APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1980, p. 1106-1114 Vol. 40, No. 6
0099-2240/80/12-1106/09$02.00/0

Transformation Assay for Identification of Psychrotrophic Achromobacters

ELLIOT JUNI* AND GLORIA A. HEYM
Department of Microbiology, The University of Michigan, Ann Arbor, Michigan 48109

The finding that many psychrotrophic, gram-negative, nonmotile, oxidase-positive coccobacilli (achromobacters) are competent for genetic transformation made possible the development of a transformation assay that permits recognition of genetically related strains. It has been demonstrated that 109 independently isolated achromobacters are genetically related since deoxyribonucleic acid samples from all of these organisms were able to transform a single competent auxotrophic strain to prototrophy. Genetically interacting bacteria included strains that lacked one or more of the characteristics typical for most achromobacters. An oxidase-negative mutant of one of these strains reacted positively in the transformation assay, unlike other oxidase-negative bacteria. Achromobacters were derived from fish, poultry, irradiated foods, seawater, and other sources. One strain previously classified as *Micrococcus cryophilus* has been shown to be related to the achromobacters. Two achromobacters had an optimum growth temperature of 35°C and behaved as typical mesophiles. The moraxellae and *Acinetobacter* were shown to be unrelated to the achromobacters by using the transformation assay. The ready demonstration of genetic relatedness provides a new basis for taxonomic grouping of the psychrotrophic achromobacters.

The present study is concerned with the identification of certain gram-negative, nonmotile, nonpigmented, oxidase-positive psychrotrophic coccobacilli frequently found in fish, in poultry, and in other food products. These *Moraxella*-like bacteria have, in the past, been designated as strains of *Achromobacter* (8, 36). In general, these bacteria do not possess a sufficient number of distinguishing characteristics to make it possible to assign an isolated strain to this group in an unequivocal manner. Studies of a series of such organisms in our laboratory have revealed that many of these strains are highly competent for genetic transformation. This finding suggested the possibility of devising an interstrain transformation assay system which could be used for determining genetic relatedness of suspected bacterial strains of this kind, similar to the systems already reported for identification of strains of *Acinetobacter calcoaceticus* (24), *Moraxella osloensis* (25), *Moraxella urethralis* (26), and *Neisseria gonorrhoeae* (22).

It has been demonstrated that deoxyribonucleic acid (DNA) samples from achromobacters isolated from a variety of sources were all able to transform auxotrophs of a highly competent strain to prototrophy, whereas DNA samples from other organisms possessing many similar characteristics were unable to do so. This new transformation assay represents a simple and rapid diagnostic procedure for definitive identification of these *Moraxella*-like organisms. The ability to ascertain genetic relatedness of the achromobacters also provides a basis for clarification of the taxonomy of these bacteria. A preliminary report of some of the results of the present study has been presented (E. Juni and G. A. Heym, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, I87, p. 126).

MATERIALS AND METHODS

Bacterial strains. The achromobacters examined in the present study are listed in Table 1. Most strains were obtained from individuals, as indicated, or from the American Type Culture Collection. Some achromobacters (strains 86 to 105) were isolated by washing several fish heads, obtained from a local fish market, with sterile saline solution and streaking this material on heart infusion agar (Difco), followed by overnight incubation at 20°C. Colonies that appeared were tested for oxidase reaction (37) and for motility (observation of wet-mount preparations). Oxidase-positive, nonmotile coccobacilli or rods were Gram stained, and those that were gram negative were further tested using the transformation assay described below. All psychrotrophic achromobacters were grown at 20°C. Mesophilic organisms were grown at 34°C. Strains were stored as described previously (26).

Growth media. All strains studied were routinely grown on heart infusion agar (Difco). The medium used to detect prototrophic transformants of auxotrophic mutant strains was prepared by adding the following chemicals, one at a time, to approximately 150 ml of distilled water: casein hydrolysate (vitamin

TABLE 1. *Achromobacter strains tested*

Strain no.	Strain	Received from	Isolated from
1	NCMB 98	S. D. Henriksen	Lemon sole (gills)
2	NCMB 131	S. D. Henriksen	Arctic cod (skin)
3	NCMB 132	R. E. Levin	
4	NCMB 200	S. D. Henriksen	Skate (skin)
5	NCMB 307	S. D. Henriksen	Arctic cod (skin)
6	ATCC 15174	ATCC[a]	Pork sausage
7	ATCC 17955	ATCC	
8	A 189	H. Lautrop	Seawater
9	A 191	H. Lautrop	Seawater
10	A 192	H. Lautrop	Seawater
11	A 193	H. Lautrop	Seawater
12	A 333	S. D. Henriksen	
13	A 348 (phenon 3)	S. D. Henriksen	Poultry
14	A 351 (phenon 3)	S. D. Henriksen	Poultry
15	A 353	S. D. Henriksen	Pus from abcess
16	A 365	S. D. Henriksen	Guinea pig (autopsy)
17	MJT F4/11/5 (phenon 4)	M. J. Thornley	Poultry
18	MJT F5/158 (phenon 3)	M. J. Thornley	Poultry
19	MJT F5/199A (phenon 4)	M. J. Thornley	Poultry
20	MJT F5/211 (phenon 2)	M. J. Thornley	Poultry
21	MJT F5/239 (phenon 4)	M. Mandel[b]	Poultry
22-25	M-1 to M-4	R. E. Levin	Fish pen slime
26-29	M-6 to M-9	R. E. Levin	Fish pen slime
30-32	A 101 to A 103	R. E. Levin	Fish
33	A 151	R. E. Levin	Fish
34-38	A 401 to A 405	R. E. Levin	Fish
39-40	A 4211, A 4212	R. E. Levin	Fish
41-42	A 4214, A 4215	R. E. Levin	Fish
43-44	I-I-1, I-I-2	R. E. Levin	Fish
45	I-I-6	R. E. Levin	Fish
46	I-I-8	R. E. Levin	Fish
47-50	I-I-10 to I-I-13	R. E. Levin	Fish
51-53	I-I-17 to I-I-19	R. E. Levin	Fish
54-56	I-II-6 to I-II-8	R. E. Levin	Fish
57-58	I-II-10, I-II-11	R. E. Levin	Fish
59	II-II-18	R. E. Levin	Fish
60	II-II-20	R. E. Levin	Fish
61-62	II-II-11, II-II-12	R. E. Levin	Fish
63	II-II-14	R. E. Levin	Fish
64	II-II-20	R. E. Levin	Fish
65-73	1975-1983	M. Catsaras	Poultry
74-75	A-4, A-5	H. Ito	Vienna sausage, irradiated
76	A-10	H. Ito	Vienna sausage, irradiated
77	M1-4	H. Ito	Vienna sausage, irradiated
78	M2-6	H. Ito	Vienna sausage, irradiated
79	S2-3	H. Ito	Vienna sausage, irradiated
80	33.1	J. G. Niemand	Ground beef, irradiated
81	107.1	J. G. Niemand	Ground beef, irradiated
82	120.1	J. G. Niemand	Ground beef, irradiated
83	121.1	J. G. Niemand	Ground beef, irradiated
84	136.1	J. G. Niemand	Ground beef, irradiated
85	149.1	J. G. Niemand	Ground beef, irradiated
86-88	A55-A57	This laboratory	Fish
89-105	1588-1604	This laboratory	Fish
106	*M. nonliquefaciens* H	M. Mandel[b]	
107	*M. duplex* H	M. Mandel[b]	
108	S38	M. Mandel[b]	
109	S131	M. Mandel[b]	

[a] ATCC, American Type Culture Collection.
[b] DNA sample obtained from M. Mandel.

free, salt free, ICN Pharmaceuticals), 3.2 g; Na_2HPO_4, 1.12 g (or $Na_2HPO_4 \cdot 7H_2O$, 2.12 g); KH_2PO_4, 0.4 g; NaCl, 2.0 g; and $MgSO_4$, 0.09 g (or $MgSO_4 \cdot 7H_2O$, 0.18 g). The final volume was adjusted to 200 ml with distilled water, and the medium was sterilized by Millipore membrane filtration or by autoclaving for 20 min. The entire volume of this liquid medium (200 ml, medium at room temperature) was poured into 200 ml of recently melted (90 to 100°C) sterile 3% agar (Difco) in distilled water and mixed, followed by addition of 3.2 ml of 50% glucose (sterilized by Millipore filtration) and 2.0 ml of 60% sodium lactate (commercial solution, sterilized by autoclaving). This so-called medium M9A was then mixed thoroughly and distributed into 20

sterile petri dishes. Medium M9A plates can be stored at room temperature in plastic bags, to retard evaporation, until ready for use.

Isolation of mutant strains. Hypoxanthine and tryptophan auxotrophs of competent strain 14 and oxidase-negative mutants of strain 7 were obtained after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) or with diethyl sulfate (Aldrich Chemical Co.) (24). Mutant Hyx-7 was used as the recipient strain in the present study.

Preparation of crude transforming DNA. A rapid method for preparation of crude transforming DNA has been described (25). A small amount of cell paste, clearly visible to the naked eye, from growth on a heart infusion plate of a strain to be assayed was removed on the edge of a bacteriological loop and transferred to 0.5 ml of lysing solution (0.05% sodium dodecyl sulfate in 0.15 M sodium chloride–0.015 M trisodium citrate) contained in a 13-mm screw-capped test tube. The exact amount of cell paste used was not critical. The cells were suspended completely by stirring with an orbital mixer and the suspension was heated at 60 to 70°C in a deep water bath or heating block for 1 h. Heating for longer periods did not affect the transforming ability of the preparation. This treatment lysed the cells releasing DNA and other cell constituents and resulted in a sterile solution. Once crude DNA samples have been prepared they can be stored in the refrigerator for several years and still retain transforming ability, provided they are not frozen or permitted to evaporate to dryness.

Transformation assay. Strain Hyx-7, grown overnight at 20°C on heart infusion agar, was the recipient strain used to assay DNA samples from all the strains listed in Table 1 plus the following: *Acinetobacter calcoaceticus* (NCTC 7976), *Branhamella catarrhalis* (ATCC 23246), *Flavobacterium meningosepticum* (ATCC 13253), Group IIj, (one of the unnamed groups of gram-negative bacteria classified according to phenotypic characteristics by the Center for Disease Control) (CDC 2706 and 5839), *Moraxella atlantae* (CDC 5118), *Moraxella bovis* (ATCC 10900), *Moraxella lacunata* (ATCC 17952 and 17970), *Moraxella nonliquefaciens* (ATCC 17953 and 19975), *Moraxella osloensis* (ATCC 10973), *Moraxella phenylpyruvica* (ATCC 23333 and CDC 9158), *Moraxella urethralis* (ATCC 17960), *Neisseria caviae* (ATCC 14659), and *Neisseria ovis* (ATCC 17575). A heart infusion plate was marked as indicated in Fig. 1, and cell paste of Hyx-7 was placed in the six labeled squares as shown. In this particular example, crude DNA samples prepared from five achromobacter strains were assayed as follows: a loopful (using a 2-mm-diameter loop) of the DNA preparation from each strain was mixed with the cell paste of Hyx-7 on a square bearing the strain designation, suspending and spreading the cell paste over an area approximately 5 to 8 mm in diameter. During spreading of Hyx-7 the sodium dodecyl sulfate present in the crude DNA preparation diffused rapidly into the agar, whereas the high-molecular-weight DNA present remained on the surface to interact with the growing cells. A loopful of the same DNA preparation was placed in the square adjacent to the one where the cell-DNA mixture using this DNA had been made (Fig. 1) and streaked to verify that the DNA sample was sterile. Cell paste of Hyx-7 in one of the squares (Fig. 1A) was spread in this square without addition of DNA and this material served as a non-DNA-treated control.

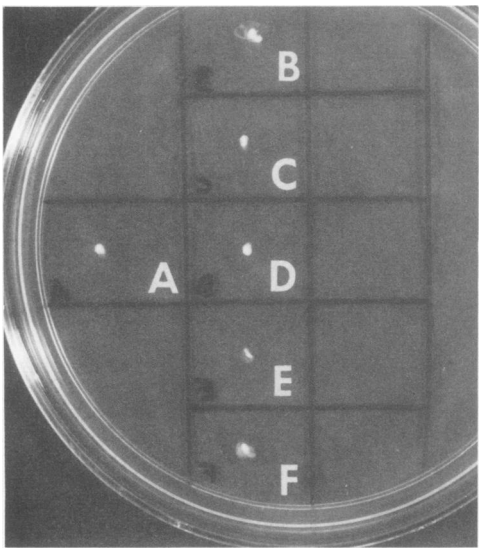

FIG. 1. *Placement of recipient cells (Hyx-7) for preparation of transformation mixtures. The bottom of a heart infusion plate was marked as indicated. Cell paste of Hyx-7 from growth overnight at 20°C on a heart infusion plate was placed in squares A to F. The approximate amount of cell paste per square used in this assay is shown.*

After completion of the above operation for all five DNA samples, the plate was incubated overnight (about 16 h) at 20°C. Shorter incubation times were shown to be possible; incubation for 8 h gave excellent results, and incubation for periods as short as 3 h gave satisfactory transformations but did result in significantly fewer transformant colonies observed on the final assay plate. Since optimum results were obtained after overnight incubation, this time was used routinely in the standard assay procedure.

After overnight incubation the plate containing the DNA-Hyx-7 mixtures appeared as shown in Fig. 2. Since there was no growth in the DNA control areas, all DNA samples tested must have been sterile. Using a sterile loop, cell paste from each of the growth areas on the plate in Fig. 2 was removed and streaked uniformly on a sector of a medium M9A plate. Figure 3 illustrates the amount of cell paste removed from each growth area of the plate in Fig. 2 and used for such streakings. After incubation at 20°C for 3 days, the streaked medium M9A plate appeared as shown in Fig. 4. The sector of this plate streaked with non-DNA-treated Hyx-7 shows only the original cell paste spread over this area (Fig. 4A). All the other streaked sectors (Fig. 4B to F) contain colonies derived from cells of Hyx-7 that were transformed to prototrophy during growth in the presence of DNA samples derived from the achromobacters used in this example. The numbers of transformant colonies that appeared were found to vary considerably and to depend on the

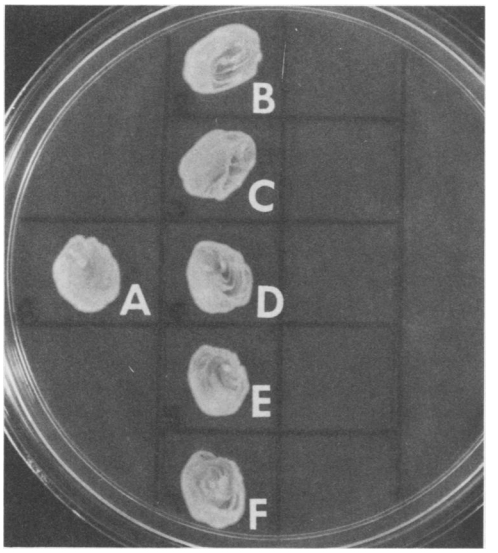

FIG. 2. *Appearance of Hyx-7 DNA mixtures after growth on heart infusion agar at 20°C for 16 h. The growth areas contained: (A) no DNA; (B) DNA from strain 89; (C) DNA from strain 72; (D) DNA from strain 70; (E) DNA from strain 68; (F) DNA from strain 67. Each DNA sample used was also spread in the square immediately to the right of the respective Hyx-7-DNA mixture to verify that the DNA preparation was sterile.*

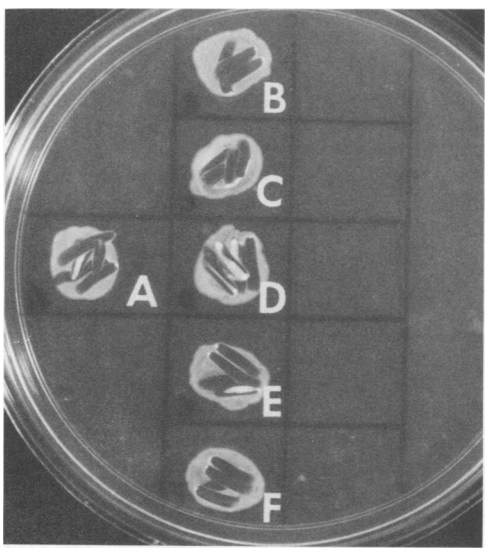

FIG. 3. *Sampling of Hyx-7-DNA mixtures for evidence of transformation to prototrophy. Cell paste from each of the six growth areas of the plate shown in Fig. 2 was removed with a sterile loop and spread on a sector of a medium M9A plate. The streak marks give an indication of the approximate amount of cell paste that was removed for this purpose.*

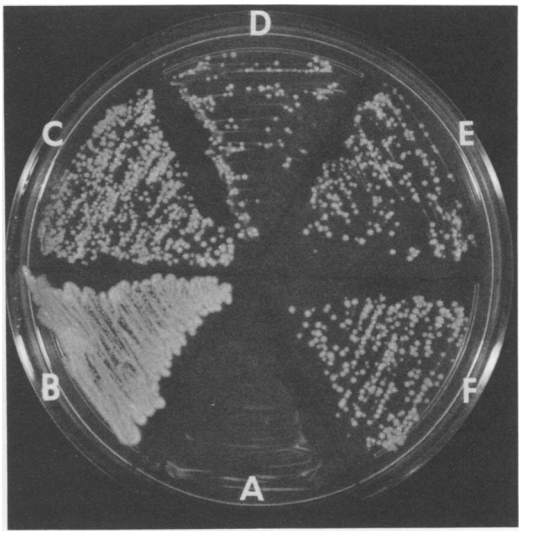

FIG. 4. *Interstrain transformation of Hyx-7 to prototrophy. Cell paste from the growth areas of the plate shown in Fig. 2 was removed as shown in Fig. 3, spread on sectors of a medium M9A plate, and incubated at 20°C for 3 days. The letters in this figure correspond to the similarly lettered growth areas of Fig. 2 and 3. Each colony observed was derived from a prototrophically transformed cell of Hyx-7 that was able to grow on the medium M9A plate. Only the original amount of cell paste of Hyx-7 streaked on sector A (non-DNA-treated control) is visible.*

source of each particular DNA sample. DNA samples from individual strains were found to give approximately the same number of transformant colonies when assays were repeated. Transformant colonies could generally be observed after 2 days of incubation, particularly when the plate was observed with a low-power dissecting microscope. Incubation for 3 days, however, gave optimum results. A negative result was recorded if no transformant colonies were observed after incubation for 3 days.

Although Hyx-7 is relatively stable, it was found to give rise to spontaneous revertants on rare occasions. For this reason it was important to test carefully those samples that resulted in small numbers of transformant colonies on the streaked sectors of the medium M9A plate to be certain that these colonies were not rare spontaneous revertants. In such cases, three to six separate DNA-Hyx-7 mixtures, as well as an equal number of non-DNA-treated Hyx-7 controls, were prepared. When this was done for the DNA sample obtained from strain 70, for example, which resulted in a small number of transformant colonies (Fig. 4D), testing of duplicate DNA-Hyx-7 mixtures always resulted in the appearance of similar numbers of prototrophic transformants in all streaked sectors. By contrast, no colonies appeared on the duplicate sectors streaked with non-DNA-treated Hyx-7.

Test for competence. Achromobacters were tested for competence by treating each strain with DNA derived from a streptomycin-resistant mutant of

that strain in the same manner described above for transformation of auxotrophic mutants. DNA-cell mixtures incubated overnight on heart infusion agar were streaked on sectors of heart infusion plates containing 100 μg of dihydrostreptomycin sulfate per ml and incubated at 20°C for 3 days. Competent strains showed numerous streptomycin-resistant transformant colonies in contrast to the non-DNA-treated controls.

An incubation temperature of 20°C for cell-DNA mixtures was found to be optimal for competence of psychrotrophic strains. Higher incubation temperatures resulted in significantly fewer transformant colonies on streaked sectors of medium M9A plates.

Determination of DNA composition. The guanine plus cytosine (G+C) content (moles percent) of the DNA of strain 14 was determined by using a modification of the methods of Meyer and Schleifer (32) and of Gibson and Ogden (15). DNA compositions of other strains used in this study and also examined by other investigators were obtained from the literature.

Photomicrographs. Photographs were taken of wet-mount preparations by using a Bausch and Lomb phase-contrast microscope equipped with a photomicrographic camera. Cultures to be photographed were grown overnight at 20°C on heart infusion plates.

RESULTS

Development of a transformation assay for achromobacters. We first became interested in the psychrotrophic *Moraxella*-like organisms when we examined a series of 36 unclassified gram-negative bacteria that resembled the moraxellae in several ways and were the subject of a study by members of the Subcommittee on *Moraxella* and Allied Bacteria of the International Committee on Systematic Bacteriology (5). Eight of the 36 strains were oxidase negative, and these were all demonstrated to be acinetobacters by using the *Acinetobacter* transformation assay (24). When we found that six of the nine oxidase-positive psychrotrophic strains in this collection were competent for genetic transformation, we prepared auxotrophs of one of these strains (strain 14). Several auxotrophs requiring either hypoxanthine or tryptophan were examined, and one of these (Hyx-7) was chosen as a suitable transformation indicator strain because of its inherent stability, its high frequency of transformation (to prototrophy) with homologous wild-type DNA, and its consistent transformation with heterologous DNA samples from genetically related strains.

Transformation assay of strains studied. The strains assayed using the transformation of Hyx-7 to prototrophy as an indicator of genetic relatedness are listed in Table 1 and above. Figures 1 to 4 illustrate the various steps used in testing DNA samples from five of the strains listed in Table 1. The number of prototrophic transformant colonies of Hyx-7 in each streaked sector of the transformation indicator plate depended upon the DNA sample used (Fig. 4). Although the transformation procedure employed is not a quantitative one, it is, nevertheless, highly reproducible, and particular DNA samples that resulted in an obviously smaller number of transformant colonies than were obtained with another DNA sample always showed the same relative proportion of transformant colonies when the procedure was repeated. The results in Fig. 4 show the range of transformant colonies obtained with several DNA samples and includes the DNA sample that consistently gave the smallest number of transformant colonies per streaked sector (Fig. 4D) for all strains listed in Table 1. Even in this case, however, the results were reproducible, with no colonies appearing on the non-DNA-treated control sector (Fig. 4A).

DNA samples from all of the 109 strains listed in Table 1 transformed Hyx-7 to prototrophy, and these strains are clearly all genetically related. Most of these strains were derived from various fish and from processed poultry although some were obtained from processed meats, from seawater, and from the tissues of animals. For a few strains (strains 21, and 106 to 109), only DNA samples (obtained from M. Mandel) were available for testing. DNA samples from representative *Moraxella* and *Acinetobacter* strains (see above) and from several strains of *Alcaligenes* and *Pseudomonas* all failed to transform Hyx-7 to prototrophy.

Transformation of high-level streptomycin resistance from one strain to another has also been used, particularly with the moraxellae, as an indicator of genetic relatedness, especially when the ratio of inter- to intrastrain transformation was relatively high (18). We have shown that competent achromobacters are readily transformed to streptomycin resistance with homologous as well as with heterologous DNA samples obtained from streptomycin-resistant achromobacters. It has been found, however, that DNA samples from some streptomycin-resistant strains transformed the streptomycin marker into some competent strains but failed to do so when other competent strains were used, in marked contrast to the uniformly positive transformation of nutritional markers such as Hyx-7 by DNA samples from all achromobacters examined. DNA samples from high-level streptomycin-resistant mutants of all the *Moraxella* and *Acinetobacter* strains listed in Materials and Methods failed to transform Hyx-7 to streptomycin resistance.

Properties of strains related to Hyx-7. Although many of the characteristics of all the achromobacters listed in Table 1 have not been determined, properties have been described for the nine strains (strains 1, 2, 4, 5, and 12 to 16) studied in great detail by the Subcommittee of *Moraxella* and Allied Bacteria and shown, in the present study, to be genetically related to Hyx-7 (5). The properties of some of the other strains listed in Table 1 have also been published (7, 9, 13, 14, 21, 23, 27, 31, 34, 38–41). Some of the characteristics of achromobacters are as follows. They are gram-negative, nonpigmented, nonmotile, aerobic, oxidase-positive, catalase-positive, penicillin-sensitive coccobacilli. They are psychrotrophic, with an optimal temperature near 20°C (most strains grow at 5°C). The DNA composition ranges from 43 to 45 mol% G+C (a G+C value of approximately 43 mol% was obtained for strain 14 in this laboratory; other values were obtained from the literature). Some strains form acid from sugars; some strains reduce nitrate. Most strains deaminate phenylalanine and tryptophan; most form phenethyl alcohol from L-phenylalanine and ethanol. Most strains have urease activity. Most hydrolyze Tween 80; most can grow in 7.5% NaCl. Some strains show twitching motion. Most strains grow on simple defined media, and many strains are competent for genetic transformation.

The microscopic appearance of four related strains is shown in Fig. 5. In addition to the usual diplococcus and coccobacillus forms most frequently observed, some strains displayed short rods and filamentous structures (Fig. 5C and D). All these organisms were aerobic, gram negative, nonmotile, nonpigmented, oxidase positive, and catalase positive and showed a DNA composition of approximately 44 mol% G+C.

Since most achromobacters can grow at temperatures near 0°C, they may be considered to be psychrotrophic (33). The optimal growth temperature for most strains appears to be approximately 20°C, and growth usually does not take place at 35 to 37°C. The optimum temperature for transformation has been found to be 20°C for the competent strains examined. About half of the independently isolated achromobacters studied proved to be competent for genetic transformation.

Strains with unusual properties. A mutant of strain 7 was obtained that failed to give an oxidase-positive test. To be certain that this oxidase-negative strain was indeed an achromobacter mutant and not simply an oxidase-negative contaminant, DNA from the oxidase-negative strain was tested and shown to transform Hyx-7 to prototrophy.

Unlike the majority of achromobacters examined, two of the strains (strains 7 and 16)

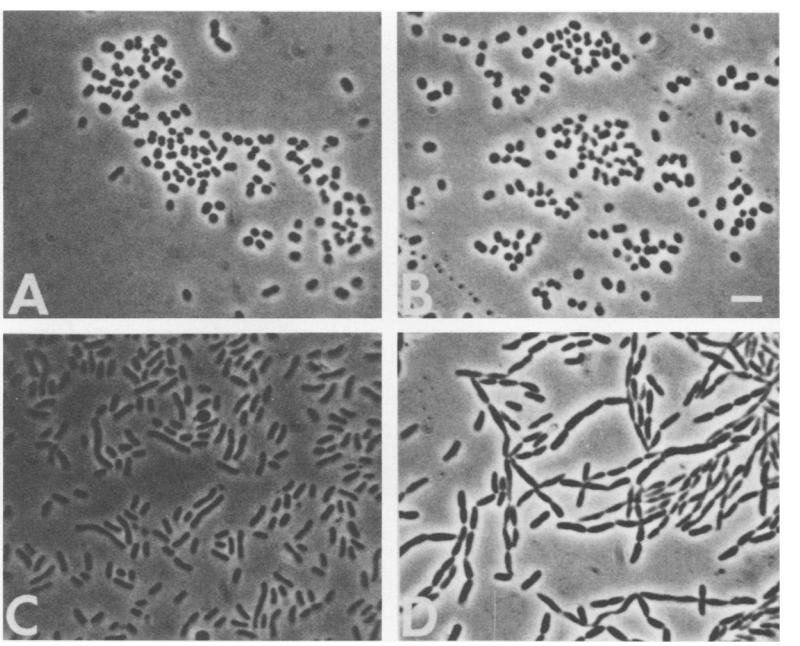

FIG. 5. *Phase-contrast photomicrograph of wet mounts of four achromobacters. (A) Strain 14; (B) strain 6; (C) strain 35; (D) strain 32. Scale marker, 3.5 μm.*

proved to be mesophilic and were shown to grow better at 35 to 37°C than at 20°C. One of these strains (strain 7) has been classified as *Moraxella phenylpyruvica* (ATCC catalog).

The use of the transformation assay has made it possible to demonstrate that *Micrococcus cryophilus* (strain 6), originally isolated from pork sausage, is genetically related to Hyx-7. Although this strain is considered to be gram positive (1), we found it to be gram negative with a tendency to retain some of the crystal violet used as the primary stain in the Gram staining procedure, a result similar to that of the original investigators of *M. cryophilus* (31).

DISCUSSION

For many years workers concerned with the microbiology of a variety of food products have isolated aerobic, gram-negative, nonmotile, psychrotrophic coccobacilli and have experienced considerable difficulties in attempting to classify these bacteria. Because of their lack of pigmentation, it has been common practice to refer to these organisms as strains of *Achromobacter*. The type species, *Achromobacter liquefaciens*, was reported to be motile, but nonmotile strains were also considered to be members of this genus (3). In 1954 Brisou and Prévot (6) suggested that the nonmotile achromobacters be classified as members of the genus *Acinetobacter*. With the introduction and widespread use of the oxidase test (2, 37), it was noted that among the bacteria classified as acinetobacters, according to the criteria of Brisou and Prévot (6), there were included both oxidase-positive and oxidase-negative strains (8). At the present time the genus *Acinetobacter* includes only oxidase-negative species (17), all members tested showing strong DNA:DNA homology (23) as well as interstrain genetic interaction (24). The taxonomic position of the oxidase-positive acinetobacters is, therefore, uncertain.

Some oxidase-positive, aerobic, gram-negative, nonmotile, mesophilic coccobacilli have been classified as species of *Moraxella* (17). The *Moraxella*-like psychrotrophic bacteria, however, appear to be unrelated to the true moraxellae. In 1960 Shewan et al. (36) called these organisms achromobacters and defined "the *Achromobacter* group as being composed of non-motile, non-pigmented, short, stout or coccoid rods, occurring singly, in pairs or short chains, forming grey or off-white slightly opaque colonies on agar, sensitive to penicillin, and usually biochemically inactive." The name *Achromobacter* has been used subsequently by many workers for bacteria having properties conforming more or less to these specifications. Ito et al. (21) used similar criteria to describe a type of bacterium intermediate between *Moraxella* and Acinetobacter. In attempts to classify organisms isolated from poultry and classified as achromobacters, Thornley (41) conducted a taxonomic study of these bacteria, as well as of others, which appeared to be members of the genus *Acinetobacter* as defined by Brisou and Prévot (6). Using morphological properties and the results of biochemical tests, organisms having a 72.5% similarity were grouped into three phenons (phenons 2, 3, and 4), each containing strains having 82.5% similarity. Oxidase-negative strains appeared chiefly in phenons 2 and 4, whereas phenon 3 was composed almost entirely (96%) of oxidase-positive strains. The high similarity of oxidase-positive and oxidase-negative strains serves to emphasize the difficulties encountered in efforts to delineate the taxonomy of these organisms.

The transformation assay for *Moraxella*-like psychrotrophic achromobacters described in the present report makes it possible to determine relatedness, or lack of relatedness, of isolated strains to the competent assay strain (Hyx-7) in a simple and unambiguous manner that is independent of the presence or absence of any particular phenotypic characteristic in the strain under study. Genetically related strains each lacking at least one of some of the typical characteristics of organisms of this type have been encountered. For example, although cells of most strains are short or coccoid rods, cells of some strains have been observed to be distinctly more rod-shaped (Fig. 5C and D).

Although 20°C is the optimal growth temperature for most achromobacters, some strains grow best at 35 to 37°C (e.g., strains 7 and 16), and mutants of strain 6 having elevated optimal growth temperatures have been isolated (39, 40). DNA samples from mesophilic mutants were capable of transforming the wild-type strain for ability to grow at higher temperatures (38).

All achromobacters tested in our laboratory gave positive oxidase tests. In addition to the oxidase-negative mutant of strain 7 described in the present report, an oxidase-negative mutant of strain 6 was reported previously (39). Oxidase-negative mutants have also been described for other oxidase-positive bacteria (19, 30). Use of the transformation assay should permit definitive identification of any possibly naturally occurring oxidase-negative achromobacters.

Chen and Levin (9) have shown that achromobacters isolated from fisheries characteristically form phenethyl alcohol from L-phenylalanine and ethanol. All achromobacters tested by these workers were capable of deaminating L-phenylalanine to phenylpyruvate (9). If, as sug-

gested by Chen and Levin (9), phenylpyruvate is the immediate precursor of phenethyl alcohol, it would be expected that achromobacters unable to deaminate phenylalanine (5) would be unable to form phenethyl alcohol from phenylalanine and ethanol.

Most achromobacters are unable to form acid from sugars (5, 9, 27), but a small proportion of achromobacters studied have been found to possess this ability (5, 27). Many achromobacters appear to be resistant to gamma irradiation and have been isolated as survivors of irradiated fish (11, 28, 29, 35), chicken (42), and meat products (21; strains 80 to 85). Achromobacters characteristically grow well in media supplemented with sodium chloride (about 10%), but also grow adequately in ordinary laboratory media without additional salt (21).

Achromobacters shown to be genetically related to Hyx-7 in the present study have been derived from a variety of sources. These include fish and fisheries, poultry, gamma-ray-treated Vienna sausage and ground beef, pork sausage, seawater, and a few unknown sources. Although DNA samples from these strains all transformed Hyx-7 to prototrophy, the efficiency of transformation was found to vary with the strain from which the DNA was derived. This finding indicates possible evolutionary change during the independent existence of different strains in a variety of environments.

A DNA composition of approximately 44 mol% G+C appears to be characteristic for strains related to Hyx-7. In their study of a variety of gram-negative coccobacilli, De Ley et al. (12) reported that a large group of nonmotile marine strains had DNA compositions of 44 to 46 mol% G+C; some if not all of these organisms probably represented strains related to Hyx-7.

Other criteria used to distinguish achromobacters from similarly appearing bacteria include analysis of cellular fatty acid composition (34), cellular wax esters (7), and electrophoretic profiles of selected enzymes (13, 14).

The ability to use the transformation assay for identification of achromobacters makes it possible for the first time to establish a new genus based on the genetic relatedness of its member strains. It has been suggested recently, however, that the generic name *Achromobacter* be rejected (16). Should this suggestion be approved, it is clear that organisms genetically related to Hyx-7 are presently not members of any known genus.

ACKNOWLEDGMENTS

The following individuals very generously supplied cultures or DNA samples used in the present study: M. Catsaras, S. D. Henriksen, H. Ito, H. Lautrop, R. E. Levin, M. Mandel, J. G. Niemand, and M. J. Thornley.

This investigation was supported by Public Health Service grant AI-10107 from the National Institute of Allergy and Infectious Disease.

LITERATURE CITED

1. **Baird-Parker, A. C.** 1974. Gram-positive cocci, p. 482-483. *In* R. E. Buchanan and N. E. Gibbons (eds.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
2. **Baumann, P., M. Dudoroff, and R. Y. Stanier.** 1967. A study of the *Moraxella* group. I. Genus *Moraxella* and the *Neisseria catarrhalis* group. J. Bacteriol. **95**:58-73.
3. **Bergey, D. H., F. C. Harrison, R. S. Breed, B. W. Hammer, and F. M. Huntoon.** 1923. Bergey's manual of determinative bacteriology, 1st ed., p. 1-442. The Williams & Wilkins Co., Baltimore.
4. **Bøvre, K., J. E. Fuglesang, N. Hagen, E. Jantzen, and L. O. Frøholm.** 1976. *Moraxella atlantae* sp. nov. and its distinction from *Moraxella phenylpyrouvica*. Int. J. Syst. Bacteriol. **26**:511-521.
5. **Bøvre, K., J. E. Fuglesang, S. D. Henriksen, S. P. Lapage, H. Lautrop, and J. J. S. Snell.** 1974. Studies on a collection of gram-negative bacterial strains showing resemblance to moraxellae: examination by conventional bacteriological methods. Int. J. Syst. Bacteriol. **24**:438-446.
6. **Brisou, J., and A. R. Prévot.** 1954. Études de systematique bacterienne. X. Revision des espéces réunies dans le genre *Achromobacter*. Ann. Inst. Pasteur (Paris) **86**: 722-728.
7. **Bryn, K., E. Jantzen, and K. Bøvre.** 1977. Occurrence and patterns of waxes in *Neisseriaceae*. J. Gen. Microbiol. **102**:33-43.
8. **Buttiaux, R., and P. Gagnon.** 1959. Au sujet de la classification des *Pseudomonas* et des *Achromobacter*. Ann. Inst. Pasteur (Lille) **10**:121-149.
9. **Chen, T. C., and R. E. Levin.** 1974. Taxonomic significance of phenethyl alcohol production by *Achromobacter* isolates from fishery sources. Appl. Microbiol. **28**: 681-687.
10. **Clowes, R. C., and W. Hayes.** 1968. Amino acid, vitamins and purine/pyrimidine pools used in the determination of auxotrophic requirements, p. 228-229. *In* R. C. Clowes and W. Hayes (ed.), Experiments in microbial genetics. Blackwell Scientific Publications, Oxford.
11. **Corlett, D. A., J. S. Lee, and R. O. Sinnhuber.** 1965. Application of replica plating and computer analysis for rapid identification of bacteria in some foods. II. Analysis of microbial flora in irradiated Dover sole (*Microstomus pacificus*). Appl. Microbiol. **13**:818-822.
12. **De Ley, J., N. Bain, and J. M. Shewan.** 1967. Taxonomy of the *Achromobacter* and allied species. Nature (London) **214**:1037-1038.
13. **Fox, R. H., and D. E. McClain.** 1974. Evaluation of the taxonomic relationship of *Micrococcus cryophilus, Branhamella catarrhalis*, and neisseriae by comparative polyacrylamide gel electrophoresis of soluble proteins. Int. J. Syst. Bacteriol. **24**:172-176.
14. **Fox, R. H., and D. E. McClain.** 1975. Enzyme electrophoretograms in the analysis of taxon relatedness of *Micrococcus cryophilus, Branhamella catarrhalis* and atypical neisserias. J. Gen. Microbiol. **86**:210-216.
15. **Gibson, D. M., and I. D. Ogden.** 1979. A rapid method for purifying bacterial deoxyribonucleic acid. J. Appl. Bacteriol. **46**:421-423.
16. **Hendrie, M. S., A. J. Holding, and J. M. Shewan.** 1974. Emended descriptions of the genus *Alcaligenes* and of *Alcalgenes faecalis* and proposal that the generic name *Achromobacter* be rejected: status of the named species of *Alcaligenes* and *Achromobacter*. Int. J. Syst. Bacteriol. **24**:534-550.
17. **Henriksen, S. D.** 1973. *Moraxella, Acinetobacter,* and the *Mimeae*. Bacteriol. Rev. **37**:522 561.

18. **Henriksen, S. D.** 1976. *Moraxella, Neisseria, Branhamella,* and *Acinetobacter.* Annu. Rev. Microbiol. **30:** 63-83.
19. **Hoffman, P. S., T. V. Morgan, and D. V. DerVartanian.** 1979. Respiratory-chain characteristics of mutants of *Azotobacter vinelandii* negative to tetramethyl-p-phenylenediamine oxidase. Eur. J. Biochem. **100:**19-27.
20. **Ingram, M., and J. M. Shewan.** 1960. Introductory reflections on the *Pseudomonas-Achromobacter* group. J. Appl. Bacteriol. **23:**373-378.
21. **Ito, H., T. Sato, and H. Iizuka.** 1976. Study of the intermediate type of *Moraxella* and *Acinetobacter* occurring in radurized Vienna sausages. Agr. Biol. Chem. **40:**867-873.
22. **Janik, A., E. Juni, and G. A. Heym.** 1976. Genetic transformation as a tool for detection of *Neisseria gonorrhoeae.* J. Clin. Microbiol. **4:**71-81.
23. **Johnson, J. L., R. S. Anderson, and E. J. Ordal.** 1970. Nucleic acid homologies among the oxidase-negative moraxellas. J. Bacteriol. **101:**568-573.
24. **Juni, E.** 1972. Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. J. Bacteriol. **112:**917-931.
25. **Juni, E.** 1974. Simple genetic transformation assay for rapid diagnosis of *Moraxella osloensis.* Appl. Microbiol. **27:**16-24.
26. **Juni, E.** 1977. Genetic transformation assays for identification of strains of *Moraxella urethralis.* J. Clin. Microbiol. **5:**227-235.
27. **Lahellec, C., C. Meurier, G. Bennejean, and M. Catsaras.** 1975. A study of 5920 strains of psychrotrophic bacteria isolated from chickens. J. Appl. Bacteriol. **38:** 89-97.
28. **Laycock, R. A., and L. W. Regier.** 1970. Pseudomonads and achromobacters in the spoilage of irradiated haddock of different preirradiaton quality. Appl. Microbiol. **20:**333-341.
29. **Licciardello, J. J., L. J. Ronisvalli, and J. W. Slavin.** 1967. Effect of oxygen tension on the spoilage microflora of irradiated and non-irradiated haddock (*Melanogrammus aeglefinus*) fillets. J. Appl. Bacteriol. **30:**239-245.
30. **Marrs, B., and H. Gest.** 1973. Genetic mutations affecting the respiratory electron-transport system of the photosynthetic bacterium *Rhodopseudomonas capsulata.* J. Bacteriol. **114:**1045-1051.
31. **McLean, R. A., W. L. Sulzbacher, and S. Mudd.** 1951. *Micrococcus cryophilus,* sp. nov.: a large coccus especially suitable for cytologic study. J. Bacteriol. **62:**723-728.
32. **Meyer, S. A., and K. L. Schleifer.** 1975. Rapid procedure for the approximate determination of the deoxyribonucleic acid base composition of micrococci, staphylococci, and other bacteria. Int. J. Syst. Bacteriol. **25:**383-385.
33. **Morita, R. Y.** 1975. Psychrophilic bacteria. Bacteriol. Rev. **39:**144-167.
34. **Nishimura, Y., H. Yamamoto, and H. Iizuka.** 1979. Taxonomical studies of *Acinetobacter* species—cellular fatty acid compostion. Z. Allg. Mikrobiol. **19:**307-308.
35. **Pelroy, G. A., J. P. Seman, and M. W. Eklund.** 1967. Changes in the microflora of irradiated petrale sole (*Eopsetta jordani*) fillets stored aerobically at 0.5 C. Appl. Microbiol. **15:**92-96.
36. **Shewan, J. M., G. Hobbs, and W. Hodgkiss.** 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.
37. **Steel, K. J.** 1961. The oxidase reaction as a taxonomic tool. J. Gen. Microbiol. **25:**297-306.
38. **Tai, P.-C., and H. Jackson.** 1969. Apparent transformation of growth temperature character in *Micrococcus cryophilus.* Can. J. Microbiol. **15:**1119-1120.
39. **Tai, P.-C., and H. Jackson.** 1969. Mesophilic mutants of an obligate psychrophile, *Micrococcus cryophilus.* Can. J. Microbiol. **15:**1145-1150.
40. **Tai, P.-C., and H. Jackson.** 1969. Growth and respiration of an obligate psychrophile, *Micrococcus cryophilus,* and its mesophilic mutants. Can. J. Microbiol. **15:**1151-1155.
41. **Thornley, M. J.** 1967. Taxonomic study of *Acinetobacter* and related genera. J. Gen. Microbiol. **49:**211-257.
42. **Thornley, M. J., M. Ingram, and E. M. Barnes.** 1960. The effects of antibiotics and irradiation on the *Pseudomonas-Achromobacter* flora of chilled poultry. J. Appl. Bacteriol. **23:**487-498.