Mycolic Acid-Containing Actinomycetes Associated with Activated Sludge Foam

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Mycolic acid-containing actinomycetes associated with extensive foaming in the aeration basin of the activated sludge process were isolated and analyzed by phenotypical, chemotaxonomical and phylogenetic methods. Whole cell sugar patterns of two isolates were pattern A. The nearly complete sequences of the 16S rRNA genes (rDNAs) of the isolates were determined and compared by using several tree-making algorithms. With polyphasic methods, strain SCNU1 was identified as *Gordona sputi*, and strain SCNU5 assigned to the genus *Tsukamurella*. The presence of opportunistic pathogens of chronic lung infections within foams can cause public health problems and render waste-treatment processes inefficient.

Key words: Mycolic acid, foaming, *Gordona sputi*, *Tsukamurella*

Bulking and foaming are the most common problems in the activated sludge process. Bulking is a macrostructure failure due to the presence of large numbers of filamentous microorganisms in activated sludge. These organisms interfere with the compaction and settling of activated sludge either by producing a very diffuse floc structure or by growing in profusion beyond the confines of the activated sludge floc into the bulk medium thereby causing bridging between flocs (1, 26).

The formation of scum or foam in the activated sludge process is also caused by filamentous bacteria, notably *Microthrix parvicella*, and mycolic acid containing actinomycetes. *Nocardia* sp., *Gordona* sp., *Tsukamurella* sp., *Actinomadura* sp., *Micromonospora* sp., *Mycobacterium* sp., and some *Streptomyces* sp. are known to produce foam in activated sludge (1, 2, 6, 25). These bacteria have hydrophobic cell surfaces and when present in sufficient numbers in activated sludge, they render flocs hydrophobic and hence amenable to the attachment of air-bubbles. The air bubble-floc aggregate is less dense than water and hence floats to the surface of the sludge. The hydrophobic flocs tend to stay at the surface of

the sludge where they accumulate to form a thick, chocolate-brown coloured foam or scum. Activated sludge foams cause a number of problems, namely extra-house keeping on the part of the operator; blockage of scum removal systems; reduction of oxygen transfer at the surface of mechanically aerated basins; carriage and dispersal of pathogens in wind blown scum; drying of scum with resultant cleaning and possibly odour problems, and reduction of effluent quality through an increase in effluent suspended solids and biological oxygen demand if the scum reaches the final effluent (26).

The primary aim of the present study was to isolate, characterize and identify mycolic acid-containing actinomycetes associated with extensive foaming in the aeration basin. First, the chemical composition and physiolgical properties of the strains were investigated. Then, the almost complete sequences of the 16S rRNA genes (rDNAs) of isolates were determined and compared by using several tree-making algorithms (11, 13, 18).

Materials and Methods

Isolation of actinomycetes associated with foam

The foams used in this study were obtained from

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three full-scale plants in Sunchon, Korea. Two pro-
cedures such as micromanipulation and direct plat-
ing were applied to isolate the foam-forming strains.
Approximately 0.5 mg of foams were diluted with
10 ml of distilled water. The mixture was stirred in
the tube mixture for 20 minutes and the flocs were
allowed to settle. Then, an aliquot (0.5 ml) of the
supernatant obtained from the surface was used as
an inoculum. First, supernatant was smeared on
the sloppy agar (0.5%, w/v) slice, and the micro-
organisms on the agar slice was picked with a
micromanipulator (Narishigae) attached to an inver-
ted microscope (Nikon). Otherwise, a direct plating
technique was used to isolate actinomycetes. Sev-
eral agar media were used to isolate the actino-
mycetes. R2A (22), Tween media (BSM, PRM-1,
EPM-1, EPM-2, and NTM; 16), SCY, I, Sludge hy-
drolysate (6), Tryptone-yeast extract-glucose agar
(TYGA;10) and MP (25) medium were prepared as
described previously. Inoculated plates were incu-
bated for 3 weeks at 28°C. The colonies were exam-
ined daily using a microscope. For the subsequent
cultural work, glucose yeast extract agar (GYEA)
medium and the same incubation conditions were
used. Isolated strains were maintained as mycelial
fragment suspensions (20% [v/v] glycerol) at –30°C.

Morphology

Mycelial fragmentation, aerial mycelium color,
pigmentation and colony morphology were observed
on GYEA, oatmeal agar (ISP3), and inorganic salts-
starch casein agar (ISP4) medium after incubation
at 30°C daily for 7 days (24). Melanin production
was observed on peptone-yeast extract-iron agar
(ISP6) and tyrosine agar (ISP7).

Mycolic acid determination

Biomasses were obtained from liquid cultures in
Sauton's broth medium (Difco) at 28°C for 5 days,
freeze dried, and kept refrigerated for further anal-
ysis. Extraction of mycolic acids was performed
using alkaline methanolysis (20). Thin layer chro-
matography, using Kieselgel 60 Aluminium plate
(Merck), was used to detect the mycolic acids. A sin-
gle development with petroleum ether (bp 60~
80°C)-toluene (70:30, v/v) was used. Detection was
performed with a 5% ethanolic molybdophosphoric
acid spray followed by heating at 180°C for 5 min.

Menaquinone analysis

About 50 mg of dried biomasses was treated with
chloroform/methanol (2:1, v/v) by shaking overnight.
The extracts were concentrated in vacuo using an
electric aspirator and the cell debris were removed
by centrifugation. The solvents were evaporated

completely, and the remnants were resuspended in
hexane. High performance liquid chromatographic
separation of the quinones was done with an ODS
Hypersil column (2004.6 mm, particle size 5 μm,
Hewlett Packard) and acetonitrile/tetrahydrofuran
(70:30, v/v) as the mobile phase. The flow rate was
1 ml/min at 37°C, and detector was a UV detector
operated at 254 nm.

Whole cell sugar analysis

Whole cell sugars extracted as alditol acetates (7)
were analyzed using a gas chromatograph (Hewlett
Packard 5890A) fitted with a flame ionization
detector. Separation was achieved using a 0.53 mm
× 30 m SP 2380 (Supelco) fused silica capillary col-
umn. The temperature was programmed to hold at
160°C for 2 min, then to rise by 5°C/min. The Injec-
tor temperature was held at 250°C, and the detec-
tor at 300°C.

Phenotypic tests

The strains were examined for 83 unit characters
including pH- and temperature-dependent growth,
enzyme synthesis, resistance to antibiotics and
chemicals, antibiosis, utilization of carbon and
nitrogen sources, and acid production from sugars
(10, 29).

DNA extraction and amplification of 16S
rDNA

Chromosomal DNA was isolated from the test
strains using a procedure (3) slightly modified from
that of Pitcher et al. (21). PCR amplification of 16S
rDNA was performed using a capillary Rapidcycler
(Idaho Tchnology). The following conditions were
used for amplification using primers 27f and 1525r:
denaturation at 94°C for 1 min, annealing at 55°C
for 1 min and elongation at 72°C for 3 min for each
cycle. A total of 30 cycles were performed followed
by a final elongation step at 72°C for 10 min and
cooling at 25°C for 1 min.

Sequencing of 16S rDNA

The amplified fragments were directly sequenced
using a Taq DyeDeoxy terminator Cycle Sequencing
Kit (Applied Biosystems) and previously described oli-
gonucleotide primers (3). Sequencing gel electro-
phoresis was carried out and nucleotide sequences
were automatically obtained using an Applied Bio-
systems DNA sequencer (model 373A) with soft-
ware provided by the manufacturer.

Phylogenetic analysis

The two 16S rDNA sequences were aligned man-
ually with mycolic acid containing actinomycete

Table 1. Chemotaxonomic characteristics of strain SCNU1 and SCNU5

Strain	Wall diamino acid	Wall sugars	Wall chemotype[a]	Major menaquinones
SCNU1	*meso*-DAP	Galactose, arabinose	IV	MK-8(H_2), MK-9(H_2)
SCNU5	*meso*-DAP	Galactose, arabinose	IV	MK-9(H_0)

[a] Wall chemotype as described by Lechevalier and Lechevalier(17).

nucleotide sequences derived from the Ribosomal Database Project (19) and EMBL/GenBank database using the AL 16S program (4). The reference sequences had the following accession numbers: Z46753 (*Corynebacterium blutamicum*), X79290 (*Dietzia maris*), X80633 (*Gordona aichiensis*), X80635 (*G. amarae*), X79287 (*G. bronchialis*), X80632 (*G. rubropertincta*), X80634 (*G. sputi*), X79286 (*G. terrae*), X52917 (*Mycobacterium tuberculosis*), X57949 (*Nocardia asteroides* DSM43005), X79288 (*Rhodococcus rhodochrous*), Z46751 (*Tsukamurella paurometabola*), X87340 (*G. hydrophobica*), X93485 (*G. hirsuta*), Z35435 (*Skermania piniformis*), X92981 (*T. pulmonis*), and Y12246 (*T. tyrosinosolvens*). 16S rDNA sequences of strain SCNU1 and SCNU5 were deposited in the GenBank under accession number AF150493 and AF150494, respectively. Evolutionary trees for the datasets were inferred by using three algorithms, namely the neighbour-joining (23), least squares (19) and maximum-parsimony (15) methods. Evolutionary distance matrices for the neighbour-joining and least-squares methods were generated as described by Jukes and Cantor (12). The PHYLIP package (8) was used for making trees with the three algorithms. The resultant unrooted tree topologies were evaluated by carring out bootstrap analysis of the neighbour-joining method data based on 1000 re-samplings using the SEQBOOT and CONSENSE programs in the PHYLIP package.

Results

Estimation of isolation medium

Several agar media were used for the isolation of actinomycetes associated with activated sludge foam. Most colonies which developed on the media were non-actinomycete bacteria. While large numbers of filamentous and mycelial fragments were observed microscopically in the foam and diluent, the recovery rate was relatively poor on the agar plate. Actinomycetes were recovered only on I and TYGA medium, while other media were not suitable for the growth of actinomycetes.

Chemotaxonomy

Two mycolic acid-containing strains were pure cultured. They contained meso-diaminopimelic acid (*meso*-DAP). TLC analysis of mycolic acids confirmed that strain SCNU1 and SCNU5 belong to the genus *Gordona*, and *Tsukamurella*, respectively. Characteristic sugars and major menaquinone composition of strains were shown in Table 1. The two strains contained the same whole cell sugars such as arabinose and galactose (whole cell sugar pattern A). Strain SCNU5 contained menaquinone of 9 isoprene units, designated as MK-9, without hydrogenation (MK-9(H_0)), while strain SCNU1 had menaquinones of different isoprene units but the same degree of hydrogenation, designated as MK-8(H_2) and MK-9 (H_2).

Phenotypic properties

The properties of the strains were described in Table 2. The strains did not produce aerial mycelium and diffusible pigment. They could grow both at pH 4 and 9. They did not produce melanin. They all degraded gelatin and esculin, but not starch, casein, elastin and xanthine. They were sensitive to streptomycin, gentamicin, and vancomycine, but resistant to bacitracin. The strains utilized succinate, pyruvate, and propionate, but not lactate and citrate. They did not produce acid from most of the sugars tested. The strains did not show antimicrobial activity.

Phylogenetic analysis

The almost complete 16S rDNA sequences of the strains were compared with the corresponding nucleotide sequences of mycolic acid containing actinomycetes by using three tree-making algorithms (i.e. the neighbour-joining, least squares and maximum-parsimony as shown in Fig. 1). 16S rDNA of strain SCNU1 was identical to that of *G. sputi* except for one nucleotide. Nucleotide sequences of strain SCNU5 showed 99.73% similarity with *T. pulmonis* and 99.66% with *T. tyrosinosolvens* (Table 3).

Comparison of *Tuskamurella* sp. strain SCNU5 with related species

The isolate SCNU5 was compared with the most related species, *T. pulmonis* and *T. tyrosinosolvens*. Physiologically, SCNU5 differs from *T. pulmonis* in i) its inability to degrade urea, ii) its ability to liquify gelatin, iii) its sensitivity to

Table 2. Phenotypic properties of the mycolic acid-containing actinomycete strain SCNU1 and SCNU5 isolated from activated sludge foam

Unit characters	SCNU1	SCNU5	Unit characters	SCNU1	SCNU5
Substrate mycelium color	Pink	–	D-Melibiose	–	–
Melanin production	–	–	Cellobiose	–	–
Degradation of:			Maltose	–	–
Hypoxanthine	–	–	Trehalose	–	–
Elastin	–	–	Mannitol	–	+
Xanthine	–	–	Salicin	–	–
Casein	–	–	Arabinose	–	–
Urea	+	–	Adonitol	–	–
Starch	–	–	Melezitose	–	–
Tyrosine	–	–	Inositol	–	–
Guanine	–	–	Xylose	–	–
Gelatin	+	+	Fructose	–	–
Esculin	+	+	Mannose	–	–
Growth in the presence of (%):			Sucrose	–	–
NaCl (4)	+	+	Erythritol	–	+
NaCl (7)	+	–	Galactose	–	–
NaCl (10)	+	–	D-Sorbitol	–	+
Sodium azide (0 01)	+	–	Glucose	–	–
Sodium azide (0.02)	+	–	Dulcitol	–	–
Phenol (0.1)	–	–	Xylitol	–	–
Phenylethanol (0.1)	+	–	Dextrin	–	–
Phenylethanol (0.3)	–	–	Utilization of nitrogen sources:		
Thallous acetate (0.001)	+	–	DL-α-Amino-n-butyric acid	–	–
Thallous acetate (0.01)	+	–	L-Cysteine	–	+
Crystal violet (0.0001)	+	+	L-Valine	–	+
Resistance to antibiotics (μg/ml):			L-Hydroxyproline	–	–
Rifampicin (50)	+	–	L-Histidine	–	–
Penicillin G (10 i.u.)	–	+	L-Phenylalnine	–	+
Streptomycin (10)	–	–	Antimicrobial activity against:		
Gentamicin (10)	–	–	A. niger	–	–
Bacitracin (10 i.u.)	+	+	B. subtilis	–	–
Vancomycin (30)	–	–	C. albicans	–	–
Neomycin (30)	–	–	E. coli	–	–
Utilization of organic acid:			P. fluorescence	–	–
Oxalate	–	–	S. cerevisiae	–	–
Succinic acid	+	+	Enzyme synthesis:		
Pyruvic acid	+	+	Lipolysis	–	–
Lactic acid	–	–	Pectin hydrolysis	–	–
Mucic acid	–	–	Nitrate reduction	+	–
Propionic acid	+	+	Production of hydrogen sulfide	+	+
Gluconic acid	–	–	Growth at:		
Citric acid	–	–	pH 4	+	+
Acid production from sugars:			pH 9	+	+
Lactose	–	–	10°C	+	–
L-Rhamnose	–	–	37°C	+	+
Raffinose	–	–	45°C	–	–

rifampicin and streptomycin, and iv) its ability to utilize gluconic acid. SCNU5 differs from *T. tyrosinosolvens* in i) its inability to degrade hypoxanthine and xanthine, ii) its ability to liquify gelatin, and iii) its ability to utilize citric acid.

Discussion

For the success of selective isolation of foam-asso-

ciated microorganisms, isolation procedures and selective media were tested. Only two media, I and TYGA were found to be adequate for the isolation of actinomycetes. Using the micromanipulation procedure, few filamentous microorganisms were recovered. Finally, two mycolic acid-containing actinomycetes were isolated. Further studies on the choice of isolation medium corresponding to the status of sludge composition and operation style should be carried out.
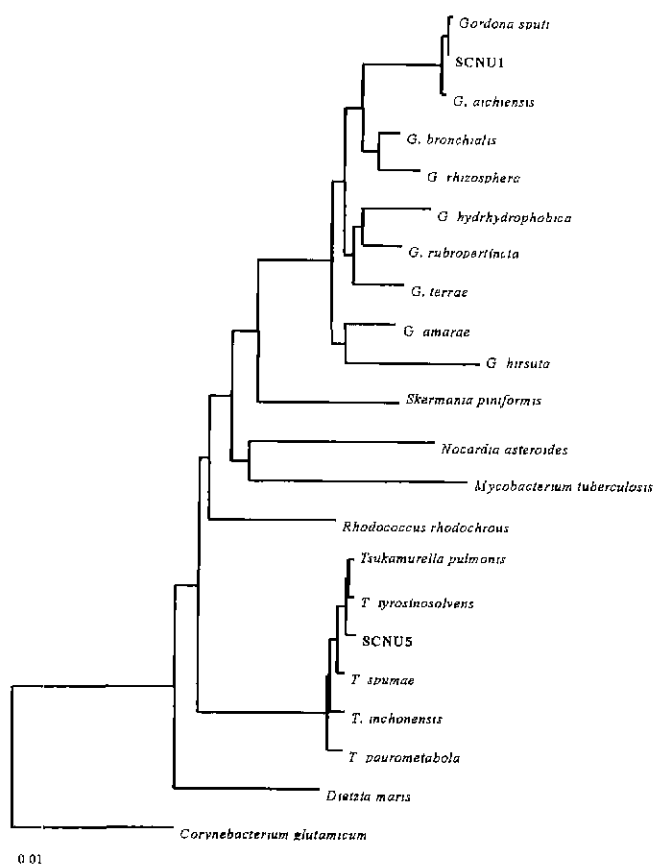
Fig. 1. A neighbour-joining tree (23) based on nearly complete 16S rRNA sequences of mycolic acid-containing bacteria and representative species of related taxa. The scale bar indicates 0.01 nucleotide substitutions per nucleotide position.

In this study, we isolated two mycolic acid-containing actinomycetes, then analyzed and identified them with different categories of phenotypic, chemotaxonomic and phylogenetic methods. Two isolates were different each other in their mycolic acid profile. Whole cell sugar and menaquinone composition were determined by the chromatographic method. They all contained arabinose and galactose as characteristic whole cell sugars. Thus, the two strains were classified as wall chemotype IV. In contrast, their major menaquinones were different each other. From the chemotaxonomical

Table 4. Differential physiological characteristics of strain SCNU5, *T. pulmonis* and *T. tyrosinosolvens*

Unit characters	SCNU5	*T. pulmonis*[a]	*T. tyrosinosolvens*[b]
Diffusible pigment	–	–	–
Degradation of:			
Hypoxanthine	–	–	+
Elastin	–	–	N
Xanthine	–	–	+
Casein	–	–	N
Urea	–	+	N
Tyrosine	–	–	+
Guanine	–	–	N
Gelatin	+	–	–
Esculin	+	+	N
Resistance to antibiotics (μg/l):			
Rifampicin (50)	–	+	N
Streptomycin (10)	–	+	N
Utilization of organic acid:			
Lactic acid	–	–	–
Gluconic acid	–	+	N
Citric acid	–	–	+
Enzymatic activity:			
Nitrate reduction	–	–	N
Growth at:			
37°C	+	+	N
45°C	–	–	–

Source of data from [a]Yassin *et al.* (30) and [b]Yassin *et al.*(31). N, data not available.

analysis, strain SCNU1 and SCNU5 were assigned to the genus *Gordona* and *Tsukamurella*, respectively.

Properties of the isolates were determined against 83 unit characters including morphology, degradation, pH- and temperature-dependent growth, enzyme synthesis, resistance to antibiotics and chemicals, antibiosis, utilization of carbon and nitrogen sources, and acid production from sugars. The two strains did not produce acid from most organic acids. In addition, their antimicrobial activity was not found.

The almost complete sequences of the 16S rRNA genes (rDNA) of two strains were determined and compared by several tree-making algorithms. Strain SCNU1 was almost identical to *G. sputi*. 16S rDNA

Table 3. Levels of 16S rDNA similarity between the strain SCNU5 and related species

Microorganism	% 16S rDNA similarity					
	SCNU5	*T. spumae*	*T. wratislaviensis*	*T. inchonensis*	*T. pulmonis*	*T. paurometabola*
T. spumae	99.66					
T. wratislaviensis	95.79	95.72				
T. inchonensis	99.12	99.52	95.72			
T. pulmonis	99.73	99.52	95.88	99.25		
T. paurometabola	99.18	99.39	95.85	99.46	99.25	
T. tyrosinosolvens	99.66	99.45	95.95	99.38	99.73	99.38

nucleotide sequence of SCNU5 differed with that of *T. pulmonis* and *T. tyrosinosolvens* by 4 and 5 nucleotides, respectively.

The similarity levels between species in the genus *Tsukamurella* were known to be relatively high (30, 31). In contrast to 16S rRNA analysis, the physiological tests revealed some differences between strain SCNU5 and the other two strains. Thus, the SCNU5 strain can be placed in a new species of the genus *Tsukamurella*. For the complete identification of strain SCNU5, molecular fingerprinting techniques, such as random amplified polymorphic DNA (RAPD) PCR and DNA-DNA hybridization, should be carried out.

G. amarae have been frequently isolated from activated sludge foam in previous studies (27). In contrast, *G. sputi* associated with sludge foam was not yet reported . In particular, *Gordona sputi* and a number of species in the genus *Tsukamurella* are infectious agents, and have been commonly isolated from the sputum of patients with chronic lung infections or from the blood of patients with cardiac pacemaker implants (27, 30, 31). The isolation of *G. sputi* in this study confirmed the existence of bacterial pathogens in activated sludge foam. Based on these results mycolic acid-containing actinomycetes can not only make the waste-treatment process inefficient by foaming, but also render the pathogens to wind dispersal.

Acknowledgment

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