	-	-	-	++	-	+	-	-	-
24	-	+++	-	-	-	++	-	+++	+++	-	-	++	-
31	-	+++	-	-	-	+++	-	+++	+++	-	-	++	-
35	+++	+++	+++	++	-	++	+	+++	++	++	+	++	+++
38	+++	-	-	-	-	++	-	++	+++	-	+++	-	-
39	+++	+	-	-	-	++	-	++	+++	-	+++	-	-
Orange													
4	-	++	-	-	-	-	-	+++	+++	+	-	-	-
* 5	-	-	-	-	-	-	-	++	++	+	-	-	-
6	-	-	-	-	-	-	-	+++	++	+	-	-	-
Pink													
8	-	++	-	+++	+	+++	-	-	+	++	-	-	-
25	++	++	+	-	-	++	-	+++	++	++	++	+++	-
*34	+	++	-	-	-	++	-	++	++	+	++	++	-
Yellow													
3	-	-	-	-	-	-	-	+++	+++	++	-	-	-
7	-	+	-	-	-	-	-	+++	+++	+	-	-	-
9	-	-	-	-	-	-	-	+++	+++	+	-	-	-
10	-	-	-	-	-	-	-	+++	+++	-	-	-	-
*11	-	-	-	-	-	-	-	+++	+++	+	-	-	-
12	-	-	-	-	-	-	-	+++	+++	-	-	+	-
13	-	-	-	-	-	-	-	+++	+++	-	-	-	-
14	-	-	-	-	-	-	-	+++	+++	+	-	-	-
17	-	-	-	-	-	-	-	+++	+++	-	-	-	-
19	-	-	-	-	-	-	-	+++	+++	-	-	-	-
21	-	-	-	-	-	-	-	+++	+++	-	-	-	-
22	-	-	-	-	-	-	-	+++	+++	++	-	+	-
23	-	-	-	-	-	-	-	+++	++	-	-	+	-
26	-	-	-	-	-	-	-	+++	+++	-	-	-	-
27	-	-	-	-	-	-	-	+++	+++	-	-	-	-
28	-	-	-	-	-	-	-	+++	+++	-	-	++	-
29	++	-	-	-	-	-	-	+++	+++	++	-	++	-
30	-	-	-	-	-	++	-	+++	+++	+++	-	++	-
32	-	-	-	-	-	-	-	++	++	-	-	-	-
33	-	+++	-	-	-	-	-	++	+++	-	-	-	-
36	-	-	-	-	-	-	-	++	+++	-	-	-	-
37	-	-	-	-	-	-	-	++	++	++	-	+	-
40	-	-	-	-	-	-	-	+++	+++	-	-	-	-
41	-	-	-	-	-	-	-	+++	+++	-	-	-	-

Table 26. Antibiotic resistance patterns.[1]

Pigmentation	Number Tested	NfNa	NfNaSu	NfNaPe(L)	NfSuPe(L)	NfNaSu Pe(L)	StNfNaSu	StOtNf NaPe(L)	Others
White	10		1 (10)		1 (10)			2 (20)	6 (60)
Pink	3								3 (100)
Orange	3		2 (67)				1 (33)		
Yellow	24	11 (46)	3 (13)	3 (13)	1 (5)	2 (8)	1 (4)		3 (13)
All	40	11 (28)	6 (15)	3 (8)	2 (5)	2 (5)	2 (5)	2 (5)	12 (30)

[1]Percentage given in parentheses.

the most common resistance pattern in that it not only included resistance to Nf and Na but also it was shared solely by the yellow-pigmented isolates. Thus, 50% (20/40) of the isolates tested fell into three resistance patterns and the remaining 50% (20) were scattered among 16 other resistance patterns, none of which was shared by more than two isolates. Three of the four pigment groups exhibited considerable diversity; the orange group differed in sharing an identical resistance to Nf + Na + Su except that one isolate adds resistance to St. The pink-pigmented group is the most heterogeneous with three isolates and three resistance patterns; it is followed closely by the non-pigmented (white) group which exhibited nine resistance patterns among ten isolates. In between these extremes was the yellow-pigmented group with 46% (11/24) sharing one resistance pattern and the other 54% (13/24) showing eight different patterns.

The fact that 40 isolates exhibited 19 different resistance patterns, only three of which were shared by at least three isolates, again points to the heterogeneity which exists within the genus Arthrobacter as well as within a group of isolates from a common origin. As a group, these marine Arthrobacter species have a rather low resistance to antibiotics in general with the exceptions of Nf and Na. Even this is not remarkable, since the current (1978) edition of the Physicians Desk Reference describes Nf as owing its effectiveness to the inhibition of carbohydrate metabolism, which may be only a secondary energy source for many Arthrobacter species (Hagedorn, 1975), and reference to the bacteriocidal effectiveness of Na only mentions gram negative organisms.

SUMMARY AND CONCLUSIONS

Marine Arthrobacter species are a collection of heterogeneous bacteria within a diverse genus. They possess an ability to utilize inorganic nitrogen, function without growth factors, procure nutrients in a very dilute environment, form storage energy sources, and withstand reasonably high extremes of pH, salinity and temperature which enables them to subsist quite well under adverse environmental conditions. Biodegradative activities are also varied and some marine Arthrobacter species are capable of hydrolyzing proteins, peptides, lipids, and starch. Although these Arthrobacter species possess a fairly high resistance to potassium sorbate, their resistance to other antimicrobial agents such as QAC and iodophor disinfectants, is unremarkable. Resistance to antibiotics is generally low. Their growth rate at psychrotrophic temperature is about average for marine organisms and they can be classified as facultative psychrotrophs. They are capable of growth at 2°C but their optimum growth temperature is near 25°C.

Diversity within this group of marine Arthrobacter isolates was demonstrated in nearly every biochemical, morphological, physical, growth and nutritional experiment to which they were subjected. This included rate and extent of the morphological cycle, pigmentation, nitrogen preference, carbohydrate utilization, proteolysis, lipolysis, pH tolerance, salt tolerance, growth temperature, heat resistance, and the ability to grow in dilute nutrients. Their sensitivity to antimicrobial agents such as sorbate, disinfectants and antibiotics are also variable. Perhaps most illustrative of this diversity are their

antibiotic resistance patterns; there were 19 separate patterns of resistance to 12 antibiotics shown by the 40 isolates tested, and the largest group sharing the same resistance pattern was 11 isolates, representing 28% of the total.

Arthrobacter in its native environment may thrive by its low demand on nutrients and ability to subsist for long periods on stored energy reserves. In spite of this ability to sustain a viable population, they are not considered active spoilers of seafood even though they may comprise a significant portion of the initial microbial flora. Their lower growth rate, and perhaps lack of motility, causes them to lag behind the well-known spoilage bacteria such as Pseudomonas species.

The origin of Arthrobacter species found in fresh seafood may be the water column, bottom mud, terrestrial soil, and the hold of ships. The first two represent the same environment and pose a question of degree rather than exclusion; likewise the microbial flora of fish holds would reflect the three others. Conceivably, the holds could become inoculated from airborne dust while in port or through soil tracked in by the fishermen when working in the holds. Terrestrial soil is dominated by Arthrobacter which routinely comprises the largest single group of organisms and sometimes account for more than 50% of all colonies isolated (Mulder, 1963; Veldkamp, 1970; Hagedorn, 1975a, 1975b, 1975c). Likewise, residual matter from previous catches could easily adhere to the walls, ceiling and bottom of the hold and act as inoculum for fresh catches. In the latter case, the microbial flora of the hold would certainly reflect that of the previous catch except when conditions in the hold were altered considerably from those of

the ocean environment. This would occur as a result of bad weather forcing the boats to stay in port during which time the hold would either retain its original microbial flora or the microbial flora would be altered by washing and sanitizing. Arthrobacter species were shown to be sensitive to disinfectants and they should not be found in large proportions in well-cleaned fish holds. The evidence of the present study and other (Crombach, 1974; Mulder, 1966; Vanderzant, 1970) is against the Arthrobacter species isolated from seafood being of terrestrial origin. There are distinct similarities between soil and marine Arthrobacter species but a sufficient difference exists to imply that our isolates represent a distinct sub-group of Arthrobacter. We thus are left with two possible origins of the Arthrobacter isolates: either from the marine water column or from the bottom mud. Shrimp, crab and scallops are bottom-dwelling creatures and when taken with nets part of the bottom mud would attach to the animals. The catch may then be washed thoroughly and sorted on deck or inadequately cleaned before being transferred to the hold. Washing is done with ocean water. Thus, if the primary source of the marine Arthrobacter was the bottom mud, a cleaner catch should show less Arthrobacter and vice versa. In the muddier waters of the Gulf of Mexico and in shallow earthen ponds Vanderzant (1970) found coryneforms to predominate over a number of other marine microorganisms. It seems apparent, then, that marine Arthrobacter are found in greater numbers in the bottom mud and a high incidence of Arthrobacter may be related to inadequate separation of mud from the catch.

Marine Arthrobacter species therefore can be characterized as a

diverse group of microorganisms sharing some characteristics with soil Arthrobacter. Although they possess certain advantages for survival in the environment, they are not active in fish spoilage and are only isolated from fresh seafoods.

The presence of Arthrobacter in seafoods, therefore, may show the inadequacy of raw product cleaning or inadequate heat treatment but will not show the extent of microbial growth of spoilage.

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