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^{2}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[21,22]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[23]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%[24]，0.8%[25]，1%[26]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 ℃で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[13]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[6,7]，API[5,10]，Biolog[8]，VITEK[9]，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%[20]，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%[24]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）でき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[27]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.[2]	P [7]	Rod	β	*Bacillus cereus* G8639 (AY138271)	99	*Arcanobacterium pyogenes*	97
6	Spring water	R.S.F.[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.[2]	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 [2] : 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 [29] 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 [30] 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 [27] 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 [27]。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[28]。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[29]，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[29]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[29]。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[30]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[31]，β溶血性を示す[32]。*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[33]。*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, 13, 15, 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[33]。*M. fortuitum* は迅速発育群に属し[28]，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^{3} 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, 23]が水道水から検出している。

グラム陰性菌では *Cupiavidus 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[13]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。

（原稿受付　2008 年 3 月 6 日）
（原稿受理　2008 年 8 月 11 日）

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3

Biocontrol Science, 2007, Vol. 12, No. 2, 39–46

Original

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

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Received 23 October, 2006/Accepted 1 February, 2007

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 wittlariensis* 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-6 (*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 frederiksbergense*	*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	78.6
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

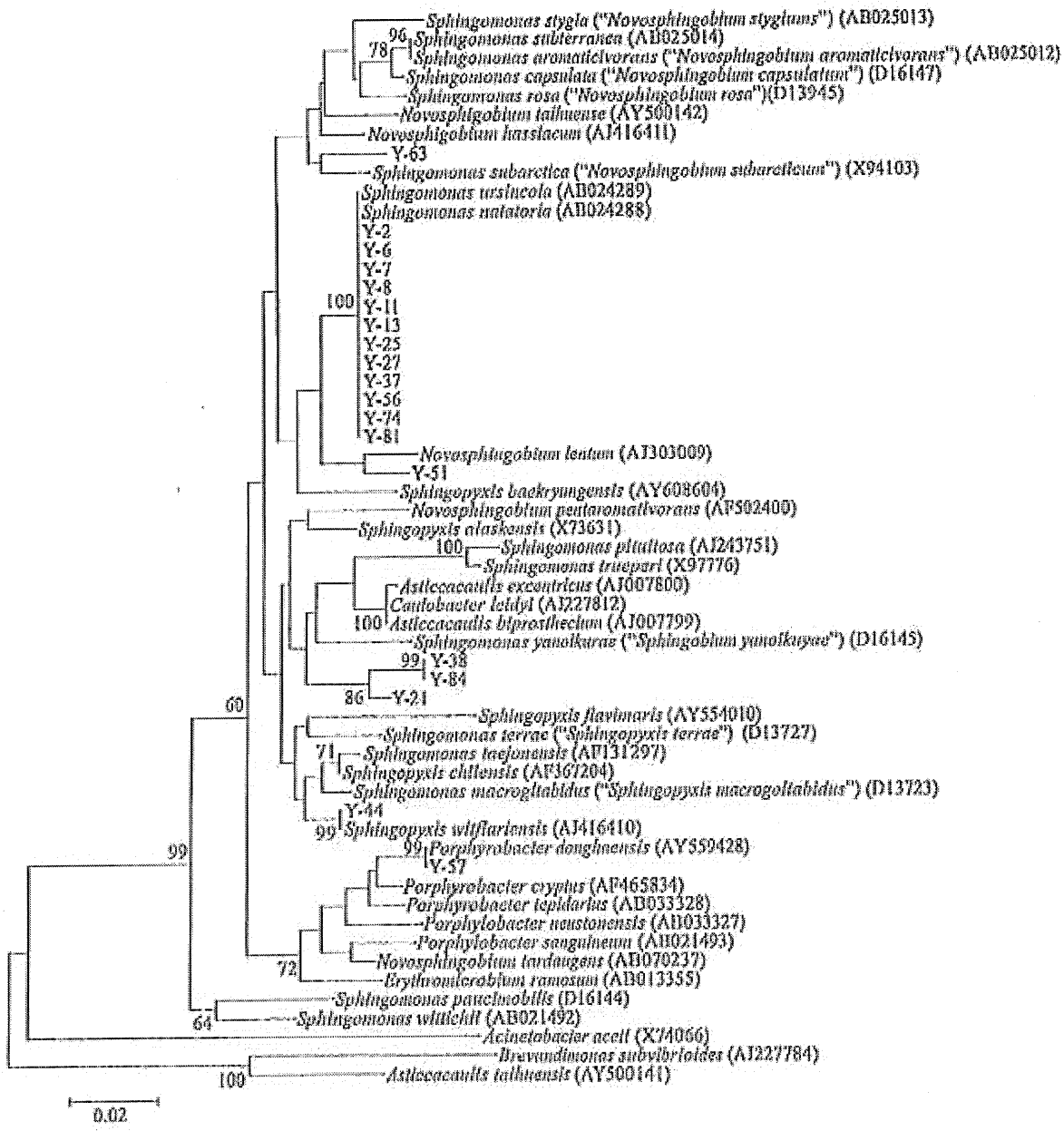

FIG. 1. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial reagion sequences (405bp). *Brevundimonas subvibrioides* (AJ227784) and *Asticcacaulis taihuansis* (AY500141) served as the out groups. The data set was resampled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

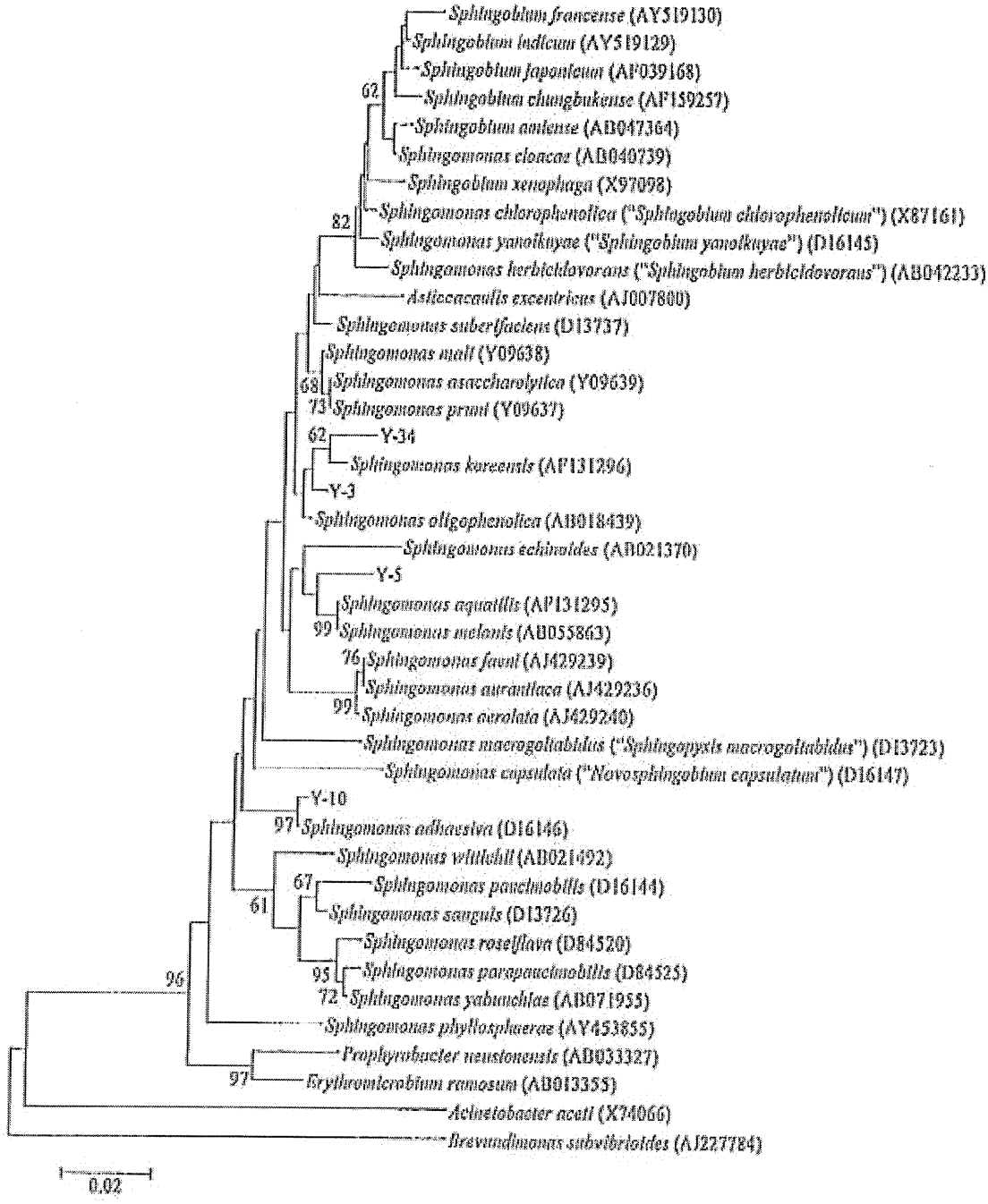

FIG. 2. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial reagion sequences (406bp). *Brevundimonas subvibrioides* (AJ227784) served as the out group. The data set was resampled 1,000 times by using the bootstrap option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

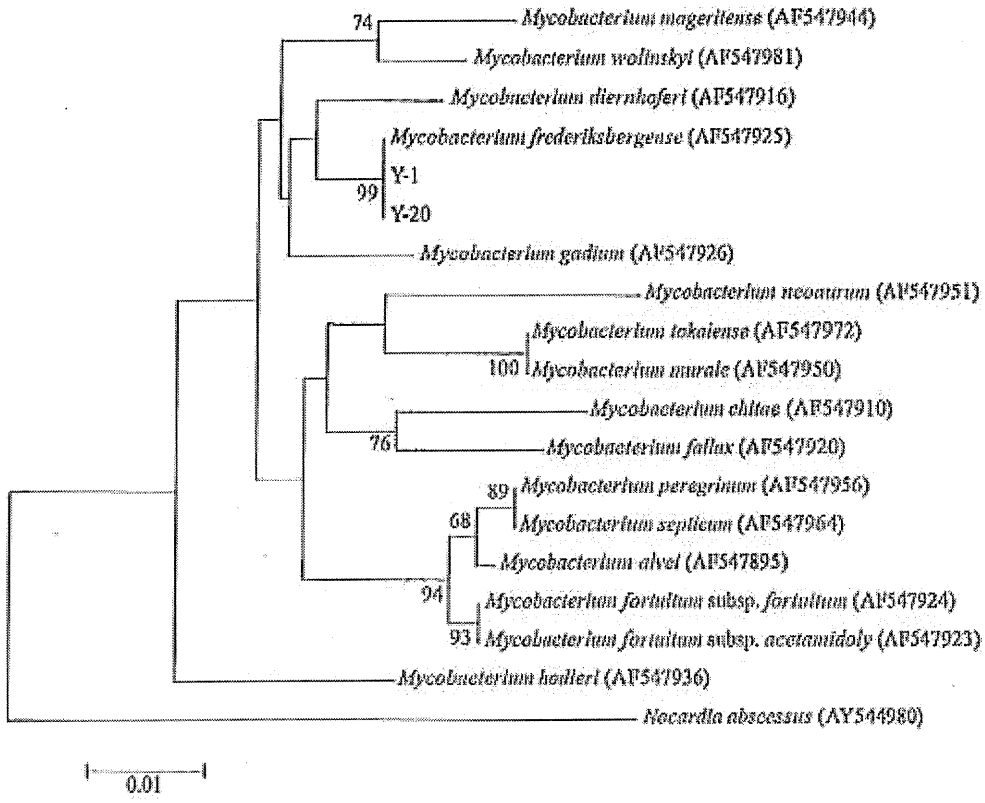

FIG. 3. Phylogenetic tree, based on neighbor-joining (Saitou and Nei, 1987), derived from an alignment comprising 16 S rDNA 5' end partial reagion sequences (439bp). *Nocardia abscessus* (AY544980) served as the out group. The data set was resampled 1,000 times by using the bootstrap option and the percentage 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 wilfiariensis*, 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 wilfiariensis* 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 chorine 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.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 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 frederiksbergense* was also identified. *M. frederiksbergenso* is an acid-fast bacterium newly named by Williumsen 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. frederiksbergense* 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. frederiksbergense* 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 10^3 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; Furuhata 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.

ACKNOWLEDGEMENT

This work was supported in part by Health and Labour Sciences Research Grants (H18-Kenki-Ippan-008) from the Ministry of Health, Labour and Welfare.

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4

Microbiol. Immunol., 50(1), 11–17, 2006

Isolation and Identification of *Methylobacterium* Species from the Tap Water in Hospitals in Japan and Their Antibiotic Susceptibility

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Received August 22, 2005; in revised form, October 20, 2005. Accepted October 20, 2005

***Abstract*: Contamination of tap water by *Methylobacterium* species has become a serious concern in hospitals. This study was planned to examine the distribution of *Methylobacterium* species inhabiting tap water used in Japanese hospitals and antibiotic sensitivity of the isolates in 2004. Species identification of 58 isolates was performed based on the homology of a partial sequence of 16S rDNA. The dominant *Methylobacterium* species in hospital water were *M. aquaticum* and *M. fujisawaense*. To examine the biochemical properties of these isolates, a carbon source utilization was tested using an API50CH kit. The phenotypic character varied widely, and was not necessarily consistent with the results of phylogenic analysis based on the partial 16S rDNA sequence, suggesting that the biochemical properties are not suitable for identification of *Methylobacterium* species. The isolates were also subjected to antibiotic sensitivity tests. They were resistant to 8 antibiotics, but highly sensitive to imipenem (MIC_{90}=1 μg/ml) and tetracycline (MIC_{90}=8 μg/ml). These findings concerning the isolates revealed the presence of *Methylobacterium* species with resistance to multiple antibiotics in hospital tap water.**

***Key words*: *Methylobacterium* spp., Antibiotic susceptibility, Tap water, Hospital**

The investigations of the water used for handwashing before surgery have revealed the absence of a significant difference on the effects of handwashing using sterilized water and tap water (5). Consequently, there has been a move to save on the costs for infection control by removing equipment installed for sterilized water and switching to the use of tap water. Although the chlorine sterilization of tap water is mandatory in Japan, *Methylobacterium* species resistant to the residual chlorine of disinfectants widely inhabit tap water piped through water supply facilities such as water tanks of high-rise buildings (7, 11). In 1990, Furuhata et al. (6) isolated *Methylobacterium* strains from hospital tap water in high frequency, showing that hospital tap water is not free from the contamination.

The genus *Methylobacterium* was initially proposed by Patt et al. in 1976 (16), and 19 species have been reported so far (4). This pink pigment-producing bacteria is commonly isolated from various natural environments, including aqueous environments. The *Methylobacterium* strains may be environmental indigenous bacteria, and have been considered to have weak pathogenicity.

However, *Methylobacterium* species have also recently been attracting attention as opportunistic pathogens in the clinical field (19), and isolation from clinical materials has been reported (12, 14). Hospital tap water is considered to be a source of *Methylobacterium* infection, and sufficient monitoring of this genus is necessary. Under these circumstances, the expansion of *Methylobacterium* infection due to the change of water for washing hands from sterile water to tap water is a concern.

With the aim of contributing to hygienic control, this study investigates the distribution of *Methylobacterium* species in hospital tap water, and their drug sensitivity.

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Abbreviations: MIC, minimum inhibitory concentration; UPGMA, unweighted pair group method average.

Materials and Methods

Bacterial strains and cultivation. In 2004, 271 samples of tap water in hospitals in 13 prefectures of Japan were cultured on R2A agar medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 30 C for 7 days. Totally 58 colonies with pink pigmentation which were formed by Gram-negative rod-shaped bacteria were collected and stored at −80 C. The 58 strains were confirmed to belong to genus *Methylobacterium* according to the definition given by Patt et al. in 1976 (16). The stored strains were cultured on R2A agar medium at 30 C for 7 days and used for further study. Residual free chlorine concentrations in tap water was simultaneously assayed by the colorimetry method with the Aquacheck LC (Nissan Chemical Industries, Ltd., Tokyo).

Biochemical tests. Forty-nine biochemical properties were tested using API50CH (bioMériux, Marcy-l'Etoile, France) according to the attached instructions. AUX medium (bioMériux) was used as the test medium, and positive reaction was judged based on the turbidity of the medium after incubation at 30 C for 14 days. The results were analyzed using analytical software, Bio Numerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium), and a phylogenetic tree was prepared using the UPGMA method (18).

Identification by partial 16S rDNA sequencing. Genomic DNA was extracted and purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the protocols of the manufacturer. Using the extracted DNA solution as the template for PCR, of a 5' end partial region about 500-bp of the 16S rRNA gene was amplified by PCR using a MicroSeq 500 16S rDNA PCR Kit (Applied Biosystems, Foster City, Calif., U.S.A.). The PCR products were purified using PCR Kleen Spin Columns (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). The sequencing reactions of the PCR products were performed using a 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 310 Genetic Analyzer (Applied Biosystems) was used for sample electrophoresis and data collection. The obtained sequence data were compared with reference data from GenBank, and a phylogenetic tree was prepared by the neighbor-joining method of Saitou and Nei (1987) (17). Identification of the species was performed by the more than 99% similarity of the partial sequence of 16S rDNA.

Susceptibility testing. Drug sensitivity tests were performed using E-test (AB BIODISK, Dalvägen, Solna, Sweden) according to the kit instructions. Antibiotics tested were ampicillin (ABPC), cefuroxime (CXM), gentamicin (GM), erythromycin (EM), vancomycin (VCM), tetracycline (TC), imipenem (IPM), chloramphenicol (CP), ofloxacin (OFLX) and fosfomycin (FOM). A bacterial suspension of each test strain (0.5 ml) was dripped on R2A agar medium (Wako Pure Chemical Industries, Ltd.) (60 ml in a 150-mm dish (Corning, Inc., U.S.A.)) and smeared over the surface using a Conradi stick, and an E-test strip was closely attached to the medium. The plates were cultured at 30 C for 7 days, and the growth inhibition zone formed around the strip was observed. MIC was judged by macroscopically reading the graduation at the point where the end of the growth inhibition zone and the strip crossed.

Results

Diversity of Biochemical Properties of Tap Water-Derived Methylobacterium *Strains*

The free residual chlorine concentrations of hospital tap water where 58 strains were isolated are shown in Table 1. The 58 strains were tested for 49 biochemical properties (Table 2). Fifty-four strains were positive for

Table 1. The free residual chlorine concentrations of hospital tap water where 58 strains of *Methylobacterium* spp. were isolated

Free residual chlorine (mg/liter)						Total
0	0.1–0.2	0.3–0.4	0.5–0.6	0.7–0.8	UN[a]	
6	13	17	2	2	18	58

[a] Unknown.

Table 2. Positive characteristics of *Methylobacterium* spp. isolated from the tap water in hospitals using the API50CH test

No.	Characteristics	No. of positive strains (%)
1	Glycerol	42 (77.8)
2	Erythritol	3 (5.6)
3	D-Arabinose	14 (25.9)
4	L-Arabinose	23 (42.6)
5	Ribose	1 (1.9)
6	D-Xylose	17 (31.5)
7	L-Xylose	4 (7.4)
10	Galactose	12 (22.2)
11	Glucose	9 (16.7)
12	Fructose	31 (57.4)
16	Dulcitol	3 (5.6)
37	Glycogen	1 (1.9)
41	D-Lyxose	13 (24.1)
43	D-Fucose	17 (31.5)
44	L-Fucose	5 (9.3)
47	Gluconate	31 (57.4)
48	2-Ketogluconate	2 (3.7)

n=54 strains.

some properties, and 4 strains were negative for all properties. Positive reaction was detected only in 17 (34.7%) of the 49 biochemical properties, and the number of positive properties was generally low. The properties shared by most strains were the utilization of glycerol (42 strains, 77.8%), followed by fructose and gluconate (31 strains each, 57.4%), and L-arabinose (23 strains, 42.6%), D-xylose and D-fucose (17 strains each, 31.5%), D-arabinose (14 strains, 25.9%), D-lyxose (13 strains, 24.1%), galactose (12 strains, 22.2%) and glu-

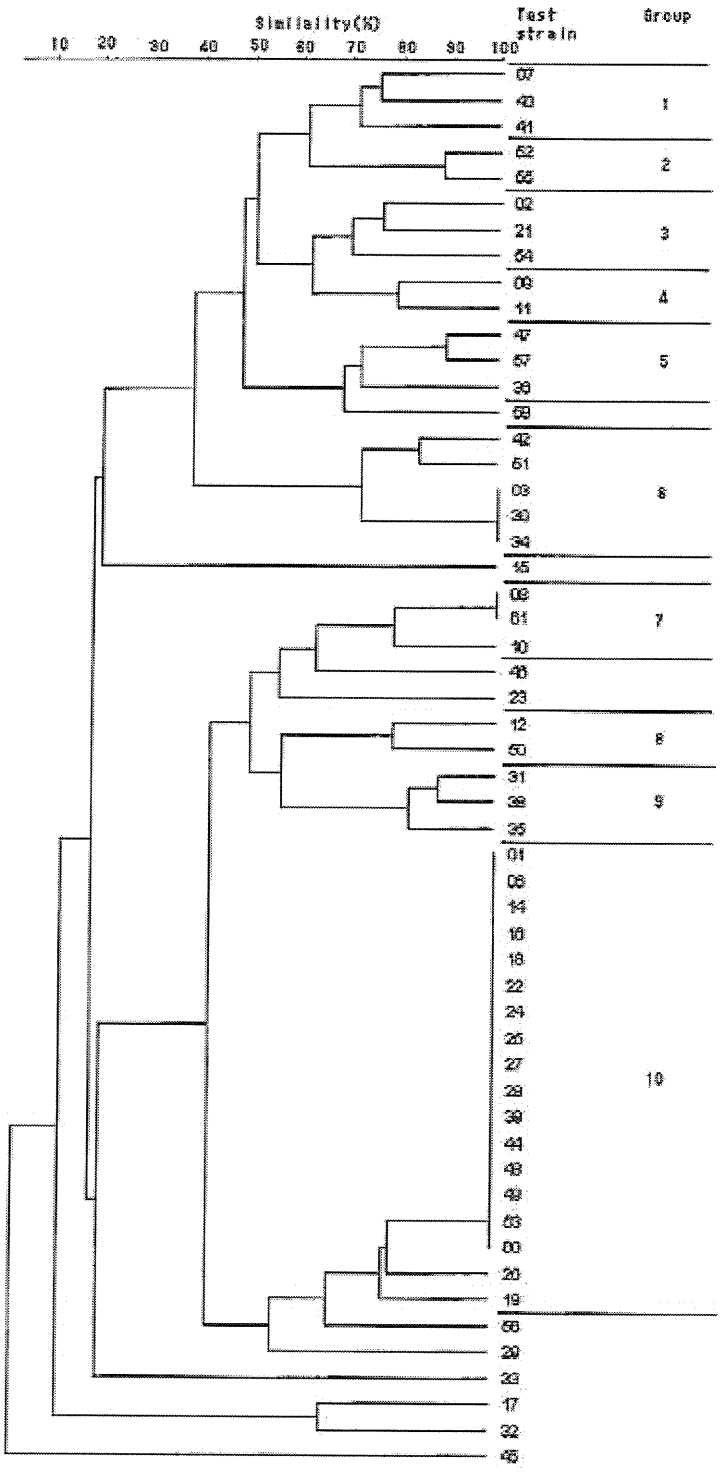

Fig. 1. Dendrogram of 54 *Methylobacterium* strains isolated from hospital tap water based on 49 biochemical characteristics.

cose (9 strains, 16.7%). In addition, positive reaction for L-fucose was detected in 5 strains (9.3%), L-xylose in 4 (7.4%), erythritol and dulcitol in 3 each (5.6%), 2-ketogluconate in 2 (3.7%), and ribose and glycogen in 1 each (1.9%).

The results of cluster analysis based on the biochemical properities are presented as a dendrogram in Fig. 1. When the strains were grouped at a similarity of 70%, 48 strains (82.8%) were grouped into 10 clusters (Groups 1 to 10), but 10 strains (17.2%) were not grouped with any strains, showing considerable diversity. The highest number of strains (18 strains, 33.3%) was included in Group 10, and the similarity of characteristics was 100% in 16 of these strains. Five strains (9.3%) were included in Group 6, and the similarity was 100% in 3 strains.

Identification of Tap Water-Derived Methylobacterium *by 16S rDNA Sequence Analysis*

From the results of the similarity analysis based on the partial sequence of 16S rDNA, 36 of the test strains (62.1%) were identified as *Methylobacterium* species (Table 3). *M. aquaticum* and *M. fujisawaense* were most frequently identified (11 strains each, 19.0%), followed by 6 strains (10.3%) each of *M. mesophilicum* and *M. radiotolerans*. One strain (1.7%) each were identified as *M. aminovorans* and *M. hispanicum*. Multiple candidate species were considered for 13 strains (22.4%), and determination based only on the results of this study was difficult. *M. rhodinum* and *M. suniense* were candidates for 3 strains (5.2%).

The results of phylogenic analysis of the isolates compared with sequence data of known species are shown in Fig. 2. Arbitrarily, the species were divided into Group A, with very high homology with allied species, and Group B with low homology with allied species. Group A consisted of 12 species: *M. zatmanii*, *M. thiocyanatum*, *M. rhodesianum*, *M. lusitanum*, *M. populum*, *M.aminovorans*, *M. suomiense*, *M. rhodinum*, *M. chloromethanicum*, *M. extorquens*, *M. dichloromethanicum* and *M. organophilum*, and 15 isolates (25.9%). Group B consisted of 6 species: *M. hispanicum*, *M. radiotolerans*, *M. fujisawaense*, *M. nodulans*, *M. aquaticum* and *M. mesophilicum*, and 43 isolates (74.1%).

Because the similarities of 9 strains (15.5%) (Strain No. 6, 8, 18, 22, 23, 26, 30, 31, 61) to the sequences of type strains (Fig. 2) were low (less than 99%), the possibility of their being new species was considered for these unidentified strains.

On comparison of these identification results with the above biochemical property clusters, strains identified to be the same species belonged to multiple biochemical property clusters (Table 3), showing that identification based on biochemical properties alone is difficult.

MIC Distributions of Tap Water-Derived Methylobacterium *Species*

Table 4 shows the ranges of MIC, 50% MIC values (MIC_{50}), and 90% MIC values (MIC_{90}) of 10 antibiotics for the test strains. On comparison of the MIC_{90} of the drugs tested, IPM showed the highest antibacterial activity (1 μg/ml) among the 10 drugs, followed by TC (8 μg/ml) and GM (128 μg/ml), but the MIC_{90} of the other drugs was the highest concentration tested (>32 μg/ml, >256 μg/ml, >1,024 μg/ml).

The range of MIC of TC, EM and GM was wide and the distribution was unimodal. The susceptibility was also various in ABPC, VCM, CP, and OFLX, and the number of strains in the highest concentration of MIC tested was 16 strains (>256 μg/ml, 27.6%), 41 strains

Table 3. *Methylobacterium* species identified by 16S rDNA sequence analysis, and their groups by biochemical properities

Species	No. of strains (%)	Groups of characteristics[a]
M. aquaticum	11 (19.0)	3, 10, UC[b], NG[c]
M. fujisawaense	11 (19.0)	1, 2, 4, 5, 6, 8, 9, UC
M. mesophilicum	6 (10.3)	1, 2, 6, NG
M. radiotolerans	6 (10.3)	3, 4, 5, 6, UC
M. aminovorans	1 (1.7)	10
M. hispanicum	1 (1.7)	7
M. rhodinum or *M. suomiense*	3 (5.2)	10
M. lusitanum or *M. populum* or *M. rhodesianum* or *M. thiocyanatum* or *M. zatmanii*	9 (15.5)	8, 10
M. extorquens or *M. chloromethanicum* or *M. dichloromethanicum*	1 (1.7)	UC
Methylobacterium sp.	9 (15.5)	6, 7, 9, 10, UC
Total	58 (100.0)	

[a] Groups in Fig. 1.
[b] Unclassified.
[c] No growth.

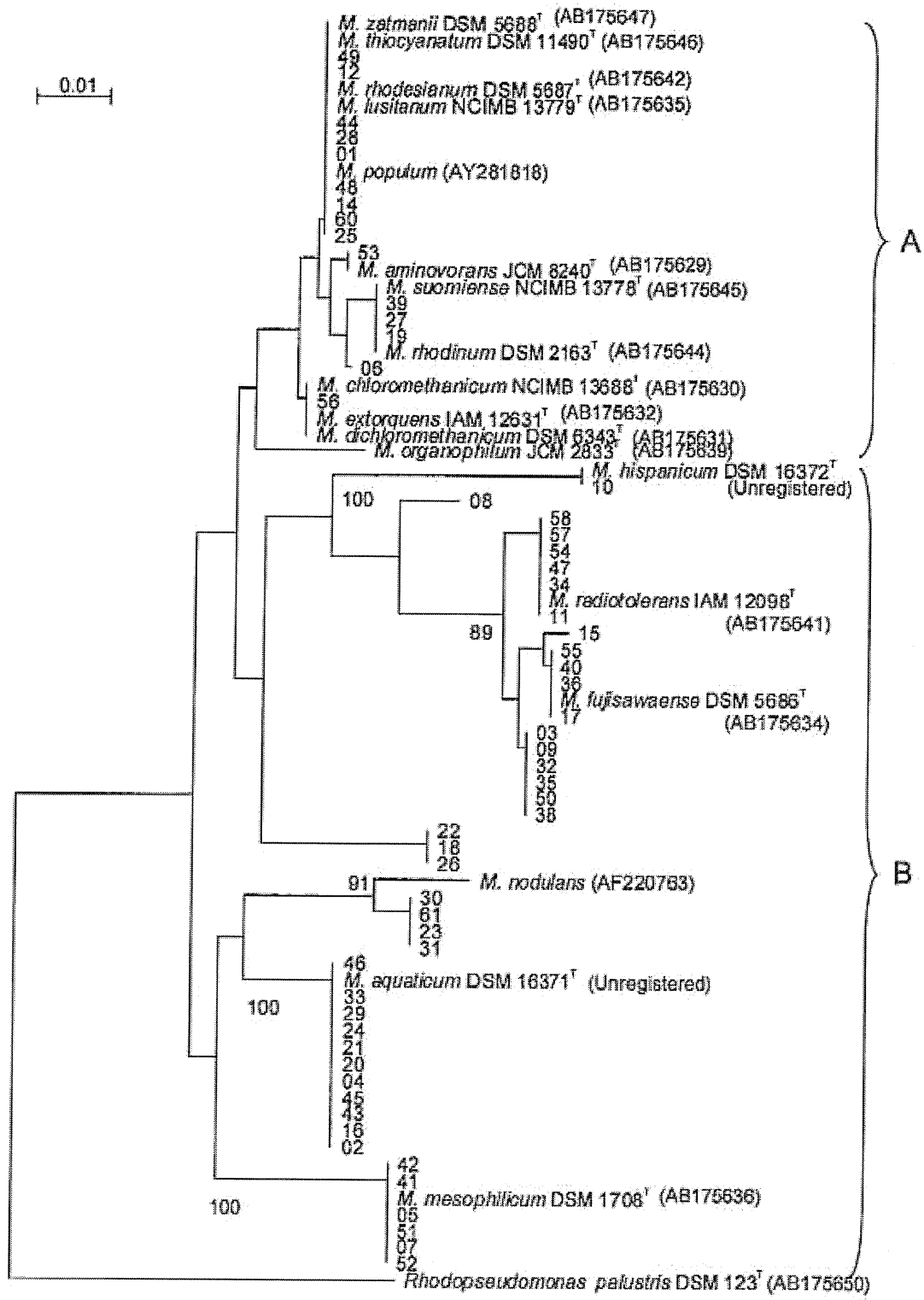

Fig. 2. Distance matrix tree showing phylogenetic relationships among the *Methylobacterium* strains within the type strains. *Rhodopseudomonas palustris* was used as a member of outgroups. The scale bar indicates the number of substitutions per nucleotide position.

Table 4. Susceptibility of *Methylobacterium* strains isolated from the tap water in hospitals to 10 antimicrobial agents using the E-test

Antibiotic	MIC (μg/ml)			
	Range		MIC_{50}	MIC_{90}
Ampicillin	8 –	>256	128	>256
Cefuroxime	1 –	>256	128	>256
Gentamicin	1 –	>256	16	128
Erythromycin	0.032 –	>256	4	>256
Vancomycin	8 –	>256	>256	>256
Tetracycline	0.064 –	32	2	8
Imipenem	0.032 –	8	0.25	1
Chloramphenicol	1 –	>256	256	>256
Ofloxacin	0.25 –	>32	8	>32
Fosfomycin	2 –	>1,024	64	>1,024

n=58 strains.

(>256 μg/ml, 70.7%), 21 strains (>256 μg/ml, 36.2%) and 20 strains (>32 μg/ml, 34.5%) respectively, showing high resistance. In contrast, IPM, CXM and FOM showed bimodal MIC distribution. Regarding the number of drugs for which the MIC value was the highest concentration tested, there were 7 drugs for 1 strain (1.7%), followed by 6 drugs for 3 strains (5.2%), 5 drugs for 2 strains (3.4%), 4 drugs for 9 strains (15.5%) and 3 drugs for 8 strains (5.2%), showing that many strains were multidrug resistant. As a result, the susceptibility varied in all drugs, and sensitivities varied markedly among the strains. Excluding IPM and TC, the cumulative MIC distribution of the other drugs was considerably shifted to the resistance side (higher concentration of MIC).

The drug sensitivities of these test strains were not related to species. There was no association between the drug sensitivity and the site of isolation or the free residual chlorine concentration in the tap water from which the bacteria were isolated.

Discussion

The objective of this study was to elucidate which species of *Methylobacterium* inhabit tap water in hospitals. Since *Methylobacterium* species were isolated from various samples, including aqueous environments (11, 15), it was showed that the bacteria were widely distributed in natural environments. Because of the diversity in the properties among the strains, new species have been reported continually since 2000 (1, 8, 13). Currently, 19 species belong to the genus *Methylobacterium* (4). This study showed that various *Methylobacterium* species inhabit hospital tap water in Japan, and that *M. aquaticum* and *M. fujisawaense* are dominant. *M. aquaticum* was isolated from tap water in Spain by Gallego et al. (8) and reported as a new species in 2005. *M. fujisawaense* was proposed as a new species by Green et al. in 1988 (10), and it was found that these bacteria were isolated in Fujisawa, Kanagawa Prefecture, Japan, by Kouno and Ozaki.

In the phylogenetic analysis, since the strains belonging to Group B formed 74.1%, it became clear that there are many strains belonging to Group B in tap water, and it was in agreement with results reported by Hiraishi et al. (11). These isolates were investigated with regard to the relationship with the isolated areas and the residual chlorine concentration in tap water, but no marked tendencies were noted in geographical differences or with regard to chlorine resistance.

Since the strains belonging to Group A had a high homology with the partial sequence of 16S rDNA, more detailed investigation is necessary for identification of the species. The 9 strains (No. 6, 8, 18, 22, 23, 26, 30, 31, 61) suggested to be new species could be divided into 4 groups.

Excluding strain 12 and 56, all other strains belonging to Group A were included in phenotypic Group 10 in Fig. 1, suggesting that the phenotypes of Group A species are similar. Although 5 strains (No. 16, 18, 22, 24, 26) belonging to Group B were also included in phenotypic cluster 10, no 16S rDNA group-specific or species-specific phenotypic characteristics were found. Based on the above findings, it may be difficult to identify *Methylobacterium* species based on the biochemical properties alone.

The isolation of *Methylobacterium* species from clinical materials has been known from the past (9). Because *Methylobacterium*-associated sepsis has recently been noted occasionally in AIDS patients (20) and bone marrow-transplanted patients (2), it has attracted attention as an etiological agent of opportunistic infection (19). Thus, information on the drug sensitivity of clinical isolates is important for therapy. Brown et al. (3) reported that in drug sensitivity tests of 15 clinical isolates and 3 standard strains, 100% of the strains were sensitive to amikacin and gentamicin even at a low concentration, but 9 isolates (60%) were resistant to β-lactams due to β-lactamase production. Zaharatos et al. and ourselves performed a similar drug sensitivity test of clinical isolates with imipenem and meropenem, using an E-test, and the MIC of imipenem was 0.25–1 μg/ml, showing high sensitivity, but the MIC of meropenem was >32 μg/ml, showing very strong resistance (21).

In the present study, the tap water-derived isolates exhibited strong resistance to 8 drugs, including the β-lactams except for imipenem and tetracycline. Regarding imipenem, the MIC_{90} was 1 μg/ml, showing high

sensitivity, as reported by Zaharatos et al. (21). However, the MIC_{90} of gentamicin was 128 μg/ml, showing low sensitivity, in contrast to the results of Brown et al. (3). Brown et al. obtained MIC using the agar plate dilution method, but we used the E-test, and the difference may have been due to methodological differences. Since a high resistance to β-lactams such as ampicillin and cefuroxime was noted, β-lactamase production by the test isolates was investigated using the disc method. β-Lactamase production was detected in 51.7% of the test isolates (data not shown), and it was in agreement with results reported by Brown et al. (3). Since *Methylobacterium* strains were multidrug resistant, it will also be necessary to elucidate the tolerance mechanisms.

As mentioned above, since the classification of *Methylobacterium* species is unclear in many regards, it is thought that a reconsideration of the taxonomy based on gene techniques will be even more necessary from now on.

From the fact that *Methylobacterium* species which are pathogenic bacteria of opportunistic infection inhabit hospital tap water in Japan, careful follow-up study is necessary to draw a conclusion that tap water can be used in stead of sterilized water for handwashing before surgical operations.

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(Bokin Bobai Vol.34, No.6, pp.323–328, 2006)

【報文】

病院内水道水からの貧栄養細菌の分離状況

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Isolation of Oligotrophic Bacteria from Hospital Tap Water

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To investigate the distribution of oligotrophic bacteria in hospital tap water, tap water samples were subjected to pour culture at 25℃ for 7 days using R2A agar medium. Bacteria were detected in 222 of 271 samples (81.9%) collected nationwide, mainly in the Kanto district, revealing that oligotrophic bacteria are present at a very high frequency in hospital tap water. The distribution was investigated in relation to the geography or form of hospital management, but no marked tendency was noted. The number of isolated oligotrophic bacteria varied from 1.0×10^3 CFU/ml to 2.8×10^1 CFU/ml among the samples, but the number was less than 1.0×10^1 CFU/ml in 54 samples accounting for the highest ratio (24.3%). Of 538 oligotrophic isolates, 457 isolates (84.9%) were gram-negative rods, and 108 isolates (23.6%) were *Methylobacterium*, ranking the highest, followed by 71 isolates of *Pseudomonas* (15.5%). However, it was not possible to identify 274 isolates (60.0%). Gram-positive rods, *Bacillus* and *Corynebacterium*, and cocci, *Micrococcus* and *Staphylococcus*, were also isolated, although the frequencies were low. Based on these findings, it should be noted that hospital tap water contains oligotrophic bacteria which may cause opportunistic infections in susceptible patients.

(Accepted 16 February 2006)

Key words : Oligotrophic bacteria (貧栄養細菌)/Tap water (飲料水)/Hospital (病院).

緒　　言

日本の水道水では塩素消毒が義務付けられていることから消化器系病原微生物などは殺菌されている。しかしながら，建築物において受水槽等の給水施設を経由した後に配水される水道水中には残留塩素に抵抗性を示す様々な貧栄養細菌が広く生息していることはすでに報告したとおりである[1]。こうした細菌は36℃，24時間の培養条件で試験される水道法の水質基準項目である一般細菌としては検出されないため，その生息が十分に認識されているとは言い難く，病院内の水道水における貧栄養細菌の生息状況は明らかにされていない。

これら貧栄養細菌のなかでも *Methylobacterium* 属菌は日和見感染原因菌として臨床材料からの分離報告が散見される[2-4]。本菌感染症の場合はその感染源が水道水の可能性もあることから，易感染者が病院内の水道水を直接利用することなどにより *Methylobacterium* 属感染症の拡大が危惧される。

そこで著者らは上述のことを加味し，微生物生態学的視点から病院内水道水における貧栄養細菌の生息実態を調査した。

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0385-5201/2006/0610-0323　$02.00/0