Original

Identification of Yellow-Pigmented Bacteria Isolated from Hospital Tap Water in Japan and Their Chlorine Resistance

KATSUNORI FURUHATA^{1*}, YUKO KATO², KEIICHI GOTO², KEIKO SAITOU³, JUN-ICHI SUGIYAMA³, MOTONOBU HARA⁴, AND MASAHUMI FUKUYAMA¹

¹*School of Environmental Health, Azabu University,
1-17-71, Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*

²*Food Research Laboratories, Mitsui Norin Co., Ltd.,
223-1, Miyahara, Fujieda, Shizuoka 426-0133, Japan*

³*Research and Investigation Department, Building Management Education Center,
1-4-28, Mita, Minato, Tokyo 108-0073, Japan*

⁴*School of Veterinary Medicine, Azabu University,
1-17-71, Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*

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Twenty-five yellow chromogenic strains isolated from hospital tap water samples collected nationwide were identified by partial 16S rDNA sequencing. In addition, the chlorine resistance of the isolates was experimentally investigated. The results showed that of the strains tested, 12 strains (48.0%) were *Sphingomonas ursincola/natatoria*, which was most frequently identified, followed by 2 strains (8.0%) of *Mycobacterium frederiksbergense* and 1 strain (4.0%) each of *Sphingomonas adhaesiva*, *Sphingopyxis witflariensis* and *Porphyrobacter donghaensis*. The other strains were not identified clearly but they belonged to the order of *Alphaproteobacteria*. On the other hand, the identification results by sequencing and biochemical property testing were not consistent in any of the strains, showing that it was difficult to accurately identify the yellow chromogenic bacteria in tap water based on only their biochemical properties.

When the 25 isolates were exposed to 0.1 mg/l residual free chlorine for 1 minute, 22 isolates (88.0%) survived. When the CT (Concentration Time) value killing 99.99% of the bacteria was investigated in 6 of these survivors, *M. frederiksbergense* (Y-1 strain) was most resistant to chlorine with the CT value of 32 mg · min/l, followed by *S. ursincola/natatoria* (Y-7 strain) with the CT value of 3.3 mg · min/l. The CT values of Y-5 (*Sphingomonas* sp.), Y-27 (*S. ursincola/natatoria*) and Y-21 (*Asticacaulis* sp.) were within the range of 0.9-0.1 mg · min /l. Of the 6 strains, *S. adhaesiva* (Y-10) showed the weakest resistance with the CT value of 0.03 mg · min/l. It was clarified that most yellow chromogenic bacteria isolated from hospital tap water were *Sphingomonas* spp., and these bacteria were experimentally resistant to chlorine.

Key words : Yellow-pigment/*Sphingomonas* sp./Tap water/Hospital/Identification/
Chlorine resistance.

*Corresponding author. Tel : +81-42-754-7111, Fax : +81-42-754-6215.

INTRODUCTION

Since washing hands with sterile water before surgery is not required and a switch to tap water had been approved in Japan, we investigated oligotrophic bacteria in hospital tap water in Japan (Furuhata and Fukuyama, 2006). The result showed that oligotrophic bacteria were isolated from about 80% of hospital tap water samples collected nationwide, and no significant regional difference was noted. The residual chlorine concentration in tap water from which oligotrophic bacteria were isolated was 0.1-0.8 mg/l, and oligotrophic bacteria were isolated from 80% of tap water samples containing residual free chlorine, although the chlorine concentration varied. Most isolates were gram-negative rods, but only about 40% of these could be identified clearly by biochemical property testing alone (*Methylobacterium* spp. and *Pseudomonas* spp.).

Furthermore, many of the other unidentified isolates produced water-insoluble yellow pigments.

In 1977, Holmes et al. performed a taxonomic study of yellow chromogenic gram-negative rods derived from human clinical specimens and hospital environments, and proposed a new species, *Pseudomonas paucimobilis*. O'Brien (1992) investigated biochemical properties of yellow chromogenic non-fermentative gram-negative rods isolated from disinfected tap water, but he could not identify the bacteria and reported it as a new *Pseudomonas* species. Since these yellow chromogenic bacteria have been suggested to be causative bacteria of opportunistic infections, a taxonomic study of the unidentified isolates from hospital tap water has become necessary.

In this study, yellow chromogenic bacteria isolated from hospital tap water were subjected to identification by genetic analysis. Since many yellow chromogenic bacteria were isolated from hospital tap water containing residual chlorine, the chlorine resistance of the isolates was also experimentally confirmed.

MATERIALS AND METHODS

Bacterial strains and cultivation

In 2004, 271 samples of tap water in hospitals in 18 prefectures of Japan were cultured on R2A agar medium (0.5 g pepton, 0.5 g yeast extract, 0.5 g caseamino • acid, 0.5 g glucose, 0.5 g soluble starch, 0.3 g K₂HPO₄, 0.05 g MgSO₄•7H₂O, 0.3 g sodium pyruvate and 15 g agar per liter, pH7.0-7.4; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 30°C for 7 days. In total, 25 colonies with yellow pigmentation

TABLE 1. Isolated regions of test strains, and the concentration of free residual chlorine when isolated from the tap water in hospitals.

Prefecture	Free residual chlorine (mg/l)					Total
	0	0.1-0.2	0.3-0.4	0.5-0.6	UN*	
Miyagi	1**	0	0	0	0	1
Tokyo	0	4	1	0	2	7
Kanagawa	0	0	0	2	3	5
Chiba	0	1	0	0	0	1
Saitama	0	1	1	1	0	3
Gunma	0	0	0	0	1	1
Ibaraki	0	0	0	0	1	1
Nagano	0	0	1	0	0	1
Shizuoka	0	1	0	0	0	1
Osaka	0	0	0	0	1	1
Tokushima	0	1	0	0	0	1
Fukuoka	0	0	1	0	0	1
Okinawa	0	0	0	0	1	1
Total	1	8	4	3	9	25

* : Unknown

** : Number of strains

which were formed by Gram-negative and positive rod-shaped bacteria were collected and stored at -80°C (Table 1). The stored strains were cultured on R2A agar medium at 30°C for 7 days and used for further study.

Biochemical tests

20 biochemical properties were tested using API20NE (bioMérieux, Marcy l'Etoile, France) following the protocol of the manufacturer. The results were analyzed using the analytical software, APIWAB Ver 1.1.0 (bioMérieux).

Identification by partial 16S rDNA sequence analysis

Genomic DNA was extracted and purified by using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the protocol of the manufacturer. Using the extracted DNA solution as the template for PCR, the 5' end partial region of 16S rRNA gene (about 500bp) was amplified using the MicroSeq 500 16S rDNA PCR Kit (Applied Biosystems, Foster City, CA, USA). The PCR products were purified using the Quantum Pre PCR Kleen Spin Columns (Bio-Rad Laboratories, Hercules, CA, USA). The sequencing reactions of the PCR products were performed using the MicroSeq 500 16S rDNA sequencing Kit (Applied Biosystems) and the reaction products were purified with AutoSeq™ G-50 (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). A model ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) was used for

TABLE 2. Comparison of species identified by 16S rDNA sequence analysis with those by using the API20NE system.

Strains	Species identified by 16S rDNA sequence analysis	Species identified by using the API20NE system	API profile	ID %
Y-2	<i>Sphingomonas ursincola/natatoria</i>	<i>Pasteurella</i> sp.	1000004	47.9
Y-6	<i>Sphingomonas ursincola/natatoria</i>	<i>Aeromonas salmonicida</i>	1000204	60.9
Y-7	<i>Sphingomonas ursincola/natatoria</i>	<i>Pasteurella</i> sp.	1000200	73.3
Y-8	<i>Sphingomonas ursincola/natatoria</i>	<i>Moraxella</i> sp.	0000004	82.2
Y-11	<i>Sphingomonas ursincola/natatoria</i>	<i>Acinetobacter lowffii</i>	0000200	38.4
Y-13	<i>Sphingomonas ursincola/natatoria</i>	<i>Aeromonas salmonicida</i>	1040204	91.2
Y-25	<i>Sphingomonas ursincola/natatoria</i>	<i>Brevundimonas vesicularis</i>	0000204	58.3
Y-27	<i>Sphingomonas ursincola/natatoria</i>	<i>Comamonas testosteroni</i>	1204440	77.5
Y-37	<i>Sphingomonas ursincola/natatoria</i>	Unknown	0000000	
Y-56	<i>Sphingomonas ursincola/natatoria</i>	Unknown	0000000	
Y-74	<i>Sphingomonas ursincola/natatoria</i>	<i>Aeromonas salmonicida</i>	1000204	60.9
Y-81	<i>Sphingomonas ursincola/natatoria</i>	<i>Acinetobacter lowffii</i>	0000200	38.4
Y-1	<i>Mycobacterium frederiksbergense</i>	<i>Comamonas testosteroni</i>	1004440	56.1
Y-20	<i>Mycobacterium frederiksbergense</i>	Unknown	0005440	
Y-10	<i>Sphingomonas adhaesiva</i>	<i>Sphingomonas paucimobilis</i>	0442164	78.0
Y-44	<i>Sphingophyxis witflariensis</i>	<i>Brevundimonas vesicularis</i>	0400200	99.7
Y-57	<i>Porphyrobacter donghaensis</i>	<i>Sphingomonas paucimobilis</i>	0062204	49.9
Y-3	<i>Sphingomonas</i> sp.	Unknown	0000000	
Y-5	<i>Sphingomonas</i> sp.	<i>Sphingomonas paucimobilis</i>	0463341	97.7
Y-34	<i>Sphingomonas</i> sp.	<i>Pasteurella haemolytica</i>	0020004	84.6
Y-63	<i>Sphingomonas</i> sp.	<i>Sphingomonas paucimobilis</i>	0461640	99.8
Y-21	<i>Asticcacaulis</i> sp.	<i>Brevundimonas vesicularis</i>	0460205	52.3
Y-38	<i>Asticcacaulis</i> sp.	<i>Brevundimonas vesicularis</i>	0440004	99.6
Y-84	<i>Asticcacaulis</i> sp.	<i>Brevundimonas vesicularis</i>	0400000	78.6
Y-51	<i>Novosphingobium</i> sp.	<i>Moraxella</i> sp.	0000004	82.2

sample electrophoresis and data collection. The obtained sequence data were compared with reference data from GenBank/EMBL/DDBJ, and a phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (1987). The isolated strains were identified on the basis of more than 99% similarity values that the same group or species showed.

Choline resistance tests

As an assumption test, 10^6 CFU/ml bacteria were exposed to 2 ml of 0.1 mg/l residual free chlorine solution for 1 minute, followed by the immediate addition of 2 ml of nutrient broth, for chlorine consumption. The bacteria were then kept at 30°C for 7 days, and the turbidity was observed. The CT value

was measured as follows: Sodium hypochlorite dilutions were added to 200 ml of sterile distilled water to prepare 0.1-1.0 mg/l experimental solutions. The test bacteria were cultured on R2A agar medium at 30°C for 5 days beforehand, and isolated colonies were suspended with sterile distilled water to prepare a bacterial suspension of about 10^8 CFU/ml. The bacteria were seeded in the experimental solutions to adjust the concentration to 10^6 CFU/ml. After exposure to chlorine with stirring at room temperature for the specified time, 3 ml of this solution was added to a sterile tube containing 50 μ l of 0.3 N sodium thiosulfate solution to neutralize the residual chlorine. After completion of the series of experiments, each solution was diluted, and 0.1 ml was smeared on R2A agar medium and cultured at 30°C for 7 days, and the

TABLE 3. CT values (99.99%) of yellow pigmented bacteria isolated from the tap water in hospitals.

Strains	Species	CT value (mg•min/l)
Y-1	<i>Mycobacterium frederiksbergense</i>	32
Y-7	<i>Sphingomonas ursincola</i> or <i>S. natatoria</i>	3.3
Y-5	<i>Sphingomonas</i> sp.	0.87
Y-27	<i>Sphingomonas ursincola</i> or <i>S. natatoria</i>	0.45
Y-21	<i>Asticcacaulis</i> sp.	0.095
Y-10	<i>Sphingomonas adhaesiva</i>	0.03

FIG. 1. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial region sequences (405bp). *Brevundimonas subvibrioides* (AJ227784) and *Asticcacaulis taihuensis* (AY500141) served as the out groups. The data set was resampled 1,000 times by using the bootstrap option and the percentage values are given at the nodes. The scale bar indicates the number of substitutions per nucleotide position.

FIG. 2. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial region sequences (406bp). *Brevundimonas subvibrioides* (AJ227784) served as the out group. The data set was resampled 1,000 times by using the bootstrap option and the percentage values are given at the nodes. The scale bar indicates the number of substitutions per nucleotide position.

FIG. 3. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial region sequences (439bp). *Nocardia abscessus* (AY544980) served as the out group. The data set was resampled 1,000 times by using the bootstrap option and the percentage values are given at the nodes. The scale bar indicates the number of substitutions per nucleotide position.

CFU were measured. The CT value was calculated by multiplying the residual chlorine concentration killing 99.99% of bacteria by the exposure time.

RESULTS

Phylogenetic analysis of tap water-derived yellow-pigmented bacteria by partial 16S rDNA sequence analysis

Phylogenetic trees based on the 16S rDNA sequences of the test strains and related bacteria are shown in Figs. 1-3. According to these trees, 12 isolates (Y-2, Y-6, Y-7, Y-8, Y-11, Y-13, Y-25, Y-27, Y-37, Y-56, Y-74 and Y-81) belonged to the *Sphingomonas ursincola* and *S. natatoria* group. Three isolates (Y-21, Y-38 and Y-84) were most related to the previously known species of the genus *Asticcacaulis* with less than 97.0% similarity values, and those levels of similarity were novel species. Y-44 was identified as *Sphingopyxis witflariensis*, and Y-57 was identified as *Porphyrobacter donghaensis*. Y-51 and Y-63 clustered with species of the genera *Novosphingobium* and *Sphingomonas* respectively.

Y-10 was clustered with *Sphingomonas adhaesiva*. Y-3, Y-5 and Y-34 clustered with *Sphingomonas* species, but they could not be identified clearly. Y-1 and Y-20 were identified as *Mycobacterium frederiksbergense*.

Comparison with species identified by 16S rDNA sequence analysis and that of using the API20NE system

The results of identification by 16S rDNA sequence analysis are shown in Table 2. Twelve strains (48.0%) were identified as *Sphingomonas ursincola*/*natatoria* by the 16S rDNA analysis, which was the highest population, followed by 2 strains (8.0%) identified as *Mycobacterium frederiksbergense*. One strain each (4.0%) was identified as *Sphingomonas adhaesiva*, *Sphingopyxis witflariensis* and *Porphyrobacter donghaensis*.

Some species identified using the API20NE system were shown in Table 2. When identification probability (ID%) were more than 80%, two strains each (8.0%) were respectively identified as *Brevundimonas vesicularis*, *Sphingomonas paucimobilis* and

Moraxella spp.. One strain each (4.0%) was identified as *Aeromonas salmonicida* and *Pasteurella haemolytica* respectively. The other strains were not identified clearly.

The identification results by sequence analysis and biochemical property testing were not consistent in any of the strains, clarifying that accurate identification results cannot be obtained by biochemical property testing alone.

Chlorine resistance of test strains

When 25 isolates were exposed to 0.1 mg/l residual free chlorine for 1 minute, the medium became turbid in 22 isolates (88.0%), showing their survival. No turbidity of medium was noted in 3 strains (12.0%), indicating that the bacteria were completely killed. Table 3 shows the 99.99% CT values of 6 strains. *M. frederiksborgense* (Y-1 strain) was most resistant to chlorine with the CT value of 32 mg•min/l, followed by *S. ursincola/natatoria* (Y-7 strain) with the CT value of 3.3 mg•min/l. The CT values of Y-5 (*Sphingomonas* sp.), Y-27 (*S. ursincola/natatoria*) and Y-21 (*Asticacaulis* sp.) were within a range of 0.9-0.1 mg•min/l. Among the 6 strains, resistance of the Y-10 strain (*S. adhaesiva*) was the weakest, and the CT value was 0.03 mg•min/l.

DISCUSSION

Out of oligotrophic bacteria isolated from hospital tap water, yellow chromogenic bacteria were subjected to identification, and many of these strains belonged to the gram-negative genus *Sphingomonas*. Although surveys of oligotrophic bacteria in tap water have been reported (Carter et al., 2000), no study has identified isolates in detail. Many oligotrophic bacterial strains produce various water-insoluble pigments, which are important properties for differentiation. Oligotrophic yellow chromogenic bacteria in tap water identified as *Pseudomonas* sp. (O'Brien, 1992) and *Flavobacterium* sp. (Reasoner, et al., 1989) based on the pigment colors have been reported, but our report is the first study which identifies the genus *Sphingomonas*. The most frequently identified species in this study, *Sphingomonas ursincola/natatoria*, was proposed as a new *Sphingomonas* species by Yabuuchi et al. (1999, 2002). A gram-positive bacteria, *Mycobacterium frederiksborgense* was also identified. *M. frederiksborgense* is an acid-fast bacterium newly named by Williamsen et al. (2001). Isolation of non-tuberculous mycobacteria from tap water has been reported (Miyamoto et al., 2000), and its influence is a health matter. Chang et al. (2002) detected non-tuberculous mycobacteria in hospital tap water at

a rate of 20.4%, showing the risk of infection in hospitals.

Chlorine resistance of isolates was also investigated in this study. Mir et al. (1997) compared the chlorine resistance of 6 isolates from water samples collected from water purification processes in which residual chlorine was present, and found that gram-positive bacteria, such as *Bacillus* sp. and *Micrococcus* sp., were more resistant to chlorine than gram-negative *Pseudomonas* sp.. In our study, the CT value (99.99%) of gram-positive *M. frederiksborgense* was the highest (32 mg•min/l), in good accordance with the findings reported by Mir et al. (1997). Since the CT value of *E. coli* was about 0.01 mg•min/l, the chlorine resistance of *M. frederiksborgense* may be more than 1,000 times that of *E. coli*. Strong chlorine resistance of acid-fast bacteria has been clarified by Grabow et al. (1983, 1984). They exposed *M. fortuitum* to 0.4 mg/l residual free chlorine for 15 minutes, but most of the seeded bacteria at 10³ CFU/ml were not killed. We previously reported that *Methylobacterium* widely present in tap water was relatively resistant to chlorine among gram-negative bacteria (Hiraishi et al., 1995; Furuhashi et al., 2006). In the *Sphingomonas* spp. investigated in this study, the CT value (99.99%) of some strains was 3.3 mg•min/l, showing that their chlorine resistance was as strong as that of *Methylobacterium*, although the bacteria were gram-negative, and their isolation from tap water containing residual chlorine was convincing.

Kelley et al. (2004) recently detected *Sphingomonas* spp. on a shower curtain, and recommended its rapid removal in consideration of the possibility that it could be a pathogen causing opportunistic infection. When Oie et al. (2006) investigated microbial contamination of the ultrasonic nebulizer solution used in hospitals, *Sphingomonas* spp. was isolated. Thus, they recommended a thorough bactericidal treatment taking a shorter time. Similarly, the possibility of *Sphingomonas* spp. isolated from hospital tap water to cause opportunistic infection cannot be ruled out. Since complete elimination of this species from tap water is impossible at present, recognition of its presence in tap water, and specific considerations for handling tap water in hospitals are necessary.

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