PSYCHROPHILIC BACTERIA FROM ARIZONA FOOD ENVIRONMENTS

by

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ABSTRACT

Samples collected from selected Arizona environments were plated and the plates incubated at 6, 24±2, and 32 C to obtain enumerations of the microorganisms present. The highest organism counts were obtained from these samples on the plates incubated at 24±2 C. This indicates that some bacteria are being excluded by using 32 C, the currently recommended temperature, for the enumeration of organisms. The counts at 6 C varied from 0 organisms recovered per 0.1 ml of rinse to greater than one million per square inch at some stations. The most commonly encountered genera were *Achromobacter*, *Flavobacterium*, and *Pseudomonas*.

Since many of these organisms grew at 0 C and were either proteolytic or lipolytic, there is a definite potential for food contamination from these environments and possible spoilage when contaminated products are held at refrigeration temperatures.

INTRODUCTION

Psychrophilic bacteria have been shown to be widely distributed. However, few studies have been done in warm climates such as in Arizona. Here, as in other areas, refrigeration is used very extensively for food storage.

The purpose of this study is to determine the presence and prevalence of psychrophilic bacteria in selected habitats in Arizona and to determine what types are found. It is also desirable to know what potential they have for causing defects in food, which includes information on growth rates at different temperatures.

The practical importance of knowing the characteristics of these organisms and how they can affect food storage is obvious.

LITERATURE REVIEW

The existence of psychrophilic bacteria has been known since Forster first reported them in 1887. Disagreement about the validity of the term psychrophilic has existed since it was first introduced. The term psychrotrophic (13) has been suggested and is used to a considerable extent in the food industry. Stokes (28) prefers the term psychrophilic, due to its long usage. The criteria for determining what is a psychrophilic organism vary among the different workers. Berry and Magoon (6) defined as psychrophilic organisms only those able to grow most rapidly at 0 C or below. No organism has been reported that meets this definition. The ability to multiply sufficiently rapidly to become a significant contributor to the microflora at a given low temperature is a less precise but more practical definition used by Schultze and Olson (24). Most definitions used currently list growth at a given temperature within a specified time, such as growth at 0 C within two weeks (17). Standard Methods for the Examination of Dairy Products (1) presently recommends 7$\pm$1 C for 10 days for incubating plates for enumeration of these organisms, but this has changed with each edition (13). Ingraham and Stokes (17)

suggest that the term facultative psychrophilic be used for organisms that grow at 0 C and the term obligate psychrophilic be reserved for organisms that grow most rapidly at less than 20 C.

Gram negative rods have been the most common psychrophilic organisms isolated by most workers, with Pseudomonas being the most common genus. Witter (30) lists 19 references where members of the genus Pseudomonas were the most commonly isolated organisms. He mentions that earlier investigators found Achromobacter to be predominant, but the shift toward Pseudomonas was probably due to nomenclature changes in newer editions of Bergey's Manual of Determinative Bacteriology (7). The investigations of Druce and Thomas (12) suggest that the predominance of gram negative rods may not be true for all types of habitats, since they found only one-third of the psychrophilic isolates from soil to be this type.

The classification of gram negative rods varies depending on the criteria used. There is as much discussion about classification as there is on terminology. Weeks (29) states that Flavobacterium is more a historic concept than a taxonomic reality and that the group probably originated not because the members form a natural group but because color was conspicuous. He states that many non-motile bacteria that have been designated Flavobacterium are Cytophaga on the basis of their mole

percent guanine plus cytosine ratios. Bean and Everton (5) found that only 16 of 132 cultures identified as *Flavobacterium* resembled any of the 26 species in Bergey's Manual. Numerical taxonomy using 28 characters indicated that the isolated did not constitute a homogenous group. This was confirmed by Byrom (10), who studied 250 flavobacteria and used 96 morphological, colonial, phsyiological, and biochemical tests to compare these organisms for homogeneity. He obtained three pleista with eight sub-pleista that contained 102 of the 250 types. One trend emerging from Byrom's study was that morphological features appear to have no major importance in differentiation, while physiological and biochemical tests are of paramount importance. Hendrie, Mitchell, and Shewan (16) state that many of the problems in the identification of gram negative, yellow pigmented rods are due to the inadequate characterization of the genus *Flavobacterium*. They list some of the tests that have proved useful for generic differentiation to include gram stain, oxidase, motility, oxidative-fermentative attack on carbohydrates, mole percent guanine plus cytosine ratios, and others. They suggest two sub-groups of *Flavobacterium*. Sub-group A is the non-motile, gram negative rods, not identifiable as *Cytophoga*. Sub-group B consisted of motile organisms (usually a few peritrichous flagella) which are gram negative and attack sugars oxidatively; these may belong in

some other group. McMeekin, Patterson, and Murray (20) state that although many characteristics have been investigated as an initial approach to the taxonomy of yellow pigmented gram negative rods, no definite conclusions about separation of cytophagous types from flavobacteria can be drawn. No criterion which can be used simply in the laboratory will give positive classification.

Flavobacterium is not the only group whose classification is uncertain. Ingram and Shewan (18) state that it is not known if a distinction can be made between *Alcaligenes* and *Achromobacter* and that most of the *Pseudomonas* and *Achromobacter* species described in Bergey's Manual are illusory. They list some changes in the taxonomy of gram negative rods through the years and various editions of Bergey's Manual. The change in classification is further illustrated by Brown and Weideman (9) who checked and re-identified the isolates of Empey and Scott. Originally, Empey and Scott in 1939 placed 90 percent of the isolates in the genus *Achromobacter* but, due to changes in classification, Brown and Weideman changed the majority to the genus *Pseudomonas*. Many workers have ceased trying to identify organisms of this group as to species.

Ingram and Shewan (18) mention that the identification of organisms will depend on the classification scheme used. Using the current edition of Bergey's Manual, *Pseudomonas* and *Achromobacter* are in different orders but Brisou

and Prévot (8) place them in the same family. This is due to the selection of different characteristics as the basis of classification.

Shewan, Hobbs, and Hodgkiss (26) working with gram negative isolates from marine environments, used a classification scheme based on motility as a basic characteristic to separate the pseudomonads from Achromobacter and Flavobacterium. Dempster (11) used this scheme to identify organisms isolated from dairy environments.

In an attempt to obtain faster enumeration of possible psychrophilic bacteria present, Freeman, Nanavati, and Glenn (14) tested 58 chemicals looking for one that would be selective for gram negative bacteria and thus allow incubation at 32 C. The basis for the experiment was that most psychrophilic bacteria of practical importance to the dairy industry are gram negative rods. None of the chemicals tested gave satisfactory results.

The potential of psychrophilic microorganisms to cause spoilage and defects is mentioned by several workers (4,25, 30). Olson, Parker, and Muller (23) state that the type of bacteria present is more important than the number present. Witter (30) states that psychrophilic bacteria are usually strongly proteolytic, and cites one study where 90 percent of the psychrophilic bacteria tested were either proteolytic or lipolytic and 66 percent were both. Bean and Everton (5) found all potential spoilage organisms

hydrolyzed casein but not all isolates that hydrolyzed casein produced spoilage.

METHODS AND MATERIALS

Sampling Locations and Procedures

Samples were collected at the following locations:

Station 1. The floor of the cold room at the University of Arizona dairy farm. This room is adjacent to the room where the bulk tank is located and occasionally is used to store 10 gallon cans of raw milk. The temperature is approximately 6 C.

Station 2. The floor drain under the sinks of the University of Arizona dairy farm. This drain is in the room where the bulk tank is located and receives some of the rinse water when the tank is rinsed. The cold room (Station 1) also has an outlet that enters this drain. This room is unheated and uncooled.

Station 3. The interior side wall about 1 foot below the lid of the bulk milk tank at the University of Arizona dairy farm.

Station 4. The exterior surface of the bulk tank at the University of Arizona dairy farm. The area around the lid hinge was sampled.

Station 5. The floor drain in a cold storage room of the Agricultural Sciences building on the University of Arizona campus. This has a 5 inch grate leading to a

3.5 inch pipe. The grate was removed and the area between the lip and the 3.5 inch pipe was sampled. This room is maintained at approximately 3 C.

Station 6. The floor of a cold storage room of the Agricultural Sciences building on the University of Arizona campus. The area sampled was adjacent to the drain. The temperature was approximately 3 C.

Station 7. Water entering the drain in Station 2 from rinsing of the bulk tank at the University of Arizona dairy farm. No refrigeration was used.

Station 8. The floor in the cold room of the dairy plant on the University of Arizona campus. This room is used to store the processed milk and is maintained at 2.5 C.

Station 9. The drain in cold room of the dairy plant on the University of Arizona campus. This has a 3.25 inch grate leading to a 1.5 inch pipe. The grate was removed and the area between the lip and the pipe was sampled. This is in an area refrigerated at approximately 2.5 C.

Station 10. The floor below the electrical switches of the west wall of the meat laboratory at the University of Arizona farm was sampled. This is the floor of the killing room which is kept thoroughly washed but unrefrigerated.

Station 11. The floor of the first cold room at the University of Arizona meat laboratory. Temperature about 4 C.

Station 12. The floor of the second cold room at the University of Arizona meat laboratory. Temperature about 3 C.

Station 13. The floor of the fruit storage room of a produce distributor in Tucson, Arizona. Temperature was 4 C.

Station 14. The floor of the vegetable storage room of a produce distributor in Tucson, Arizona. Temperature was 6 C.

Sterile cotton swabs were used to collect the samples. Usually areas of 8 square inches were swabbed. At stations with irregular surfaces, such as drains and grates, the area was estimated. The sterile swabs were dipped in tubes containing 5 ml of sterile buffered water (1). The excess was pressed out against the side of the tube. Each area was swabbed twice, once in each direction. The swab was then placed in the tube, breaking off the part that had been touched. The tubes were placed on ice for transportation to the laboratory.

The samples were plated at the laboratory using two replicate smear plates at each of several dilutions to cover the probable range of count. Standard Methods for the examination of Dairy products (1) was followed in preparing dilutions and pipetting procedures. Each sample was plated on Plate Count Agar (PCA) and Potato Dextrose Agar (PDA) and a set of plates poured with each medium was incubated for 48±4 hours at 24±2 and at 32 C. These plates were also observed at 24 hours. A set of plates of each medium was incubated at 6 C for 10 days. Some

of these 6 C plates were recounted after 14 days, but no significant changes were noted.

Media Used in Enumeration

Standard Methods for the examination of Dairy Products (1) recommends Plate Count Agar (Standard Methods Agar) for the enumeration of bacteria and Potato Dextrose Agar (acidified) for the enumeration of yeasts and molds. The media for this study were prepared from the dehydrated media of the Difco Laboratories. The Plate Count Agar was control number 545719 and the Potato Dextrose Agar control number 539912. Both media were prepared in 3 or 4 liter quantities, sterilized and stored in 100 ml milk dilution bottles. When needed, it was melted, cooled to 46±1 C and poured into plastic petri dishes. The PDA was acidified to pH 3.5 by the addition of 1.2 ml of 10% (wt/vol) d-Tartatic acid per 100 ml of medium. This acid was added just prior to pouring the agar into petri dishes.

An additional set of plates using T-soy (BBL) agar was made for the first enumerations and isolations, but this was discontinued as a routine, since the counts were comparable with the counts obtained on the Plate Count Agar.

Isolation and Purification

The colonies selected for isolation from the 6 C plates were picked with an inoculating needle and transferred to a fresh PCA plate using the streak plate method for isolation. After incubation at room temperature the plates were examined for purity of growth and a well isolated colony was selected and transferred to a PCA slant and a gram stain was made to observe the cellular morphology and also the purity. This process was repeated until a pure culture was obtained. When selected each isolate was assigned a number. The first part of the number gives the station from which it was isolated and the second part is the isolate number from that station. For example, 1-3 was the third isolate from station 1.

The isolates were picked from 6 C plates on the basis of colonial morphology and pigmentation. When one type of colony represented 25 percent or more of the total number of colonies on a plate, then more than one of that type was isolated to verify that similar organisms were actually the same.

All bacteria isolated at 6 C were checked for growth at 0 C, room temperature (24 ± 2 C) and 32 C. The 0 C test was done by inoculating PCA slants and packing them in a styrofoam ice chest filled with ice. The ice chest was held in a cold room at 3 C and a thermometer

placed in the ice chest was checked daily and was always below 1 C. The tubes were checked for growth at 10, 23, and 30 days. The room temperature tubes were incubated in the laboratory which was 24±2 C, and the 32 C tubes in a water jacked incubator.

Identification

The media and tests used in identification and classification of the organisms isolated at 6 C were as follows:

1. Motility test medium (3)
2. Simmons citrate agar (3)
3. Nutrient gelatin (3)
4. Nitrate broth (3)
5. MR-VP medium (2)
6. Indole production (27)
7. Triple sugar iron (TSI) agar (3)
8. Milk
9. Litmus milk (27)
10. Gelatin liquefaction (3)
11. Starch agar (3)
12. Lipase activity (15)
13. Acid and gas production (3) in:
    - A. Glucose 1%
    - B. Lactose 1%
    - C. Maltose 1%

D. Mannitol 1%

E. Sucrose 1%

14. Gram stain (Hucker Modification) (27)

15. Catalase (27)

16. Growth at 0, 6, 24±2, and 32 C.

The colonial and cellular characteristics were also used in identification. Some organisms also were checked for growth at 37 C. Bergey's Manual (seventh edition) was used as the basis for classification, although some attempt was made to reconcile such classification with current literature concerning organisms of the types encountered. Since the new edition of Bergey is not yet available, use of the seventh edition seemed necessary, as current literature leaves room for numerous differences of opinion which need to be resolved to some degree in an authoritative publication.

The tests were run in duplicate with a control tube being incubated with each test. If the duplicate tubes gave different results, the test was repeated using two more tubes after checking the purity of the culture. If a difference was obtained a second time the organism was listed as variable for that test.

Growth Curves

Growth curve experiments were conducted on several representative isolates. These were done at 6, 24±2, and

32 C to obtain the generation times of the organisms at different temperatures. The cells were prepared for the growth curve by heavily inocultaing an agar slant to give solid growth on the slant. After 18-24 hours at 24±2 C, the growth was removed from the slant by pipetting 1-2 ml of sterile buffered water onto the slant. Using the pipette, the cells were gently scraped from the agar and the suspension returned to the water blank. To prepare a more dilute suspension, a further 1:100 dilution was made from the suspension and 0.1 ml was placed in a flask containing 30±3 ml of T-soy broth (BBL). This gave a starting count in the range of 10^4 cells per ml. One flask was inoculated for each temperature. Samples were taken from the flasks at various times starting with T_o until the log phase had been sampled enough times to calculate the generation time. The samples held at 24±2 and 32 C were plated at 2 hour intervals and the sample held at 6 C was plated at 12 hour intervals. The final plating for all samples was made when the count was in the range of 10^8 per ml. The generation times were computed using actual counts from two samples taken during the log phase of growth.

RESULTS AND DISCUSSION

The total viable organism counts for each station, as determined at the different temperatures of plate incubation, are shown in Table 1. The counts at 6 C were less than the counts at 32 C at four stations from which nine samples were obtained and greater at six stations from which 13 samples were obtained. At eight stations from which 19 samples were obtained the counts at 6 C were less than the counts at 24±2 C. Only at two stations, 5 and 13, did the counts at 6 C equal or exceed the counts at 24± C. The counts at 24±2 C were greater than the counts at 32 C at ten stations, based on the majority of samples when multiple samples were taken from some of the several stations.

The counts at 24±2 C might be expected to be greater than the count at 6 or 32 C, since the majority of both psychrophilic and non-psychrophilic organisms would grow at 24±2 C. No organisms were recovered at stations 3, 4, 7, or from the second sample at stations 10 and 12 at the dilutions used. The bulk milk tank where stations 3 and 4 were located received regular and thorough washings, which explains the absence of organisms on the plates inoculated with 0.1 ml. The sample at station 7 was the rinse water from one of the bulk

TABLE 1

Organism counts per square inch of sample area on plate count agar incubated at several temperatures.

Station	Sample	Temperature of incubation		
		6 C.	24±2 C.	32 C.
1	A	6.1×10^3	1.4×10^5	1.2×10^4
	B	1.0×10^5	1.7×10^5	1.9×10^5
	C	5.3×10^4	1.5×10^5	1.2×10^5
2	A	1.0×10^3	5.0×10^4	1.7×10^4
	B	5.0×10^4	1.1×10^6	6.3×10^5
	C	1.4×10^4	4.5×10^5	3.3×10^5
3	A	10^a	10^a	10^a
4	A	10^a	10^a	10^a
5	A	6.9×10^5	6.9×10^5	5.0×10^2
	B	8.7×10^5	7.5×10^5	4.9×10^2
6	A	1.8×10^2	10^a	10^a
7	A	10^a	10^a	10^a
8	A	5.1×10^5	5.3×10^5	4.6×10^4
	B	1.2×10^4	1.9×10^4	7.5×10^3
	C	2.9×10^3	1.7×10^4	4.6×10^4
	D	5.1×10^3	1.8×10^4	4.4×10^3
9	A	2.9×10^6	4.1×10^6	2.1×10^5
	B	3.0×10^5	3.1×10^5	5.5×10^4
	C	1.9×10^6	2.2×10^6	3.2×10^5
	D	7.5×10^5	8.0×10^5	2.7×10^5

Table 1 (Cont'd)

Station	Sample	Temperature of incubation		
		6 C.	24±2 C.	32 C.
10	A	7×10^5	1.1×10^6	1×10^6
	B	10[a]	10[a]	10[a]
11	A	3.0×10^3	3.5×10^3	1.1×10^3
	B	9.2×10^3	1.1×10^4	1.5×10^3
12	A	1.1×10^2	2.9×10^2	1.2×10^2
	B	10[a]	10[a]	10[a]
13	A	8×10^5	8×10^5	2.1×10^5
14	A	3.5×10^6	4.3×10^6	3.0×10^6

[a] No organisms recovered from 0.1 ml amount of sample at these stations.

tank washings and also was low in bacteria, having no organisms in a 0.1 ml sample. Washing of the sample area also occurred at stations 10 and 12 just prior to the second sample being taken. The areas had been washed with a detergent and flushed thoroughly with water. No organisms were recovered from 0.1 ml aliquots placed on PCA plates. Psychrophilic organisms were present in all samples from which micro-organisms were recovered. The counts of psychrophilic organisms ranged from less than 10 at some stations to 3.5 million per square inch at station 14. The majority of these stations had counts of psychrophilic organisms which exceeded 1,000 per square inch. As shown by the absence of psychrophilic organisms at some stations, it is possible to reduce these populations in the environments in which foods are processed and stored and thus significantly reduce the possibility of food contamination from floors, drains, and similar areas which foods ordinarily would not contact but which may be a source of contamination through splashing of water, contact with equipment and similar circumstances. In areas that have a high population of psychrophilic bacteria a high potential for contamination with these organisms exists. Psychrophilic organisms were present in all samples from which microorganisms were recovered. At 60 percent of the stations the number of colonies that developed at 6 C was greater than the number of colonies that developed at 32 C.

Table 2 shows the counts at 6 C as a ratio of the counts at 24 ± 2 C and 32 C and the counts at 24 ± 2 C as a percentage of the counts at 32 C. At stations 1 and 8 the relationship of the 6 and 24 ± 2 C counts showed a large fluctuation between samples. At station 1 this could be accounted for by the irregular use of the cold room for storage of milk in 10-gallon cans. Sample 1B was taken while the room was being used. The increased traffic in and out of the room may have caused changes in the microorganism populations. Sample 8C was taken a few hours after the sample area was washed. This would be expected to minimize organism recovery. The values of the 6 C/32C vary more than do the 6C/24 ± 2 C values. Station 8 is the only station that had a mixture of percentages above and below 100 percent. This may be a result of the washing mentioned above. The temperature at station 2 was not controlled and varied according to the ambient temperature. The temperature range noted during sampling was 15 to 35 C. These higher temperatures would not be selective for psychrophilic organisms and the 6/24 ± 2 and the 6/32 ratios are lowest at this station.

The types of colonies that grew on the 24 ± 2 C and the 32 C plates were the same as grew on the 6 C plates, except there were more molds and actinomycetes at the higher temperatures. No molds grew at 6 C within the time allotted for plate incubations.

TABLE 2

The ratios of total organism counts at the different incubation temperatures.

Station	Sample	6C/24±2C	6C/32C	24±2C/32C
1	A	.04	.51	12
	B	.59	.53	.9
	C	.35	.44	1.2
2	A	.02	.06	2.9
	B	.04	.08	1.7
	C	.03	.04	1.4
5	A	1.00	1400	1400
	B	1.20	1800	1500
8	A	.96	11	12
	B	.63	1.6	2.5
	C	.17	.06	.37
	D	.28	1.2	4.1
9	A	.71	1.4	20
	B	.97	5.4	5.6
	C	.86	5.9	6.9
	D	.94	2.8	3.0
10	A	.64	.7	1.1
11	A	.86	2.7	3.2
	B	.84	6.1	7.3

Table 2 (Cont'd)

Station	Sample	6C/24±2C	6C/32C	24±2C/32C
12	A	.38	.92	2.4
13	A	1.00	3.8	3.8
14	A	.81	1.2	1.4

Various differential tests were used to identify the isolates. None of the organisms produced acid from lactose, maltose, mannitol, or sucrose and all gave negative results on the methyl red and Voges-Proskauer tests. Table 3 contains the results of the other tests made on the individual cultures isolated. Using the data in Table 3, the organisms were classified according to Bergey's Manual. The majority of the bacteria did not match a described species in Bergey's Manual, and some of the isolates matched the descriptions for more than one species equally well. When an organism could not be classified using Bergey's Manual, other literature was consulted. Shewan et al.(26) give a classification scheme that was applied to some gram negative rods isoalted for marine environments. This scheme was used to help classify some of these isolates. Of the 32 isoaltes, 23 were bacteria, eight were yeasts and one was an actinomycete.

The bacteria are predominantly gram negative rods that are inactive biochemically. The isolates include both pigmented and non-pigmented, motile and non-motile organisms. These characteristics fit several genera of bacteria, including Pseudomonas, Achromobacter, and Flavobacterium. Two of the isolated organisms were gram positive rods which were identified as members of the genus Brevibacterium. No sporeforming bacteria were found, nor were any true cocci.

TABLE 3

The results from selected differential tests

Isolate number	gram stain	motility	gelatin liquefaction	nitrate reduction	indole	citrate	triple sugar iron agar	litmus milk	catalase	oxidase	glucose	starch hydrolysis	lipolysis	Growth 0 C 30 D	Growth 32 C 48 Hr	Growth 37 C 48 Hr
1-1	-	+	-	-	-	+	AkS	AR	+	+	A	-	-	+	+	-
1-2	-	+	+	-	-	+	AkS	R	+	+	A	-	+	+	+	-
1-3	-	-	-	-	-	+	AkS	AR	+	+	A	-	+	+	+	-
1-4	-	-	+	-	-	-	AkS	AkR	+	-	-	-	-	+	-	-
2-1	-	+	+	+	-	+	AkS	AkP	+	+	-	-	+	+	+	-
2-2	-	+	+	+	-	+	AkS	AkP	+	+	A	-	+	+	+	-
5-1	-	-	+	-	-	+	AkS	AkP	+		-	+		+	+	+
5-2	+	-	-										-	+	-	-
5-3	+	-	-	+	-	+	AS	R	-	+	-	-	-	+	+	-
6-1	+	-	-	+	-	+	AS	R	-	+	-	-	-	+	+	-
8-1	-	-	-	-	-	-		AR	+	+	-	-	-	+	-	-
8-4										-			-	+	±	-
9-1	+	-	+	+	-	-	AkS	R		-	-	-	-	+	-	-
9-2	-	-	+	-	-	-	AkS	AkR	+		-	±		+	+	
9-3	-	+	+	-	+	+	Ak	AkP	+	-	A	-	+	+	+	-
9-4	-	-	-	-	-	-	A	AkP		-	A	-	-	+	+	-
9-6	-	-	+	-	-	-	NC	P	+	+	-	-	-	+	+	
10-1	-	-	+	-		+	NC	RC	+	-	-	-	+	+	+	+
10-2	-	+	+	-		+	AkS	AkP	+	+	-	-	+	+	+	+
11-1	-	-	-	+	-	-	A	NC	+		-	-		+	-	-
11-2	+	-	-	-	-	-	Ak	Ak		-	-	-	-	+	-	-
11-3	-	-	-	-	-	-	Ak	R			-		-	+	-	-
11-5	+	-	-	-	-	-	Ak	Ak		-	A	-	-	+	+	-
12-1	+	-	-	-	-	+	Ak	Ak		+	A	-	-	+	+	±
13-1	+	-	-	-	-	-	A	Ak		-	A	-	-	+	-	-
13-2	+	-	-	-	-	-	NC	Ak			A	-	-	+	+	±
13-3	-	-	+	-	-	-	NC	NC			A	-	-	+	±	-
14-1	-	-	-	-	±	-	NC	NC		+	-	-	-	+	-	-

Table 3 (Cont'd)

Isolate number	gram stain	motility	gelatin liquefaction	nitrate reduction	indole	citrate	triple sugar iron agar	litmus milk	catalase	oxidase	glucose	starch hydrolysis	lipolysis	Growth		
														0 C 30 D	32 C 48 Hr	37 C 48 Hr
14-4	-	-	+	-	-	-	AkS	NC	+	+	-	-	-	+	+	
14-7	-	-	+	-	±	-	AkS	R	+	+	-	-	-	+	+	+
14-8	-	-	-	+	-	-	AkS	NC	+	-	-	-	-	+	+	
14-11	-	+	+	-	-	+	NC	P		+	-	-	+	+	+	

A = acid
Ak = alkaline
NC = no change
\+ = positive
± = uncertain

P = peptonization
R = reduction
C = coagulation
\- = negative
S = slant

Blank space = no data

The yeasts were differentiated on the basis of colony pigmentation and cell morphology only. The yeasts that grew on PDA were compared with those that grew on PCA and found to be the same; therefore, separate data are not presented for isolates from the PDA plates.

All 32 isolates grew at 0 C, but 30 days were required for some of them to show growth. Twenty of the isolates grew by the twenty-third day. Nine of the 32 isolates did not grow and two others showed poor growth at 32 C in 48 hours. At 37 C only four of the 32 isolates showed definite growth, with two others being doubtful at 48 hours.

Eighteen of the 23 bacteria, or 78 percent, were proteolytic as measured in litmus milk, and eight bacteria were lipolytic on corn oil.

Some of the isolates differed from other isolates by one or two reactions, or in some cases their colonial and cellular characteristics were different. These differences may or may not have caused them to be classified as different species depending on which tests were the ones that differed. The results from the motility and gelatin liquefaction fests were considered more valuable for identification of these organisms than were the IMViC tests.

An example of the difficulties encountered in classifying and identifying these organisms is represented by organisms 2-1 and 2-2. These two isolates gave identical

results on all tests except glucose, 2-2 producing a slight acid reaction. The results from 2-1 on most media were obtained faster and were more intense than those of 2-2. These two organisms also varied in colonial and cellular characteristics. According to these differences they were classified as different organisms. Other organisms, such as 1-4 and 5-1, that differed in some results were classified as the same organism.

Using the data in Table 3, the types were identified. The bacteria are listed in Table 4 and the other micro-organisms are listed in Table 5. These tables also show the percentage each organism represents of the total organisms at the station from which it was isolated. Several organisms were not assigned to a species. This was done when the isolate did not match a species listed in Bergey's Manual closely enough to be considered the same or else it matched more than one species equally well and more information was needed for definite placement. The isolates that were classified as to species were done so on a best fit basis using Bergey's Manual. The 32 isolates were grouped into four genera of bacteria, two kinds of yeasts, and one actinomycete. Table 6 lists these groups, with the number of isolates in each, and the percentage each group is of the total number of isolates. The number of samples taken at each station and the number of isolates and types obtained from these samples are shown in Table 7.

TABLE 4

The identification and prevalence of the bacterial isolates.

Isolate number	Identification	Percent of total organisms recovered at station
1-1	Achromobacter superficialis	35-40
1-2	Achromobacter liquefaciens	30-35
1-3	Achromobacter delmarvae	30-35
1-4	Flavobacterium sp.	3- 5
2-1	Pseudomonas sp.	50-55
2-2	Achromobacter sp.	45-50
5-1	Flavobacterium sp.	20-25
8-1	Achromobacter parvulus	99
9-1	Brevibacterium vitarumen	5-10
9-2	Flavobacterium sp.	30-35
9-3	Pseudomonas schaykilliens	10-15
9-4	Pseudomonas eisenbergii	40-45
9-6	Flavobacterium sp.	5-10
10-1	Achromobacter butyri	90-95
10-2	Achromobacter delicatulus	5-10
11-1	Brevibacterium sp.	15-20
11-3	Flavobacterium sp.	10-15
13-3	Flavobacterium peregrinum	5-10
14-1	Flavobacterium breve	70-75
14-4	Flavobacterium sp.	10-15
14-7	Flavobacterium sp.	3- 5
14-8	Achromobacter parvulus	10-15
14-11	Achromobacter xerosis	1- 2

TABLE 5

Incidence of yeasts and actinomycetes on Plate Count Agar plates incubated at 6 C

Isolate number	Organism	Percent of total organisms recovered at station
5-2	pink yeast	65-70
5-3	white yeast	10-15
6-1	white yeast	99
11-2	pink yeast	55-60
11-5	white yeast	5-10[a]
12-1	white yeast	99
13-1	pink yeast	55-60
13-2	white yeast	30-35
8-4	actinomycete	1

[a] This yeast represented greater than 95 percent of the colonies on Potato Dextrose agar.

TABLE 6

The number and percentage of the isolated organisms belonging to each group

Organism type or genus	Number of types	Percent of total types
Achromobacter	9	28.2
Flavobacterium	9	28.2
Pseudomonas	3	9.4
Brevibacterium	2	6.3
White yeasts	5	15.3
Pink yeasts	3	9.4
Actinomycete	1	3.1
Total	32	100

TABLE 7

The number of samples taken per station and the number of isolates selected from each station with the number of different types these isolates represented

Station	Number of samples taken at station	Number of isolates from station	Number of types
1	3	10	4
2	3	5	2
3	2	0[a]	0[a]
4	2	0[a]	0[a]
5	2	5	3
6	1	1	1
7	1	0[a]	0[a]
8	4	5	2
9	4	7	5
10	2	2	2
11	2	6	4
12	2	3	1
13	1	6	3
14	1	11	5
Totals	30	61	32

[a] No colonies developed on plates containing 0.1 ml of sample.

From the 30 samples taken, 61 colonies were selected that appeared to be different organisms. These colonies were selected due to their differences in colony characteristics such as size, texture, and pigmentation. When two isolates selected from the same station gave the same results on the biochemical tests, but varied in their colonial characteristics, they were considered to be the same organism. After elimination of the duplicate organism from each station 32 isolates were left. These 32 isolates do not represent all different types, as the same organism may have been isolated from more than one station.

The predominant genera isolated from the sample locations were _Achromobacter_ and _Flavobacterium,_ with each genus comprising 28 percent of the total types. Both of these genera are difficult to identify and much work has been done on their taxonomy since the seventh edition of Bergey's Manual. _Pseudomonas_ was the third most common type of organism isolated, being nine percent of the total. The predominance of _Achromobacter_ and _Flavobacterium_ over _Pseudomonas_ disagrees with some data in the literature. Witter (30) gives seven references where _Pseudomonas_ was the predominant psychrophilic organism found in dairy environments and products. Dempster (11) also found _Pseudomonas_ to be the most common in a dairy environment; however in the rinse water the most common organisms were _Achromobacter_ and _Flavobacterium_. Since only selected

areas associated with dairy environments and no products were sampled, the data presented here are not directly comparable with other studies.

The classification scheme used could affect the distribution of organisms. If the previously mentioned scheme of Shewan et al. (26) is used, the genus *Achromobacter* is considered non-motile and some of the isolates in this study, identified as belonging to this genus, would be classified as pseudomonads.

Nine of the 32 types isolated were placed in the genus *Flavobacterium*, making it one of the most common genera isolated. Although several types of flavobacteria were isolated, only a relatively small number of each type was present. Station 14 is the only station where *Flavobacterium* represented the greatest number of organisms.

The generation times for selected isolates at different temperatures are shown in Table 8. The isolates selected give some comparisons between organisms of the same genus at the same and different locations and also between different genera at the same station. The three organisms from station 1 have generation times that are almost equal at 6 C, but they vary greatly in the percentage of the total organisms they represent at station 1. The two *Achromobacter* types each represnet about 35 percent, while organisms assigned to the genus *Flavobacterium* represent only 3-5 percent, as seen in Table 4. Organism 9-2 has

TABLE 8

The generation times of selected isolates at different temperatures.

Isolate number	Genus of organism	Generation time in minutes at 6 C	24±2 C	32 C
1-1	*Achromobacter*	360	57	110
1-2	*Achromobacter*	400	100	90
1-4	*Flavobacterium*	400	84	NG[a]
2-1	*Pseudomonas*	300	71	61
8-1	*Achromobacter*	530	100	NG[a]
9-2	*Flavobacterium*	310	90	130
9-3	*Pseudomonas*	640	72	47
10-1	*Achromobacter*	550	56	43
14-1	*Flavobacterium*	490	100	110

[a] Organism did not grow in T-soy broth at 32 C.

a generation time of less than one-half that of organism 9-3, but it also represents a larger percentage of the total population at station 9. The data from stations 1 and 9 indicate that a shorter generation time may aid an organism to become dominant in its evnironment, but there are other factors involved. Five of the nine organisms for which comparisons can be made had shorter generation times at 24 ± 2 C than at 32 C. When comparing generation times with dominance, it must be remembered that in a natural habitat conditions may be such that the generation time possibly will not be the same as in a laboratory.

This study demonstrates the presence of psychrophilic bacteria in a variety of refrigerated and unrefrigerated environments in Arizona. Some locations that are refrigerated are selective for psychorphilic bacteria and such bacteria were found in greater numbers than bacteria that grew at 32 C. This could be an example of the suggestion of Stokes (28) that the ability to grow at both low and moderate temperatures may give psychrophilic bacteria a competitive advantage over mesophilic bacteria.

The highest counts usually were obtained at 24 ± 2 C and not at 32 C. This suggests that an incubation temperature of 32 C or higher may exclude some psychrophilic bacteria, resulting in a lower total count of organisms than would be obtained when using a more nearly optimum incubation temperature. In a refrigerated food these

bacteria may be capable of increasing to numbers much in excess of one million per gram during storage when all conditions are favorable. Since the majority of psychrophilic bacteria isolated from dairy environments have been shown to be proteolytic, a high potential exists for defect production in the stored products.

The recommended temperature of 32 C for detection of psychrophilic bacteria may be too high to give accurate counts. Nelson and Baker (22) recommended plates be incubated at 21 C for 4 days or 25 C for 3 days for the detection of milk having a high count of psychrophilic bacteria.

Although Larkin (19) did not recover any psychrophilic bacteria during the summer in Louisiana, they appear to be ubiquitous and have the potential to cause defects at low temperature. There is a great need for a rapid and accurate method for enumerating and identifying them.

CONCLUSIONS

Organisms capable of growth at refrigeration temperature are commonly encountered. At many of the stations in this study they exceeded one thousand per square inch and at some stations were in excess of one million per square inch. The genera most commonly found are *Achromobacter*, *Flavobacterium*, and *Pseudomonas*. Many of the organisms do not match described species, and for some of the bacteria even the genus to which they belong is in doubt. Of the 23 bacterial isolates studied in some detail, six would not grow at 32 C and would be missed in enumeration studies done at that temperature, which is currently recommended. Numerous psychrophilic bacteria that do not grow at 32 C are active in a cold environment and could be a hazard to food products stored at refrigeration temperatures.

Although no psychrophilic bacteria have been shown to be pathogenic, their potential to cause defects in food at low temperature creates a need for additional study of their characteristics, taxonomy, and methods of enumeration.

REFERENCES CITED

1. American Public Health Association. 1967. Standard methods for the examination of dairy products. 12th Ed. American Public Health Association, Inc. New York, N.Y.

2. Anonymous. 1968. BBL Manual of products and laboratory procedures. 5th Ed. Baltimore Biological Laboratory Division of BioQuest, Division of Becton, Dickinson and Co. Cockeysville, Md.

3. Anonymous. 1968. Difco Supplementary Literature. Difco Laboratories. Detroit, Mich.

4. Baumann, D. P. and G. W. Reinbold. 1963. Enumeration of psychrophilic microorganisms, a review. J. Milk Food Technol. 26:81-86.

5. Bean, P. G. and J. R. Everton. 1969. Observations on the taxonomy of chromogenic bacteria isolated from cannery environments. J. Appl. Bacteriol. 32:51-59.

6. Berry, J. A. and C. A. Magoon. 1934. Growth of microorganisms at and below 0 C. Phytopathology 24:780-796.

7. Breed, R. S., E. G. D. Murray and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th Ed. The Williams & Wilkins, Co., Baltimore, Md.

8. Brisou, J. and A. R. Prévot. 1954. Etudes de systématique bactérienne X. Revision des espéces reunies dans le genre Achromobacter. Ann. Inst. Pasteur. 86:722-728.

9. Brown, A. D. and J. F. Weideman. 1958. The taxonomy of the psychrophilic meat-spoilage bacteria: A reassessment. J. Appl. Bacteriol. 21:11-17.

10. Byrom, N. A. 1971. The Adonsonian taxonomy of some cannery flavobacteria. J. Appl. Bacteriol. 34: 339-346.

11. Dempster, J. F. 1968. Distribution of psychrophilic microorganisms in different dairy environments. J. Appl. Bacteriol. 31:290-301.

12. Druce, R. G. and S. B. Thomas. 1970. An ecological study of the psychrophilic bacteria of soil, water, grass and hay. J. Appl. Bacteriol. 33:420-435.

13. Eddy, B. P. 1960. The use and meaning of the term 'Psychrophilic.' J. Appl. Bacteriol. 23:189-190.

14. Freeman, T. R., N. V. Nanavati, and W. E. Glenn. 1964. Faster enumeration of psychrophilic bacteria in pasteurized milk-- A preliminary report. J. Milk Food Technol. 27: 304-307.

15. Hammer, B. W. and F. J. Babel. 1957. Dairy Bacteriology. 4th Ed. John Wiley and Sons, Inc. New York, N. Y.

16. Hendrie, M. A., T. G. Mitchell, and J. M. Shewan. 1968. The identification of yellow pigmented rods. In Identification Methods for Microbiologists. Part B., B. M. Gibbs ed. Academic Press. London.

17. Ingraham, J. L. and J. L. Stokes. 1959. Psychrophilic Bacteria. Bacteriol. Rev. 23:97-106.

18. Ingram, M. and J. M. Shewan. 1960. Introductory reflections on the _Pseudomonas-Achromobacter_ group. J. Appl. Bacteriol. 23:286-291.

19. Larkin, J. M. 1970. Seasonal incidence of bacterial temperature types in Louisiana soil and water. Appl. Microbiol. 20:286-291.

20. McMeekin, T. A., J. T. Patterson, and J. G. Murray. 1971. An initial approach to the taxonomy of some gram negative yellow pigmented rods. J. Appl. Bacteriol. 34:699-716.

21. Mitchell, T. G., M. S. Hendrie, and J. M. Shewan. 1969. The taxonomy, differentiation and identification of Cytophaga species. J. Appl. Bacteriol. 32:40-50.

22. Nelson, F. E. and M. P. Baker. 1954. The influence of time and temperature of plate incubation upon bacterial counts of market milk and related products, particularly after holding under refrigeration. J. Milk Food Technol. 17:95-100.

23. Olson, J. C. Jr., R. B. Parker, and W. S. Muller. 1955. The nature, significance and control of psychrophilic bacteria in dairy products. J. Milk Food Technol. 18: 200-203.

24. Schultze, W. D. and J. C. Olson, Jr. 1960. Studies on psychrophilic bacteria. I. Distribution in stored commercial dairy products. J. Dairy Sci.. 43:346-350.

25. Shewan, J. M. 1971. The microbiology of fish and fishery products. A progress report. J. Appl. Bacteriol. 34:299-315.

26. Shewan, J. M., G. Hobbs, and W. Modgkiss. 1960. A determinative scheme for the identification of certain genera of gram-negative bacteria with special reference to the Pseudomonadaceae. J. Appl. Bacteriol. 23:379-390.

27. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co. Inc. New York, N. Y.

28. Stokes, J. L. 1962. General biology and nomenclature of psychrophilic microorganisms. Recent Progress in Microbiology VII:187-192.

29. Weeks, O. B. 1969. Problems Concerning the Relationship of Cytophagas and Flavobacterium. J. Appl. Bacteriol. 32:13-18.

30. Witter, L. D. 1961. Psychrophilic bacteria, A Review. J. Dairy Sci. 44(1):983-1015.

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