資　料

表1　文献一覧

1）微生物汚染実態に関する文献

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2	猪又明子	東京都健康安全研究センター	水道水中の従属栄養細菌の同定におけるDNA塩基配列解析法と表現性状試験との比較	水環境学会誌	31(10)	609-614	2008
3	Furuhata, K	麻布大学	Identification of yellow-pigmented bacteria isolated from hospital tap water in Japan and their chlorine resistance （病院水道水から単離した黄色色素細菌の同定）	Biocontrol Science	12(2)	39-46	2007
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5	古畑勝則	麻布大学	病院内水道水からの貧栄養細菌の分離状況	防菌防黴	34(6)	323-328	2006
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No.	著者	所属	タイトル	掲載誌	巻、号	ページ	発行年
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Original

Identification of Heterotrophic Plate Count Bacteria Isolated from Drinking Water in Japan by DNA Sequencing Analysis

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Heterotrophic plate count (HPC) test has been employed to indicate the effectiveness of water treatment processes and the microbiological condition of the distribution system. In Japan, because the majority of HPC bacteria are supposed to be harmless and all tap water should maintain residual chlorine, there are few studies of the virulence of HPC bacteria. In this study, we examined HPC bacteria isolated from finished and tap water for hemolytic activity to determine their virulence potential. 34 of 39 colonies expressing hemolytic activity were identified by partial 16S rDNA sequencing, but some of their percent identity were relatively low. This may have been due to the mismatching of the primer pair with some strains, or these strains may be unidentified new species. A total of 30 of 34 isolates identified have been reported to be opportunistic pathogens or food poisoning bacteria. To control the growth of these opportunistic pathogens among HPC bacteria, appropriate water quality control must always be done and residual chlorine must be maintained in every tap for a safe water supply.

Key words : HPC bacteria/Drinking water/Hemolysis assay/16S rDNA sequencing/Opportunistic pathogen.

INTRODUCTION

The heterotrophic plate count (HPC) test has been considered to be a good indicator of the effectiveness of water treatment processes and the microbiological condition of the drinking water distribution system because the number of HPC bacteria in drinking water is greater than coliforms or standard plate count bacteria, and also HPC bacteria increase in the cases of the loss of residual disinfectant or the stagnation of water in the distribution system. In Japan, there have been few studies on the virulence of HPC bacteria because all tap water is regulated to maintain residual chlorine by law and, the majority of HPC bacteria found in tap water are supposed to be harmless (Japan Water Works Association, 2001). However, in Europe, United States, and South Africa, many studies on the virulence of HPC bacteria have been carried out. In these studies, some virulence factors were researched. To produce disease, bacteria must often attach to, penetrate, and multiply in epithelial cells or produce toxins or other substances that disrupt the normal metabolism of the attacked cells. In these countries, HPC bacteria expressing virulence factors such as hemolysis, cytotoxicity, invasiveness, and adherence, were isolated from drinking water (Lye and Dufour, 1991; Payment, Coffin and Paquette, 1994; Edberg et al., 1997; Pavlov et al., 2002; Stelma et al., 2004; Lye and Dufour, 2006). To evaluate the health risks of HPC bacteria, individual virulent HPC bacterium isolated from drinking water should be identified accurately. Nonetheless, in these previous studies, various phenotypic tests could not successfully identify most of the virulent HPC bacteria at the

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species level (Payment, Coffin and Paquette, 1994; Edberg et al., 1997; Pavlov et al., 2002). It is considered that the scale of the database from phenotypic tests used is insufficient for identification of HPC bacteria in the environment because phenotypic tests have been developed for the identification of pathogenic bacteria.

Recently, 16S rDNA sequencing analysis has been applied as an easy and reliable tool for the identification and classification of bacteria (Furuhata et al., 2007; Ultee et al., 2004; Williams et al., 2004; Tokajian et al., 2005; Singh et al., 2003). In the present study, we examined the growth ability at 37℃ and hemolytic activity of isolated HPC bacteria to determine the virulence potential of HPC bacteria in finished and tap water. Then, we identified hemolytic HPC bacteria by partial 16S rDNA sequencing. We also considered the health effects of HPC bacteria isolated from drinking water.

MATERIALS AND METHODS

Water Samples

Finished water (28 samples) and tap water (9 samples) from a small water supply utility in the Tokyo metropolitan area were collected from their water purification plants and water supply area, respectively. At the sampling point, the concentration of residual chlorine (free or combined) was measured. The water was then sampled in a sterile plastic bottle (200ml volume) containing sodium thiosulfate for neutralization of residual chlorine in drinking water.

Heterotrophic plate count (HPC) test

The HPC test was performed according to the established method in our laboratory (Hosaka and Maki, 2001). Briefly, a 1ml water sample was spread onto R2A agar (MERCK, Darmstadt, Germany). Then, it was incubated at 25℃, and colonies were counted after 7 days.

Virulence potential assay

We examined the 1) growth ability at 37℃, 2) growth ability at 44.5℃, and 3) hemolytic activity of HPC bacteria to determine their virulence potential. All HPC colonies showing morphological differences were selected. They were inoculated onto R2A agar again and incubated at 37℃ and 44.5℃. After 24 hours, growth ability at 37℃ and 44.5℃ was evaluated. Each strain grown at 37℃ was then inoculated into sheep blood agar (Poa Media, Eiken Chemical, Tokyo, Japan) to assay hemolytic activity. After 24 hours incubation at 37℃, the type of hemolysis was recorded as alpha, beta, or negative.

Identification of hemolytic HPC bacteria

HPC bacteria isolates expressing alpha or beta hemolysis were identified by partial 16S rDNA sequencing. Genomic DNA was extracted by alkali dissolution method according to Jiwa et al. (1989). Using the extracted DNA solution as the template for PCR, the 5' end partial region of 16S rRNA gene (about 500 bp) was amplified with MicroSeq 500 16S rDNA Bacterial Identification PCR Kit (Applied Biosystems, Foster City, CA, USA). The PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA, USA). The sequencing reaction of the PCR products was performed using MicroSeq 500 16S rDNA Bacterial Identification sequencing Kit (Applied Biosystems) and the reaction products were purified with CentriSep Spin Column (Applied Biosystems). A model ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) was used for sample electrophoresis and data collection. The obtained sequence data were compared with reference data from NCBI/EMBL/DDBJ. The closest matching sequences in NCBI/EMBL/DDBJ were identified by using basic local alignment search tool (BLAST).

RESULTS and DISCUSSION

HPC bacteria in water samples

HPC bacteria have been added to the water quality management target items since 2008, and the target level is 2,000 colonies or less in 1 ml. Microbiological and Biological Standard Methods for Examination of Water Investigative Committee of Japan Water Works Association (2006) performed the national survey of HPC bacteria in drinking water. In the report, the ratio of HPC bacteria positive samples was 46% in finished water at 0 to 89 cfu/ml and 64% in tap water at 0 to 600 cfu/ml.

The results of HPC bacteria detection and residual chlorine concentration of water samples are shown in TABLE 1. HPC bacteria were detected from 25 of 28 (89%) finished water samples ranging at 0 to 180 cfu/ml (average 27.9 cfu/ml) and from all 9 tap water samples at 2 to 42 cfu/ml (average 11.1 cfu/ml). The positive rates and the level of HPC bacteria in this study are almost same as those in the 2006 report. HPC bacteria in drinking water in Japan therefore seems to be found at 10^0 to 10^2 cfu/ml.

FIG. 1 shows the distribution of the number of HPC bacteria in finished and tap water. HPC bacteria at 1 to 9 cfu/ml were most frequently found in our study, which agrees with other reported results (Microbiological and Biological Standard Methods for Examination of Water Investigative Committee of

TABLE 1. Distribution of HPC bacteria isolated from finished and tap water and the free or combined residual chlorine concentration of the water samples.

Type of water	No. of sample examined	Positive sample No.	(%)	Heterotrophic bacteria count range			(average)	Residual chlorine concentration (mg/l) Free 0.1	0.2	0.3	0.4	0.5	0.5<	Combined 1.5
Finished water	28	25	(89.3)	0	–	180	(27.9)	0	8	5	8	2	1	1
Tap water	9	9	(100)	2	–	42	(11.1)	2	2	3	0	1	1	0
Total	37	34	(91.9)	0	–	180	(23.6)	2	10	8	8	3	2	1

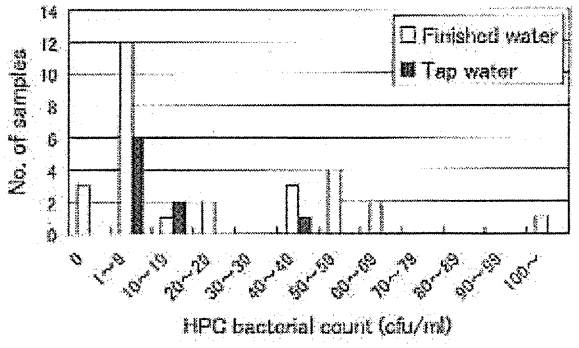

FIG. 1. Number of HPC bacteria in finished and tap water.

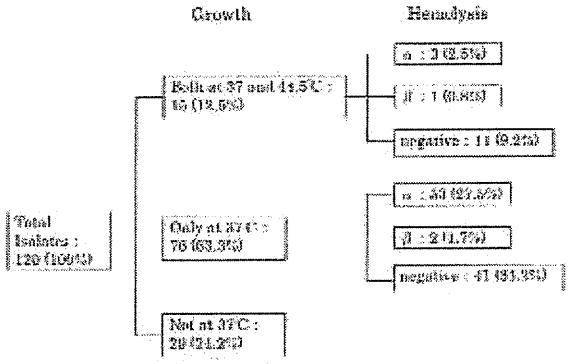

FIG. 2. Growth ability at 37 and 44.5℃ and hemolytic activity of isolated HPC bacteria.

Japan Water Works Association, 2006; Furuhata and Fukuyama, 2006).

Because all water samples in which HPC bacteria were isolated contained residual chlorine, those isolated HPC bacteria are thought to be resistant to chlorine disinfection. It is suggested that HPC bacteria can increase in biofilms on the surface of drinking water distribution systems because the positive rate of HPC bacteria in tap water was higher than in finished water.

Virulence potential assay

It is important to study the ratio of HPC bacteria showing virulence potential to evaluate their effects on health. FIG. 2 shows the growth ability at 37 and 44.5℃ and the hemolytic activity of isolated HPC bacteria. 91 isolates of 120 examined could grow at 37℃. This means the majority of HPC bacteria which were thought to prefer relatively low temperatures can multiply at body temperature. Among the isolates that grew at 37℃, 36 isolates revealed alpha hemolytic activity and 3 isolates revealed beta. The ratio of hemolytic isolates among all isolated HPC bacteria was 32.5%, which agrees with a previously reported ratio (36%) (Lye and Dufour, 2006). In reported results for another virulence potential cytotoxic assay, the ratio of cytotoxic isolates was extremely low such as 0.2%, 0.8%, and 1% (Lye and Dufour, 1994; Payment, Coffin and Paquette, 1994.; Lye and Dufour, 2006). Therefore, the hemolytic assay is a useful tool to determine virulent HPC bacteria.

On the other hand, thermotolerant HPC bacteria that grew at 44.5℃ were limited (12.5%) and hemolytic isolates were only 3.3% of all isolates. Hosaka and Maki (2001) showed that HPC bacteria isolated on oligotrophic media (R2A or PGY) are more numerous than those isolated on tryptone-glucose-yeast extract agar even if the incubation temperature is low. Therefore, HPC bacteria might be a different bacterial group from Enterobacteriaceae in terms of their thermotolerance and minimum nutrient requirements.

Identification of hemolytic HPC bacteria

Identification results from the 16S rDNA sequencing analysis and their hemolysis type are summarized in TABLE 2. Recently, the advances of DNA sequencing technology have enhanced the identification of bacteria, and especially 16S rDNA sequencing analysis has been applied to the identification and classification of bacteria (Furuhata et al., 2007; Ultee et al., 2004; Williams et al., 2004; Tokajian et al., 2005; Singh et al., 2003). 16S rDNA sequencing analysis is superior to phenotypic tests in terms of its objectivity and reproductivity. Furthermore, as the sequence data recorded in public database are numerous, 16S rDNA sequencing analysis can be a useful tool for identification of bacteria to the species level.

We used the MicroSeq 500 kit to amplify the first 500 bp fragment that is most variable region of the 16S rDNA, but some isolates (i.e. No.2, No.3, No.6, No.8, No.12, No.23, No.32, No.33, and No.35) could not be identified to the species level. Furthermore, 5 of 39 isolates (i.e. No.5, No.7, No.16, No.22, and No.36) could not be amplified. Furuhata et al. (2007) reported several strains had been given 2 species name when using the MicroSeq 500 kit to identify yellow pigmented bacteria isolated from drinking water. We experienced that some *Legionella* isolates could not be amplified by MicroSeq 500 kit. In addition, the percent identity of 17 isolates was lower than 97%. Stackebrandt and Goebel (1994) proposed that 16S rDNA sequences with less than 97% identity were most likely to belong to different species. According to this hypothesis, the results of identified isolates with less than 97% identity were questionable. There are many unidentified species among the HPC bacteria, so these strains may be new species. Also, the MicroSeq 500 primer pair might not be matched with these isolates showing a low percent identity. Though the MicroSeq 500 kit has these shortcomings, this kit can perform 16S rDNA sequencing analysis simply and quickly. In this way, this kit is useful in analyzing many samples such as in a field survey. If some isolates cannot be amplified or identified by the MicroSeq 500 kit, then these isolates might be amplified by using another universal primer pair, or another gene (e.g. *hsp65*) or DNA gyrase B that shows more rapid evolution than 16S rDNA.

In our study, the obtained sequence data were compared with the reference data from NCBI/EMBL/DDBJ. The closest matching sequences in NCBI/EMBL/DDBJ were identified by using BLAST. However, there was not enough sequence data for HPC bacteria in NCBI/EMBL/DDBJ compared with that for pathogenic bacteria. For pathogenic bacteria, numerous sequences of known species such as ATCC strains have been recorded in NCBI/EMBL/DDBJ, and it is easy to get the closest matching species sequences. In the cases of HPC bacteria, the closest matching sequences are occasionally unidentified bacteria sequences. For example, strain No.38 and No.39 were most closely matched with 'Uncultured alpha proteobacterium'. Furthermore, some recorded sequences were identified at the genus level (i.e. No.1, No.9, No.10, No.13, No.14, No.19, No.21, and No.27).

Though the classification of bacteria has changed as the DNA sequencing analysis has progressed, some sequences and species registered in NCBI/EMBL/DDBJ have not been changed yet. That is why the same sequence is sometimes recorded as different species. For example, the sequence of No.6 isolate showed the closest matching with *Bacillus simplex* and *B. macroides*. Heyrman et al. (2005) found that the strains identified as *B. macroides* in different collections do not belong to the same species. He suggested that some strains should be allocated to the species *B. simplex*. Thus, the sequence of No.6 isolate had the closest matching with those two species. In the same way, the sequence of No.33 isolate had the closest matching with *Mycobacterium fortuitum* subsp. *fortuitum* and *M. porcinum*. *M. porcinum* was described in 1983 as a causative agent of submandibular lymphadenitis in swine (Tsukamura, Nemoto and Yugi, 1983). Because of almost complete phenotypic and molecular similarity in identity between *M. fortuitum* ATCC49404 and *M. porcinum*, Wallace Jr. et al. (2004) suggested that they belong to the same species. They reported 100% identity of the first 500 bp beginning at the 5' end between *M. porcinum* and *M. fortuitum*. Therefore, it was a natural result that the sequence of No.33 isolate showed the closest matching with these two species. To enhance the effectiveness of 16S rDNA sequencing analysis to identify HPC bacteria, many researchers should register the DNA sequence data of environmental bacteria.

Evaluation of the health effects of identified hemolytic HPC bacteria

We identified 34 isolates of hemolytic HPC bacteria. As far as we know, this is the first report on an isolation and identification trial of hemolytic HPC bacteria from finished and tap water in Japan. To evaluate the presence of HPC bacteria in drinking water, we must discuss the health effects and occurrence of identified hemolytic HPC bacteria.

The most frequently found genus was *Sphyngomonas* (total 13 isolates). *Sphyngomonas* species can act as opportunistic pathogens and have caused meningitis, bacteremia, septicemia, peritonitis, and neonatal infections in intensive care units (Yabuuchi and Kosako, 2005).

The second most frequently found genus was *Staphylococcus* (6 isolates). Identified species were *S. pasteuri*, *S. warneri*, *S. cohnii*, and *S. hominis*, which are coagulase-negative staphylococci. They are commonly found on human skin, can cause bacteremia related to indwelling devices, central nervous system shunt infections, infective endocarditis, urinary tract infections, and endophthalmitis (Huebner and Goldmann, 1999).

The third most frequently found genus was *Mycobacterium* (4 isolates). Identified species were *M. fortuitum* subsp. *fortuitum* (or *M. porcinum*), *M.*

TABLE 2. Identification of hemolytic HPC bacteria.

Isolate No.	Type of hemolysis	16S rDNA sequence	Accession No.	Identity (%)
1	β	Uncultured *Staphylococcus* sp.	EF547991	84
2	α	*Sphyngomonas ursicola*	AB024289	96
		Sphyngomonas natatoria	AB024288	96
3	β	*Staphylococcus pasteuri*	AJ717376	100
		S. warneri	AY186059	100
4	β	*Bacillus cereus*	AY138271	99
5	α	Not amplified		
6	α	*Bacillus simplex*	EF690433	96
		B. macroides	AF501307	96
7	α	Not amplified		
8	α	*Staphylococcus pasteuri*	AJ717376	95
		Staphylococcus warneri	EF442317	95
9	α	*Sphyngomonas* sp.	AB033949	93
10	α	*Brevibacterium* sp.	EU099374	96
11	α	*Sphyngomonas xenophaga*	AY611716	93
12	α	*Sphyngomonas ursicola*	AB024289	95
		Sphyngomonas natatoria	AB024288	95
13	α	*Sphyngomonas* sp.	DQ340653	96
14	α	*Mycobacterium* sp.	AM990744	88
15	α	*Acidovorax defluvii*	EU434539	90
16	α	Not amplified		
17	α	*Mycobacterium cosmeticum*	AY449729	94
18	α	*Staphylococcus cohnii*	AB009936	99
19	α	*Sphyngomonas* sp.	AB033949	100
20	α	*Micrococcus luteus*	AF501366	99
21	α	*Caulobacter* sp.	EF433769	93
22	α	Not amplified		
23	α	*Sphyngomonas ursicola*	AB024289	97
		Sphyngomonas natatoria	AB024288	97
24	α	*Staphylococcus warneri*	AY126244	100
25	α	*Staphylococcus hominis*	AJ717375	99
26	α	*Bacillus idriensis*	EF010751	95
27	α	*Sphyngomonas* sp.	EU071484	97
28	α	*Cupriavidus metallidurans**	AY860234	98
29	α	*Micrococcus luteus*	AJ309917	99
30	α	*Micrococcus luteus*	EF187229	99
31	α	*Mycobacterium mucogenium*	AM884316	97
32	α	*Sphyngomonas ursicola*	AB024289	95
		Sphyngomonas natatoria	AB024288	95
33	α	*Mycobacterium porcinum*	AY012581	99
		M. fortuitum subsp. *fortuitum*	AY457068	99
34	α	*Sphyngomonas xenophaga*	X94098	98
35	α	*Sphyngomonas macrogoltabida*	AB372254	95
		Sphyngomonas chilensis	EF203909	95
36	α	Not amplified		
37	α	*Sphyngomonas xenophaga*	X94098	97
38	α	Uncultured alpha proteobacterium	EF702819	90
39	α	Uncultured alpha proteobacterium	AF446303	95

*: Synonym of *Ralstonia metallidurans*

cosmeticum, *M. mucogenium*, which are nontuberculous mycobacteria. *M. fortuitum* can cause intractable pulmonary disease (Mangione et al., 2001), as well as surgical wound infections, osteomyelitis, and catheter-related infections (Wallace Jr. et al., 2004). *M. cosmeticum* has been isolated from footbath drains and a sink, and from a granulomatous subdermal lesions (Cooksey et al., 2004). Nontuberculous mycobacteria have high chlorine resistance and are capable of biofilm formation, so they are frequently isolated worldwide from water distribution systems (Primm, Lucero, and Falkinham

III, 2004; Furuhata et al., 2007; Williams et al., 2004; Tokajian et al., 2005; September, Brozel, and Venter, 2004).

Three strains of *Micrococcus luteus* were isolated. *Micrococcus* species were regarded as non-pathogenic skin commensals, but *M. luteus* has been reported as the causative agent of intracranial abscesses, meningitis, pneumonia, and septic arthritis in immunosuppressed or immunocompetent hosts (Altuntas et al., 2004). *Micrococcus* species also have high chlorine resistance. Mir, Morato, and Ribas (1997) demonstrated that *M. luteus* was inactivated only by 0.79 log after 60 minutes at 0.5 mg/l free chlorine treatment.

Three strains of *Bacillus* species were isolated: *B. cereus*, *B. simplex*, and *B. idriensis*. *B. cereus* is distributed in the environment in the soil and air, and known as a causative agent of food poisoning. *B. simplex* is also reported as a causative agent of food poisoning (Taylor et al., 2005). *B. idriensis* was isolated from the blood of newborn child with sepsis (Ko et al., 2006).

One isolated strain, *Cupiavidus metallidurans* (Synonim *Ralstonia metallidurans*), has been reported as a causative agent of pulmonary infections for cystic fibrosis patients (Goris et al., 2001).

As a result, 30 of 34 isolates identified have been reported to be opportunistic pathogens or food poisoning bacteria. We think that the existence of these bacteria in drinking water does not necessarily mean danger for human health, because the number of HPC bacteria can be extremely low when drinking water retains residual chlorine. However, HPC bacteria can increase when residual chlorine concentration is decreased, or disappears because of their high chlorine resistance and ability to form biofilm. Actually, Hirata et al. (1993) reported that the number of HPC bacteria in first flush of tap water with low residual chlorine concentration is 10~100 times higher than in tap water after being run for 2~5 minutes. In addition, though the number of HPC bacteria was usually 10^2 cfu/ml in appropriately controlled tap water, the number of HPC bacteria in the water of a polluted receiving tank sometimes exceeded 10^5 cfu/ml (Hosaka and Maki, 2001). To control the growth of these opportunistic HPC bacterial pathogens, appropriate water quality control must always be done and residual chlorine must be maintained to keep a safe water supply. The significance of opportunistic HPC bacteria is still uncertain, so further studies should be performed.

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水道水中の従属栄養細菌の同定における DNA塩基配列解析法と表現性状試験との比較

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Comparison of DNA Sequencing Analysis with Phenotypic Test for Identification of Heterotrophic Bacteria Isolated from Drinking Water

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Abstract

Heterotrophic bacteria isolated from finished and tap water samples were examined for hemolysis activity to determine their virulence factors. Eleven colonies expressing hemolysis activity were identified by partial 16S rDNA sequencing and phenotypic test using Biolog™. All eleven strains were identified at the genus level and eight strains were identified at the species level by 16S rDNA sequencing. On the other hand, phenotypic test could identify only two strains at the genus level and four strains at the species level. Furthermore, three of four strains identified at the species level by phenotypic test might have been misidentified. Therefore, it was suggested that phenotypic test is inadequate identifying heterotrophic bacteria in drinking water, whereas 16S rDNA sequencing is a useful identification tool for them. The eleven strains identified have been reported to be food poisoning bacteria or opportunistic pathogens. Since these opportunistic pathogens exist in heterotrophic bacteria, both appropriate water quality control and further study should be continued for safe tap water supply.

Key words: heterotrophic bacteria, drinking water, hemolysis assay, 16S rDNA sequencing, opportunistic pathogen

1．はじめに

平成 20 年4月1日から従属栄養細菌が水質管理目標設定項目に追加された[1]。その目的は「水道施設の健全性を判断するため，また，わが国における従属栄養細菌の存在量等必要な情報，知見の収拾を図るため[2]」であり，当面の目標値として「1 ml の検水で形成される集落数として 2,000 以下（暫定）[2]」が示された。従属栄養細菌は水道水中に一般細菌よりも著しく多く存在することから，浄水処理過程や消毒過程での細菌の挙動を知る上で一般細菌よりも優れており，また配水施設内での水の滞留や塩素の消失に伴って増加することから，配水施設における水質管理上の指標としても利用できる[3]。

このように従属栄養細菌の量に着目した水質指標性については，従来から一定の意義付けがされているが，質的な面に関しては情報が極めて乏しい。従属栄養細菌の中には日和見感染菌が含まれることが示唆されている[4]が，従属栄養細菌のヒトへの健康影響を評価するためには，病原性状だけでなく，菌種名の同定が必要である。米国，ヨーロッパ，南アフリカ等では従属栄養細菌の病原性に関する調査が数多く行われており，溶血性，細胞毒性，細胞凝集性，細胞侵襲性等を持つ細菌の分離が報告されている[5-10]。これらの研究においては，病原因子を持つ菌の同定に種々の表現性状試験が用いられたが，種まで分類できない菌が多く報告されている[6-8]。また，わが国では水道水中の従属栄養細菌に関する研究はきわめて少ないことから，Hirata ら[11]は水道水中の従属栄養細菌を調査し，それらの分離株を表現性状試験に基づいて属までの分類を試みた。その結果，一部の菌株において日和見感染菌を含む属が同定されたが，大部分の株が同定不能であったため，従属栄養細菌を分類する試験法の必要性を指摘した。

従属栄養細菌は低温，低栄養条件下で生育する菌が多く，表現性状を用いた市販同定キットによる正確な同定が困難であるものが多い。これは，同定に用いた表現性状試験の多くが病原細菌検出用に開発されたものであり，環境由来の非病原性細菌や日和見感染菌のデータベースが不十分であるためと考えられる。細菌の厳密な同定には，DNA-DNA 相同性，DNA の GC %，細胞壁のペプチドグリカン組成，菌体脂肪酸組成等を調べる必要がある。しかし，分離した菌株すべてについてこれらのデータを得るには，煩雑な作業と膨大な時間が必要となり，実用的でない。一方，近年細菌の 16S rDNA 塩基配列を利用した分類や同定手法が適用され始めており，特

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に変異の高い領域である上流側500塩基対を対象とした塩基配列解析が数多く行われている[15-16]。

そこで，本研究では浄水場の浄水及び給水栓水から分離した従属栄養細菌について，病原因子の指標として37 ℃での生育能及び溶血性の有無を検査し，これらの因子保有状況を調査した。また，溶血性を示した菌の簡便な同定法として，市販キットを用いた16S rDNA塩基配列解析法と表現性状試験とを比較，検討した。さらに，同定された菌種について，ヒトへの健康影響等を考察した。

2．方　　法

2.1　試料採取

平成18年5～6月に都内小規模浄水場の浄水28検体，給水栓水9検体を採取し，調査に用いた。採水時に現場で残留塩素濃度（遊離又は結合）を測定した。

2.2　細菌試験

1）一般細菌：標準寒天培地（栄研化学）を用いて上水試験方法[17]に従って試験した。

2）従属栄養細菌：R2A 寒天培地（MERCK）を平板固化し十分に乾燥させた後，検水1 ml を塗布し，25 ℃で7日間培養[18]後，形成された集落数を計数した。

2.3　病原因子調査

1）体温下，高温下増殖能：従属栄養細菌の集落を目視による色・大きさで分類・計数し，各分類群から1集落を釣菌して再度 R2A 培地に画線し，37 ℃（体温下）及び44.5 ℃（高温下）で24時間培養し，増殖能の有無を調べた。

2）溶血性試験：37 ℃で増殖した菌を羊血液寒天培地（栄研化学）に穿刺し，37 ℃で24時間培養後，溶血性（α，β）の有無を判定した。

2.4　細菌の同定

溶血性を示した菌株について，下記の2方法により同定を試みた。

1）16S rDNA塩基配列解析：Jiwa らの報告[19]に準拠したアルカリ溶解法で菌体から DNA を抽出した。16S rDNA の上流側500塩基対を対象とする MicroSeq 500 16 S rDNA Bacterial Identification PCR Kit（Applied Biosystems）を用いて反応液を調整後，DNA サーマルサイクラー（GeneAmp 9600: Perkin-Elmer）を用いて PCR を行った。PCR 産物の精製は Montage PCR Centifugal Filter（Millipore）を用いた限外ろ過により行った。精製した PCR 産物を MicroSeq 500 16S rDNA Bacterial Identification Sequencing Kit（Applied Biosystems）を用いてシークエンス反応を行った後 ABI PRISM 3130（Applied Biosystems）を用いて塩基配列の決定を行った。決定した塩基配列について Genbank を利用した BLAST（basic local alignment search tool）解析を行い，最も高い一致率を示したものを菌種として決定した。

2）表現性状試験：生化学的性状試験には，Biolog システム（Biolog Inc）を用い，マニュアルにしたがって試験した。羊血を加えた Biolog Universal Growth Agar で37 ℃，24 時間前培養した後，菌体を所定の濃度に懸濁し，グラム陰性菌は GN2 プレート，グラム陽性菌は GP 2 プレートに接種した。24 時間後の測定で種または属を決定した。

3）16S rDNA塩基配列解析と表現性状試験結果の比較：2種類の同定法で菌種名が異なった場合には，表現性状試験結果により同定された菌種の16S rDNA塩基配列を GenBank から取得し，MEGA3[20]を用いた多重アライメントを行い，16S rDNA の塩基配列解析により得られた塩基配列と比較した。

3．結果と考察

3.1　従属栄養細菌と一般細菌の検出状況

水道水中の従属栄養細菌の実態については，日本水道協会水質試験方法等調査専門委員会微生物・生物部会による全国規模の調査報告がある[21]。この報告によると，一般細菌の検出率は浄水で4％，給水栓水で2％であった。従属栄養細菌の検出率は浄水で 46%，給水栓水で64%であり，その菌数は浄水で0～89 cfu·ml^{-1}，給水栓水で0～600 cfu·ml^{-1}であった。

本研究では，一般細菌は浄水 28 検体中1検体（検出率 3.6%）から1 cfu·ml^{-1}検出され，給水栓水9検体からは全て不検出であり，上記の調査報告と同様の結果であった。

従属栄養細菌の検出率は Table 1 に示すように，浄水で 89%，給水栓水で 100%と上記の報告値よりも高い値であった。検出された細菌数は，浄水で0～180 cfu·ml^{-1}，給水栓水で2～42 cfu·ml^{-1}であり，上記の報告値

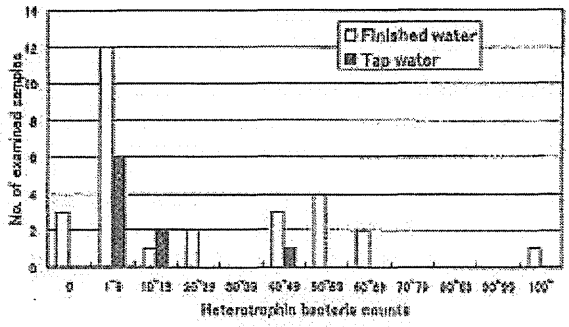

Fig. 1　Number of heterotrophic bacteria in finished and tap water.

Table 1　Distribution of heterotrophic bacteria isolated from finished and tap water and the free or combined residual chlorine concentration of the water samples.

Type of water	No. of sample examined	Positive sample No.	(%)	Heterotrophic bacteria count range	(average)	Residual chlorine concentration (mg·l^{-1}) Free 0.1	0.2	0.3	0.4	0.5	0.5<	Combined 1.5
Finished water	28	25	(89.3)	0 - 180	(27.9)	0	8	5	8	2	1	1
Tap water	9	9	(100)	2 - 42	(11.1)	2	2	3	0	1	1	0
Total	37	34	(91.9)	0 - 180	(23.8)	2	10	8	8	3	2	1

と同様であった。したがって，浄水・給水栓水の従属栄養細菌数は 10^{0}～10^{3}オーダーと考えられる。また，本研究ではFig. 1に示すように，浄水・給水栓水とも1~9 cfu·m/ $^{-1}$の試料が最も多く、既報の研究結果[31,32]と同様であった。

従属栄養細菌が検出された浄水・給水栓水は全て残留塩素が存在していた（Table 1）ことから，これらの従属栄養細菌は塩素耐性を持つと考えられる。本研究結果及び既報の研究結果[33]により，浄水よりも給水栓水での従属栄養細菌検出率が高いことから，塩素耐性を持つ細菌が給配水施設内で生物膜を形成し給水栓から流出していることが推察される。

3.2　従属栄養細菌における病原因子保有状況

本研究では，従属栄養細菌の病原因子として，1）体温下（37 ℃）での増殖能（体内での増殖可能性），2）高温下（44.5 ℃）での増殖能（腸管内での増殖可能性），3）溶血性（赤血球の溶解），についてそれぞれの保有状況を調査した。

調査した120株中91株（75.8%）が37 ℃で増殖可能であり，低温で生育するといわれる従属栄養細菌の多くが体温下で増殖可能であることがわかった。体温下増殖株のうち，39株（42.9%）に溶血性が見られ，そのほとんど（36株）がα溶血性を示した。全菌株に占める溶血菌株の割合（32.5%）は既報値（36%[10]）と同程度であった。細胞毒性菌株の分離報告値は0.2%[34]，0.8%[35]，1%[36]ときわめて低いことから，溶血性試験は細胞毒性試験に比べて高感度かつ簡便であり，病原因子を持つ菌のスクリーニングに有効な方法と考えられる。

一方，体温下増殖株のうち15株(16.5%)が44.5 ℃の高温下で増殖したが，この中で溶血性を持つものは4株だけであった。この結果から，腸管内温度で増殖できる従属栄養細菌は全体の12.5%と少なく，溶血性を合わせ持つものはわずか3.3%に過ぎないことがわかった。従属栄養細菌は，低温度下での培養においても標準寒天培地より低栄養の培地で多く分離される細菌群集であること[10]から，温度耐性及び至適栄養濃度において腸内細菌とは異なると考えられる。

3.3　菌同定におけるDNA塩基配列解析法と表現性状試験との比較

溶血性を示した菌株のうち，11株についてDNA塩基配列解析及び表現性状試験を行った。供試菌株のグラム染色性，細胞の形状，溶血性の種類（α，β），16S rDNA塩基配列解析及び表現性状試験の結果を，水源の種類及び浄水処理方法とともにTable 2に示した。

従属栄養細菌のヒトへの健康影響を評価するためには，その病原性状だけでなく，正確な菌種名を同定することが必要である。病原因子を持つ従属栄養細菌の同定には，これまで様々な表現性状試験が試みられた。具体的には，Bergey's manual of systematic bacteriology [6, 37]，API [5, 10]，Biolog [31]，VITEK [31]，Crystal [9]等であるが，これらの表現性状試験では種の同定に至らず，属レベルの同定や同定できなかった菌株が多く報告されている。これらは病原細菌検出用に開発されたものであり，環境由来細菌のデータベースが不十分であるために同定できなかったと考えられる。市販キットのデータベースを比較すると，Biologがグラム陰性菌501種，グラム陽性菌318種と，VITEK（グラム陰性菌104種，グラム陽性菌49種），API（グラム陰性菌180種，グラム陽性菌105種），Crystal（グラム陰性菌105種，グラム陽性菌140種）に比較して最も多くの種を含んでいる。このため本研究では市販の表現性状試験キットの中からBiologを選択し，従属栄養細菌の同定を試みたが，11株中4株しか同定できなかった（Table 2）。Biologで歯科用給水装置の生物膜構成細菌の同定を試みたSinghらも55株中14株しか同定できなかったことを報告している[10]。しかし，腸内細菌科を含むグラム陰性菌をBiologで同定した研究では266株中75.6%[38]，再生水中のグラム陰性菌を対象とした研究では45株中93%[39]が同定可能であった。この違いは，Biologのデータベースが腸内細菌等のグラム陰性菌については充実しており，同定精度が高いためと考えられる。また，Biologのプロトコールでは，前培養24時間以内の菌を懸濁して検査することになっているが，従属栄養細菌の中には発育の遅い菌が含まれるという問題もある。Table 2に示したNo.33の*Mycobacterium*は24時間の培養では検査に必要な菌量が得られず，数日間培養後に検査せざるを得なかった。このため，メーカーのプロトコール通りに検査できず，正しい同定ができなかったと考えられる。

一方，近年の分子生物学的手法の進展により，細菌の16S rDNAを対象としたDNA塩基配列解析法による同定や分類も行われるようになってきた[15-18]。16S rDNA塩基配列解析法は，表現性状試験に比べて客観性や再現性に優れており，公共データベースに登録されているデータ数も充実していることから，菌種の分類，同定にきわめて有効な方法と考えられる。本研究においても，Table 2に示すように，11株全てを属レベルまで，うち8株を種レベルまで同定することができた。Biologによる同定結果が16S rDNA塩基配列解析法と一致しなかった4株について，Biologにより同定された菌種名で登録されている16S rDNA塩基配列を，分離された菌株の塩基配列と比較した結果，両者の塩基配列は明らかに異なっており，Biologによる同定結果が誤りである可能性が示された。Biologにより同定された4株のうち，正しく同定されたものはわずか1株であり，Probability が97%以上の2株（No.4,28）でさえも誤同定であったことから，Biologは従属栄養細菌の同定には適さないと考えられる。

しかし16S rDNA塩基配列解析法による従属栄養細菌の同定方法にも，いくつかの問題点が存在する。今回使用した市販の塩基配列解析キット（Microseq 500）は，16S rDNAの中で最も変異の高い部位である上流側500塩基対を対象としているが，この塩基配列では種まで特定できない菌株（No.3，6，33）が存在した。同じキットを用いて水道水中の黄色色素産生菌の同定を行ったFuruhataら[10]の研究でも2種の菌名が併記された菌株が多数報告されている。このような短所はあるものの，Microseq 500は試薬がプレミックスされており，簡便かつ迅速に16S rDNA塩基配列解析が行える利点があるため，多数の検体を分析する実態調査等に適している。Microseq 500で同定できなかった場合には，対象とする16S rDNA塩基配列をより長くしたり，別の遺伝子（*hsp 65*等）や進化速度の速いDNA gyrase Bを対象とした塩

Table 2 Identification of hemolytic heterotrophic bacteria isolated in this study with 16S rDNA sequencing analysis and phenotypic test.

Strain No.	Water resouce	Water treatment	Gram stain	Cell form	Type of Hemolysis	Results of identification: 16S rDNA sequence (Accession No.)	Identity (%)	Biolog (24hr)	Probability (%)
3	Shallow well	R.O.[1]	P [7]	Cocci	β	*Staphylococcus pasteuri* CV5 (AJ717376)	100	*Staphylococcus* spp.	---
						S. warneri PB1 (AY186059)	100		
4	Surface water	S.S.F.[3]	P [7]	Rod	β	*Bacillus cereus* G8639 (AY138271)	99	*Arcanobacterium pyogenes*	97
6	Spring water	R.S.P.[3]	P [7]	Rod	α	*Bacillus simplex* M3-4 (EF690433)	96	Not identified	---
						B. macroides HAMBI2406 (AF501367)	96		
18	Deep well	C.[4]	P [7]	Cocci	α	*Staphylococcus cohnii* ATCC49330T (AB009936)	99	*S. arlettae*	76
20	Deep well	C.[4]	P [7]	Cocci	α	*Micrococcus luteus* HAMBI2408 (AF501366)	99	*M. luteus*	100
24	Surface water	U.F.[5]	P [7]	Cocci	α	*Staphylococcus warneri* ES1 (AY126244)	100	Not identified	---
25	Surface water	S.S.F.[3]	P [7]	Cocci	α	*Staphylococcus hominis* CV21 (AJ717375)	99	Not identified	---
28	Shallow well	N.F.[6]	N [8]	Rod	α	*Cupriavidus metallidurans* [9] AU4057 (AY860234)	98	*Ralstonia paucula*	98
29	Shallow well	C.[4]	P [7]	Cocci	α	*Micrococcus luteus* dtb113 (AJ309917)	99	Not identified	---
30	Shallow well	C.[4]	P [7]	Cocci	α	*Micrococcus luteus* AUH1 (EF187229)	99	Not identified	---
33	Surface water	R.S.F.[3]	P [7]	Rod	α	*Mycobacterium porcinum* W6236 (AY012581)	99	*Rhodococcus* spp.	---
						M. fortuitum subsp. *fortuitum* ATCC49404 (AY457068)	99		

[1]: Reverse osmosis [4]: Slow sand filtration [3]: Rapid sand filtration [4]: Chlorination only
[5]: Ultrafiltration [6]: Nano-filtration [7]: Positive [8]: Negative
[9]: Synonym for *Ralstonia metallidurans*

基配列解析を行う等によって，同定を試みる必要があろう。

また，今回は決定した塩基配列について，Genbank を利用した BLAST 解析を行い，最も高い一致率を示したものを種としたが，従属栄養細菌についてはこのデータベースにおける ATCC 株等の標準菌株の塩基配列データが不十分であるという問題もある。病原細菌では標準菌株のデータが上位を占めるため，最も一致率の高い菌名が容易に得られるが，従属栄養細菌では最も一致率の高い菌として，Uncultured bacterium clone，Unidentified bacterium，Swine manure pit bacterium，Soil bacteirum 等が上位を占め，種名の明らかな菌との一致率が低くなる場合もあるなど，16S rDNA 塩基配列のデータベースにも表現性状試験と同様の問題が存在している。

さらに，遺伝子解析の進展により細菌の分類が変化しているが，一度 Genbank に登録された菌種名と塩基配列はそのまま存在しているため，異なる菌種名で同じ塩基配列が登録されている例も見られた。たとえば，本研究で分離された No. 6 株は *B. simplex* 及び *B. macroides* との塩基配列一致率が最も高かった。Heyrman ら[19]は *B. macroides* と同定された多数の菌株を遺伝子解析した結果，*B. simplex* と同一の遺伝子を持つ菌株が複数存在することを発見し，これらの菌株を *B. simplex* に移すよう提案しており，No. 6 株がこの 2 種の塩基配列と高い一致率となったのは，このような状況があるためと考えられた。また，No. 33 株は *Mycobacterium fortuitum* subsp. *fortuitum* 及び *M. porcinum* との塩基配列一致率が最も高かった。*M. porcinum* はブタのリンパ節炎の原因菌として新種記載された[20]が，表現性状や各種遺伝子の塩基配列が *M. fortuitum* の ATCC49404 株とほぼ完全に一致することから，この 2 種を同一とする研究者[21]もいる。両者の 16S rDNA の最初の 500 塩基配列は全く同一であることが報告されている[21]。したがって No. 34 株が *M. fortuitum* subsp. *fortuitum*（ATCC49404）及び *M. porcinum* の塩基配列に対して同じ一致率を示したのは，当然の結果と考えられた。

今後，16S rDNA 塩基配列解析法の有効性をさらに高めるためには，標準菌株のデータを充実させるとともに，多くの研究者が環境由来細菌のデータを調査，登録していく必要があろう。

3.4　同定された従属栄養細菌のヒトへの健康影響

本研究で同定された従属栄養細菌について，ヒトへの健康影響を考察する。

Staphylococcus 属が 4 株と最も多く分離され，*S. pasteuri*, *S. warneri*, *S. cohnii*, *S. hominis* が同定された。これら 4 種は全てコアグラーゼ陰性ブドウ球菌群であり，ヒトの皮膚に常在し，ときに尿路感染症，感染性心内膜炎，カテーテル菌血症，日和見感染症の原因となる[20]。コアグラーゼ陰性ブドウ球菌群には β 溶血を示す菌株が存在し[20]，またウシ乳房炎から分離された *Staphylococcus* 属 12 種の 272 株すべてが α 溶血を示した[20]報告例もある。水道水からの *Staphylococcus* 属の分離報告例は多く[6~8, 11, 15, 22]，水道水中に広く生息する菌である。

次いで，*Micrococcus luteus* が 3 株分離された。*Micrococcus* 属は自然界に広く生息し，病原的意義は低いが免疫不全患者への感染例の報告もある[23]。本研究の 3 株は全て地下水（深井戸 1，浅井戸 2）を塩素処理しただけの水道水から分離された。Mir ら[20]は浄水場の塩素処理水（遊離残留塩素濃度 2.00mg·l^{-1}）から分離した *M. luteus* を用いて，塩素による不活化試験を行った。その結果，塩素濃度 0.5mg·l^{-1}で 60 分間接触後でも 0.79 log の不活化に留まり，*M. luteus* が高い塩素耐性を持つことが示された。したがって，今回の検出は原水中に存在していた菌が塩素処理後も生残した可能性が高いと考えられる。*M. luteus* 及び *Micrococcus* 属も水道水からの検出例が多い[6~8, 11, 13, 15, 22]。

Bacillus 属は *B. cereus* と *B. simplex*（又は *B. macroides*）の 2 株が分離された。*B. cereus* は土壌，空中など自然界に広く分布し，食中毒の原因菌として知られており[21]，β 溶血性を示す[22]。*B. simplex* は *B. cereus* と同様の加熱に強い毒素を産生し，食中毒の原因となることが報告されている[23]。*B. cereus* 及び *Bacillus* 属の水道水からの検出例は数多く報告されている[6~9, 11, 15, 16, 22]。

非結核性抗酸菌（NTM）の *Mycobacterium fortuitum* subsp. *fortuitum*（又は *M. porcinum*）が 1 株検出された。NTM は塩素抵抗性が高く，水道管内表面に生物膜を形成して水道水中に広く存在することが知られている[32]。*M. fortuitum* は迅速発育群に属し[33]，創傷感染，カテーテル菌血症，肺炎等の感染を起こす[33]。汚染された浴槽水からのエアロゾル暴露による難治性肺炎の原因となることも報告されている[34]。Wallace Jr.ら[35]は給水栓水から *M. porcinum* を検出し，本種がカテーテル菌血症の原因となるのは，カテーテルが水道水に暴露されるためではないかと推定している。また，Grabow ら[36]は *M. fortuitum* を用いて塩素抵抗試験を行い，遊離残留塩素濃度 0.4mg·l^{-1}で 15 分間接触後の菌数は初期濃度（10^{7} cfu·ml^{-1}）とほとんど変わらないことを報告した。このように NTM は塩素抵抗性が高いことから，多くの研究者[12, 14, 15, 32]が水道水から検出している。

グラム陰性菌では *Cupriavidus metallidurans*（Synonim *Ralstonia metallidurans*）が分離された。本種は重金属耐性の強い細菌である[37]が，嚢胞性線維症患者の肺炎原因菌としての分離報告例[38]もある。Schmeisser ら[39]は配水施設内の生物膜から本種の DNA 検出を報告しており，水道施設内での存在が示唆されている。

このように本研究で分離した菌株は，全て人への感染例があり，病原性を持つ種であることがわかった。残留塩素が十分確保されている水道水であれば，従属栄養細菌数は非常に少なく制御されていることから，これらの菌の存在が直ちにヒトへの危険性を意味するわけではない。しかし，これらの菌の多くが高い塩素抵抗性を持ち，生物膜を形成することから，残留塩素の低下や消失により繁殖する可能性がある。実際に，Hirata ら[10]は残留塩素濃度の低い早朝の給水栓初流水において，2~5 分流水後の水よりも 10^{1}~10^{2}多い従属栄養細菌を検出したことを報告している。また，適正に管理された給水栓水の従属栄養細菌数は 10^{2} cfu·ml^{-1}程度であるが，汚れた受水槽から採種した水では 10^{6} cfu·ml^{-1}を越える菌数となった例もある[13]。したがって，これまで無害と考えられていた従属栄養細菌にこのような日和見感染菌等が存在することを前提として，水道水の水質管理を実施していく必要があると考えられる。

4．ま と め

浄水場浄水及び給水栓水から分離した従属栄養細菌について，ヒトへの病原因子の指標として 37 ℃での生育能及び溶血性を調査した。溶血性を示した菌の簡便な同定法として，市販キットを用いた 16S rDNA 塩基配列解析法と表現性状試験とを比較した。さらに同定された菌のヒトへの健康影響について考察を行った。本研究から得られた主な結論を以下に示す。

1）37 検体全てにおいて残留塩素が検出され，一般細菌は 1 検体で 1 cfu·ml^{-1}検出された以外は不検出であった。従属栄養細菌は 34 検体（92%）から検出された。

2）溶血性を持つ従属栄養細菌 11 株を市販キットによる 16S rDNA 塩基配列解析法と表現性状試験により同定した。両検査法の結果が一致したのは 1 株のみであった。表現性状試験で同定された菌種名で登録されている 16S rDNA 塩基配列を分離された菌株の塩基配列と比較した結果，両者の塩基配列は異なっていたことから，表現性状試験による従属栄養細菌の同定は困難であり，塩基配列解析法による同定が有効であることが示された。

3）16S rDNA 塩基配列解析法により同定された 11 株は，いずれも食中毒原因菌あるいは日和見感染菌であった。従属栄養細菌の中にこのような日和見感染菌等が存在することを前提として，水道水の水質管理を実施していく必要があると考えられる。

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Original

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

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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 wittfariensis* 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.

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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 casamino • acid, 0.5 g glucose, 0.5 g soluble starch, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.3 g sodium pyruvate and 15 g agar per liter, pH 7.0-7.4; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 30°C for 7 days. In total, 25 colonies with yellow pigmentation 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.

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

Biochemical tests

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

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	*Sphingomonas ursincola/natatoria*	*Pasteurella* sp.	1000004	47.9
Y-6	*Sphingomonas ursincola/natatoria*	*Aeromonas salmonicida*	1000204	60.9
Y-7	*Sphingomonas ursincola/natatoria*	*Pasteurella* sp.	1000200	73.3
Y-8	*Sphingomonas ursincola/natatoria*	*Moraxella* sp.	0000004	82.2
Y-11	*Sphingomonas ursincola/natatoria*	*Acinetobacter lowffii*	0000200	38.4
Y-13	*Sphingomonas ursincola/natatoria*	*Aeromonas salmonicida*	1040204	91.2
Y-25	*Sphingomonas ursincola/natatoria*	*Brevundimonas vesicularis*	0000204	58.3
Y-27	*Sphingomonas ursincola/natatoria*	*Comamonas testosteroni*	1204440	77.5
Y-37	*Sphingomonas ursincola/natatoria*	Unknown	0000000	
Y-56	*Sphingomonas ursincola/natatoria*	Unknown	0000000	
Y-74	*Sphingomonas ursincola/natatoria*	*Aeromonas salmonicida*	1000204	60.9
Y-81	*Sphingomonas ursincola/natatoria*	*Acinetobacter lowffii*	0000200	38.4
Y-1	*Mycobacterium frederiksborgense*	*Comamonas testosteroni*	1004440	56.1
Y-20	*Mycobacterium frederiksbergense*	Unknown	0005440	
Y-10	*Sphingomonas adhaesiva*	*Sphingomonas paucimobilis*	0442164	78.0
Y-44	*Sphingophyxis wittlariensis*	*Brevundimonas vesicularis*	0400200	99.7
Y-57	*Porphyrobacter donghaensis*	*Sphingomonas paucimobilis*	0062204	49.9
Y-3	*Sphingomonas* sp.	Unknown	0000000	
Y-5	*Sphingomonas* sp.	*Sphingomonas paucimobilis*	0463341	97.7
Y-34	*Sphingomonas* sp.	*Pasteurella haemolytica*	0020004	84.6
Y-63	*Sphingomonas* sp.	*Sphingomonas paucimobilis*	0461640	99.8
Y-21	*Asticcacaulis* sp.	*Brevundimonas vesicularis*	0460205	52.3
Y-38	*Asticcacaulis* sp.	*Brevundimonas vesicularis*	0440004	99.6
Y-84	*Asticcacaulis* sp.	*Brevundimonas vesicularis*	0400000	78.8
Y-51	*Novosphingobium* sp.	*Moraxella* 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.

Chroline 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℃ 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℃ for 5 days beforehand, and isolated colonies were suspended with sterile distilled water to preparea bacterial suspension of about 10^6 CFU/ml. The bacteria were seeded in the experimental solutions to adjust the concentration to 10^5 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 μl 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℃ 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	*Mycobacterium frederiksbergense*	32
Y-7	*Sphingomonas ursincola* or *S. natatoria*	3.3
Y-5	*Sphingomonas* sp.	0.87
Y-27	*Sphingomonas ursincola* or *S. natatoria*	0.45
Y-21	*Asticcacaulis* sp.	0.095
Y-10	*Sphingomonas adhaesiva*	0.03

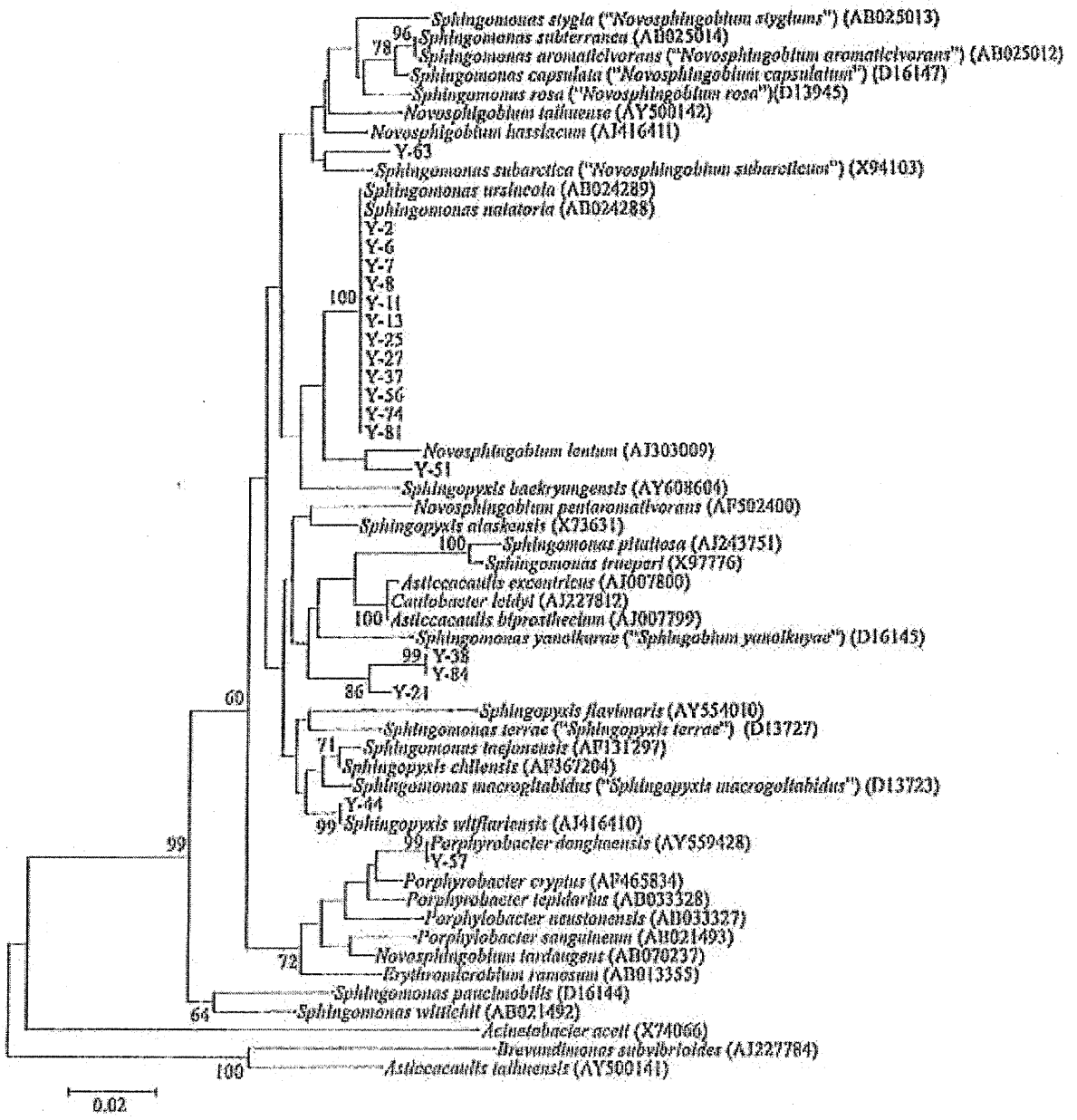

FIG. 1. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial reagion sequences (405bp). *Brevundimonas subvibrioides* (AJ227784) and *Asticcacaulis taihuensis* (AY500141) served as the out groups. The data set was resampled 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.

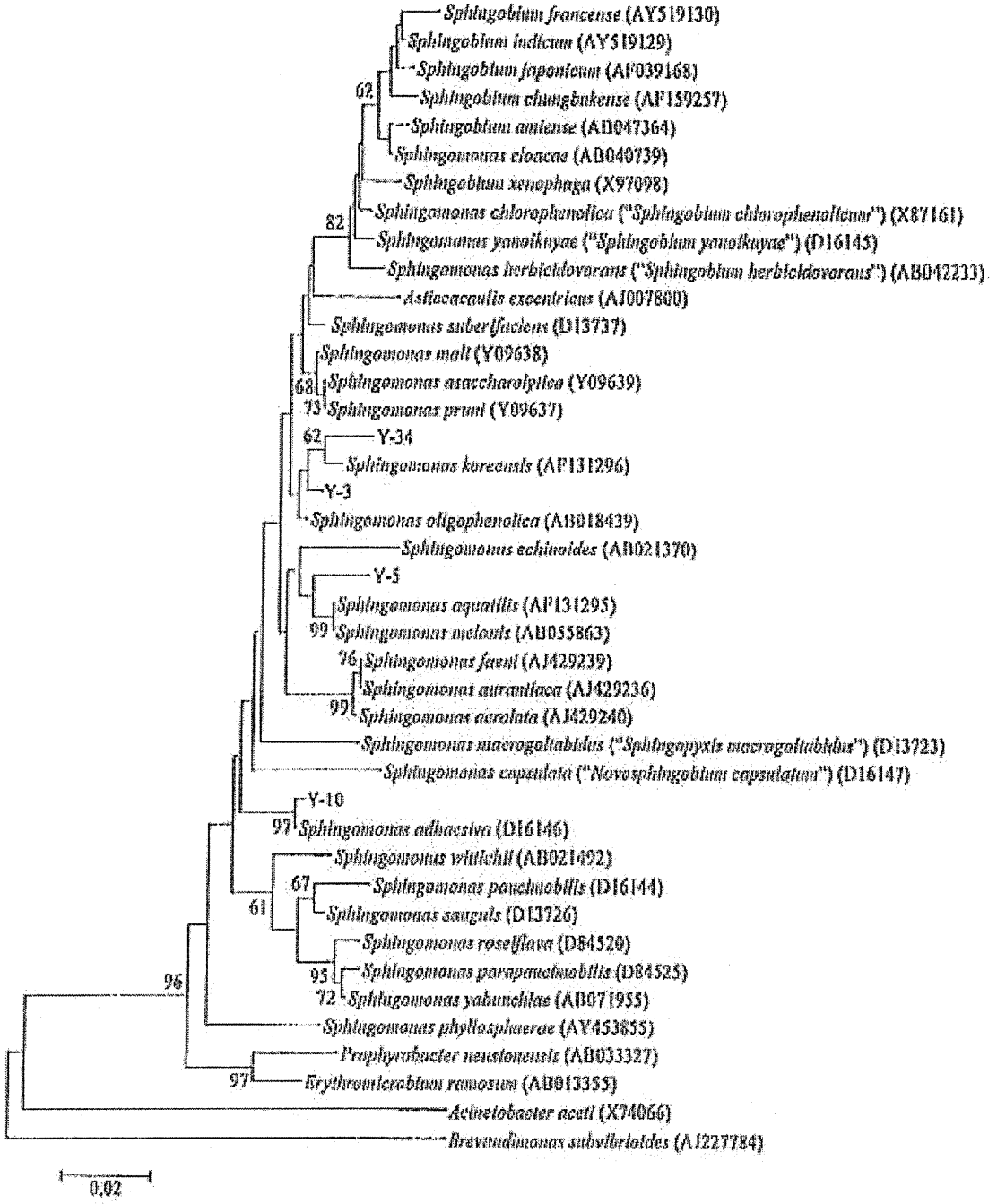

FIG. 2. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial reagion sequences (406bp). *Brevundimonas subvibrioides* (AJ227784) served as the out group. The data set was resampled 1,000 times by using the bootstrap option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