

**Morphological and molecular identification of
filamentous microorganisms associated with bulking
and foaming activated sludge**

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Ankia Marleen Wagner

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I certify that the thesis hereby submitted to the University of Pretoria for the degree of M.Sc. Microbiology has not previously been submitted by me in respect of a degree at any other University.

Signature: *Hagner*

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God has never given us a dream without also giving us the ability of making it true.... We might have to work for it though!

Unknown

Opgedra aan die mense wat ek liefhet

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**MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF FILAMENTOUS
MICROORGANISMS ASSOCIATED WITH BULKING AND FOAMING
ACTIVATED SLUDGE**

by

ANKIA MARLEEN WAGNER

PROMOTER: Prof. T. E. Cloete

DEPARTMENT: Microbiology and Plant Pathology

DEGREE: MSc. Microbiology

SUMMARY

The activated sludge process comprises a complex and enriched culture of a mixture of generalist and specialist organisms. The lack of knowledge on species diversity of microbial communities is due to the simplicity of bacterial morphology and the phenotypic characters, and the unculturable portion of microbial cells in natural habitats. Although a wide range of bacteria can be isolated using conventional microbiological techniques of sample dilution and spread plate inoculation, many well-known activated sludge bacteria can not be isolated using them.

The individual microbial cells in activated sludge grow in aggregates that consist of floc-forming organisms together with filamentous microorganisms that form the backbone of the activated sludge flocs. Overgrowth of these filamentous microorganisms often causes settling problems called bulking and foaming. These problems consist of slow settling, poor compaction of solids and foam overflow into the effluent.

Although methods for the isolation of filamentous bacteria from mixed liquor samples have been investigated, the attempts have been largely unsuccessful.

In this study we investigated bulking and foaming activated sludge to identify the dominant filamentous organisms using microscopy and molecular techniques. Using microscopy, the dominant filament associated with the foaming sample was "*Microthrix parvicella*" and in the bulking sample was *Nocardia* spp. The foaming sample was investigated using molecular techniques that involved 16S rDNA sequencing. Although some of the clones isolated from the sludge foam were associated with filamentous bacteria causing foam, no positive identification could be made.

In the part of the study that was conducted in Australia, a rRNA-targeted oligonucleotide probe was designed for the identification of a filamentous organism occurring in activated sludge foam. This organism resembled Eikelboom Type 0041 and was classified in the candidate bacterial division TM7.

The discrepancy that the sequence data did not indicate the dominant filamentous organisms observed by microscopy, highlights the fact that natural microbial communities need to be studied using a combination of techniques since none of the techniques available are sufficient to determine the complete community structure of complex communities such as activated sludge.

**MORFOLOGIESE EN MOLEKULÊRE IDENTIFIKASIE VAN
FILAMENTAGTIGE MIKROËRGANISMES GEASSOSIEER MET UITDYENDE
SLYK EN SLYK SKUIM IN GEAKTIVEERDE SLYK STELSELS**

deur

ANKIA MARLEEN WAGNER

PROMOTOR: Prof. T. E. Cloete

DEPARTEMENT: Mikrobiologie en Plantpatologie

GRAAD: MSc. Mikrobiologie

OPSOMMING

Die geaktiveerde slyk proses bestaan uit 'n komplekse en verrykte kultuur van 'n mengsel van algemene en spesialis bakterieë. Die gebrek aan kennis oor spesie diversiteit van mikrobe gemeenskappe is as gevolg van die eenvoud van bakteriële morfologie en fenotipiese karakters, asook die onkweekbare gedeelte van mikrobiële selle in natuurlike habitate. Hoewel 'n wye reeks bakterieë geïsoleer kan word met behulp van die konvensionele mikrobiologiese tegnieke soos monsterverdunning en uitplating, kan baie bekende geaktiveerde slyk bakterieë nie hierdeur geïsoleer word nie.

Die individuele mikrobiële selle in geaktiveerde slyk groei in aggregate wat bestaan uit vlokvormende bakterieë saam met filamentagtige mikroörganismes wat die ruggraat van die geaktiveerde slyk vlokke vorm. Wanneer die filamentagtige mikroörganismes oorgroei, veroorsaak dit dikwels besinkings probleme naamlik uitdyende slyk en skuimvormende slyk. Hierdie probleme behels stadige besinking, swak verdigting van vastestowwe en skuim wat oorfloei in die uitvloeiende.

Hoewel metodes vir die isolasie van filamentagtige bakterieë vanuit die slykvloeistof ondersoek is, was die pogings grootliks onsuksesvol.

In hierdie studie het ons uitdyende- en skuivormende slyk ondersoek om die dominante filamentagtige organismes met behulp van mikroskopie en molekulêre tegnieke te identifiseer. Mikroskopie het getoon dat die dominante filament geassosieer met die skuim monster "*Microthrix parvicella*" was en in die uitdyende slyk was *Nocardia* spp. oorheersend teenwoordig. Die skuim monster is ook ondersoek met behulp van molekulêre tegnieke wat 16S rDNA basisvolgorde bepaling ingesluit het. Sommige van die klone wat verkry is vanuit die slyk skuim was geassosieer met skuimvormende filamentagtige bakterieë maar geen positiewe identifikasie kon gemaak word nie.

In die deel van die studie wat in Australië gedoen is, is 'n rRNA gerigte oligonukleotied peiler ontwerp vir die identifikasie van 'n filamentagtige organisme wat in gekultiveerde slyk voorkom. Hierdie organisme het ooreengekom met Eikelboom Tipe 0041 en is geklassifiseer in die kandidaat bakteriese divisie, TM7.

Die teenstelling dat die basisvolgorde data nie die dominante filament, soos aangewys deur mikroskopie, uitgewys het nie, verskerp die feit dat natuurlike mikrobiële gemeenskappe met behulp van 'n kombinasie van tegnieke behoort bestudeer te word. Dit is aangesien geen van die tegnieke beskikbaar voldoende is om die algehele gemeenskapstruktuur van komplekse gemeenskappe soos gekultiveerde slyk te bepaal nie.

LIST OF ABBREVIATIONS

BOD	biological oxygen demand
°C	degrees celsius
ca.	approximately
CFB	Cytophaga-Flavobacter-Bacteroides
CLUSTALX	Cluster analysis version X
CY	carbocyanines
dH ₂ O	distilled water
DNA	deoxyribonucleic acids
dNTP	deoxyribonucleic-5"-triphosphate
DO	dissolved oxygen
EDTA	ethylenediaminetetra-acetic acid
F/M	food to microorganism ratio
FIG	figure
FISH	fluorescent <i>in situ</i> hybridisation
g	gram
g/l	gram per litre
h	hour
HRT	hydraulic retention time
l	litre
M	molar
mg	milligram
milliQ H ₂ O	double distilled water from milliQ system
min	minutes
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
mM	millimolar
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHB	poly-β-hydroxybutyric acid
RAS	return activated sludge
RFLP	restriction fragment length polymorphism
RPM	revolutions per minute
rRNA	ribosomal Ribonucleic acid
s	seconds
SDS	sodium dodecyl sulphate
μl	micro litre
WWTP	waste water treatment plant

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CHAPTER 1

INTRODUCTION

Treatment of wastes with the activated sludge process represents a component of the largest biotechnology industry of the world. All of the substrates in the activated sludge system needs to be dispersed so that the microbes can make intimate contact with them, and these microbes must grow as three-dimensional aggregated communities called flocs. The flocs need to have good settling properties to allow them to separate efficiently in the clarifiers from the liquid supernatant (Seviour and Blackall, 1999).

Filamentous organisms will always be present in small numbers in healthy activated sludge plants (Seviour and Blackall 1999). Filamentous microorganisms form the backbone of the activated sludge flocs (Sezgin *et al.*, 1987). The overgrowth of filamentous microorganisms often causes settling problems in activated sludge called bulking and foaming (Bitton, 1994). Bulking sludge is defined as one which only settles slowly and compacts poorly because of an excessive growth of filamentous bacteria associated with flocs (Eikelboom and van Buijsen, 1981). Foaming is the formation of a thick, viscous, stable foam (Jenkins *et al.*, 1993). Among other problems foam may be responsible for reduction of oxygen transfer at the surface of aeration basins and an increase in suspended solids in the effluent (Bitton, 1994; Jenkins *et al.*, 1993).

The routine recognition of filamentous microorganisms is based on microscopic features, which is not a satisfactory system for reliable identification. This problem is exacerbated by an inability to grow many of these organisms in pure culture (Seviour and Blackall, 1999). Many features used for the identification, like PHB and polyphosphate inclusion bodies and attached cells, may only be produced by cells growing under certain culture conditions, and there is no way of knowing how these conditions might change within or between plants (Wanner, 1994b). Proper identification of filamentous bacteria is important to

predict what kind and to which extent the bulking and foaming problems is to be expected (Wanner and Grau, 1989).

A correct finding of the taxonomic position is necessary for the documentation of a microorganism and for its preservation in microbiological collections (Wanner and Grau, 1989). Little taxonomically useful information has been obtained from most of the studies where attempts were made at the characterisation of filaments using their morphological and physiological properties (Williams and Unz, 1985). Most of the filamentous bacteria have not been properly named following the rules of the International Code of Nomenclature. This situation is improving as molecular characterisation techniques like 16S rDNA sequence analysis begin to be applied to these bacteria, and their taxonomic positions become clearer (Seviour and Blackall, 1999).

The application of group-specific, rRNA directed probes may help to determine to which phylum in the Bacteria Domain they belong (Kämpfer, 1997). More discriminating subgroup probes (Amann, 1995) can then be employed together with *in situ* typing or molecular fingerprinting to provide identifiatory information.

The aims of this study were to determine the dominant filamentous organisms in bulking and foaming activated sludge systems using microscopy and molecular identification techniques. In the part of the study conducted in Australia an attempt was made to develop a fluorescently labelled rRNA-targeted oligonucleotide probe for the identification of a filamentous microorganism in a foaming activated sludge sample.

CHAPTER 2

LITERATURE REVIEW

1. Introduction

Activated sludge has become the most widely used process for the treatment of both domestic and industrial wastewaters (Gray, 1990). The activated sludge process also represents the most widespread technology for wastewater purification (Wanner, 1994). The activated sludge process relies on a dense microbial population being mixed in suspension with the wastewater. This process happens under aerobic conditions, which together with the sufficient supply of food, enable the microorganisms to utilise the organic matter present or to support the growth of microorganisms (Gray, 1990).

The individual microbial cells are not separated in the cultivation medium; they grow in aggregates (Wanner, 1994). These aggregates usually consist of floc-forming organisms together with filamentous microorganisms that form the backbone of the activated sludge flocs (Sezgin *et al.*, 1987). The overgrowth of these filamentous microorganisms often causes settling problems in activated sludge, called bulking and foaming (Bitton, 1994).

Since the activated sludge process was first developed, bulking and foaming sludge have been observed (Kämpfer, 1997).

Sludge bulking is one of the major problems affecting biological wastewater treatment (Bitton, 1994). The bulking problem consists of slow settling and poor compaction of solids in the clarifier of the activated sludge system (Jenkins and Richard, 1985).

Foaming creates a number of serious problems. The problems according to Bitton (1994) are as follows:

- Excess scum formation which can overflow into walkways and cause slippery conditions, leading to hazardous situations for plant workers.
- Excess scum may pass into activated sludge effluent, resulting in increased BOD and suspended solids in the effluent.
- Foaming causes problems in anaerobic digesters.
- Foaming can produce the nuisance of odours, especially in warm climates. Foam carries the potential of infection of wastewater workers with opportunistic pathogenic actinomycetes such as *Nocardia asteroides*.

The conditions in wastewater treatment plants are continuously changing, and therefore it seems unlikely that specific parameters will cause the proliferation of certain types of filamentous organisms (Kämpfer, 1997). A comprehensive knowledge of the growth requirements of the filamentous bacteria is necessary for successful control of bulking and foaming of activated sludge (Kämpfer *et al.*, 1994).

Attempts to isolate filamentous bacteria from mixed liquor samples by direct plating were largely unsuccessful (Ziegler *et al.*, 1990). Methods for the isolation and cultivation have been investigated (Van Veen, 1973; Williams and Unz, 1985; Ziegler *et al.*, 1990; Tandoi *et al.*, 1992; Blackall *et al.* 1995). Although some methods have been used successfully, the need for further investigation is still required.

According to Wanner and Grau (1989) a proper identification of filamentous microorganisms is required to:

- predict what kind and to which extent the problem is to be expected,
- estimate the causes of the presence of the filaments in the biocenosis,
- remediate bulking and foaming problems.

The filamentous bacteria found in bulking and foaming activated sludge are normally identified and classified into morphotypes according to Eikelboom (Eikelboom, 1975; Eikelboom and van Buijsen, 1981).

A correct finding of the taxonomic position is necessary for the documentation of a microorganism and for its preservation in microbiological collections (Wanner and Grau, 1989). The lack of availability of pure cultures of most filaments and the limited amount of characterisation data available for them, means that our current understanding of their taxonomic position is very poor and their relationship to other bacteria are not known (Blackall *et al.*, 1996b).

Classification based on morphological, developmental and phenotypic characteristics does not correlate well with phylogenetic relationships (Lane *et al.*, 1985). The comparative analysis of rRNA gene sequences is the preferred method for the classification of bacteria (Amann *et al.*, 1997).

Specific oligonucleotide probes can be synthesised for a unique segment of the 16S rRNA gene of a particular organism (Blackall *et al.*, 1996b). The use of probes in activated sludge allows the genotypic diversity of the filament types to become known, and filament levels to be monitored while altering the wastewater treatment plant (WWTP) conditions.

In this literature review the following topics will be discussed in detail:

- the activated sludge process in general
- the microbiology of activated sludge
- bulking and foaming activated sludge
- types of filamentous organisms
- foam microbiology
- factors that support the excessive growth of filamentous organisms
- isolation and cultivation of filamentous organisms in bulking and foaming activated sludge

- identification techniques including morphological and molecular methods
- the ribosomal RNA approach
- ribosomal RNA
- PCR amplification of 16S rDNA and cloning thereof
- Sequencing and analysis
- in situ hybridisation with examples of oligonucleotide probes for filamentous bacteria

2. Activated sludge

Activated sludge is a suspended-growth process and it represents the most widespread technology for wastewater purification (Bitton, 1994; Wanner, 1994). It is used throughout the world for the treatment of both domestic and industrial wastewaters (Gray, 1990).

The activated sludge process is an aerobic process whereby organic and inorganic substances are transformed by an enrichment culture consisting of an assemblage of micro- and macroorganisms (Richard, 1984a). This process consists of an aerobic treatment that oxidises organic matter to CO_2 , H_2O , NH_4 , and new cell biomass. The microbial cells form flocs that settle in a clarification tank (Bitton, 1994).

The conventional activated sludge system involves the following (Bitton, 1994):

- *Aeration tank:* Aerobic oxidation of organic matter is carried out in the tank. Primary effluent is introduced and mixed with return activated sludge (RAS). This mixture is called the mixed liquor which contains 1,500-2,500 mg/L of suspended solids. In this first stage of purification, the particulate and colloidal material of the wastewater is rapidly absorbed or agglomerated onto the microbial floc. Air is provided mechanically and the aeration supplies oxygen and mixes the floc and wastewater continuously. This continuous mixing action ensures adequate organic nutrients and a maximum oxygen concentration gradient to enhance mass transfer and to help disperse metabolic end products from within the floc (Gray, 1990). A large proportion of the biomass is recycled. A large number of microorganisms is maintained in this way, and these microorganisms can effectively oxidise organic compounds in a relatively short time (Bitton, 1994).
- *Sedimentation tank:* In the tank, sedimentation of the microbial flocs (sludge) produced during the oxidation phase takes place. A large portion of the sedimented sludge is returned to the aeration tank. The

remainder of the sludge is wasted to control the food-to-microorganism (F/M) ratio (Bitton, 1994).

2.1 Operational parameters commonly used in activated sludge (Bitton, 1994):

- *Mixed liquor suspended solids (MLSS)*: The total amount of organic and mineral suspended solids, including microorganisms, in the mixed liquor.
- *Mixed liquor volatile suspended solids (MLVSS)*: The organic portion of MLSS, which comprises non-microbial organic matter as well as dead and live microorganisms and cellular debris (Nelson and Lawrence, 1980).
- *Food-to-microorganism ratio (F/M)*: Indication of the organic load into the activated sludge system and is expressed in kilogram biological oxygen demand (BOD) per kilogram MLSS per day (Curds and Hawkes, 1983; Nathanson, 1986).
- *Hydraulic retention time (HRT)*: The average time spent by the influent liquid in the aeration tank of the activated sludge process; it is the reciprocal of the dilution rate D (Sterrit and Lester, 1988).
- *Sludge age*: The mean residence time of microorganisms in the system.

2.2 The microbiology of activated sludge

Activated sludge should be understood as an artificial living ecosystem under continuous influence of biotic and abiotic factors (Wanner, 1994). According to Wanner (1994), the microorganisms in activated sludge can be divided into two major microbiological groups:

- *Decomposers*, which are represented mainly by bacteria, fungi and colourless cyanophyta. The organisms from this group are responsible for biochemical degradation of polluting substances in wastewater.
- *Consumers*, which consist of phagotrophic protozoa and microscopic metazoa. These organisms utilise bacterial and other microbial cells as substrates.

Bacteria constitute the major component of activated sludge flocs and are present as: individual free-swimming cells dispersed in the liquid phase, floc-forming bacteria and dispersed, non-floc-forming bacteria associated with the floc (Bitton, 1994; Gray, 1990). Heterotrophic bacteria form the basis of the flocs (Gray, 1990). The type of wastewater being treated will, to a large extent, determine the type of bacteria present in the system (Gray, 1990). Other factors in the aeration tank, such as pH, temperature, dissolved oxygen (DO), nutrient concentration, degree of turbulence, and the operating factors also determine which bacteria will be present (Gray, 1990). As the oxygen level in the flocs is diffusion-limited, the number of active aerobic bacteria decreases as the floc size increases (Hanel, 1988).

The flocs in an activated sludge system, where only floc-forming bacteria are present, are small and relatively weak (Jenkins *et al.*, 1986). They can easily be sheared in the turbulent environment of the aeration tank. This condition is called "pinpoint floc" (Jenkins *et al.*, 1986). These small flocs do not settle well (Bitton, 1994). Normal flocs occur when there is a balance between floc-forming and filamentous bacteria, these flocs are strong and keep their integrity in the aeration basin and settle well in the sedimentation tank (Bitton, 1994).

Among other bacterial species, a wide variety of filamentous bacteria is always present in activated sludge systems (Gray, 1990). The filamentous bacteria facilitate adhesion to floc-forming bacteria, in this way the floc macrostructure is formed (Bux and Kasan, 1993).

Two major separation problems, filamentous bulking and foaming, can occur as a result of an increased occurrence of filamentous microorganisms in the

biocenoses of activated sludge. (Wanner). When filamentous microorganisms occur at lower frequencies, they can contribute to the formation of large, firm activated sludge flocs exhibiting good settling properties. However, according to Wanner, when their numbers exceed a certain level, a number of problems can occur:

- Low zone settling velocities.
- Excessive volumes of the settled activated sludge
- The settled sludge is diluted, which affects both the operation of the aeration basin and the processing of waste activated sludge.

2.3 Bulking activated sludge

Sludge bulking is one of the major problems affecting biological wastewater treatment (Bitton, 1994). The bulking problem consists of slow settling and poor compaction of solids in the clarifier of the activated sludge system (Jenkins and Richard, 1985). Bulking sludge is often light brown, grey or white in colour and sometimes has a sweetish odour (Pipes, 1967).

When filamentous organisms predominate, sludge bulking can occur (Bitton, 1994). According to van Veen (1973), unicellular bacteria present in certain bulking-sludge samples appeared to be attracted by the protruding multicellular filaments.

There are major physiological differences between floc-forming and filamentous bacteria (Bitton, 1994). Filamentous bacteria can survive better under low oxygen concentrations and low-nutrient conditions than their floc-forming counterparts, due to the fact that the former have a higher surface-to-volume ratio (Bitton, 1994). The filamentous bacteria have a low half-saturation constant and have a high affinity for substrates, thus behaving as oligotrophs and surviving well under starvation conditions (Bitton, 1994).

2.4 Foaming activated sludge

Although foaming of activated sludge caused by filamentous microorganisms is a common problem encountered in many wastewater treatment plants around the world (Bitton, 1994), filamentous bulking occurs more frequently (Wanner, 1994). However, it does create a number of serious problems where it does occur.

The problems according to Bitton (1994) are as follows:

- Excess scum (activated sludge foam) can overflow into walkways and cause slippery conditions, leading to hazardous situations for plant workers.
- Excess scum may pass into activated sludge effluent, resulting in increased BOD and suspended solids in the effluent.
- Foaming causes problems in anaerobic digesters.
- Foaming can produce the nuisance of odours, especially in warm climates.
- Foam carries the potential of infection of wastewater workers with opportunistic pathogenic actinomycetes such as *Nocardia asteroides*.

2.4.1 Mechanisms of foam production

Although the causes and mechanisms of foam production are not well understood, Sodell and Seviour (1990) suggested the following mechanisms:

- Gas bubbles produced by aeration or metabolism (e.g., N_2) may assist in flotation of foam organisms.
- The hydrophobic nature of the cell walls of foam microorganisms helps their transport to the air-water interface.

- Biosurfactants produced by foam microorganisms assist in foam formation.
- Foam is associated with relatively long retention times (>9 days), with temperatures (>18°C)(Pipes, 1978), and with wastewater rich in fats (Eikelboom, 1975).

2.4.2 Foam microbiology

The brown viscous scum in activated sludge is mainly associated with the presence of actinomycete bacteria from the genus *Nocardia* (Jenkins *et al.*, 1986; Bittton, 1994). Microorganisms identified in foams are *Nocardia* (now *Gordona*) *amarae* (Klatte *et al.*, 1994), *N. rhodochrous*, *N. asteriodes*, *N. caviae*, *N. pinensis* now called *Skermania piniformis* (Chun *et al.*, 1997), *Streptomyces* spp, *Microthrix parvicella*, *Micromonospora*, Type 0675 and *Rhodococcus* (Blackall *et al.*, 1989; Goddard and Forster, 1987a; Lechevalier and Lechevalier, 1974, 1975; Lemmer and Kroppenstedt, 1984; Pujol *et al.*, 1991; Seviour *et al.*, 1990; Sezgin and Karr, 1986; Sezgin *et al.*, 1988). *Nostocoida limicola* and Type 0041 can also cause foaming (Goddard and Forster, 1987; Wanner and Grau, 1989).

The most frequently observed microorganisms in the foam from nutrient removal activated sludge plants in South Africa are: Type 0092, "*M. parvicella*" and Type 0041 (Blackbeard *et al.*, 1988). In a microbiological survey of ten activated sludge plants, done by Bux and Kasan (1993), it was evident that there was a high frequency of occurrence of "*M. parvicella*", Type 0041 and *Nocardia* spp. They stated that the reason for this was the ability of the bacteria to adapt and withstand environmental stress.

It has been shown that "*M. parvicella*" is a deep branching member of the actinomycetes subphylum (Blackall *et al.* 1995). The phylogenetic position of "*M. parvicella*" in the high mol% G+C Gram positive subphylum (the actinomycetes) was determined by 16S rRNA comparative analysis (Blackall *et al.* 1995).

Several studies indicated that sludge foaming is often caused by an increase in the amount of nocardioform actinomycetes (Schuppler *et al.*, 1995). Since nocardioform actinomycetes and other Gram-positive bacteria with high G+C content occur preferentially in sewage treatment plants with enhanced biological phosphorous removal (EBPR), plants of this type are especially susceptible to developing scum. Isolation and identification of these filamentous, nocardioform actinomycetes are hampered by their fastidious nature (Schuppler *et al.*, 1995). Most species are still unculturable and their taxonomy is unresolved (Schuppler *et al.*, 1995).

It is important to note that *Nocardia* spp. are only nuisance organisms when they result in foaming. It appears that *Nocardia* spp. can remove organic matter from wastewater so that their presence, without causing foaming, does not compromise the efficiency of secondary treatment (Jenkins *et al.*, 1986).

Isolates of the genus *Haliscomenobacter* are always present in activated sludge flocs, sometimes in large amounts (Mulder and Deinema, 1992). Only one species has been described so far, *Haliscomenobacter hydrossis* (Kampfer, 1995). Despite their frequent detection in activated sludge flocs by microscopic investigations, these filamentous bacteria are difficult to isolate and cultivate (Kampfer, 1995).

Table 1. Filamentous organisms in activated sludge adapted from Eikelboom, 1975.

Group I	Sheath forming, Gram negative bacteria	Problems caused in activated sludge
	1. <i>Sphaerotilus natans</i>	Bulking, foaming
	2. Type 1701	Bulking
	3. Type 1702	Bulking
	4. <i>Haliscomenobacter hydrossis</i>	Bulking, foaming
	5. Type 0321	
Group II	Sheath forming, Gram-positive bacteria	
	6. Type 0041	Bulking, foaming
	7. Type 0675	Bulking, foaming
	8. Type 1851	Bulking, foaming
Group III	Sheathless curled, multicellular bacteria resembling blue-green algae	
	9. Type 021N	Bulking, foaming
	10. <i>Nostocoida limicola</i>	Bulking, foaming
	11 Cyanophyceae	Bulking
Group IV	Slender, coiled bacteria	
	12. <i>Microthrix parvicella</i>	Bulking, foaming
	13. Type 0581	Bulking, foaming
	14. Type 0192	
Group V	Straight, multicellular, Gram-negative bacteria	
	15. Type 0803	Bulking, foaming
	16. Type 1091	
	17. Type 0092	Bulking, foaming
	18. Type 0961	Bulking
Group VI	Filamentous bacteria motile by gliding	
	19. Type 0914	Bulking, foaming
	20. <i>Beggiatoa</i> spp	Bulking
	21. Type 1111	
	22. Type 1501	
Group VII	Additional types	
	23. Type 1863	Bulking, foaming
	24. Type 0411	Bulking
	25. Fungi	Bulking
	26. <i>Nocardia</i> spp.	Bulking, foaming

2.5 Types of filamentous organisms

According to Kämpfer (1997) the classical approach for the identification of filamentous bacteria is that of Eikelboom (1975) who studied more than 1100 activated sludge samples by phase contrast microscopy. Twenty-six types were distinguished and grouped into seven assemblages (table 1). The identification and classification techniques used by Eikelboom (1975) and Eikelboom and van Buijsen (1981) was further modified by Jenkins *et al.* (1984) and Wanner and Grau (1989). The organisms are classified into the types according to the following features (Wanner, 1994): filament shape, filament colour, location of the filament, presence or absence of a sheath or slime coating, attached growth, branching (true or false), motility, filament length and diameter, cell shape, cell size, septa between cells, Gram stain, Neisser stain and intracellular granules (sulphur, poly- β -hydroxybutyric acid (PHB), polyphosphate).

Eikelboom's types can be classified into four groups according to their morphological, physiological and metabolic similarity, their occurrence under the same operational conditions and the problems they could cause (Wanner and Grau, 1989).

- *Group I: Sphaerotilus-like microorganisms.* These filaments are composed of rod- or square-shaped cells contained in a clear sheath. False branching might occur. Types 1701, 0041 and 0657 can also be included in this group (Wanner and Grau, 1989). Type 1701 may be related to the genus *Sphaerotilus* (Williams and Unz, 1985a).
- *Group II: Leucothrix, Thiothrix and Eikelboom's type 021N strains.* It can be concluded from the extensive studies conducted by Williams and Unz (1985a and 1985b), Shimizu (1985) and Williams *et al.* (1987) that Type 021N is not identical with either the genus *Leucothrix* or the genus *Thiothrix* (Wanner and Grau, 1989). In a study conducted by Kanagawa *et al.* (2000) it was also illustrated that although 15 isolates of Eikelboom Type 021N were morphologically clearly different from *Thiothrix* species, comparative 16S rDNA sequence analysis indicated

that all the strains formed a monophyletic cluster with all recognised *Thiothrix* species. The investigation further indicated that the type 021N isolates were subdivided into three distinct groups (I-III) demonstrating a previously unrecognised genetic diversity hidden behind the uniform morphology of the filaments. Both the genera *Leucothrix* and Type 021N are connected with the filamentous bulking caused by readily biodegradable wastewater, high sludge retention time and probably by nutrient deficiency (Wanner and Grau, 1989).

- Group III: This includes "*Microthrix parvicella*" and other microorganisms capable of utilising substrate not only under oxic conditions. "*Microthrix parvicella*" is also able to accumulate a significant portion of degradable substrate and is thus able to compete against floc-forming bacteria (Baték, 1988; Wanner and Grau, 1988). The filamentous microorganisms from this group can be generally characterised as microorganisms which are able to accumulate or utilise substrate under anoxic conditions (anoxic respiration, denitrification) or to utilise substrate under anaerobic conditions. This is connected with the phenomenon of polyphosphate formation and degradation (Wanner and Grau, 1989). However, in a study conducted by Andreasen and Nielsen (2000) results indicated that "*Microthrix parvilcella*" was not a typical phosphorus accumulating organism, and that the presence and availability of the preferred substrate, long chain fatty acids and lipids were the key factors determining the extent of growth in treatment plants.
- Group IV: *Foam-forming microorganisms*. Abundant branched hyphae of actinomycetes form a net that entraps oil droplets and gas bubbles, this causes the formation of a chocolate-brown, viscous and stable scum (Wanner and Grau, 1989). The actinomycete bacteria of the genus *Nocardia* is reported to be connected to activated sludge foaming (Wanner and Grau, 1989). *Nostocoida limicola*, which is an actinomycete bacterium, could also cause foaming, and according to Goddard and Forster (1987) a stable foam was produced when this organism and Type 0041 were present in the biocenosis of activated

sludge. Essentially, there are three morphotypes for '*Nostocoida limicola*' (Eikelboom and van Buijsen, 1981). After a study to better describe '*N. limicola*' II, it was proposed that these organisms be named as '*Candidatus Nostocoida limicola*' (Blackall *et al.*, 2000).

Eikelboom's types can also be classified into four groups of filamentous microorganisms on the basis of their similarity, occurrence under the same operational conditions and causing of the same problems (Wanner and Grau, 1989):

- Oxidic zone growers S (*Sphaerotilus*-like organisms)
- Oxidic zone growers C (Cyanophyceae-like microorganisms, i.e., *Leucothrix*, *Thiothrix* and Type 012N)
- All zone growers A ("*Microthrix parvicella*" and other heterotrophic microorganisms capable of utilising substrate not only under oxidic conditions)
- Foam-forming microorganisms F (*Nocardia* spp, *Nostocoida limicola*, Type 0092 and other microorganisms producing biosurfactants).

Richard (1984b) studied 29 activated sludge samples in South Africa and discovered the following filaments to be prevalent (in order of significance):

Type 0092, Type 0041, Type 0675, *Nocardia* spp., Type 0914, Type 0803, Type 1851, "*M.parvicella*", *N.limicola* II, Type 021N, *Thiothrix* spp., *H. hydrossis* and Type 1701.

"*Microthrix parvicella*" was recently elevated to "*Candidatus Microthrix parvicella*," a standing created to accommodate bacteria for which there is not enough information to validly name them (Blackall *et al.*, 1996a).

2.6 Factors which support the excessive growth of filamentous organisms

(Wanner, 1994):

- *Wastewater composition:* This is a source rich in substrates, nutrients and micronutrients for the microbial consortium, including filamentous bacteria, of activated sludge.
- *Readily biodegradable substrates:* Compounds with simple molecules can usually be directly utilised by bacterial cells. The concentration of these compounds (monosaccharides, alcohol, volatile fatty acids and amino acids) in the mixed liquor of activated sludge is not very high, which is a favourable factor for the growth of some filamentous microorganisms.
- *Particulate, slowly biodegradable substrates:* Most utilisable organic compounds in municipal wastewater can be described as particulate substrate. Some kinds of particulate substrates (e.g., fats and grease that is concentrated in foams) can support the growth of filamentous organisms.
- *Inoculation of activated sludge systems from wastewater:* The sewage network and wastewater treatment plants from one system, and the processes in sewers may significantly affect the composition of activated sludge consortia. Although filaments will grow in sewers, it is always useful to know whether the activated sludge system is inoculated with floc-formers or with filaments.
- *Biomass retention time:* According to their growth and decay rates, the distribution of individual microbial species in the consortium of activated sludge is affected by the biomass retention time (sludge age). At low biomass retention time, the slow growing species might wash out. The safe values of retention time, when all filamentous microorganisms are certainly washed out, are too low to be applied as a common bulking control measure.
- *Actual substrate concentration in reactor:* Floc-formers can take advantage of their r-strategy of substrate utilisation over the filaments

which are mostly K-strategists, if the return activated sludge is mixed with the wastewater in such a way that a substrate concentration gradient between the inlet and outlet part of the reactor is created.

- *Dissolved Oxygen, nutrients, pH and temperature in aeration basins:*

⇒ Dissolved oxygen (DO). A great affinity is exhibited by some filamentous microorganisms for a low dissolved oxygen concentration because of their low values of half-saturation constant K_0 . The mixed liquor from preceding anaerobic or anoxic conditions should be converted to oxic conditions as soon as possible by the aeration equipment in oxic zones of nutrient removal activated sludge systems.

⇒ Nutrients. Some filaments exhibit higher affinity for nutrients (i.e., nitrogen, phosphorus and micronutrients).

⇒ pH. None of the common filamentous organisms present in bulking and foaming exhibits a special preference to extreme pH values.

Temperature. Elevated temperatures will support the growth of filamentous microorganisms connected with low DO concentrations. Surveys revealed significant seasonal shifts in the dominance of individual filamentous types.

- *Cultivation conditions:*

⇒ Selection in altering anaerobic and oxic conditions. The alteration of anaerobic and oxic conditions is a prerequisite for the enhanced biological phosphorus removal. Filamentous organisms with the ability to utilise substrate in this way, at a rate comparable to that of non-filamentous bacteria, are "*Microthrix parvicella*", Type 0092 and possibly *Nostocoida limicola*.

Selection in anoxic conditions. Most filamentous microorganisms are not able to use nitrogen as a final electron acceptor, at least not with a rate enabling them to compete with floc-formers for substrate under anoxic conditions.

Other advantages of actinomycetes over other wastewater bacteria are their higher resistance to desiccation and UV irradiation and their ability to store polyphosphates and PHB (Lemmer and Baumann, 1988b). Anionic surfactants and their biodegradation products can also significantly enhance foaming in *Nocardia*-containing activated sludge (Ho and Jenkins, 1991). The excessive growth of *N. amarae* in activated sludge can be explained by the production of biosurfactants and selective utilisation of hydrophobic compounds such as hydrocarbons (Lemmer and Baumann, 1988a). These characteristics are essential for foam production and transport of the cells to the bubble phase (Blackall and Marshall, 1989; Blackall *et al.*, 1988).

2.7 Isolation and cultivation of filamentous organisms in bulking and foaming activated sludge

The isolation of filamentous microorganisms, occurring in bulking sludge, is not an easy task due to the presence of large numbers of more rapidly growing non-filamentous bacteria in the flocs (Van Veen, 1973).

Isolation of filamentous microorganisms from activated sludge is still difficult (Kämpfer, 1997; Ziegler, Lange and Dott, 1990). In spite of this difficulty the isolation of these organisms is a prerequisite for the investigation of nutritional and growth parameters (Kämpfer, 1997). Both Kämpfer (1997) and Ziegler *et al.* (1990) as well as Williams and Unz (1985) discovered that in most cases, direct plating onto culture media was unsuccessful since the majority of filamentous organisms are overgrown by more rapid growers, or their nutritional requirements are not provided by the chosen medium (Kämpfer, 1997).

Another method for isolating filamentous bacteria is micromanipulation. During this procedure a drop of mixed liquor or foam diluted with water is placed on the surface of a plate of the medium to be used under . Special microtools, like the hook formed by the method described by Skerman (1968), attached to a 32 X long working distance objective lens, is then used to drag filaments which can be identified under the microscope away from the surrounding biomass to the edge

of the plates. Filamentous bacteria isolated from bulking or foaming sludge using micromanipulation includes - among others - "*Microthrix parvicella*", Type 0092, Type 0803, Type 0411 and Type 1863 (Blackall *et al.*, 1995, 1996b; Bradford *et al.*, 1996; Kocianova *et al.*, 1994; Rosetti *et al.*, 1997), *Gordona amarae* and *Skermania piniformis* (formerly *Nocardia amarae* and *Nocardia pinensis*) (Blackall *et al.*, 1994). According to Seviour and Blackall (1999) micromanipulation should be used if pure culture studies is contemplated, since it is the only method of isolation, where particular morphological filament types can be found under the microscope, and specifically physically isolated (Blackall, 1991).

Foam microorganisms can use several substrates and these can be added to growth media in attempts to enhance isolation. Examples of substrates are: sugars, high-molecular weight polysaccharides, proteins and aromatic compounds (Lemmer, 1986; Lemmer and Kroppenstedt, 1984).

Kämpfer *et al.* (1994) studied 68 strains of filamentous bacteria belonging to the genera *Sphaerotilus* and *Haliscomenobacter*, Type 021N and 1701, some nocardioform organisms and unidentified, Gram-negative isolates qualitatively and quantitatively for their growth requirements and growth response to different nutrients. They found a strict dependency for the presence of calcium and phosphate in the media for all isolates (no strain grew in absence of one of these substances). With the exception of the nocardioform organisms Kämpfer *et al.* (1994) found that most of the isolates developed optimally at calcium concentrations ranging from 0.05-0.4g/L, and at magnesium concentrations ranging from 0.05-1g/L. Williams and Unz (1985) found that ammonia was the best nitrogen source for the filamentous bacteria in their study. Kämpfer *et al.* (1994) and Williams and Unz, (1985) both observed better growth at low concentrations (<0.1g/L) of nitrate. The filamentous organisms also grew in absence of nitrate, sulphide and thiosulfate (Kämpfer, 1994).

There is thus wide diversity in the nutrition and physiology of the pertinent filamentous bacteria. Various classes of carbon and nitrogen compounds, potentially present in wastewaters, can be utilised.

2.8 Identification techniques for filamentous organisms

Conventional techniques for the identification of filamentous microorganisms are difficult and time-consuming due to their slow growth and difficulty in obtaining pure cultures from activated sludge samples (Wanner and Grau, 1989). Although the difficulty in obtaining pure cultures can sometimes be overcome by use of micromanipulation, filamentous microorganisms are characterised mainly by microscopic examination as discussed earlier in this chapter.

A more recent approach is the molecular characterisation of filamentous microorganisms. Recombinant DNA and molecular phylogenetic methods provide means for identifying microorganisms in microbial communities without the need for cultivation (Hugenholtz *et al.*, 1998).

2.9 Molecular characterisation of filamentous microorganisms in activated sludge

Until now, the diversity and dynamics of activated sludge have been analysed by culture dependent methods (Manz *et al.*, 1994). Culture dependent methods are time consuming and the number of colony forming units is often only a minor fraction (less than 10%) of the cell counts determined by direct microscopic techniques (Manz *et al.* 1994).

Wagner *et al.* (1993) also stated that recoveries from activated sludge even with optimised media are only between 5 and 15%, and that often the microscopically prominent microorganisms, like the filamentous bacteria in foaming activated sludge, cannot be found by standard cultivation procedures. The percentage recovery for filamentous organisms from sludge can be significantly improved if micromanipulation is used.

The key developed by Eikelboom (1975) and Eikelboom and van Buijsen (1981) enabled the identification of predominant filamentous bacteria in bulking activated sludge on the basis of morphological characteristics. However,

filaments occurring in low numbers are easily missed using this key. Morphology and staining reactions of microbial cells can also vary in a broad range depending upon environmental conditions like nutrient status (Wagner *et al.*, 1994). Non-filamentous forms have been reported for filaments such as *Haliscomenobacter hydrossis*, *Sphaerotilus natans* and "*Microthrix parvicella*". Some filamentous bacteria can also show variable Gram stain reactions.

Culture-dependent methods have thus proved to be inadequate to determine species diversity of microorganisms in their natural habitat. Close morphological similarity between bacteria does not necessarily reflect the true relatedness at a genomic level. Knowing the 16S rDNA sequence for a particular organism means that a unique segment of that gene can be recognised. A specific probe can be synthesised for this gene in the laboratory (Blackall *et al.* 1996b). This probe can be tagged and used specifically to identify only that organism in a complex natural community of bacteria (Amann *et al.*, 1995).

The quantitative and qualitative biases introduced by cultivation-dependent methods can only be avoided by the use of *in situ* techniques (Manz *et al.*, 1994). *In situ* identification and enumeration of microorganisms harbouring a certain rRNA sequence, requires the specific detection of the rRNA within morphologically intact cells in their natural microhabitat. This technique is referred to as whole-cell or *in situ* hybridisation (Amann *et al.*, 1995).

2.9.1 The rRNA approach

Traditionally, the classification of organisms has been based on similarities in their morphological, developmental, and nutritional characteristics (Lane *et al.*, 1985). It is now clear that a classification system based on these criteria does not necessarily correlate well with natural (i.e., evolutionary) relationships, as defined by macromolecular sequence comparisons (Lane *et al.*, 1985).

The comparative analysis of rRNA gene sequences is the preferred method for the classification of bacteria and has created a stable framework in which

microorganisms are no longer lumped into artificial taxonomic entities, but placed according to their true evolutionary relationships (Amann 1997).

In order to interpret results of rRNA sequencing studies, the multiple steps involved in today's standard technique for rRNA sequence retrieval from an environmental sample have to be considered. The nucleic acid sequencing analytical procedure involves extraction of nucleic acids, PCR-mediated amplification of a 16S rRNA gene fragment, sequence determination, and computer-aided analysis (Böttger, 1996). The technique is illustrated in figure 1 (Amann *et al.*, 1995).

The results will be dependent (I) on the optional cell enrichment, (II) on the efficacy of DNA extraction, (III) on the PCR amplification of the different rRNA genes (rDNA), (IV) on the cloning of the different rDNAs, and (V) on the number of clones which are screened and sequenced (Amann *et al.*, 1997).

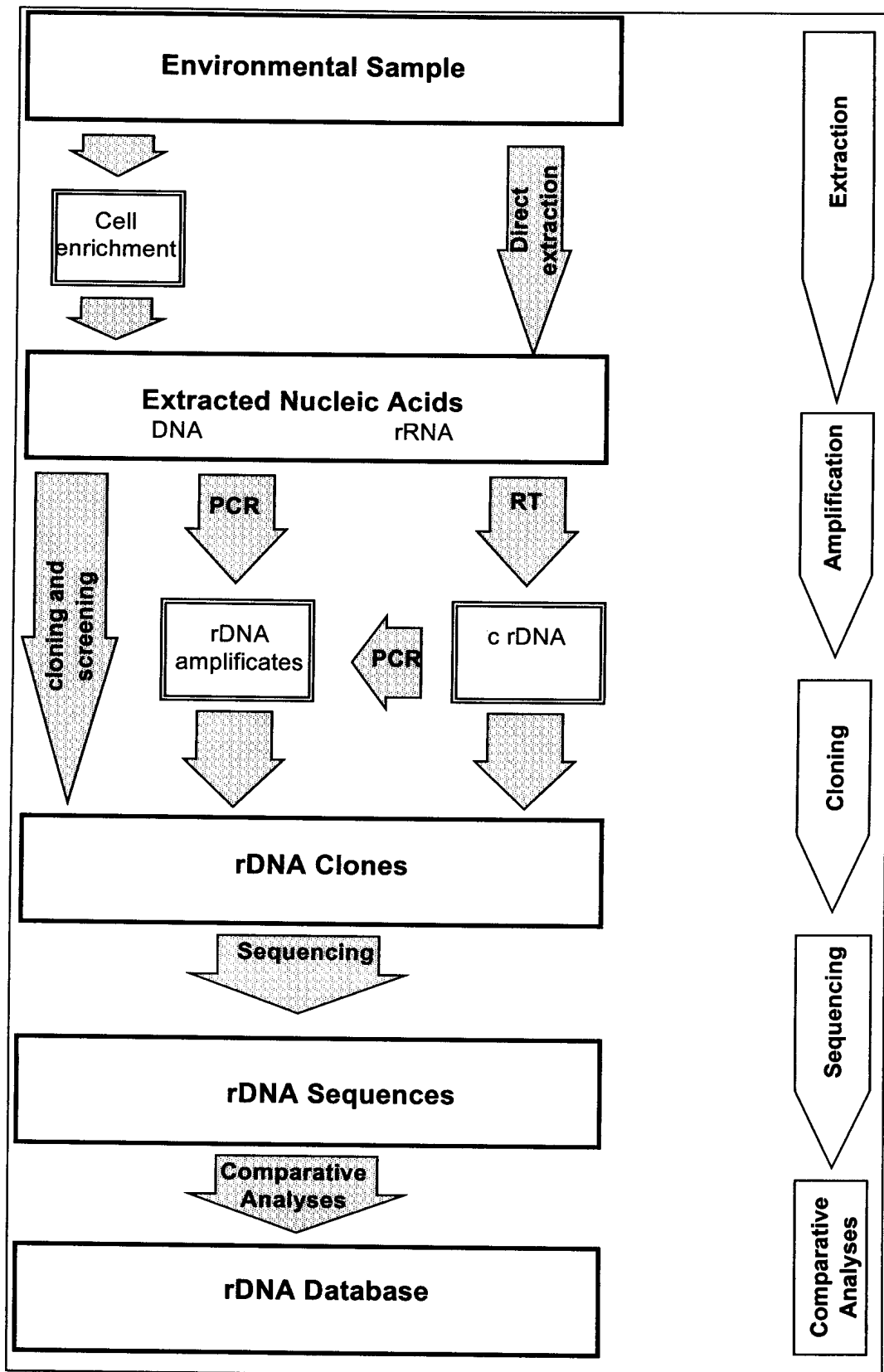


Figure 1. Flow chart showing the different possibilities to characterize an environmental sample by comparative rRNA sequence analysis (Amann *et al.* 1995).

2.9.2 Ribosomal RNA

Ribosomal DNA (rRNA genes) forms the template for rRNA. Ribosomal RNA (rRNA) molecules are present in all living cells in quite high copy numbers (Amann, 1997). They are an integral part of the ribosomes. Bacterial cells have three different size classes of rRNA molecules, the 5S, 16S and the 23S rRNA (Amann, 1997). They are evolutionarily quite conserved but contain, like a patchwork, regions with variable degrees of conservation (Amann, 1997). An average bacterial 16S rRNA molecule has a length of ca. 1500 nucleotides and when fully, or almost fully (>1000 nucleotides) analysed, contain sufficient information for reliable phylogenetic analyses (Amann *et al.*, 1995).

It is therefore possible to define so-called signature sites that may be unique for such large domains as bacteria (Amann, 1997). Based on a signature analysis, microorganisms can be affiliated to phylogenetic groups. rRNA allows differentiation down to the species level, but not always to the strain level (Amann, 1997).

For identification purposes, the variable and conserved residues of the rRNA molecules are equally important (Amann *et al.*, 1994). The highly conserved regions can serve as primer binding sites for *in vitro* amplification by PCR. It can serve as targets for universal probes that react with all living organisms to discriminate between broad phylogenetic domains such as the *Bacteria*, *Archaea* and *Eucarya*. The more variable sequence regions of the rRNA genes are appropriate targets for genus-, species-, and sometimes even subspecies-specific hybridisation probes (Amann *et al.*, 1995). Another advantage of using small subunit (SSU) rRNA is that it is part of the larger ribosome, which is central to the functioning of the cell and as a result cannot be transferred between species (Woese *et al.*, 1985).

The structure of the rRNA operon is illustrated in figure 2. The highly conserved regions are shown as the dark blocks. These are the regions which need to be sequenced to identify the organisms. The sequences of regions 1-4 in figure 2

are very similar in all bacteria. Consequently, primers can be synthesised and used in the PCR reactions to amplify the 16S rDNA. As a result, large quantities of the 16S rDNA will be produced and this PCR product can then be used for sequencing of the 16S rDNA (Gurtler and Stanisich, 1996).

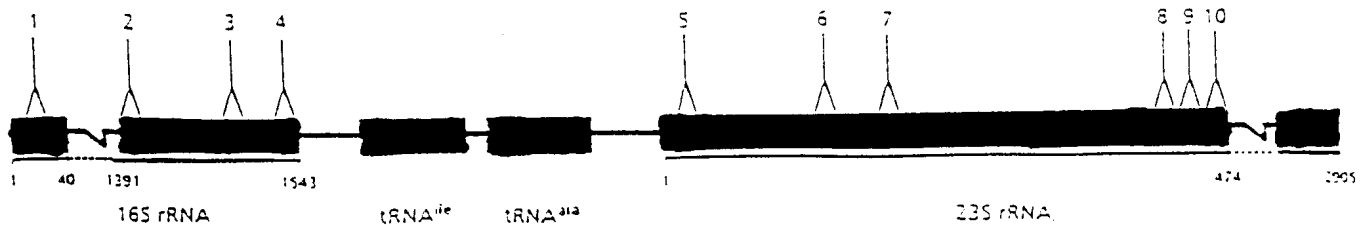


Figure 2. Position of conserved regions within the rRNA operon. The boxed areas represent the various genes of the bacterial rRNA operon. The dark lines represent the spacer regions that separate the various rRNA genes and the dark jagged lines represent breaks in the 16S and 23S genes which have not been shown fully. The 5S rRNA gene that occurs to the right of the 23S rRNA gene is not shown. The thin numbered line shows the nucleotide numbering of the 16S and 23S rRNA genes of *E.coli* with breaks shown as dashed lines. The numbers 1-10 represent the following nucleotide sequence blocks. 16S rRNA gene: 1, 8-27; 2, 1390-1407; 3, 1491-1506. 23S rRNA gene: 5, 21-38; 6, 115-132; 7, 188-208; 8, 422-437; 9, 441-460; 10, 456-474. The sequences (5'-3') of regions 1-4 are AGAGTTTGATCCTGGCTCAG, TTGTACACACCGCCCGTC, AAGTCGTAACAAGGTA and GGCTTGGATCACCTCCTT, respectively (Gürtler and Stanisich, 1996).

2.9.3 PCR amplification of 16S rDNA

An approach currently being used in many laboratories utilises the polymerase chain reaction (PCR) to amplify rDNA (Wisozetsky, 1990).

PCR enables specific regions of DNA to be amplified from the total DNA of an organism using specific primers which are complementary to known sequences of the DNA (Hiraishi, 1992). Sufficient amounts of material (DNA) are generated and as a result the need to culture the bacteria is eliminated. Any region of any DNA can be chosen as long as the sequences at the borders of the region are known (Brown, 1995).

2.9.4 Cloning

The 16S rRNA from mixed microbial communities are normally amplified, and should theoretically result in 16S rRNA sequences from each organism present in that environment. Cloning of the product into a suitable vector has proven to be a reliable approach (Head *et al.*, 1998). Once a 16S rDNA gene library has been obtained, the clones can be sequenced directly. Screening techniques such as colony hybridization and restriction fragment length polymorphisms (RFLP) can be used to identify specific sequences within the sample, to reduce the number of clones to be sequenced. Denaturing gradient gel electrophoresis is another technique which separates the PCR products according to their sequence (Head *et al.*, 1998)

2.9.5 Sequencing and analysis

DNA fragments of interest are specifically amplified using PCR, and the DNA sequence is determined by inclusion of fluorescently-labelled dye-primer or dye-terminator nucleotides in the sequencing reactions, and laser scanning of the sequencing gels (Applied Biosystems).

Comparative sequence analysis of small subunit rRNA is currently one of the most important methods for the elucidation of bacterial phylogeny as well as bacterial identification (Ludwig *et al.*, 1998). A comprehensive sequence data set is available in generally accessible databases in plain or processed format, and the number of entries is permanently increasing (Ludwig *et al.*, 1998).

DNA sequence analysis is a multi-step process. It is first necessary to align the sequences, so that nucleotide sites in correspondence are being compared (Priest and Austin, 1993). The procedure involves the insertion of gaps in the sequences so as to maximise the overall similarity (Higgins and Sharp, 1988). Alignment of the conserved and the neighboring parts is a straightforward procedure that can be performed reliably using multiple alignment software such as CLUSTAL W (Ludwig *et al.* 1998) and Clustal X (Thompson *et al.*, 1997).

The classical form of visualisation of sequence-based phylogenetic analyses are additive trees. Trees can be rooted or unrooted. A rooted tree specifies the evolutionary pathway followed. An unrooted tree presents the phylogenetic relationships but does not specify the evolutionary path (Priest and Austin, 1993). The branching pattern of a rooted tree indicates the path of evolution and branch lengths indicate the phylogenetic distances. These trees are shown as radial or dendrogram format. The (phylogenetic) distances between the organisms (sequences) are expressed as the sum of the lengths of all branches connecting the two particular nodes (Ludwig *et al.*, 1998).

Three of the most common approaches to tree reconstruction are: distance matrix, maximum parsimony and maximum likelihood. Distance matrix methods utilise only the sequence distances between pairs of sequences. Cluster analysis groups sequences on the basis of how similar they are to one another or to other groups of sequences (Woese, 1987). Maximum parsimony analysis assumes that the evolutionary path is the shortest path with the least number of mutations. With maximum likelihood, the probability of obtaining the observed data, given the tree and model, is computed. The tree that maximises this probability is the maximum likelihood estimate (Priest and Austin, 1993).

2.9.6 Principles of *in situ* hybridization

rRNA-targeted oligonucleotide probes were introduced as a tool for *in situ* identification of bacteria. This approach is based on the comparative analysis of macromolecules most notably of the rRNA molecules. Phylogenetically based rRNA-targeted probes can be designed for determinative, phylogenetic and environmental studies (Amann *et al.*, 1990).

Two major *in situ* techniques are commonly used:

- The *immunofluorescence approach* (Bohlool and Schmidt, 1980) has successfully been used to identify bacteria in complex environments. The applicability of the immunofluorescence approach is limited by antibody penetration problems through extracellular polymeric

substances that are components of the activated sludge flocs, and by binding of antibodies to detritus particles and fungal spores leading to high levels of background fluorescence (Wagner *et al.*, 1994). Furthermore, specific antibodies can only be raised for culturable bacteria (Manz *et al.*, 1994).

- *Fluorescent rRNA-targeted oligonucleotide probes* are an alternative to fluorescent antibodies in the identification of bacteria (Amann *et al.*, 1990).

Oligonucleotide primers can be designed complementary to the signature sites on the rRNA. Different marker molecules can be attached to the oligonucleotides. Such labeled nucleic acids can be used to identify the complementary sequences and are referred to as oligonucleotide probes (Amann, 1997). Unlike immunoprobes, specificities of the chemically synthesised oligonucleotide probes can be adjusted to different phylogenetic levels ranging from the subspecies to the kingdom level (Wagner *et al.*, 1993). Oligonucleotide probes can discriminate changes of individual nucleotides (Amann, 1997). The specificity and sensitivity of these probes rely on optimised chemical and physical parameters that need to be held constant during the hybridisation (Amann, 1997).

The detectability of bacteria by oligonucleotide probes depends on the presence of sufficient ribosomes per cell (Manz *et al.*, 1993).

Manz *et al.* (1994) listed a few significant advantages of rRNA-targeted oligonucleotide probes:

- 16S and 23S rRNAs contain regions of highly conserved sequences. Therefore, it is possible to design probes with various specificities ranging from a species-specific probe to one that will hybridise with any bacterial cell.
- A considerable increase in sensitivity is achieved by targeting rRNA instead of DNA, since ribosomal rRNA molecules are naturally

amplified target molecules with copy numbers ranging from 10^4 to 10^5 in metabolically active cells. So the detection of individual cells can be performed with oligonucleotide probes that direct only one fluorescent dye molecule to each target molecule.

- Nucleic acid probes directed against rRNA can be applied to samples without prior cultivation and have successfully been used to detect and identify hitherto uncultured bacteria.

Fluorescent derivatives of oligonucleotide probes have been successfully used for *in situ* enumeration of defined groups of microorganisms in activated sludge (Wagner *et al.*, 1993).

Fluorochromes like fluorescein, rhodamine or esters of the carbocyanines Cy3 and Cy5 can be covalently bound to the oligonucleotide probes (Amann *et al.*, 1997). After hybridisation, fluorescence conferred by rRNA-targeted oligonucleotides can be easily analysed by epifluorescence microscopy. Several probes labelled with spectrally different fluorochromes can be simultaneously used on one sample (Amann *et al.*, 1997).

The morphology of the cells in the examined sample has to be stabilised and the cell walls and membranes have to be permeabilised for the penetration of the probes (Amann *et al.*, 1997). This can both be achieved by fixatives which are usually based on aldehydes and/or alcohols (Amann *et al.*, 1997).

2.9.7 Oligonucleotide probes and filamentous bacteria

“Microthrix parvicella” is the main filamentous microorganism responsible for bulking and foaming events in activated sludge systems (Rosetti *et al.*, 1997). *“Microthrix parvicella”* is not a valid name and the organism’s presence in activated sludge is evaluated according to morphological properties (Rosetti *et al.*, 1997). In Blackall *et al.* (1996) it has been stated that neither they nor others had succeeded in addressing the growth limitations of *“M. parvicella”* in the

foregoing decade. Fundamental information about its physiology is still incomplete and reliable biological control methods to prevent its growth on activated sludge systems, are not available. The reason for this is mainly the difficulty that is experienced in trying to culture the microorganism (Stratton *et al.*, 1996a).

Four 16S rRNA directed oligonucleotide probes (MPA probes) specific for the activated sludge bulking and foaming filamentous bacterium "*Microthrix parvicella*" were designed and evaluated for the detection and identification of this organism (Erhart *et al.*, 1997). Hybridisation stringency for the probes was empirically determined with activated sludge samples because "*M. parvicella*" cannot be cultured to give adequate amounts of biomass (Erhart *et al.*, 1997).

Erhart *et al.* (1997) have employed the "*M. parvicella*" 16S rRNA sequence to prepare specific rRNA directed, DNA-hybridisation probes for the *in situ* identification of this bacterium. They also reported the evaluation of the high mol% G+C Gram positive probe (HGC69a) for its ability to bind to "*M. parvicella*" filaments in activated sludge. In their study, none of the cells morphologically identified as "*M. parvicella*" gave positive hybridisation signals with the HGC69a probe. However, other cells in the activated sludge samples and pure cultures of *Gordona amarae* showed high fluorescence when probed with the probe HGC69a indicating that this probe was working efficiently (Erhart *et al.*, 1997).

Schuppler *et al.* (1995) screened a library of *in vitro*-amplified 16S rRNA genes of activated sludge bacteria with an oligonucleotide probe in order to detect clones with 16S rDNA signature sequences of nocardioform actinomycetes and mycobacteria, and positive clones were sequenced and analysed.

Application of the probe HHY specific for *Haliscomenobacter* spp., a member of the cytophaga-flavobacterium cluster resulted in the specific visualisation of heavily stained thin filamentous bacteria (Wagner *et al.*, 1994).

De Los Ryes *et al.* (1997) developed and characterised group-, genus-, and species-specific oligonucleotide probes targeting the small subunit rRNA of the *Mycobacterium* complex, also known as the *Corynebacterium-Mycobacterium-Nocardia* (CMN) group. In 1998 de Los Reyes *et al.* developed oligonucleotide probes for *Gordona amarae* and two subgroups within this species. The probes allowed identification and quantification of *G. amarae* strains often implicated in foaming activated sludge by membrane hybridisation and FISH (de Los Reyes *et al.*, 1998).

2.8 Concluding Remarks

The activated sludge process comprises a complex enrichment culture of a mixture of different microorganisms. The solid separation problems of bulking and foaming have compelled researchers to examine the biological component of the mixed liquor of activated sludge plants (Blackall and Hugenholtz, 1999).

Although a variety of bacteria can be isolated using conventional microbiological techniques, some organisms cannot be cultured in this way. In a means to overcome this, molecular methods classifying and identifying bacteria on a phylogenetic basis, provide results that are independent of growth conditions and media used. A much used approach is the analysis of 16S rRNA sequences (Amann *et al.*, 1994). rRNA molecules are present in all living cells, and are an integral part of the ribosomes. The comparative analysis of rRNA sequences allows microorganisms to be placed according to their true evolutionary relationships (Amann, 1997). Microorganisms can now be described by their gene sequences without the need to cultivate them (Blackall and Hugenholtz, 1999).

rRNA-targeted oligonucleotide probes can be tailored to different taxonomic levels to allow identification of subclasses, genera or species. Such probes can be labelled with flouochromes and can then be used to identify single cells directly in complex samples. This flourescent *in situ* hybridisation (FISH)

technique allows researchers to identify and monitor hitherto uncultured microbes in complex communities such as activated sludge (Amann, 1997).

Although pure culture techniques are still important, the methods of choice for studying communities of activated sludge are 16S rRNA comparative studies and *in situ* hybridisation.



CHAPTER 3

MICROSCOPIC INVESTIGATION AND IDENTIFICATION OF FILAMENTOUS BACTERIA ASSOCIATED WITH BULKING AND FOAMING IN ACTIVATED SLUDGE PLANTS

The style of this chapter is in accordance with that of *Systematic and Applied Microbiology*.

ABSTRACT

The proliferation of filamentous bacteria in activated sludge system can cause solids separation problems known as bulking and foaming. As a result, sludge recycling becomes difficult and the quality of the secondary effluent decreases. Foaming and bulking samples were collected from two activated sludge plants in Gauteng, South Africa and the Noosa waste water treatment plant in Queensland, Australia. A microscopic study was conducted to identify the filamentous microorganisms present in the samples. Both light microscopy, using various staining techniques, and scanning electron microscopy (SEM) were performed. In the South African samples, the dominant filamentous organisms present in the bulking sample were Nocardioform actinomycetes and in the foaming sample "*Microthrix parvicella*". In the Australian sample Type 0041 was most abundant, with *Nocardia* spp. also occurring.

Key words: Foaming, bulking, "*Microthrix parvicella*", *Nocardia* spp., Type 0041

Introduction

Activated sludge is a microbiological enrichment culture consisting of a mixture of generalist and specialist microorganisms (Blackall and Hugenholtz, 1999). The settling properties of activated sludge are vital to obtain good separation of the sludge from the treated wastewater. Ever since the activated sludge process was developed, several kinds of sedimentation problems have been observed, in particular bulking sludge and foaming (Eikelboom, 1999; Kämpfer, 1997).

Most activated sludge plants around the world suffer from bulking and/or foaming which can severely disrupt solids separation, with the majority of cases being associated with an abundance of one or more of 20-30 different filamentous bacteria (Jenkins *et al.*, 1993). Procedures for their identification rely on morphological characteristics and their response to a number of simple staining techniques (Eikelboom and van Buijsen, 1981; Eikelboom, 1999). A variety of FISH probes are also available for the rapid identification of filaments. Knowing the identity of the filamentous organisms that cause bulking is essential

to: predict what and to what extent the problem is to be expected, estimate the causes of the presence of the filaments in the bioscenosis and remediate bulking and foaming problems (Wanner and Grau, 1989).

Microscopic examination of activated sludge is useful for determining the structure of the activated sludge floc and for determining the abundance and types of filamentous organisms present (Jenkins *et al.*, 1986).

Using morphological criteria, Eikelboom (1975 and 1999) classified different types of filamentous bacteria and grouped them into numbered categories neglecting taxonomic principles (Ziegler *et al.*, 1990).

“Microthrix parvicella” is one of the filamentous microbes most frequently found in bulking and foaming activated sludge (Knoop and Kunst, 1998). The presence of *“Microthrix parvicella”* is evaluated according to morphological properties (Rosetti *et al.*, 1997). The occurrence of foam is also often associated with the presence of filamentous mycolic acid-containing actinomycetes such as members of the genus *Nocardia*, *Gordona* and *Rhodococcus* (Jenkins *et al.*, 1993).

Although more than 30 different species of filamentous organisms are observed in activated sludge, a number of species do not have a name yet as their properties are insufficiently known. Very little useful information is available for some strains (Eikelboom, 1999).

The aim of this chapter was to get acquainted with the microscopic methods used for identification of filamentous bacteria. We used bright field, phase contrast and scanning electron microscopy to identify the dominant filamentous bacteria in foaming and bulking activated sludge samples.

Materials and methods

Sampling

Foam samples were taken from the aerobic basin at the Centurion water purification plant in Gauteng, South Africa and the Noosa waste water treatment plant (WWTP) in Australia. Care was taken not to sample from the subsurface liquid, as this contains different microbiota from that found in the foam alone. The samples were bottled without dilution with the use of funnels.

A sample of activated sludge bulking was taken by the personnel from ERWAT (East Rand Water Board) from the Rhyndfield WWTP in Gauteng, South Africa.

Samples were kept on ice during transport and the samples were prepared and stained on the same day as sampling.

Sample preparation

For dry preparations, one drop of the sample was spread evenly over a microscope slide. These slides were allowed to air dry. For wet preparations, one drop of sample was placed on a microscope slide and covered with a cover slip.

Several staining techniques were performed on the samples. These included Gram staining, sulfur granule staining, Polyhydroxybutyrate (PHB) staining and crystal violet sheath staining, all according to the methods described in Jenkins *et al.* (1986)(Appendix). Methylene blue staining was also performed on the Noosa WWTP sample (Seviour and Blackall, 1999, Appendix).

Microscopic examination procedure

The slides were examined under phase contrast or transmitted light at 1000x and 630x magnification for the characteristics as summarised in table 1 and 2.

Table 1. Floc characteristics used to assist in the identification of the filamentous organisms.

Property	Description/ size/ extent
Size range	small (<150µm); medium (150-500µm); large (>500µm)
Floc texture	rounded and compact; irregular and diffuse; firm or weak
Presence and effect of filaments on floc structure	none; bridging (filaments extending from the flocs and bridge between flocs; open floc (floc attach to filaments leading to a large, irregularly shaped floc
Abundance of filaments	none; few; some; common; very common; abundant;excessive

Table 2. Filament characteristics used to assist in the identification of the filamentous organisms.

Property	Description/ size/ extent
Branching	true; false; absent
Motility	none; if present, describe
Filament shape	Straight; smoothly curved; bent; irregularly shaped chain of cells; coiled; mycelial
Colour	transparent; medium; dark
Location	extending form the floc; within the floc; free in the liquid
Attached growth of epiphytic bacteria	present or absent; substantial or incidental
Sheath	present; absent
Cross-walls	present; absent
Filament size	diameter and length
Cell shape	square; rectangular; oval; barrel; discoid; round-ended rods
Sulphur deposits	present; absent
PHB granules	present; absent
Staining reactions	Gram positive or Gram negative; Neisser positive or negative

Scanning electron microscopy (SEM)

The foam sample from Centurion was diluted 1:1 and the bulking sample from Rhyndfield 1:4 with sterile distilled water and both were fixed in 1,25% Glutaraldehyde for at least 24h. The samples were aspirated into the tip of a syringe and deposited onto a filter membrane. The samples - on the filter membranes - were washed 3 times in buffer solution for 15min. The samples were dehydrated in a graded ethanol series (50%, 70%, 90%), and finally, three

times with 100% ethanol for 15 min each. The samples were then submitted to critical point drying for approximately 1h. The filter membranes containing the dried samples were then mounted on studs and gold coated. Scanning electron microscopy was then performed and photographs were taken showing the most abundant types of filamentous microorganisms present in the samples.

Filamentous organism identification

The photographs taken from the light microscope images and the SEM images were compared with those published in the literature. Photographs from Jenkins *et al.* (1986), Richard (1989) and Wanner (1994) were used for the identification of the filamentous organisms present in the bulking and foaming samples.

Results And Discussion

Filamentous microorganisms identified

"Microthrix parvicella "

The filaments in the foam sample from Centurion WWTP occurred as Gram positive, irregularly coiled filaments that were found coiled in and around the floc (Fig. 1). Filaments were 0.6-0.8 μ m in diameter and 100-400 μ m in length (not illustrated). No sheath or attached growth was present and no branching occurred. Cellular inclusions were common which gave a "beaded" appearance (Fig. 1). According Jenkins *et al.* (1986) these are characteristics true to "*Microthrix parvicella*".

Nocardioform actinomycetes.

The filaments observed in the bulking sample from Rhyndfield were irregularly bent, mycelial, true branched filaments (Fig.4, 5, 6). The filaments were 1.0 μ m in diameter and 10-20 μ m in length (not illustrated), Gram positive (Fig.4, 5A) with PHB granules (not illustrated), with no sheath or attached growth. These characteristics were typical to those for *Nocardia* in Jenkins *et al.* (1986). Polyphosphate granules were observed after Methylene Blue staining (Fig. 5B).

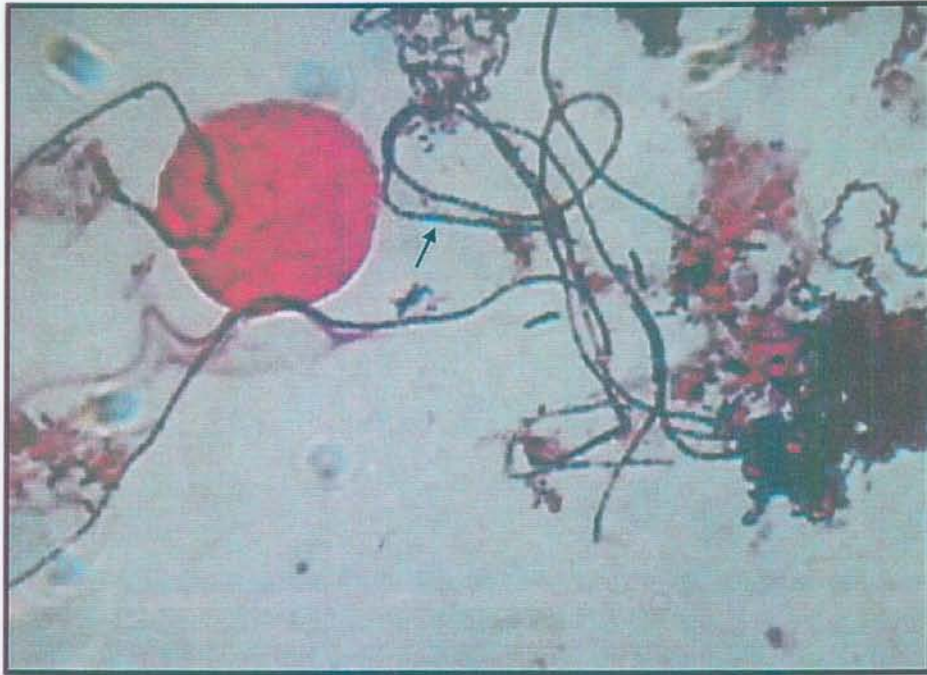
Type 0041

The following characteristics were observed in the foam samples from Centurion and Noosa WWTP, which corresponded with those mentioned in Jenkins *et al.* (1986) for type 0041. Straight, smoothly curved and bent filaments, 100-500µm in length (not illustrated), a tight fitting sheath were present. The filament stained Gram positive (Fig. 7, 8B). PHB- or sulfur granules were not observed. Heavy attached growth of epiphytic bacteria was observed (Fig. 7, 8, 9, 10).

Rod shaped cells in chains

These organisms were rarely observed and in very low abundance. The organisms had rounded rods in irregularly shaped chains of cells, found mostly at the edges of the floc. The organism stained Gram positive (Fig. 3). No sheath and no sulfur granules were observed. The characteristics corresponded with those observed for *Bacillus* spp. by Jenkins *et al.* (1986).

A



B

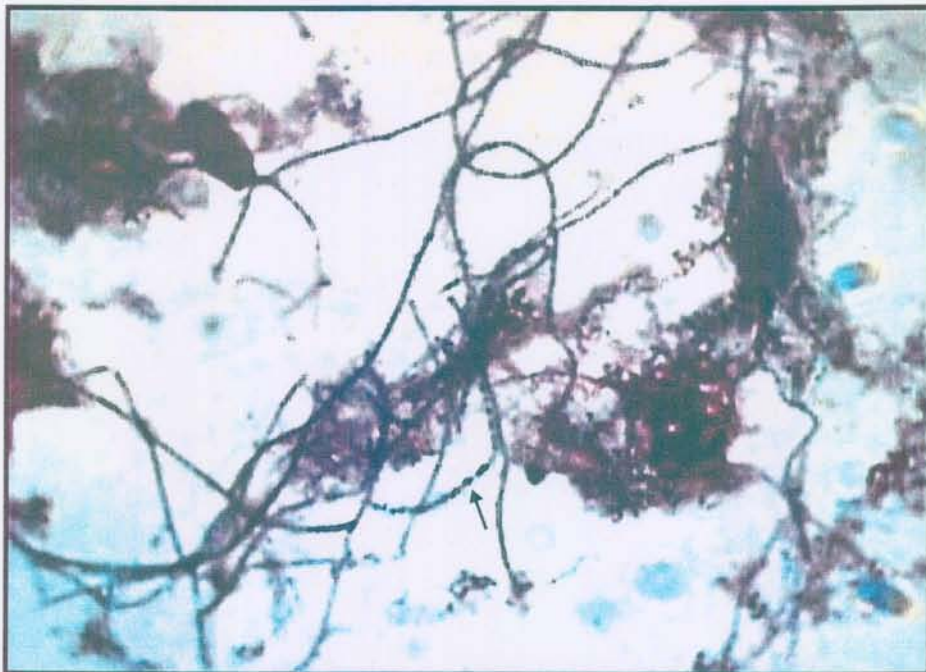
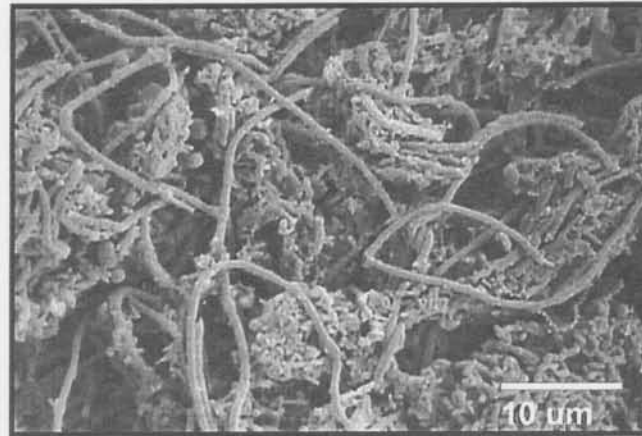


Fig. 1. Light micrographs of foam from activated sludge from Centurion WWTP. (A) Coiled, Gram positive filament "*Microthrix parvicella*" (1000x). (B) PHB granules present in "*Microthrix parvicella*" (1000x).

A



B



C

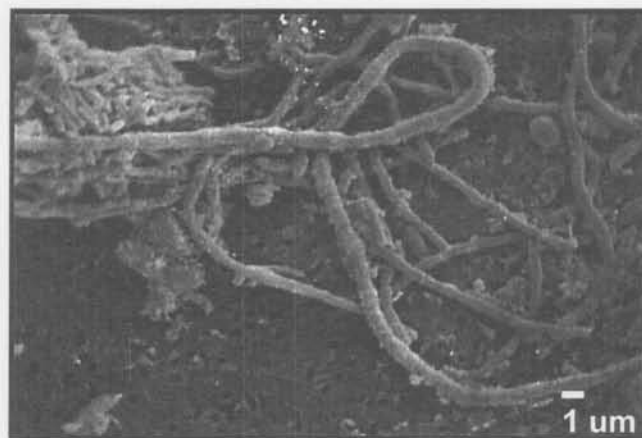


Fig. 2. SEM of foaming activated sludge flocs from Centurion WWTP. (A) Curved and coiled filaments within the floc. (B) Smoothly curved filaments free from the flocs. (C) "*Microthrix parvicella*".



Fig. 3. Rod shaped, Gram positive cells occurring in chains, *Bacillus spp.*(1000x) from the foam from Centurion WWTP.

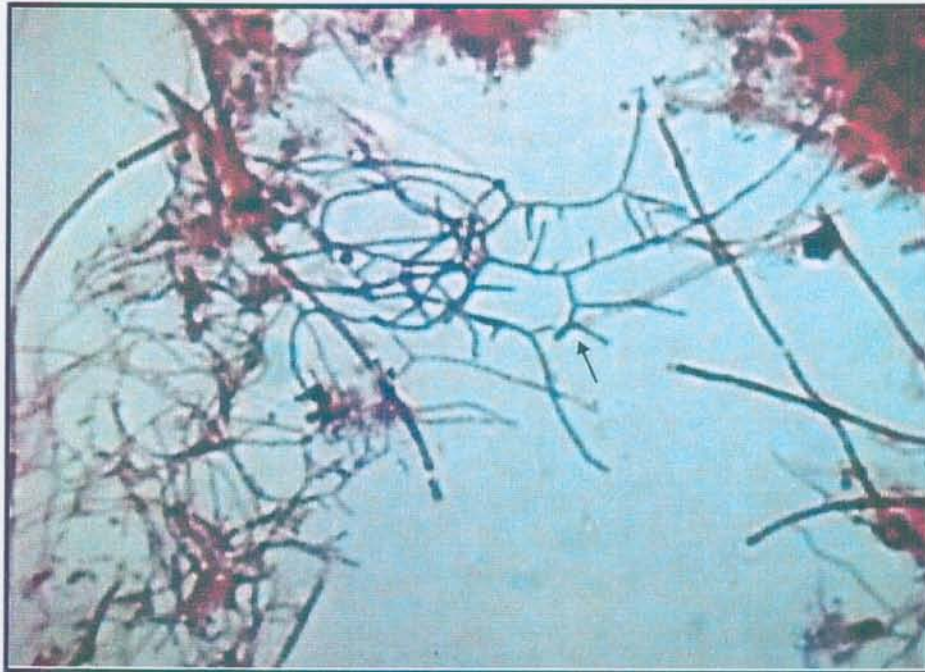
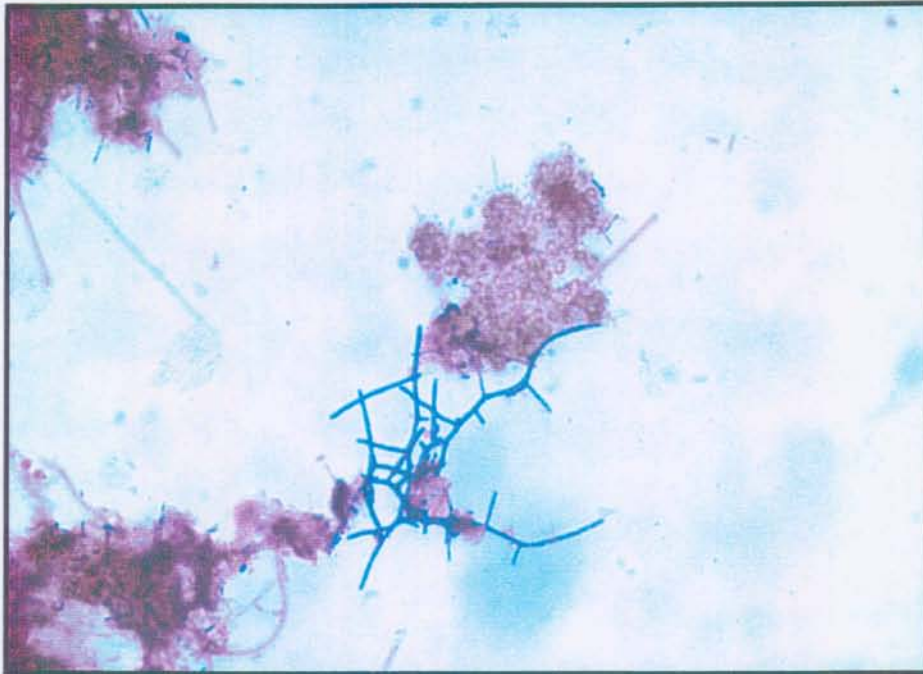


Fig. 4. Gram positive, mycelial filament, *Nocardia spp.* (1000x) from Rhyndfield WWTP bulking activated sludge.

A



B

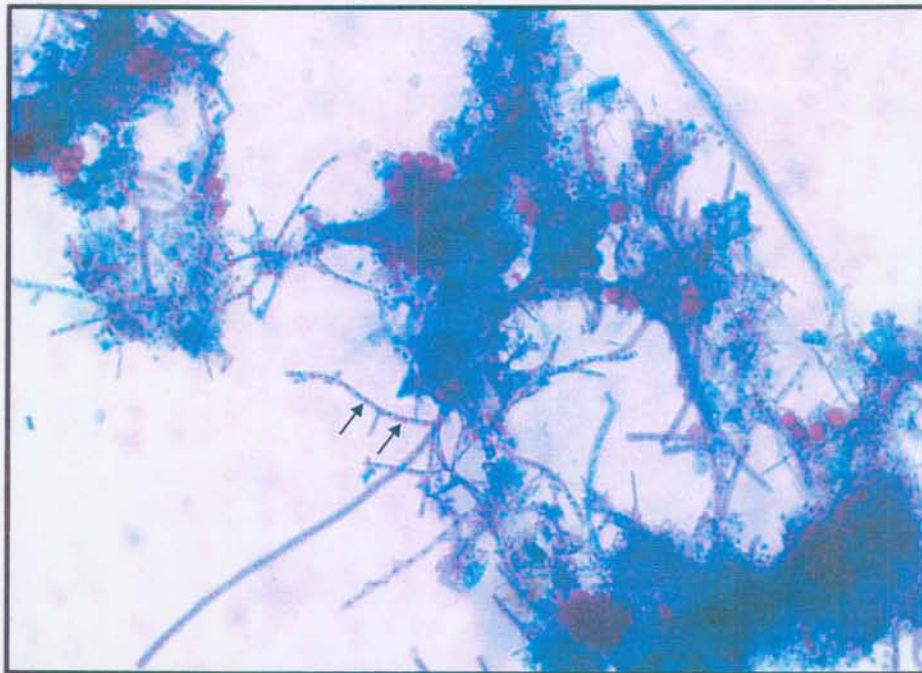


Fig. 5. Light micrograph of flocs of foaming sludge sampled from Noosa WWTP in Australia. (A) Gram positive, mycelial filament, *Nocardia* spp.(630x). (B) Methylene blue stain indicating polyphosphate molecules in *Nocardia* filaments (630X).

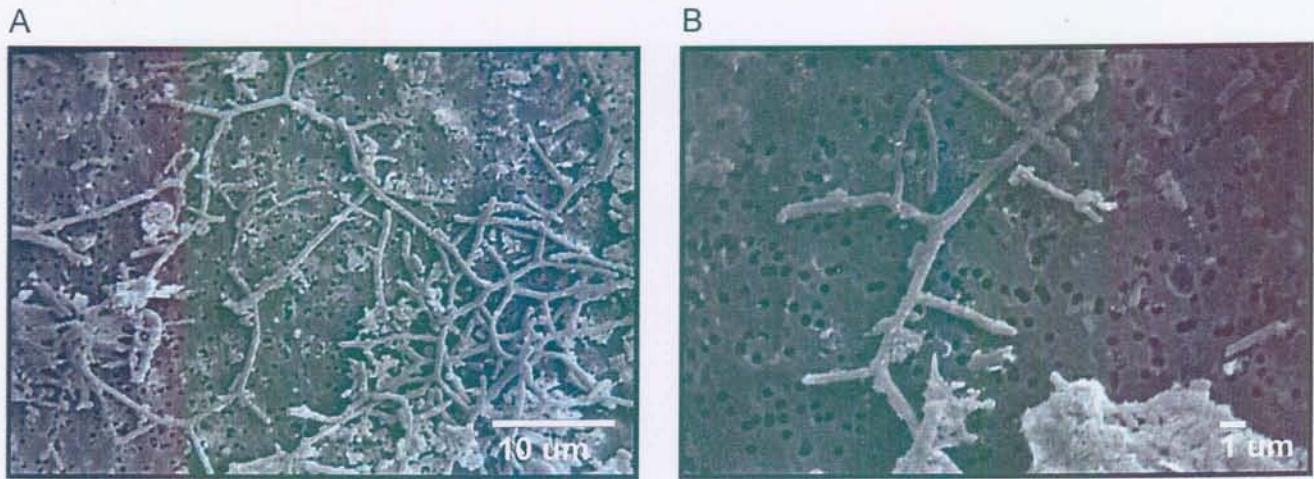


Fig. 6. SEM of bulking activated sludge from Rhyfield WWTP in South Africa. (A) *Nocardia* spp. (B) *Nocardia* spp.



Fig. 7. Gram positive filament with attached epiphytic growth, Type 0041 (1000x) from Centurion WWTP foaming activated sludge.

A



B

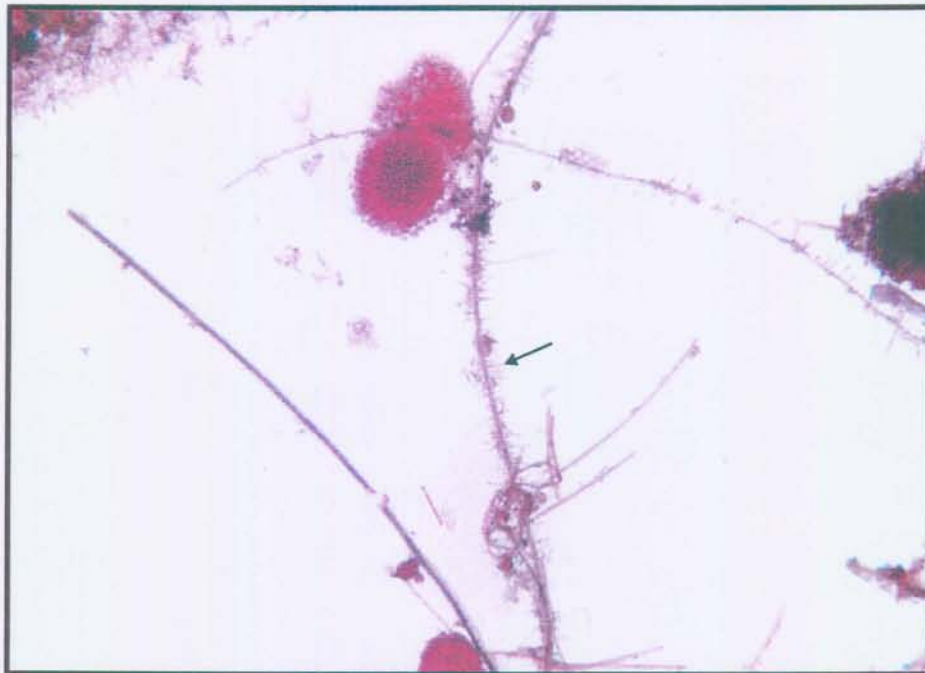
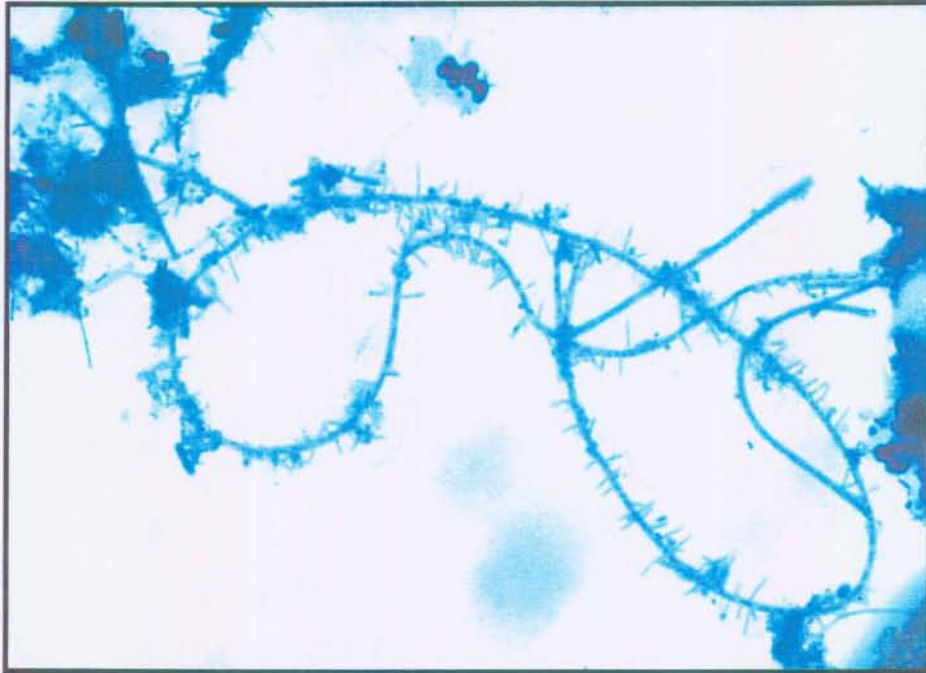


Fig. 8. Phase contrast and light micrographs of the foam of activated sludge from Noosa WWTP. (A) Type 0041 (630x phase contrast). (B) Gram positive filament with attached cells, Type 0041 (630x).

A



B

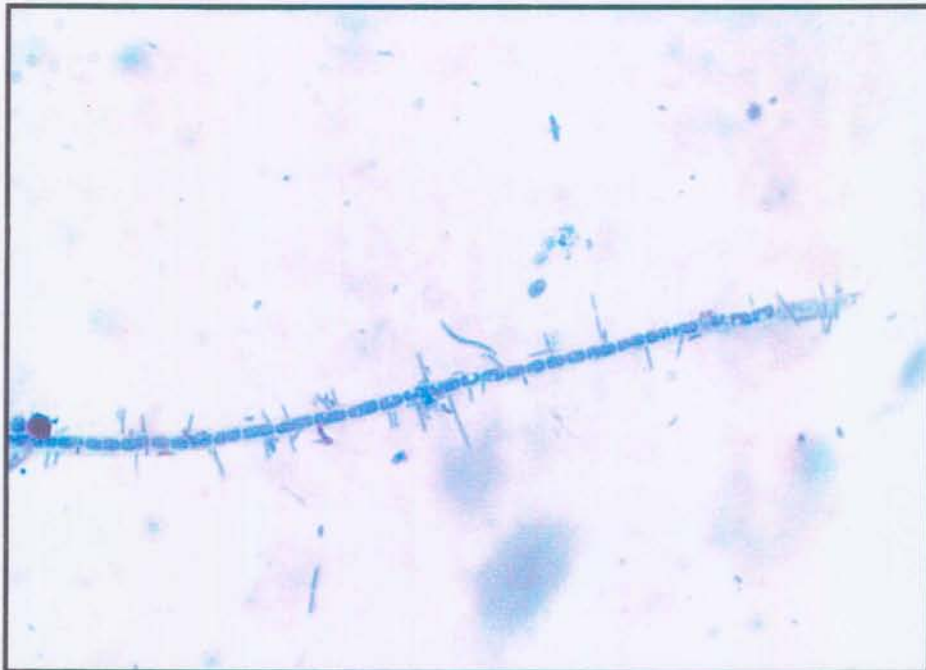
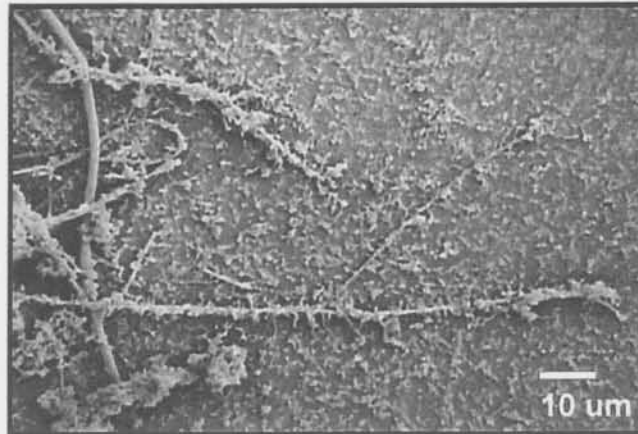


Fig. 9. Light micrographs of foam from sludge from Noosa WWTP. (A) Methylene blue stain indicating polyphosphate granules in type 0041 (630x). (B) Type 0041 with "cuff" at the end (630x).

A



B

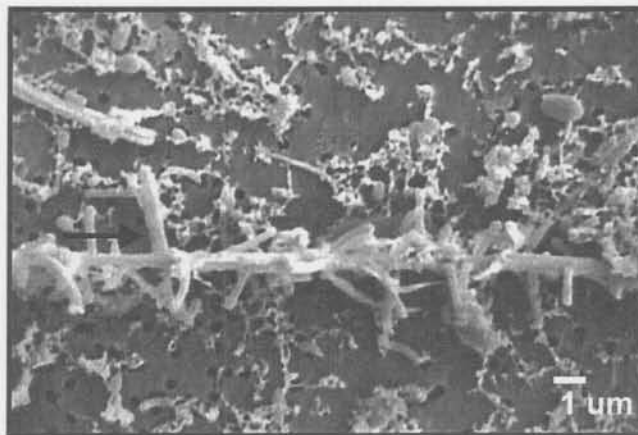


Fig. 10. SEM of the foaming activated sludge from Centurion WWTP. (A) Attached epiphytic growth on Type 0041. (B) Attached bacteria on Type 0041.

Conclusions

Filaments in the foam sample from South Africa

The filamentous microorganism most frequently observed and with the highest abundance (approximately 75%), in the Centurion WWTP foam sample was "*Microthrix parvicella*". In no order of significance, other filaments observed were Type 0041, rod shaped cells in chains and other filaments that were so infrequently observed that no positive identification could be made.

Filaments in the foam sample from Australia

Type 0041 were observed most frequently. *Nocardia* spp. were also present.

Filaments in the bulking sample from Rhyndfield WWTP

Nocardioform actinomycetes were observed most frequently and with the highest abundance (approximately 90%). "*Microthrix parvicella*" was observed in very low abundance (<5%). Other filaments were observed but so infrequently and with such low abundance (<1%) that no positive identification could be made.

Since only a few culturing methods are available for the isolation and cultivation of filamentous microorganisms associated with bulking and foaming activated sludge, microscopy and molecular taxonomy is one of the most reliable methods for identification. The microscopic study that was conducted on the bulking and foaming samples gave an indication of which filamentous microorganisms were present in the samples and which filaments were dominant. Our conclusions support the findings of the operators of the South African water purification plants from where the samples were taken, that the dominant filamentous organisms are "*Microthrix parvicella*" and Nocardioform actinomycetes respectively.



CHAPTER 4

16S rRNA SEQUENCE ANALYSIS OF BACTERIA PRESENT IN FOAMING ACTIVATED SLUDGE

The style of this chapter is in accordance with that of *Systematic and Applied Microbiology*

ABSTRACT

The formation of a stable viscous foam on activated sludge aeration basin surfaces is a worldwide problem. Foam is usually comprised of filamentous bacteria occurring predominantly in the mixed liquor. The lack of availability of pure cultures of most filaments and the limited amount of characterisation data available for them, means that our current understanding of their taxonomic position is very poor and their relationship to other bacteria are not known. 16S rDNA sequencing was used in an attempt to identify the filamentous microorganisms in a foaming sample from the Centurion Waste Water Treatment Plant in Gauteng South Africa.

Key words: 16S rDNA sequences, activated sludge foaming

Introduction

The performance of a sewage treatment plant depends upon the microbial community of the activated sludge (Gray, 1990). The analyses of the microbial community in activated sludge is important for the understanding and possible control of separation problems in sewage treatment plants (Schuppler *et al.*, 1995). Foaming is one of the solid separation problems experienced in activated sludge systems that has compelled researchers to examine the biological component of the mixed liquor of activated sludge (Blackall and Hugenholtz, 1999).

Foaming filamentous organisms are differentiated mainly using morphology (Blackall, 1994). Microorganism morphology is a poor descriptive attribute that can vary widely depending upon nutritional conditions. Because of their inadequate characterization, filamentous bacteria in activated sludge are not included in the widely used and generally accepted phenetic classifications (Seviour and Blackall, 1999).

Employing molecular biological methods provide an alternative approach for the detection of microorganisms that are difficult to identify by conventional culture techniques or microscopy (Blackall, 1994). The analysis of 16S rRNA genes, aided by using PCR to amplify target sequences in environmental samples, has enabled microbial ecologists to identify and characterise microorganisms in a natural community, like activated sludge, without prior cultivation (Schuppler *et al.*, 1995).

The taxonomic position of an organism can be determined by comparing the sequence with those of other bacteria (Seviour and Blackall, 1999). Analysis of such data has helped to resolve the taxonomic position of some of the Eikelboom filaments (Seviour and Blackall, 1999). Many of the filaments still remain to be characterised in this way and further extensive phenotypic characterisation is still needed before the organisms can be identified.

Molecular methods as mentioned above provide microbiologists with the tools to study the ecology and population dynamics of these filaments in activated sludge plants.

In this study foaming activated sludge from a water purification plant in Gauteng, South Africa was examined. The organisms in the foam were identified using molecular techniques and phylogenetically placed using the Clustal X software program.

Materials and methods

Sampling

Activated sludge foam was collected from the aerobic zone of the Centurion Wastewater treatment plant in Gauteng, South Africa.

Sample preparation

Volumes of 100 ml foam were homogenised for 10 min using 70g glass beads. The foam was pelleted by centrifugation for 10 min at 7000 rpm in a Hermle 360 K centrifuge.

DNA extraction and purification (Modified from Ehlers, 1995)

After centrifugation, the supernatant was discarded. The pellet was resuspended in volumes of 40 ml Sodium-Tris-EDTA buffer (STE, 4°C, pH 8) (Appendix) and 2 ml lysosyme (3 mg/ml of a freshly made 50 mg/ml stock solution). This was incubated on ice for 1 h. SDS (20%) and proteinase K (100 µg/ml) were added to the cell suspension. The cell suspension was incubated overnight at 50°C with slight agitation. Two gentle extractions with equal volumes phenol:chloroform/isoamylalcohol were carried out. The suspension was mixed for 30 min in a shake incubator at 100 rpm. Centrifugation was carried out at 7000 rpm for 10 min to separate the organic and aqueous phases. The aqueous DNA-containing top layer was removed with a “wide bore pipet” without disturbing the white protein-rich interface. This step was repeated until there was no more protein visible. A final extraction step with equal volumes chloroform:isoamylalcohol was carried out by centrifugation for 10 min at 7000 rpm in order to remove the residual phenol from the DNA suspension.

The aqueous DNA phase was aliquoted into sterile microcentrifuge tubes, and adjusted to 0.3 M sodium acetate (NaOAc) with a 3 M NaOAc stock solution (pH 5.2). Two volumes of cold (-20°C) absolute ethanol was added on top of the aqueous layer and mixed. The DNA was precipitated overnight at -20°C. The solution were centrifuged at 10000 rpm for 10 min at 0°C to pellet the DNA. The supernatant was discarded and the tubes were inverted on towelpaper and left to air dry. The dried pellets were resuspended in 200-300 µl Tris-EDTA (TE) buffer. The DNA was concentrated. A volume of 1 ml cold (-20°C) 100% ethanol was added again and the salt precipitated for 2 h at -20°C. The DNA was pelleted again at 10000 rpm for 10 min. A wash step to desalt the pellets, with 70% ice cold (-20°C) ethanol, was carried out and the pellets were left to air dry. TE buffer was added to the dried and desalted pellets.

The purity and concentration of the DNA was determined with a CARY 1E UV-Visible spectrophotometer of Varian (Varian Australia) at A_{260} and A_{280} .

PCR amplification of 16S rDNA

The 16S rRNA genes were amplified from total DNA using two universal bacterial primers, forward primer 27 and reverse primer 1492r (Table 1)(Blackall, 1994). The numbering of the primers is based on the *Escherichia coli* 16S rRNA gene. These primers amplify approximately 1400 to 1500 bp (Blackall, 1994). The primers were synthesised at ROCHE at a scale of 50 nmol.

The (50 μ l) PCR mixture contained 25 pmol of each primer, 2.5 mM of each dNTP (dATP, dGTP, dTTP, dCTP [Takara]), 10 X Reaction buffer (100 mM Tris/HCl, 500 mM KCl, 15 mM $MgCl_2$)(Takara), 0.5 unit of *Taq* DNA polymerase (Takara) and 10-50 ng of genomic bacterial DNA. The PCR was performed on a Perkin Elmer GeneAmp 2400 system. The DNA and ddH₂O were subjected to a denaturation step of 98°C for 2 min followed by addition of the rest of the PCR mix and 30 cycles of 93°C for 1 min, 48°C for 45 s, 72°C for 2 min; a final re-annealing at 48°C and extension at 72°C for 10 min (Blackall, 1994).

Following thermal cycling, the PCR products were visualised by agarose gel electrophoresis and UV illumination (Sambrook *et al.*, 1989). Sizes of the amplicons were assessed by comparison with a 1 kb marker (Gibco, BRL) run in the agarose electrophoresis.

PCR product purification

The amplification mix was purified using the High Pure PCR Product Purification Kit following the standard protocol as supplied by the manufacturer (Boehringer Mannheim). The purification involved adding binding buffer to the PCR mix and centrifuging it through filter tubes. After this the unincorporated nucleotides were removed by adding wash buffer and centrifugation at 10 000 rpm for 2 min. The PCR products were eluted using elution buffer and centrifugation.

Clone library construction

Clone libraries from purified PCR products were constructed using the PGemT-easy cloning kit following the standard protocol supplied by the manufacturer (Promega). Ligation of the PCR product to the vector was carried out using T4 DNA ligase, ligation buffer and a vector, all supplied by the manufacturer. The ligation was carried out at 4°C overnight. Transformation was carried out using high efficiency competent cells and recombinant colonies were selected using blue/white colour screening.

Purification of plasmids

Each of the clones was resuspended in a volume of 5 ml Luria Burtani Broth containing ampicillin (Appendix) and incubated at 37°C overnight. The cells were centrifuged at 10 000 rpm for 1min after which the supernatant was discarded. Each pellet was resuspended in 400 µl lysis buffer (Appendix) and vortexed for 5-10 s. This was incubated at room temperature for 10 min. To this, 400 µl freshly prepared NaOH/SDS (Appendix) was added and mixed thoroughly but carefully. This was left on ice for 10 min. Volumes of 300 µl cold (4°C) 7.5 M NH₄OAc (pH7.6) was added to each pellet and mixed thoroughly but carefully. This was incubated on ice for 10 min and afterwards centrifuged for 10 min at 10 000 rpm at room temperature. An aliquots of 1000 µl of the supernatant was collected in a new microcentrifuge tube and 650 µl isopropanol was added to this and mixed. The mixture was left at room temperature for 10 min and then centrifuged for 10 min at 10 000 rpm. The supernatant was discarded and 100 µl of 2 M NH₄OAc (pH 7.4) was added to the pellet and mixed thoroughly. This was left on ice for 10 min and then centrifuged for 10 min at 10 000 rpm at room temperature. The supernatant was collected and to this 110 µl isopropanol was added. This was left at room temperature for 10 min. The suspension was centrifuged at 10 000 rpm for 10 min at room temperature and the supernatant discarded afterwards. The pellet was washed with 1 ml 70% ethanol and then dried in the desiccator. Afterwards the pellet was dissolved in 25 µl dH₂O. The purified plasmids were screened for the correct sized insert using restriction enzyme digestion with EcoR1 for 1 h at 37°C, and agarose gel electrophoresis.

Amplification of 16S rDNA

PCR's were performed on the recombinant clones using the vector primers SP6 and T7 (table 1) to amplify the insert. The PCR mix consisted of 40 pmol of each primer, 2.5 mM of dNTPs, 10X Reaction buffer (Takara), 0.5 U of Taq DNA polymerase (Takara), 10-50 ng of genomic DNA and ddH₂O to make up the volume of the reaction to 25 μ l.

The PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim). The protocol for the kit was followed. Products were visualised with ultra-violet illumination after agarose gel electrophoresis (Sambrook *et al.*, 1989).

Sequencing

Sequencing of PCR amplified 16S rDNA was carried out using the ABI PRISM dye terminator cycle-sequencing ready-reaction kit (Perkin Elmer) and an ABI Prism 377 sequencer, according to the manufacturers protocol. The sequencing mix - 4 μ l Terminator ready reaction Mix, 50 ng PCR product, 1.6 pmol primer - was placed in the thermal cycler. The thermocycling protocol employed was one cycle of 96°C for 5 s, and 25 cycles of denaturation at 96°C; reannealing at 50°C for 5 s and extension at 60°C for 4 min. The vector primers SP6 and T7 (PROMEGA) was used as end primers and the internal primers used was reverse primer ii, forward primer iv and the 530 forward primer (ROCHE) (Table 1).

The excess dye terminators were removed from the reaction mixture by adding a mixture of 50 μ l of 100% ethanol and 2 μ l 3M sodium acetate (pH 5.2) to the sequencing reaction. This mixture was left on ice for 20 min, after which it was centrifuged at 10 000 rpm for 20 min at 4°C. The supernatant was tipped off, and 250 μ l 70% ethanol was added, and tipped off again. The pellet was gently flicked and the tube centrifuged for 2 min at 10 000 rpm. The excess fluid was carefully dried with a tissue, and the products vacuum dried (ca. 10 min). For analysis, the purified products were resuspended in 3.5 μ l Blue dextran/EDTA

loading buffer (PE Applied biosystems), denatured for 2 min at 90°C and loaded onto the ABI Prism model 377 DNA sequencer gel.

Table 1. Sequences of the primers used in amplification and sequencing reactions.

Name and Sequence	Sequence 5' – 3'	Reference
27 forward	GAGTTTGATCCTGGCTCAG	Blackall, 1994
1492 reverse	TACGGYTACCTTGTTACGACTT	Blackall, 1994
SP 6	TTTAGGTGACACTATAGAATAC	PROMEGA
T 7	TAATACGACTCACTATAGGCGA	PROMEGA
530 forward	GTGCCAGCMGCCGCGG	Lane, 1991
Forward nr. ii	GTGTAGCGGTGAAATGCGTAG	Kunhert <i>et al.</i> , 1996
Reverse nr. vii	CTTGCGACCGTACTCCCCAGGC	Kunhert <i>et al.</i> , 1996

Phylogenetic analysis

The sequences obtained were trimmed of uncomparative data so that only from nucleotides 28-1491 were aligned and compared. Sequences were compiled using the Sequence Navigator software package (Applied Biosystems) and compared to available databases by the use of the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) to determine approximate phylogenetic affiliations. The compiled sequences were aligned using the Clustal X software program (Thompson *et al.*, 1997).

The sequences determined in this study were phylogenetically placed by comparative analysis (715 nucleotides) with 16S rDNA sequences of various other bacteria including members of the Cytophaga-Flavobacter-Bacteroides group. Sequences (from the BLAST search) with the greatest similarity to the clone sequences were selected. These sequences were retrieved from the GenBank database. The phylogenetic tree (Fig.1) was calculated using the neighbour-joining algorithm (Saitou and Nei, 1987).



Table 2. Databank accession numbers of the 16S rRNA gene sequences of organisms used in this study

Organism	GenBank accession no.
• <i>Actinomycetaceae</i>	X87310
• <i>Actinomycete</i> LP2 gene	A3030911
• <i>Actinobacterium</i> from anoxic soil	AJ229241
The genus <i>Aquabacterium</i>	
• <i>A. aqua</i> 2	AF089858
• <i>A. aqua</i> 1b	AF089857
• <i>A. aqua</i> 2b	AF089856
• <i>A. aqua</i> 3	AF089859
The genus <i>Bacteroides</i>	
• <i>Bacteroides</i> sp. ASF519	AF157056
• <i>Bac. putredinis</i>	L16497
Beta proteobacteria	
• Beta proteobacterium A0640	AF236010
• Beta proteobacterium B4	AF035050
• Beta proteobacterium B6	AF035052
• Beta proteobacterium B8	AF035054
The genus <i>Capnocytophaga</i>	
• <i>Cap. gingivalis</i>	X67608
• <i>Cap. sputigena</i>	X67609
The genus <i>Cellulomonas</i>	
• <i>C. cellasea</i>	X83804
• <i>C. sp.</i>	Y09658
• <i>C. sp.</i> UZFB529	AF237956
The genus <i>Chryseobacterium</i>	
• <i>C. balustinum</i>	M58771
• <i>C. indoltheticum</i>	M58774
The genus <i>Cytophaga</i>	
• <i>Cy. arvensicola</i>	D12657
• <i>Cy. aurantica</i>	D12658
• <i>Cy. hutchinsonii</i>	M58768
• <i>Cytophaga</i> (type 0092)	X85210
• Denitrifying Fe-oxidising bacterium	U51102
The genus <i>Dietzia</i>	
• <i>D. maris</i>	Y8883
• <i>D. sp.</i> 147	Y17518
• <i>D. sp.</i> strain JTS6455	AB010905
Eikelboom types	
• Type 1701	L79964
• Type 0803	X86071
The genus <i>Empedobacter</i>	
• <i>E. brevis</i>	M59052
The genus <i>Flavobacterium</i>	
• <i>F. aquatile</i>	M58764
• <i>F. johnsoniae</i>	M59051



Table 2. (continued)

Organism	GenBank accession no.
• <i>F. spiriticum</i>	M58768
The genus <i>Flexibacter</i>	
• <i>Fx. canadensis</i>	M62793
• <i>Fx. elegans</i>	M58782
The genus <i>Frankia</i>	
• <i>F. sp</i>	L40622
• <i>F. sp. strain AVNNS</i>	L40613
• <i>F. sp.</i>	U60287
The genus <i>Haliscomenobacter</i>	
• <i>H. hydrossis</i>	M58790
The genus <i>Janibacter</i>	
• <i>J. limosus</i>	Y085391
• <i>J. terrae</i>	AF176948
The genus <i>Leptothrix</i>	
• <i>Leptothrix cholodnii</i>	X97070
• <i>Leptothrix discophora</i>	L33974
• <i>Leptothrix mobilis</i>	X97071
• <i>Microthrix parvicella</i>	X89774
The genus <i>Nocardia</i>	
• <i>N. abscessus</i> str IMMIB	AF218294
• <i>N. calcarea</i>	X80618
The genus <i>Nocardioides</i>	
• <i>N. simplex</i>	NS081990
The genus <i>Nostocoida</i>	
• <i>N. limicola I</i>	AF255736
• <i>N. limicola II</i>	X85212
• <i>N. limicola II</i>	X85211
• <i>N. limicola II</i>	Y14597
The genus <i>Ornithobacterium</i>	
• <i>O. rhinotracheale</i>	L19156
The genus <i>Rhodococcus</i>	
• <i>R. sp (GH557)</i>	X76704
• <i>R. coprophilus</i>	U93340
• <i>R. erythreus</i>	X79289
• <i>R. erythropolis</i>	AJ237967
• <i>R. zopfii</i>	AF191343
The genus <i>Sphaerotilus</i>	
• <i>Sphaerotilus sp. IF9</i>	AF072916
The genus <i>Sphingobacterium</i>	
• <i>S. spiritivorum</i>	M58778
• <i>S. thalophilum</i>	M58779
The genus <i>Sphingomonas</i>	
• <i>Sphingomonas sp. MBIC4193</i>	AB023290
• <i>S. paucimobilis</i>	X94100
• <i>S. yanoikayae</i>	U37524
The genus <i>Terrabacter</i>	
• <i>T. sp</i>	Y08853
• <i>T. sp. DDE-1</i>	U96645
The genus <i>Tetrasphaera</i>	
• <i>T. australiensis</i> str 109	AF12509
• <i>T. japonica</i> str T1-Y7	AF125092



Table 2. (continued)

Organism	GenBank accession no.
The genus <i>Thiothrix</i>	
• <i>Thiothrix</i> sp. OS-F gene	AB042538
The genus <i>Tsukamurella</i>	
• <i>T. paurometabolum</i>	Z36933
• <i>T. spumae</i>	Z37150
• <i>T. sp. SCUN5</i>	AF150494
Unidentified oral cavity clones	
• clone BE109	AY005446
• clone AH040	AF125204
• clone BS003	AY005448
The genus <i>Weeksella</i>	
• <i>W. virosa</i>	M93152

Results and discussion

PCR amplification of 16S rRNA genes

The 16S rRNA genes of the bacteria in the foaming sample were amplified with the 27f and 1492r primers (Table 1), and PCR produced a single band of about 1500 bp.

Clone library

After screening, 25 clones containing the correct sized insert were submitted to automated cycle sequencing.

Automated cycle sequencing

The nucleotide sequences of the 16S rRNA genes of the selected clones were determined with automated sequencing on the ABI PRISM 377 model and sequences of between 1400 and 1464 were obtained.

Phylogeny of the clones

The 25 clone sequences obtained from the foam sample were aligned and phylogenetically placed with sequences from various other bacteria that showed identity of 90 - 99% to the clone sequences in the BLAST search (Fig 1). Where similarity less than 90% were found the sequences with the greatest similarity to the clone sequence were used. The clone sequences were edited to a length of 1463 bp. After aligning these sequences to the sequences obtained from

GenBank, all of the sequences were trimmed to 715 bp to accommodate shorter sequences from GenBank.

The dendrogram indicated 7 genotypic clusters (Fig. 1).

In Group 1, clones 61, 10, 799, 53 and 4 showed similarities greater than 95 % with *Dietzia maris* which was reclassified from *Rhodococcus maris* by Rainey *et al.* (1995). *D. maris* has been isolated as a dominant microbe from activated sludge foam (Sezgin *et al.* 1988).

In Group 2 clones 14 and 17 grouped with *Nostocoida limicola* II often associated with foaming activated sludge (Seviour and Blackall, 1999). Clone 17 showed more than 95% similarity to the *Terrebacter* and *Janibacter* spp. Clone 21 seemed to be similar to the *Actinomycetaceae* species within Group 2. Clone 16 grouped alone, but had less than 4% sequence divergence from Group 2 that included *Nostocoida limicola* II, also a well known foam forming filamentous microorganism (Seviour and Blackall, 1999).

Although clone 47 falls into group 3, which comprises members of the *Cytophaga-Flavobacter-Bacteroides* (CFB) group, it only had a 90% similarity with *Flavobacterium* sp. when a sequence of 350 bp was compared (Fig. 1). Clones 7A, 6 and 10A had less than 90% similarity to the CFB group.

Group 4 included a number of unidentified bacterial clones from oral cavities. Clones 71, 75, 8A and 734 formed a cluster with this group of clones. Clone 22 also showed greater than 91% similarity with these clones. One of the unidentified clones from an oral cavity (AH040) was resolved into the third subdivision of the candidate division TM7 (Hugenholtz *et al.*, 2001). This subdivision include bacterial clones from foaming activated sludge (Hugenholtz *et al.*, 2001).

Although clone 15 showed similarities of 96% and more with the organisms in Group 5, when the sequence was compared with those of other bacteria, it also indicated a 97% homology with members of Group 7 (Fig. 1).

Group 7 indicated that the sequences of clones 8, 62 and 15A were more than 95% similar to that of a denitrifying Fe-oxidising bacterium as well as *Aquabacterium aqua* and Beta-proteobacterium B8 (Fig 1). Group 7 had less than 1% sequence divergence from Group 6 which involved the filamentous bacteria Eikelboom Type 1701 and *Sphaerotilus natans* strain IF9, both associated with activated sludge bulking and foaming.

Clone 5 did not fall into any of the groups in the dendogram (Fig. 1). However, a nucleotide sequence of approximately 440 nucleotides showed 97% similarity to members of group 1 i.e. *Dietzia*, *Rhodococcus* and *Nocardia* spp. that form part of the filamentous bacteria associated with activated sludge foam (Seviour and Blackall, 1999).

Clone 11 was not closely associated to any of the groups of bacteria examined in the study (Fig. 1).

Clone 7 had a 93% similarity with members of the genus *Frankia* (Fig. 1).

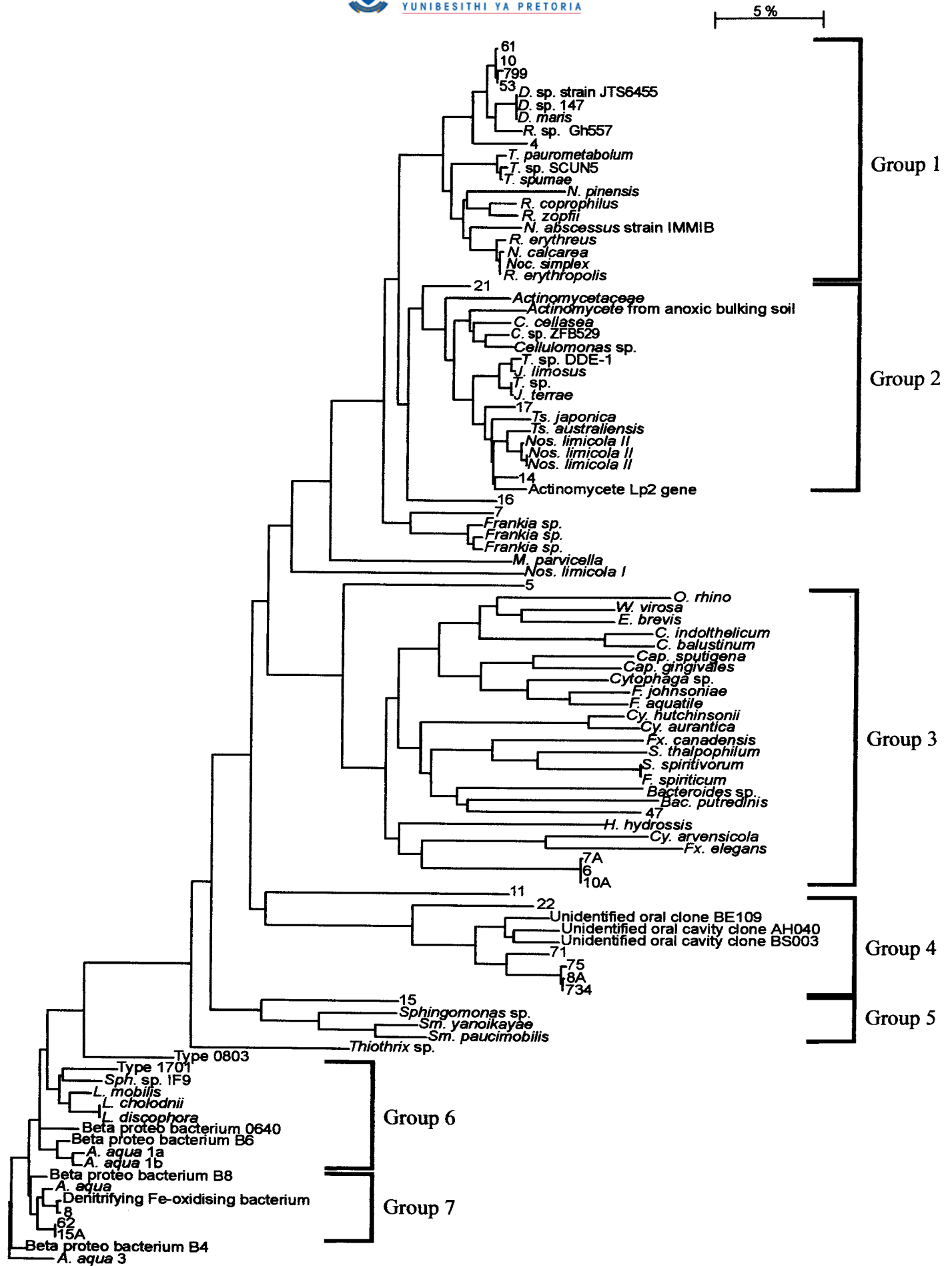


Fig. 1.

Figure 1 (Legend). Phylogenetic relationships of 25 bacterial clones, isolated from activated sludge foam, to other bacteria. The sequences were edited to 1476 bp and aligned using the CLUSTAL X software. The tree was reconstructed using the neighbour-joining algorithm. The Genbank accession numbers of the sequences are given in Table 1. The bar indicates a 5 % sequence divergence.

Conclusions

Due to the unculturability of a number of bacteria present in activated sludge, other means of identification of these bacteria need to be employed. The routine recognition of filamentous microorganisms is based on microscopic features, which is not always a satisfactory system for reliable identification (Seviour and Blackall, 1999). Molecular identification techniques permit the researcher to identify organisms present in their natural environment without prior cultivation (Schuppler *et al.*, 1995).

The sequences from the clones obtained indicates a wide variety of organisms present in the foaming sample without prior cultivation of the bacteria present in the sample. Although only a small number of clones were sequenced, the results indicated that some of the clones isolated did group with the filamentous organisms associated with foaming activated sludge.

“Microthrix parvicella” was not identified in the study and this is contrary to the finding in Chapter three of this dissertation, where it was established by microscopy that *“M. parvicella”* was the dominant filamentous organism in this sample. A possible explanation for this discrepancy could be that the primerset that was used in this study targeted a too narrow range of bacteria. Cloning can also introduce bias in that not all of the clones obtained are sequenced and in this way the organism expected could be missed. The fact that a low number of positive clone was obtained in this study could have contributed to *“Microthrix parvicella”* not being among those organisms identified from the sample.

The reason that no clear homology or no definite identification could be made in this study could be that the existing database could still be limited in terms of the

sequences available for the filamentous organisms. Hence the importance of further studies like this to expand the database on the filamentous microorganism sequences.

CHAPTER 5

MOLECULAR IDENTIFICATION OF FOAMING BACTERIA AND THE DETECTION AND IDENTIFICATION OF A FILAMENTOUS BACTERIUM RESEMBLING TYPE 0041 BY FISH WITH rRNA-TARGETED OLIGONUCLEOTIDE PROBES

The editorial style of *Systematic and Applied Microbiology* was followed in this chapter.

This study formed part of the publication: **Investigation of candidate division TM7, a recently recognized major lineage of the domain *Bacteria* with no known pure-culture representatives.** Hugenholtz *et al.*, 2001. *Applied and Environmental Microbiology*. **67**: 411-419.

ABSTRACT

16S rDNA sequence analysis were performed on clones obtained from foaming activated sludge. A fluorescently labelled oligonucleotide probe for members of the TM7 division of bacteria, TM7305 was tested on activated sludge from the Noosa waste water treatment plant in Queensland Australia. The probe confirmed the presence of two morphotypes of filaments, one resembling Eikelboom Type 0041. The other morphotype was a sausage-shaped rod. TM7-specific PCR-clone libraries prepared from the activated sludge yielded 18 novel TM7 sequences which were phylogenetically analysed and placed in a tree indicating the taxonomic position of the clones, with regard to the three subdivisions defined for TM7.

Introduction

The majority of bacterial divisions are poorly represented by cultured organisms. Reliance on the study of cultivated organisms has limited our knowledge of the extent and character of microbial diversity. Culture dependent methods are time consuming and render only a fraction of the cell counts determined by direct microscopic techniques (Manz *et al.*, 1994). The fact that no reasonable monitoring is possible by cultivation techniques, demanded the development of new methods of *in situ* detection of filamentous bacteria in activated sludge samples (Wagner *et al.*, 1994).

Knowing the 16S rDNA sequence for a particular organism means that a unique segment of that gene can be recognised (Blackall *et al.*, 1996b). When the 16S rDNA sequence of an unknown isolate is known, it can be compared with all other bacterial 16S rDNA sequences and its taxonomic position determined (Seviour and Blackall, 1999).

Woese described the bacterial domain comprising of about 12 natural relatedness groups, based mainly on analysis of cultivated organisms (Woese 1987). These relatedness groups have been called "kingdoms," "phyla," and "divisions". Hugenholtz *et al.* (1998) defined a bacterial division on phylogenetic

grounds as a lineage consisting of two or more 16S rRNA sequences that are reproducibly monophyletic and unaffiliated with all other division-level relatedness groups that constitute the bacterial domain. Culture-independent phylogenetic studies relying on sequences of rRNA genes obtained by cloning directly from environmental DNA or after amplification by PCR, led to the recognition of the now almost 40 bacterial divisions (Hugenholtz *et al.*, 1998).

Once the sequences of the unique regions of the conserved gene for an organism have been determined, a specific oligonucleotide probe for its identification can be synthesised (Seviour and Blackall, 1999).

The complex bacterial communities present in activated sludge can be readily studied with fluorescently labelled, rRNA directed oligonucleotide probes. With a set of oligonucleotide probes it is possible to affiliate the majority of the cells hybridising with the bacterial probe to distinct phylogenetic groups (Amann, 1997). Fluorescently labelled rRNA targeted oligonucleotide probes results in high resolution and rapid monitoring of defined bacterial populations in activated sludge (Wagner *et al.*, 1994).

During fluorescent *in situ* hybridisation (FISH) the target molecules remain in the whole fixed cells. The probes diffuse into the cells, bind specifically, and the probe-conferred fluorochromes can subsequently be visualised by epifluorescence microscopy (Amann, 1997).

Divisions that are characterised only by environmental sequences are termed "candidate divisions". The organisms associated with foaming and bulking activated sludge would probably fall within one or more of these divisions. For example in other ecological studies, candidate division TM7 was named after sequences obtained in an environmental study of peat bog (Rheims *et al.*, 1996). Partial-length-sequence representatives of this candidate division were also identified from activated sludge (Bond *et al.*, 1995).

Type 0041(chapter 3) is a filamentous organism often dominant in the mixed liquor of activated sludge systems. Countries surveyed include Australia (Seviour *et al.*, 1990, 1994), France (Pujol *et al.*, 1991) South Africa (Blackbeard

et al., 1986, 1988) and the Netherlands (Eikelboom 1998). The organism also occurs in large numbers in activated sludge foam (Seviour and Blackall, 1999). A highly diagnostic feature of Type 0041 is the attached growth on the filament. It has a similar morphology and occurrence pattern to Type 0675 and reliable distinction is difficult (Seviour and Blackall, 1999). Successful cultivation techniques for filament types like 1701 or 0041/0675 with attached epiphytic growth are not available yet (Seviour and Blackall, 1999) hence the need for using a molecular approach to identify Type 0041.

In this study a fluorescently labelled oligonucleotide probe for TM7 picked up a filament, which looked like Type 0041, from an activated sludge foam sample. A primer (314f) that was designed from all the TM7 sequence data was used in conjunction with the bacterial primer (1492r) to amplify DNA from the Noosa sewage treatment plant sludge for cloning. Cloning and sequencing were done to increase the database of sewage TM7 sequences so that more accurate FISH probes could be designed.

Materials and methods

Sample collection

Foaming sludge samples were collected from the Noosa wastewater treatment plant (WWTP). The samples for DNA extraction were stored at -70°C prior to use. Aliquots (0.5 ml) of the samples were fixed in paraformaldehyde (described later) and stored at 4°C.

Total DNA extraction from sludge sample

After thawing the Noosa sludge sample, the BIO 101 FastDNA[®] Spin Kit for Soil was used for the extraction of the DNA from the sludge sample. Aliquots of 1 ml of sludge were centrifuged at maximum speed for 2 min. The supernatant was discarded, and the pellet resuspended in whatever supernatant was left in the tube. The protocol for the BIO 101 kit was followed, using 500 µl of the resuspended pellet. The extracted DNA, as well as a 1 kb marker (1 kb PLUS from GibcoBRL), was run on a 1% agarose gel at 85 V for 20 min. The DNA was visualised under ultra-violet illumination (Sambrook *et al.*, 1989).



Amplification of 16S rRNA genes

Amplification of near complete 16S rRNA genes from the extracted genomic DNA was performed on the samples, using the conserved bacterial primers 314f and 1492r (Table 1). A hotstart at 96°C for 10 min was performed with DNA (1 µl) and 70.5 µl of milliQ-water (MQH₂O) in a thermal cycler (PTC-100 programmable thermal controller from MJ RESEARCH INC). The reaction mixture (28.5 µl) was added to the lysed DNA. The reaction mixture contained (as final concentrations) 1x Tth plus reaction buffer (Biotech International), 1.5 mM MgCl₂, 200 µM each dNTP, 0.3 U of Tth plus DNA Polymerase (Biotech International) and 50 ng of each forward and reverse primer. The PCR cycle included 29 cycles of denaturing at 94°C for 1 min, annealing at 48°C for 1 min and elongation at 72°C for 2 min; cycling was completed with one cycle each of 48°C for 1 min and a final elongation period of 5 min at 72°C. The amplification products were visualised using gel electrophoresis and UV illumination (Sambrook *et al.*, 1989)

Table 1. Sequences of the primers and probes used in amplification and sequencing reactions.

Name and Sequence	Sequence 5' – 3'	% formamide	Reference
Primers			
27 forward	GAGTTTGATCCTGGCTCAG	NA	Dorsch and Stackebrandt, 1992
314forward	GAGAGGATGATCAGCCAG	NA	Hugenholtz <i>et al.</i> , 2001
1492 reverse	TACGGYTACCTTGTACGACTT	NA	Blackall, 1994
SP 6	TTTAGGTGACACTATAGAATAC	NA	PROMEGA
T 7	TAATACGACTCACTATAGGCGA	NA	PROMEGA
580 forward	AYTGGGCGTAAAGAGTTGC	NA	Lane, 1991
Probes			
TM7305	GTCCCAGTCTGGCTGATC	30%	Hugenholtz <i>et al.</i> , 2001
TM7905	CCGTCAATTCCTTTATGTTTTA	20%	Hugenholtz <i>et al.</i> , 2001
EUB338	GCTGCCTCCCGTAGGAGT	20-35%	Amann <i>et al.</i> , 1990b

NA = Not applicable

Purification of the PCR products

The PCR products were purified using the CHROMA SPIN™ Columns (CLONTECH). The protocol as prescribed by the manufacturer was used. An additional elution step was added, where 50 µl of MQH₂O was added to the column and centrifuged for 5 min at 700 x g. The purified amplification product,

as well as the unpurified PCR products were visualised by gel electrophoresis and UV illumination.

Ligation and transformation

Ligation of the PCR product to the vector was carried out by adding 3 µl of the product to the ligation mix consisting of 5 µl ligation buffer, 1 µl pGEM-T vector (50 ng), 1 µl T4 DNA ligase (3 Weiss units/µl; Promega). The ligation reaction was carried out at 4°C overnight. Transformation was carried out according to the manufacturers' protocol (pGEM-T cloning kit, PROMEGA). Positive colonies were selected using blue/white colour screening.

16S rDNA clone library analysis

Amplification of clone inserts

PCR's were performed on the positive clones using the vector primers SP6 and T7 (Table 1) to amplify the insert. The same procedure was followed as described earlier in the chapter. The PCR products were purified using the QIAGEN QIAquick™ PCR Purification Kit. The protocol as prescribed by the manufacturer was followed. Products were visualised with ultra-violet illumination after agarose gel electrophoresis (Sambrook *et al.*, 1989).

Restriction fragment length polymorphism (RFLP) analysis

The PCR products of the clones containing the desired sized insert (positive clones) were subjected to a restriction enzyme analysis (REA), whereby the insert DNA was cut by two restriction enzymes, *Msp* I, and *HinP1* I (both New England Biolabs). The REA reaction mix consisted of 2.5 µl 10x NE buffer (New England Biolabs), 0.125 µl *HinP1* I, *Msp* I and 22 µl PCR product. The reaction was carried out for 2 h in a 37°C waterbath. The restriction enzyme digested fragments were visualised by gel electrophoresis on a 2% Metaphor gel that was run at 40 V for 15 min and then for 1 h at 50 V. Clones containing inserts that produced identical restriction patterns, as determined by eye upon examination of the gel photograph, were grouped into operational taxonomic units (OTUs) with the representatives of each OTU selected for 16S rDNA insert sequencing and phylogenetic analysis.

Partial sequencing of clone inserts

Plasmid inserts were sequenced with the ABI PRISM™ dye terminator cycle-sequencing ready-reaction kit (Perkin Elmer) and an ABI PRISM™ 377 sequencer (Perkin Elmer), using the vector primers SP6 and T7 as well as 580f universal primer. The 10 µl reaction volumes contained: 4 µl sequencing premix, 5 µl DNA (200 ng), 0.5 µl primer (25 ng/µl) and 0.5 µl of MQH₂O. After an initial 2 min denaturation step at 96°C, 25 cycles of 94°C for 30 s, 50°C for 15 s, and 60°C for 4 min were employed.

From the Noosa WWTP foaming sludge, 18 clones were fully sequenced.

Sequencing clean-up

The excess dye terminators were removed from the reaction mixture by adding a mixture of 50 µl of 100% ethanol and 2 µl 3M sodium acetate (pH 5.2) to the sequencing reaction. This mixture was left on ice for 20 min, after which it was centrifuged at maximum speed for 20 min at 4°C. The supernatant was tipped off, and 250 µl 70% ethanol was added, and tipped off again. The pellet was gently flicked and the tube centrifuged for 2 min at maximum speed. The excess fluid was carefully dried with a tissue, and the tube was then submitted to the speed vacuum dryer until the pellet was dry (ca. 10 min). These reactions were gel separated in the Australian Genome Research Facility (AGRF) on an ABI PRISM™ 377 automated sequencer.

Sequence analysis

Sequences were compiled using the SeqEd software package (Applied Biosystems) and compared to available databases by the use of the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations. The compiled sequences were aligned using the ARB software package (Ludwig and Strunk, 1997) and alignments were refined manually. Phylogenetic trees based on comparative analysis of the 16S rRNA genes were constructed by distance and parsimony methods.

Nucleotide sequence accession numbers

Eighteen clones sequenced in this study have GenBank accession numbers AF269010 to AF269027

Fluorescence in situ hybridisation (FISH) (Seviour and Blackall, 1999)

Paraformaldehyde (3 volumes) fixative were added to 1 volume of sample and held at 4°C for 1 to 3h. The cells were pelleted by centrifugation (5000 x g) and the fixative was removed. The cells were washed in 1 X phosphate buffered saline (PBS) (10 mM phosphate, 130 mM NaCl, pH 7.2) and resuspend in 1X PBS to give 10⁸ to 10⁹ cells/ml. One volume of ice cold ethanol was added and mixed. Fixed cells were stored at -20°C until used.

A volume of 3 µl of fixed sample was applied to the 8 mmØ wells of teflon coated microscope slides, air dried and dehydrated in ethanol series (3 min each), 50%, 80%, 98%.

At the time of use, in a 2 ml microcentrifuge tube, the following were added to a final volume of 2 ml: 360 µl of 5 M NaCl (autoclaved), 40 µl of 1 M Tris /HCl, 2 µl of 10% SDS – not autoclaved – placed in the lid of the centrifuge tube, 500 µl of formamide and 1098 µl of autoclaved milli-Q water (Table 1, Appendix).

Aliquots of 8 µl hybridisation buffer (Appendix B) were added to each well on the slide, the remainder was used to moisten a tissue paper in the 50 ml tube. Probe (0.5 µ) at 50 ng/µl was added and mixed carefully. The slide was placed in the 50 ml tube containing the moistened tissue. The tube was closed and put into a hybridisation oven at 46°C for 1 to 2 h.

After hybridization, the slides were carefully removed from the tube and splashed with prewarmed (48°C) wash buffer into a beaker. The slides were then placed into the wash buffer tube and into the water bath at 48°C for 10-15 min. Slides were rapidly transferred to prevent cooling, which could lead to non-specific probe binding.

After the wash, the slide was removed and gently rinsed in MQH₂O. After the wash step, all droplets of water were removed from the wells by directing compressed air at the sides of the slides.

Probes were commercially synthesised and 5'-labelled with the fluorochromes FITC and CY3 (Genset, France or Interactiva, Germany). The fixed samples were dual-hybridised with CY3 labelled TM7305 (Table 1) and FITC labelled bacterial probe EUB338 (Table 1) at empirically determined stringencies (Table 1). The slides were mounted with Citiflour AF1 (Citifluor Ltd, UK) and covered with a coverslip. The slides were viewed using a Zeiss epifluorescence microscope. Red and green fields were registered in Adobe Photoshop 4.0.

Results

Phylogeny of the clones

All 18 clone sequences were affiliated with division TM7. Of the 18 clone sequences six were affiliated with TM7 subdivision 1, three with subdivision 2 and eight with subdivision 3.

The rRNA-targeted oligonucleotide probe TM7305 targeted the sequences of subdivision 1, and neither subdivision 2 nor 3 (Fig 1).

TM7-specific PCR-clone libraries

Comparative analysis of the fully sequenced clones (prefix NoosaAW) enabled all the clones to be phylogenetically resolved into recognised divisions in the bacterial domain (Hugenholtz *et al.*, 1998).

PCR primers and FISH probes specific for candidate division TM7 were designed previously, based on comparative analysis of available TM7 16S rDNA sequences (Table 1). Members of the TM7 division were present in the full-scale activated sludge from Noosa WWTP as indicated by TM7-specific PCR, using TM7314f/1492r and TM7580f/1492r primers. A negative control containing

no DNA and a positive control containing the DNA of a positive clone was also subjected to PCR.

The phylogenetic positioning of the clones with the TM7 division confirms the specificity of the PCR (Fig. 1).

TM7-specific fluorescence in situ hybridisation (FISH)

Probe TM7305 revealed a filament morphotype occurring in long chains (Fig 2A). TM7305 only targets subdivision 1, hence the filament seems to belong to subdivision 1 (Fig. 1).

FISH analysis done on Noosa sludge, with TM7305, indicated two morphotypes. The two morphotypes that hybridised the TM7-specific probe, were a sheathed filament (Fig. 2) and a sausage-shaped rod occurring in short chains (Fig. 3).

Probe TM7905 (targeting nearly the entire division) was used and confirmed that the filament belonged to the TM7 division when used in combination with TM7305 (Fig. 1) (Hugenholtz *et al.*, 2001).

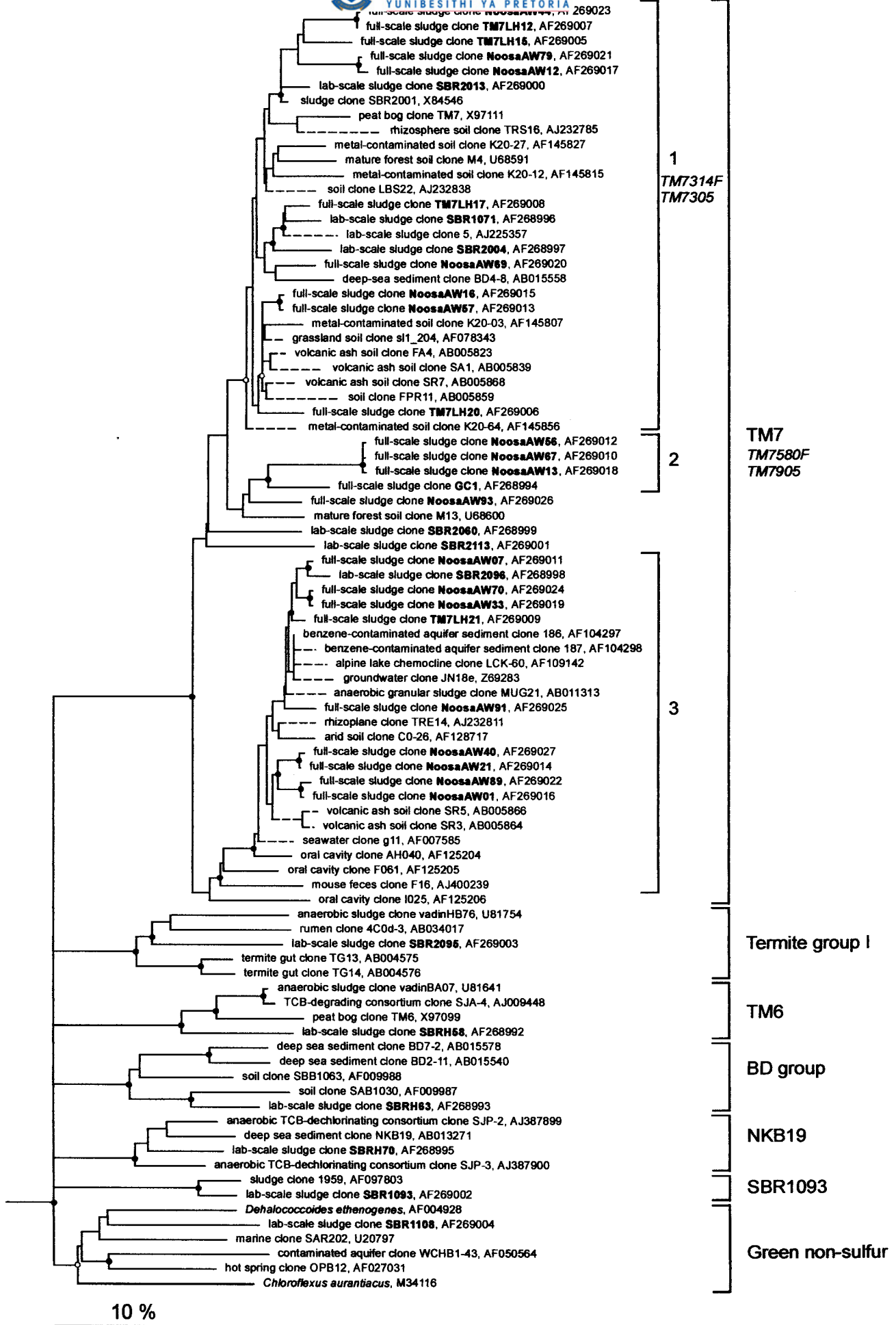
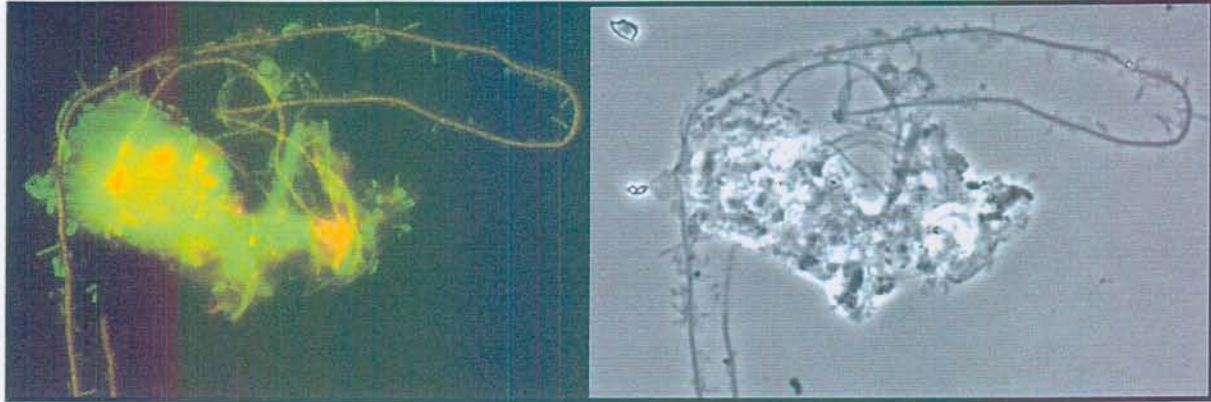


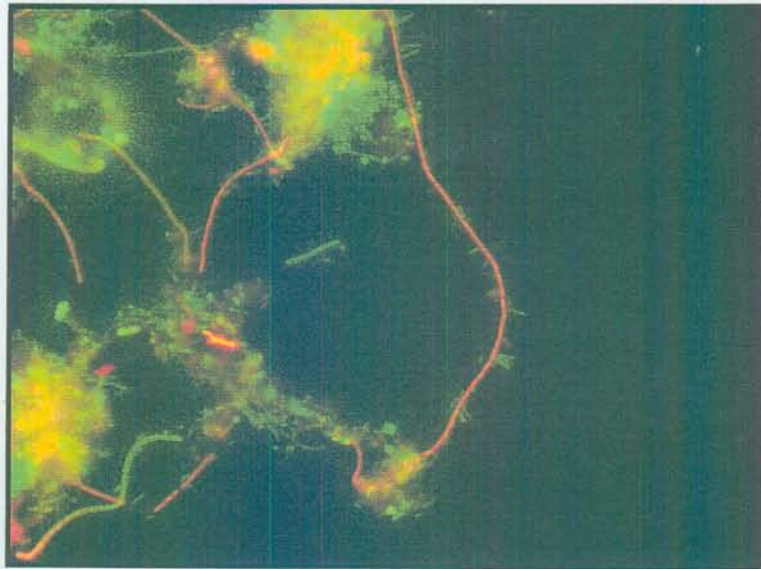
Fig. 1.

Figure 1. (Legend). Evolutionary distance dendrogram of candidate division TM7 and of the bacterial division-level groups based on comparative analysis of 16S rDNA data. Division and subdivision designations are bracketed to the right of the figure. Specificities of primers and probes designed to target the TM7 division are indicated in italics outside the brackets. Branch points supported (bootstrap values of >74 %) by all inference methods used are indicated by filled circles and those supported by most inference methods are indicated by open circles. Branch points without circles were not resolved (bootstrap values, <75 %) as specific groups in different analyses, and at the division-level were collapsed back to the next significant node. Clones sequenced in the present study are bolded and have the prefix NoosaAW. Archaeal outgroups (not shown) for the tree were *Methanococcus vanielii* (M36507) and *Sulfolobus acidocaldarius* (D14876). The bar represents 10 % estimated sequence divergence (Hugenholtz *et al.*, 2001).

A



B



C

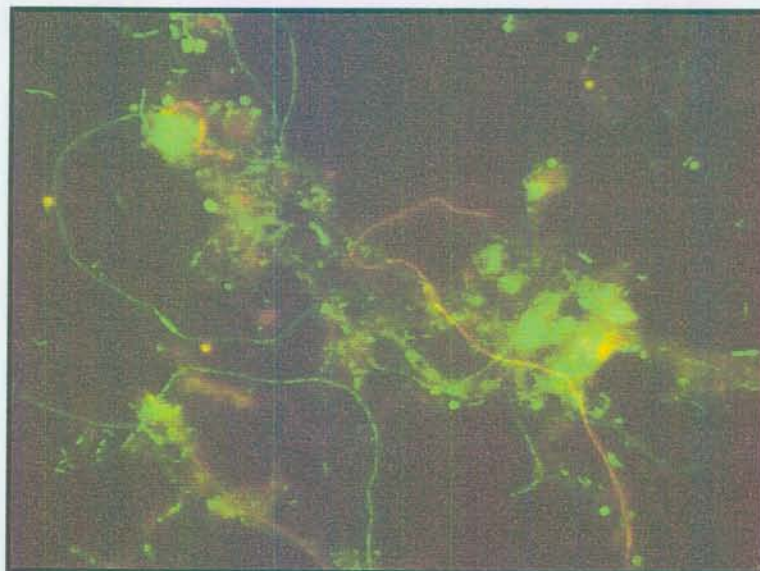
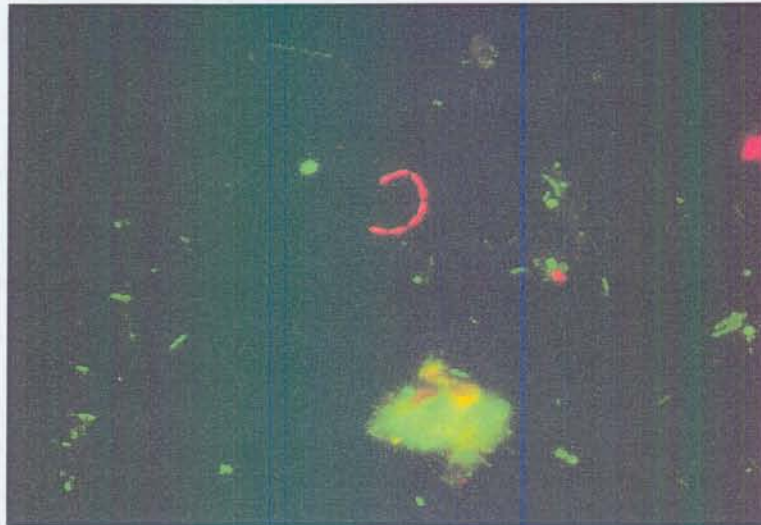
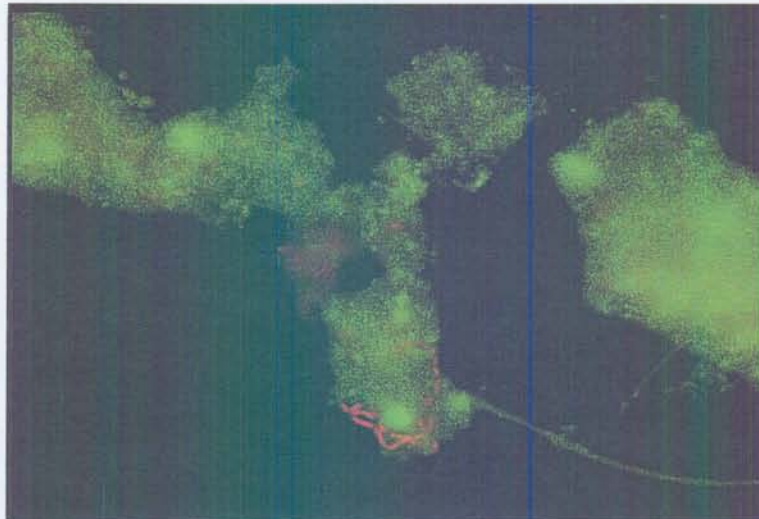


Fig. 2. Noosa WWTP sludge dual hybridised with FITC-labeled EUB338 and CY3-labeled Tm7305. The TM7 filament appear yellow-orange and the other bacteria green. A. FISH and phase contrast micrograph of the Tm7 filament.

A



B



C

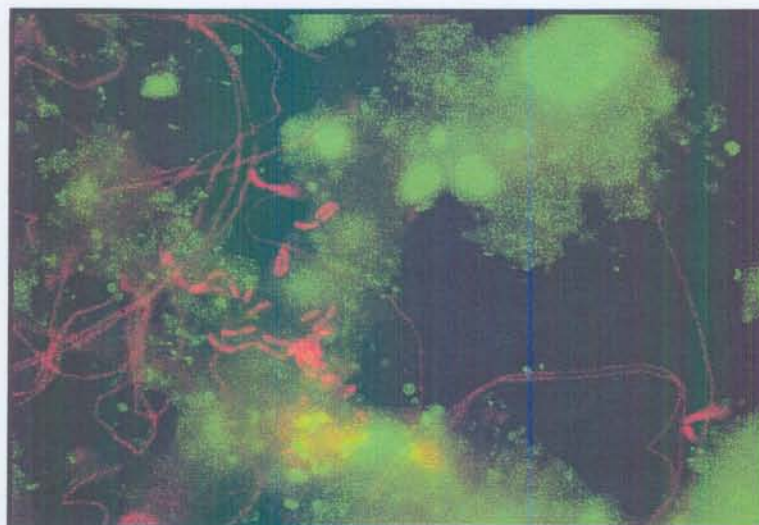


Fig. 3. Noosa WWTP sludge dual hybridised with FITC-labeled EUB338 and CY3-labeled Tm7 305. The sausage-shaped TM7 rod appear red and the other bacteria appear green.

Discussion

Based on sequence data, three subdivisions can presently be resolved in the TM7 division (Fig. 1). The sequences resulting from this study expanded the number of environmental clones associated with candidate division TM7.

Eikelboom Type 0041 is a Gram positive sheathed filamentous bacterium associated with foaming activated sludge (Seviour and Blackall, 1999). The filament that was predominantly observed in the foaming sludge from the Noosa WWTP resembled Eikelboom Type 0041.

When hybridised with the fluorescently labeled rRNA targeted probe TM7305, nearly all the filaments observed in the Noosa foam were targeted. This suggests that the filaments resembling Type 0041 were members of the TM7 division.

A sheathed filament occurring in chains as well as a sausage-shaped rod was also targeted by the TM7305 probe.

In a subsequent study (Hugenholtz *et al.*, 2001) another rRNA-targeted oligonucleotide probe (TM7905) was designed, targeting almost the entire division. When used in conjunction with TM7305, TM7905 confirmed that the filament morphotype belonged to the TM7 division. Probe TM7305 and TM7905 were also used during FISH analysis of two full scale WWTP sludges where it indicated the presence of a number of TM7 morphotypes (Hugenholtz *et al.*, 2001).

It was concluded that, since the probes target a broad phylogenetic group, the filament morphotype may comprise more than one species or genus of the TM7 division.

CHAPTER 6

CONCLUSIONS

- Microscopy indicated that the dominant filamentous microorganisms in the bulking sample was *Nocardia* spp. and in the foaming sample "*Microthrix parvicella*".
- Molecular identification techniques like DNA extraction followed by cloning and 16S rDNA sequencing performed on the foaming sample did not verify the microscopic observation that "*Microthrix parvicella*" was the dominant organism in the foaming sample. The reason for this could be that the primerset used was too narrow to target this organism.
- The dominant filamentous organism in the foaming sample from Australia resembled Eikelboom Type 0041. When the sample was hybridised with the fluorescently labelled rRNA-targeted oligonucleotide probe, nearly all the filaments were targeted. This suggests that the filaments resembling Type 0041 were members of the TM7 division of bacteria. This study indicated the usefulness of molecular techniques for the identification of filamentous organisms.
- DNA extracted from foaming activated sludge and subsequent cloning and sequencing indicated that not all the organisms grouped closely with 16S rDNA sequences of reference filamentous organisms known to cause activated sludge foaming. This indicated that no positive identification of filamentous organisms isolated from foaming activated sludge could be made using the molecular techniques, suggesting that the existing database could still be too limited in terms of the sequences available for the filamentous organisms. Hence the importance of further studies like this to expand the database on the filamentous microorganism sequences.

- The advances in molecular identification techniques is greatly expanding our knowledge of the diversity in natural communities, but this technique still needs to be investigated and refined further.
- The findings in this study highlight the fact that natural microbial communities need to be studied using a combination of techniques.

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APPENDIX

1. Staining preparations

a. Gram Stain (Jenkins *et al.*, 1986 modified)

Solution 1: Prepare the following separately, then combine:

	A		B
Crystal violet	2g	Ammonium oxalate	0.89g
Ethanol, 95%	20ml	Distilled water	80ml

Solution 2:

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Solution 3:

Safranin O	0.25g
Ethanol, 95%	10ml
Distilled water	100ml

Acetone ethanol solution

Acetone	30ml
Ethanol, 100%	70ml

Procedure

1. Prepare thin smears on microscope slides and thoroughly air dry (do not heat fix).
2. Stain 1min with solution 1; rinse with water.
3. Stain 1min with solution 2; rinse well with water.
4. Decolorize with the acetone ethanol solution for 30 sec.
5. Stain with solution 3 for 1min; rinse well with water and blot dry.

b. Sulfur oxidation test

Solution: Sodium sulfide solution ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) 1.0g/l (prepare weekly).

Procedure

1. On a microscope slide mix 1 drop of activated sludge sample and 1 drop sodium sulfide solution.
2. Allow to stand open to the air 10-20min.
3. Place a coverslip on the preparation and gently press to exclude excess solution; remove expelled solution with a tissue.
4. Observe at 1000x using phase contrast. A positive S test is the observation of highly refractive, yellow-colored intracellular granules (sulfur granules).

c. Polyhydroxybutyrate (PHB) stain

Solution 1: Sudan black B (IV), 0.3% w/v in 60% ethanol

Solution 2: Safranin O 0.5% w/v aqueous.

Procedure:

1. Prepare thin smears on a microscope slide and thoroughly air dry.
2. Stain 10min with solution 1; add more stain if the slide starts to dry out.
3. Rinse 1 sec with water.
4. Stain 10 sec with solution 2; rinse well with water; blot dry.
5. Examine under oil immersion at 1000x magnification with transmitted light: PHB granules will appear as intracellular, blue-black granules while cytoplasm will be pink or clear.

d. Crystal Violet sheath stain

Solution: Crystal Violet, 0.1 % w/v aqueous solution.

Procedure:

1. Mix one drop activated sludge sample and one drop Crystal Violet solution on a microscope slide, cover and examine at 1000x magnification phase contrast. Cell stain deep violet while the sheaths are clear to pink.

e. Methylene Blue Stain (Seviour and Blackall, 1999)

Solution: Methylene Blue, 1,6g; 95% ethanol, KOH0.01% (w/v)100µl aqueous. Prepare a saturated solution of Methylene Blue by adding the dye to the ethanol. Then add 30µl of the supernatant solution to the KOH.

Procedure:

1. Prepare a thin smear of the sample on a microscope slide and thoroughly air dry.
2. Stain 5 min with the solution and rinse.
3. Examine under oil immersion at 1000x magnification with transmitted light: polyphosphate granules will appear as intracellular, purple granules while cytoplasm will be blue.

2. Buffers and solutions for DNA and plasmid extraction

a. STE (pH 8.0)

10 mM NaCl	0.584 g
10 mM Tris/HCl	3.15 g
1 mM EDTA	2 ml
dH ₂ O	900 ml

b. Lysis buffer

50 mM Glucose	-	25 ml of 1 M Glucose
2 mM Tris HCl	-	12.5 ml of 1 M Tris.HCl
10 mM EDTA	-	10 ml of 0.5 M EDTA
		452.5 ml dH ₂ O to make up to 500 ml

Use 10 ml of the above and add 100 mg lysozyme (10 mg /ml) to this.

c. NaOH/SDS

0.2 ml of 0.2 M NaOH
0.5 ml of 20 % SDS
9.3 ml dH ₂ O

3. **In situ hybridisation preparation and application (Seviour and Blackall, 1999)**

a. **Paraformaldehyde for cell fixation**

Heat 65 ml of high purity water to 60°C. Add 4g paraformaldehyde. Add a drop of 2M NaOH solution and stir rapidly until the solution has nearly clarified (ca. 1-2min). Remove from the heat source and add 33ml of 3 X PBS. Adjust pH to 7.2 with HCL. Remove any remaining crystals by sterile filtration (0.2µm). Quickly cool to 4°C and store at this temperature.

b. **PBS**

130 mM NaCl,

10 mM sodium phosphate buffer, pH 7.2

c. **Requirements for *in situ* hybridisation**

- 50ml polypropylene screw top tubes – 1 slide per tube for hybridization.
- Tissues
- Hybridization buffer (final concentrations – 0.9 M NaCl, 0.01% SDS, 20 mM Tris/ HCl, pH 7.2)
- 2ml microcentrifuge tubes (sterile)
- *Formamide (aliquoted and frozen)
- Hybridization oven at 46°C
- Probes

*Formamide – frozen at -20°C in 2ml aliquotes – this will depend on the number of hybridizations that are done. This should have no colour. When thawed, restore at 4°C and use within a week. A range of formamide concentrations are needed for different probes. SDS is added last to the hybridisation buffer (in the lid of the tube, therefore added when lid closed and mixed) because it can interact with the concentrated NaCl and precipitate.



Table 1. Volumes of formamide and water to achieve different % of formamide solutions

Amount of formamide	%Formamide	μl of milliQ H_2O
0	0	1598
100	5	1498
200	10	1698
300	15	1298
400	20	1198
500	25	1098
600	30	998
700	35	898
800	40	798
900	45	698
1000	50	598

d. Wash Buffer

NaCl(5 M – autoclaved)	2150 μl (for 20% formamide)
Tris/HCl(1 M – autoclaved)	1ml
Milli-Q (autoclaved)	43.8 ml
SDS(10% not autoclaved – added last)	50 μl
Total volume = 50ml	

Method

NaCl was added to a new 50 ml tube, then the Tris/HCL, then the tube was filled to the 50ml mark with milliQ water. The buffer was mixed and then the SDS was added. The wash bufer was prewarmed at 48°C during the hybridization. Washing is done at 48°C.



b. LB agar (per liter)

NaCl 10 g

Tryptone 10 g

Yeast extract 5 g

Agar 20 g

Add deionised H₂O to a final volume of 1 liter

Adjust pH to 7.0 with 5 N NaOH

Autoclave

c. LB-Ampicillin plates (per plate)

Prepare 1 liter of LB agar

Autoclave

Cool to 55°C

Add 5 ml of 10mg/ml filter-sterilised ampicillin

Pour into petri dishes