TAXONOMIC STUDIES OF AEROBIC COCCOBACILLI¹ FROM SEAWATER

YUKIMASA NISHIMURA, KENJI KANBE, AND HIROSHI IIZUKA

*Institute of Applied Biology, Science University of Tokyo,
Noda 278, Japan*

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Strictly aerobic, non-motile, gram-negative coccobacilli were isolated from the seawater of Otsuchi Bay, Japan, and the Indian Ocean. They were divided into group 1, group 2 and others on the basis of morphological, physiological and chemotaxonomic characteristics. The members of group 1 were classified as a separate genus closely related to the genera *Acinetobacter* and *Moraxella*. The members of group 2 were assigned to the genus *Moraxella*. However four strains of group 2 were psychrotrophic, so we call them tentatively "psychrotrophic *Moraxella*." In the other isolates, the taxonomic positions of seven strains, having G+C contents of 50.5% to 61.8%, were unclear. Also one strain was recognized as closely related but different from the genera *Acinetobacter* and *Moraxella*. And one strain was identified as *Acinetobacter lwoffii* (Audureau 1940) Brisou and Prévot 1954. All but two of the isolates were moderately halophilic.

In studies of the strictly aerobic, non-motile, gram-negative coccobacilli from seawater (1–3), they were reported to be the members of the genus *Acinetobacter* (1,2) and the *Acinetobacter-Moraxella* group (3). We now recognized some organisms possibly belonging to these groups in the isolates from Otsuchi Bay (Iwate Prefecture, Japan) and the Indian Ocean.

In this study, we examined several taxonomic characteristics of these isolates including the ubiquinone type, the cellular fatty acid composition and the guanine plus cytosine (G+C) content of the DNA. On the basis of these observations we grouped these isolates and here we discuss their taxonomic positions.

MATERIALS AND METHODS

Microorganisms. Thirty-five strains were used in this experiment (Table 1).

¹ Abbreviations used in this text are as follows: DNA, deoxyribonucleic acid; G+C, guanine plus cytosine.

Table 1. Morphological, physiological, and

	Strains	Morphology	Oxidase	Penicillin resistance (100 IU)	Nitrate reduction
Group 1	1S-3	P	+	+	+
	15S-2	P	+	+	+
	22S-5	P	+	+	+
	22S-8	P	+	+	+
	8S-2	P	+	+	+
	14S-4	P	+	+	+
	17S-7	P	+	+	+
	5S-4	SP	+	+	+
	3S-2	SP	+	+	+
	20S-3	P	+	+	+
	16S-2	P	+	+	+
	21S-1	P	+	+	+
	13S-3	P	+	+	+
	7S-1	P	+	+	+
	6N-1	P	+	+	+
	7S-2	N	+	+	+
	22S-6	SP	+	+	—
	114-43	SP	+	+	+
	18S-1	P	+	+	—
	4S-1	P	+	+	+
Group 2	3S-7	SP	+	—	—
	9S-5	N	+	—	+
	2S-4	N	+	—	+
	I13-114	N	+	—	—
	I14-180	N	+	—	—
	6S-5	SP	+	—	—
Others	I14-112	P	+	+	+
	I14-124	P	+	+	+
	I15-81	P	+	+	+
	I14-38	P	+	+	—
	10S-2	P	+	+	—
	19S-1	N	+	—	—
	I13-163	N	+	—	+
	I1S-5	N	—	—	—
	8S-1	P	—	+	—

P: pleomorphic, SP: slightly pleomorphic, N: non-pleomorphic, +: positive, and moderate growth,

Twenty-seven strains (1S-3–22S-8) were isolates from twenty-two stations in Otsuchi Bay and eight strains (I13-114–I15-81) from the Indian Ocean were supplied by Dr. Simidu, Ocean Research Institute, University of Tokyo. The strains from Otsuchi Bay were isolated in August using the seawater agar (Bacto-Peptone (Difco) 5.0 g, Bacto-Yeast Extract (Difco) 1.0 g, agar 20.0 g, seawater 1,000 ml, pH

Table 1.

Growth at various salt concentrations						Growth
0.5%	3%	5%	7%	10%	15%	5°
+	++	++	++	-	-	+
+	+	++	++	++	-	+
+	++	++	++	+	-	+
+	+	+	++	+	-	+
+	++	++	+	-	-	+
+	+	++	++	+	-	+
+	++	+	+	-	-	+
+	++	+	+	-	-	+
+	++	++	++	+	-	+
+	+	++	++	+	-	+
+	+	++	++	+	-	+
+	++	+	+	-	-	+
+	+	++	++	-	-	+
+	+	++	++	++	-	+
-	+	+	-	-	-	+
+	++	+	+	+	-	+
-	+	+	++	-	-	+
-	+	+	++	-	-	+
+	++	+	+	-	-	+
-	+	++	-	-	-	+
-	+	++	+	+	+	+
++	++	+	-	-	-	+
-	+	++	++	+	-	+
-	++	+	+	+	-	+
-	+	++	+	+	-	+
-	++	+	-	-	-	+
-	++	+	-	-	-	+
-	++	++	++	+	+	+
-	++	+	+	+	+	+
++	+	-	-	-	-	+
+	+	+	-	-	-	+
+	+	+	-	-	-	+
+	+	++	+	+	-	+
++	+	-	-	-	-	+

nutrient) and the last number after the hyphen denotes the order of dominance (1 is the strain from the most dominant colony).

Bacteriological procedures. The cell morphology was determined in cells grown in marine broth (Marine Agar 2216 minus agar) for 3–26 hr at 30°C with shaking. The effects of the salt concentration on the growth of the isolates were examined with nutrient broth (Bacto-Beef Extract 3.0 g, Bacto-Peptone 5.0 g, distilled water 1,000 ml, pH 7.6) with the sodium chloride added. The salt

(continued)

at various temperatures			Optimal growth temperature (ca. °C)	Ubiquinone type	GC content of DNA (mol %)
20°	30°	37°			
+	++	++	34	Q-8	40.9
+	++	++	35	Q-8	44.0
+	++	++	34	Q-8	44.4
+	++	++	36	Q-8	44.6
+	++	++	36	Q-8	45.0
+	++	++	37	Q-8	45.0
+	++	++	37	Q-8	45.3
+	++	++	33	Q-8	45.4
+	++	+	31	Q-8	45.6
+	++	++	37	Q-8	45.7
+	++	++	35	Q-8	45.8
+	++	++	36	Q-8	45.8
+	++	++	33	Q-8	46.0
+	++	++	37	Q-8	46.3
+	++	++	33	Q-8	46.9
+	++	+	33	Q-8	42.5
+	++	++	34	Q-8	41.3
+	++	++	33	Q-8	44.1
+	++	++	33	Q-8	44.4
+	++	++	37	Q-8	46.1
++	+	—	27	Q-8	41.3
++	+	—	27	Q-8	43.1
++	+	—	25	Q-8	44.8
++	+	—	27	Q-8	45.7
+	++	+	33	Q-8	45.9
+	++	++	34	Q-8	46.4
++	+	—	23	Q-8	50.5
++	+	—	24	Q-8	51.4
+	++	++	35	Q-9	57.0
+	++	++	36	Q-9	59.1
++	++	—	28	Q-9	61.6
++	+	—	26	Q-10	58.1
++	++	+	32	Q-10	61.8
+	++	+	32	Q-8	47.4
++	++	—	26	Q-9	41.1

concentrations used were 0.5, 3, 5, 7, 10 and 15%. The penicillin resistance was tested by incubating the organisms in the marine broth with a specified amount of benzylpenicillin potassium added. Nitrate reduction was tested using marine broth containing 0.1% KNO₃. Oxidation-fermentation was tested using a complex medium consisting of OF basal medium prepared with half-strength artificial seawater (NaCl 13.75 g, MgCl₂·6H₂O 2.5 g, MgSO₄·H₂O 1.0 g, CaCl₂ 0.25 g, KCl 0.5 g, FeSO₄·7H₂O 0.005 g, distilled water 1,000 ml, pH 7.6). The growth of the

isolates at various temperatures was examined by incubating the isolates in marine broth for 3–7 days. The optimal growth temperature was taken to be the growth temperature giving the highest optical density at 660 nm after incubating for 5–7 hr in marine broth using Toyo Kagaku Sangyo Temperature Gradient Model TN-3.

Chemotaxonomic procedures. The cells used for the chemotaxonomic analysis were obtained with a test-tube shaking culture with marine broth at 30°C for ca. 24 hr. Ubiquinone was extracted and analyzed by the methods of CRANE and DILLEY (4), and YAMADA and KONDO (5). Cellular fatty acid composition was analyzed by gas chromatography. The details of these two procedures were described in previous papers (6, 7). The G + C contents of DNA were analyzed by high performance liquid chromatography using a 5 cm column of Shim-Pack WAX-1 (Shimadzu Co., Ltd., Kyoto, Japan) after hydrolysis of the purified DNA by nuclease P1 (Yamasa Shoyu Co., Ltd., Choshi, Japan). Phosphate buffer (50 mM, pH 2.83) was used as eluent. The method is essentially the same as the method of KATO et al. (8).

RESULTS AND DISCUSSION

Many of the isolates were pleomorphic, and the degree of pleomorphism was different in the strains. As shown in the Fig. 1, the isolates were generally plump rods in the early logarithmic phase, changing to slender rods and to coccoid form in the late logarithmic to stationary phase. Some strains were also elongated in the logarithmic phase. All of the isolates were non-motile, non-sporeforming, gram-negative, catalase positive and strictly aerobic. Cytochrome oxidase was positive in most of the isolates (Table 1). The reduction of nitrate was positive in twenty-four strains. Aerobic production of acid from carbohydrates was different in the strains, and it was efficient for grouping the isolates. Twenty-six strains were penicillin resistant (100 IU). Twenty-nine strains were Q-8 ubiquinone type, four were Q-9 and two were Q-10. Twenty-six strains were mesophiles and nine strains were psychrotrophs. The term psychrotroph was used according to the definition of MORITA (9). That most strains were mesophiles agrees with HAUXHURST et al. (3) for the *Moraxella-Acinetobacter* group. The optimal growth temperatures are listed in Table 1. The G + C content of DNA was 40.9% to 47.4% for twenty-eight strains and 50.5% to 61.8% for seven strains. On the basis of these data, the isolates were divided into group 1, group 2 and others as shown in Table 1. The members of group 1 were pleomorphic, cytochrome oxidase positive, penicillin resistant, had the Q-8 ubiquinone system, produced acid from carbohydrates, their nitrate reduction was mostly positive, and the G + C content of their DNA was 40.9% to 46.9%. Group 1 coincided with the genus *Acinetobacter* (*A. calcoaceticus*, type species) in its cell morphology, penicillin resistance and acid production from carbohydrates, and with the genus *Moraxella* in its cytochrome oxidase and ubiquinone type. For the ubiquinone types of the genera *Acinetobacter* and *Moraxella*, we previously determined that *A. calcoaceticus* ATCC23055 (type strain) and *A. lwoffii*

Fig. 1. Photographs of three strains of isolates. a, b, and c are pictures of strains 14S-4, 5S-4, and 2S-4 respectively. Strains 14S-4 and 5S-4 are pleomorphic, and strain 2S-4 is non-pleomorphic. The pictures were taken of cells cultivated for 3, 7, and 26 hr from left to right in the figure.

Table 2. Cellular fatty acid

	Strains	10:0	12:0	14:0	15:0	16:0
Group 1	1S-3		2.3	4.2	2.8	12.6
	15S-2		2.0	2.8	1.5	17.3
	22S-5		2.2	3.9	2.4	17.5
	22S-8	0.6	2.6	4.2	1.5	17.1
	8S-2		3.0	5.1	2.7	13.1
	14S-4		2.8	4.5	2.0	17.4
	17S-7		2.3	4.6	2.9	13.7
	5S-4		1.7	3.4	1.8	14.5
	3S-2	0.3	2.0	3.2	2.7	13.9
	20S-3		2.6	3.9	2.9	13.3
	16S-2		2.5	3.7	2.0	11.0
	21S-1			3.5	1.7	14.1
	13S-3		2.3	3.7	3.4	12.0
	7S-1		2.1	3.3	4.7	11.8
	6N-1		1.8	3.1	1.5	14.8
	7S-2		2.8	4.0	1.6	19.7
	22S-6		1.3	0.8	3.1	18.6
	I14-43		5.6	1.8	1.4	17.1
	18S-1			1.2		20.7
	4S-1	0.4	2.1			14.3
Group 2	3S-7			1.4	4.6	20.4
	9S-5					0.6
	2S-4		1.9			1.3
	I13-114	2.0	3.0	0.4		0.9
	I14-180	1.8	2.0	0.5		0.8
	6S-5		4.8	2.3		7.5
Others	I14-112			4.2		31.1
	I14-124	2.1	0.6	14.6		20.5
	I15-81	0.6	0.3	3.3		13.5
	I14-38		4.3	1.3	0.7	28.9
	10S-2	0.8	0.4	0.2	0.1	36.7
	19S-1	0.8		0.2		
	I13-163		0.4		0.3	2.1
	11S-5		2.3	4.3	0.6	18.0
	8S-1		5.3	0.4		17.1

Fatty acid composition is shown in %.

Number preceding colon indicates number of carbon atoms; number after colon designates degree

ATCC15309 (type strain) were Q-9, and *M. lacunata* ATCC17967 (type species and strain) *M. bovis* ATCC10900 (type strain) and *M. nonliquefaciens* ATCC19975 (type strain) were Q-8 (unpublished data). Based on these results, group 1 should be classified as a separate genus related closely to the genera *Acinetobacter* and *Moraxella*. The members of group 2 were non-pleomorphic or slightly pleomorphic, cytochrome oxidase positive, penicillin sensitive, ubiquinone type Q-8, did not

composition of the isolates.

16:1	17:1	18:1	18:2	Other compositions
42.1	4.1	30.2		1.7
42.4	6.5	25.8	0.4	1.3
40.2	5.5	25.4	0.3	2.6
40.0	4.4	26.7	0.2	2.7
41.3	3.8	28.8	0.2	2.0
41.6	4.5	25.3	0.2	1.7
43.7	4.1	26.9		1.6
41.6	5.9	27.7	0.5	2.9
44.1	5.4	20.0	0.1	8.3
40.6	5.8	29.0	0.2	1.7
41.2	4.4	30.9	0.1	4.2
42.8	6.2	28.3	0.3	3.1
43.2	6.8	27.0	0.3	1.3
40.9	9.7	25.9	0.3	1.3
40.4	7.4	27.3	0.5	3.2
45.6		23.1		3.2
39.8	13.2	20.3	0.3	2.6
34.5	13.0	23.1	1.0	2.5
30.5	6.8	35.1	0.9	4.8
41.4	3.1	19.3	0.4	19.0 (14:1, 15.9%)
48.3	12.3	11.1	0.5	1.4
7.8	7.9	81.9	1.5	0.3
22.5	8.5	63.9	0.5	1.4
6.7		84.7	0.5	1.8
8.6	1.5	81.6	0.7	2.5
37.8		45.7		1.9
50.1	0.2	12.6		1.8
53.7		5.3	0.1	2.2
6.6		67.3		8.4
26.4		34.9		3.9
16.1	14.8	15.6	0.3	15.0 (17:0, 6.2%)
		88.4		10.6
		84.1	1.2	11.9 (17:0, 3.4%; 18:0, 4.2%)
33.3		34.0		7.3
42.7		32.2		2.3

of unsaturation.

produced acid from carbohydrates, and they had the G+C contents of 41.3% to 46.4%. In these characteristics, group 2 corresponded to the genus *Moraxella*. But strains 3S-7, 9S-5, 2S-4, and I13-114 were psychrotrophic. Therefore we tentatively call these four strains "psychrotrophic *Moraxella*." JUNI and HEYM(10) used "psychrotrophic *Achromobacter*" for psychrotrophic coccobacilli (*Moraxella*-like bacteria) in their study of the transformation assay for identification. Those were

isolates from many sources including some from seawater. However, the name *Achromobacter* is not valid, so we used the above name in this paper. Our strains and the strains used by JUNI and HEYM should be examined to clarify their relationship. In the other isolates, strains I14-112 and I14-124 showed the same characteristics as group 1 except for the acid production from carbohydrates and the optimal growth temperature, and their G + C contents of DNA were higher than those of group 1. The strains 10S-2, I14-38 and I15-81 were pleomorphic, cytochrome oxidase positive, penicillin resistant, and had ubiquinone type Q-9 and G + C contents of 57.0% to 61.6%. The strains 19S-1 and I13-163 had the same phenotype as group 2 with regard to morphology, cytochrome oxidase, penicillin sensitivity etc., but they were different in ubiquinone type (Q-10) and the G + C contents of DNA. These seven strains in which the G + C content of the DNA was higher should be treated as groups far removed from the genera *Acinetobacter* and *Moraxella*, and their taxonomic positions are unclear. Strain 11S-5 was non-pleomorphic, cytochrome oxidase negative, penicillin sensitive, nitrate reduction negative, produced acid from carbohydrates, had ubiquinone type Q-8 and the G + C content was 47.4%. Accordingly, strain 11S-5 also was recognized as a different organism related closely to the genera *Acinetobacter* and *Moraxella*. Strain 8S-1 was pleomorphic, cytochrome oxidase negative, penicillin resistant, had ubiquinone type Q-9 and the G + C content was 41.1%. Therefore it belonged to the genus *Acinetobacter* and was identified as a *Acinetobacter lwoffii* (Audureau 1940) BRISOU and PRÉVOT 1954 (11) based on its failure to produce acid from carbohydrates.

The major cellular fatty acids in group 1 were $C_{16:1}$, $C_{18:1}$, and $C_{16:0}$, except in the strains 4S-1 and 18S-1. Strain 4S-1 was unique in containing 15.9% $C_{14:1}$. In group 2, the major fatty acids of strains 2S-4 and 6S-5 were $C_{18:1}$ and $C_{16:1}$, that of strains 9S-5, I14-180 and I13-114 was $C_{18:1}$, and those of strain 3S-7 were $C_{16:1}$ and $C_{16:0}$. These results may be sufficient to divide groups 1 and 2 into some subgroups. Especially, the strains that had $C_{18:1}$ as the major fatty acid are unique in the *Moraxella* species. The major fatty acids of the strain 8S-1 were $C_{16:1}$, $C_{18:1}$ and $C_{16:0}$, and this pattern agrees with that of *A. lwoffii* ATCC15309, $C_{16:1}$ (40.5%), $C_{18:1}$ (36.2%) and $C_{16:0}$ (15.8%) for the cells cultivated on nutrient broth (unpublished data).

The effects of salt concentration on growth of the isolates were shown in Table 1. The isolates, except strains 8S-1 and 10S-2, were moderately halophilic and many of them could grow in 3–10% salt concentrations. The degree of halophilism was different in the strains. For example, strains 3S-2, 4S-1, 13S-3, 17S-7, 22S-6, I14-180, I14-112, I14-124 and I14-38 grow well in 3% salt concentration and strains 3S-7, 6S-5, 9S-5 and 11S-5 grow well in 5%. This resembles the 3% optimum salt concentration for the isolates from the Arctic Beaufort Sea (2), and most of the strains isolated at 20°C tolerated a wide range of salt concentration.

Nutritionally, all of the isolates were eutrophic and could grow in the modified nutrient agar.

As mentioned in the introduction, there are some ecological studies of coccobacilli from seawater, but detailed taxonomic studies have not been conducted. SIMIDU et al. (1) reported that the strains tentatively identified as *Acinetobacter* were predominant among the bacteria isolated from Tokyo Bay, but the Kovacs' oxidase test of those strains was positive. Therefore it may resemble our group 1. KANEKO et al. (2) reviewed the numerical taxonomy of bacteria from the Arctic Beaufort Sea, and reported that many of the psychrophilic or psychrotrophic gram-negative rods with high pleomorphism could not be identified. The strains in their cluster H12 appeared to be *Acinetobacter* species. However they did not list the data of cluster H12 in their table, so we could not compare them with our data. HAUXHURST et al. (3) reported on the numerical taxonomy of bacteria from the Gulf of Alaska that the dominant bacteria in Northwest Gulf isolates were identified as being in the *Moraxella-Acinetobacter* group. The strains in cluster E23 had all the characteristics of the genus *Acinetobacter* and the strains in cluster E20 had those of *Moraxella*. Clusters E18, E19, E22 and E24 were intermediate cases which could be classified as either *Acinetobacter* or *Moraxella*. They reported that their strains were not pleomorphic, so they do not resemble our pleomorphic strains.

Several kinds of such coccobacilli have been isolated from the coasts of bays, gulfs and oceans as one of dominant bacteria. Our isolates from seawater are mostly eutrophic, halophilic and mesophilic, so they are regarded as being originally terrestrial and then adapting to seawater. However, a precise conclusion awaits further comparative and physiological studies.

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