THE ISOLATION AND CHARACTERISATION OF *CAULOBACTER* FROM MANAWATU WATER SYSTEMS

A THESIS PRESENTED IN PARTIAL FULFILMENT
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This Thesis is dedicated to
my family;

Patricia and Williamson Dunnington,
my husband Michael
and my daughter
Jamie Jessica.

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ABSTRACT

This study reports the isolation of 22 strains of *Caulobacter* from a variety of local water supplies. Most of the strains (17) were from the sewage treatment plant, while others were isolated from rivers (2), tap water (1) and stored water (2).

Conjugative plasmid transfer was demonstrated between a strain of *E. coli* and a sewage *Caulobacter* strain. Eckhardt gel analysis and antibiotic sensitivity tests confirmed that the transconjugant *Caulobacter* carried a plasmid conferring neomycin resistance when compared to the neomycin sensitive parent. *Caulobacter* isolated from sewage tended to carry more plasmids than freshwater *Caulobacter,* and showed an increase in resistance to many second generation antibiotics when compared to their freshwater counterparts.

Based on the sequence of a 260 bp fragment of 16S rDNA, the identities of the *Caulobacter* isolates were confirmed. A phylogenetic tree constructed from the sequence data showed that the *Caulobacter* isolates form a diverse group. Some of the isolates appear to be closely related to marine *Caulobacter* and were able to grow in media containing 2.5% salt. Other isolates appear to be closely related to *Pseudomonas diminuta.* A number of new *Caulobacter* strains were identifed on the basis of their 16S rDNA sequences.

The role of *Caulobacter* in the environment has not been well studied, partly due to the difficulties in detecting their presence. The use of the polymerase chain reaction to amplify the 16S rDNA sequence may help to overcome this problem, bearing in mind the diverse nature of the *Caulobacter* group.

TABLE OF CONTENTS

Table of Contents (cont.)

Table of Contents (cont.)

Table of Contents (cont.)

Table of Contents (cont.)

Table of Contents (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Plates **Page**

List of Plates (cont.)

INTRODUCTION

1. Discovery.

Caulobacter are stalked aquatic bacteria that are scavengers in nature. They were first discovered in 1935 after direct microscopic examination of glass slides that had been submerged in a lake for some time (Henrici and Johnson, 1935). Stalked bacteria were found adhered to the slides by virtue of an adhesive holdfast on the base of the stalk. It was not until the 1950's that *Caulobacter* were again noticed; this time in the water used to prepare electron microscope specimens. It was some time later in the 1960's that *Caulobacter* were actually isolated and maintained in pure culture (Poindexter, 1964).

2. Cell Structure.

Caulobacter are Gram negative polarly flagellate bacteria which physiologically resemble the aerobic chemoheterotrophic pseudomonads. (Poindexter, 1964) *Caulobacter* is unusual because cell division results in two different cell types, a stalked cell and a swarmer cell. The stalked cell is a mature cell which immediately starts replicating its chromosome in preparation for the next cell division. However, the motile swarmer cell is an immature cell which is incapable of DNA replication. In order to divide, it must differentiate by losing its flagellum and synthesising a stalk in its place. The resulting stalked cell then initiates DNA replication. *C. crescentus* provides an excellent model system for studies of the temporal control of gene expression (Ely *et al.*, 1990).

Caulobacter is one of the many genera (Gram negative and Gram positive) that elaborate a paracrystalline array surface (S) layer on their outermost surface.

S layers are nearly always composed of a single protein type. For most genera the function of these layers is unknown, but a protective barrier function is often presumed (Walker *et al.*, 1992). S layer proteins share a number of physical features including a low isoelectric point pH, absence of cysteine residues, and a high proportion of hydroxy-amino acids. In several studies it has been possible to assemble the protein in the absence of the cell surface from which it was derived (Koval and Murray, 1984). Given such similarities or capabilities, it has been suggested that some S layers were acquired by genetic exchange with other soil and aquatic bacteria and are retained because they offer a competitive advantage, analogous to antibiotic resistance or heavy metal detoxification (Walker *et al.*, 1992). Freshwater *Caulobacter* are common inhabitants of aquatic and soil environments. Most isolates have S layers that are hexagonally packed and indistinguishable from each other by gross analysis .

Typical strains (by laboratory analysis) have crescent shaped cells, and short stalks. Few rosettes are produced in culture but an elaborate hexagonal S layer is formed. (Walker *et al.*, 1992) Atypical strains have a variety of rod shapes; thin, straight, fat, short or long. They have larger rosettes, longer stalks and no visible S layer.

In natural environments, enrichment cultures, and pure cultures in diluted media (not more than 0.05% organic material) the length of the prosthecae or stalk exceeds the cell length by 5 - 40 times (Poindexter, 1981b). It is the ability to produce stalks coupled with the fact that *Caulobacter* can survive in oligotrophic environments that forms the basis of the methods for the isolation of *Caulobacter.* In richer media (at least 0.2% organic material) the stalk typically is much shorter.

Direct microscopic examination of environments with high organic content failed to detect *Caulobacter* and so it was assumed that they were not present. Also, sampling of water systems usually involves the use of saline solutions and freshwater *Caulobacter* do not grow in salinities greater than 50 to 100 mM.

3. Distribution and Ecology.

Stalked and budding bacteria are widespread in natural ecosystems; in fresh and sea water as well as soil. These groups of bacteria may represent up to one third of the total microbial biomass (Nikitin *et al.*, 1990). Because *Caulobacter* adhere to surfaces and are found in diverse locales, their role in oligotrophic environments and bacterial biofilm communities is of interest.

It has been generally assumed that *Caulobacter* are found only in environments of low organic content but they have been enriched and isolated from a variety of sewage treatment systems (MacRae and Smit, 1991). The sewage strains were relatively homogenous and could be reliably detected by gene probes derived from *C. crescentus,* a freshwater type. Most of the isolates from sewage contained one or more high molecular weight plasmids and were resistant to a number of antibiotics, characteristics not normally shared with *Caulobacter* isolated from other sources. *Caulobacter* could be detected from virtually every type of municipal waste water treatment plant from across the USA and Canada at all points in the process except for the strongly anaerobic regions of sludge digesters used by many facilities to reduce sludge volume and generate methane gas.

A recent development in waste water treatment is the 'biological' removal of phosphate from effluent. Phosphate is a key nutrient causing eutrophication of

water sources as a result of sewage discharge. The process involves the accumulation of phosphate into the bacterial population as polyphosphate (Yeoman, *et al.,* 1986). Whether *Caulobacter* are active participants in the phosphate accumulation process is being investigated (MacRae and Smit, 1991).

Strains isolated from sewage were morphologically similar to freshwater strains. The cell bodies were crescent shaped, produced few rosettes (fused holdfasts of multiple cells) and had hexagonally packed paracrystalline surfaces (see section on Cell Structure). These isolates had increased resistance to some antibiotics such as chloramphenicol, tetracycline, erythromycin, and tobomycin. Some of these antibiotics are in common clinical use, others are 'second generation' antibiotics. These resistances may be due to plasmid transfer between antibiotic resistant intestinal or human associated bacteria and *Caulobacter* in the waste water treatment systems. Freshwater *Caulobacter* generally had no plasmids but conjugation experiments between *E. coli* and freshwater *Caulobacter* isolates have demonstrated that antibiotic resistance transfer to *Caulobacter* is possible in the laboratory (Ely, 1979). Plasmid transfer between marine, freshwater *Caulobacters* and *E.coli* have also been accomplished (Ely, 1979; Anast and Smit, 1988).

Because of the ability of *Caulobacter* to survive in oligotrophic environments, the transfer of antibiotic plasmids from coliforms to *Caulobacter* could aid the persistence of these plasmids in the gene pool. The significance of these observations is that *Caulobacter* may serve as a reservoir of antibiotic resistance determinants which then persist in the environment and be transferred back to human associated bacteria. One consequence might be a reduced lifetime for antibiotics used in clinical medicine.

Some freshwater strains appear capable of survival in a marine environment. In areas where there is storm or sewer runoff into the sea, some marine *Caulobacter* isolates have features which are commonly associated with freshwater strains but are rare in marine strains (Anast and Smit, 1988).

One of the more diverse environments where *Caulobacter* have been found, apart from the gut of a millipede (Poindexter, 1964), was on unfertilised cod eggs where a long stalk was demonstrated (Hanseng and Olfasen, 1989). However, on fertilised eggs in hatching units the short stalks were more common. Reports indicate that stalked and budding bacteria were relatively abundant in intensive marine rearing units. The occurrence of *Caulobacter* on eggs dissected from the ovary indicated that eggs were colonised by bacteria before spawning but it is not known if this results from a pre-spawning invasion or represents an indigenous population in the Cod.

4. Oligotrophy.

An oligotrophic environment characteristically has a flux of nutrients at 0.1 mg of carbon/litre per day (Poindexter, 1981b). Most bacteria require a nutrient flux at least 50 fold higher than this. The fact that *Caulobacter* can survive in low nutrient environments is well established (Poindexter, 1981a). The cell can adhere to a solid surface by virtue of the adhesive material (holdfast) on the end of the stalk, allowing it to take full advantage of any nutrients which may pass by. This ability to survive in famine conditions forms the basis for the isolation of *Caulobacter* from the environment. In media containing low amounts of organic material (ie. 0.01% peptone water), the bulk of 'contaminating' bacteria fail to thrive, so *Caulobacter* eventually become the dominant population. Coupled to this, the stalk elongates in low phosphate

conditions which is in itself the main diagnostic feature for the detection and isolation of *Caulobacter.* It is known that in phosphate sufficient environments some *Caulobacter* strains do not produce the long stalks that are characteristic of the genus in phosphate limited situations, and so can be difficult to identify by light microscopy.

The concentration of at least one inorganic nutrient, phosphate, is inversely proportional to the length of the appendage (stalk), a relationship seen in other prosthecate bacteria (Poindexter, 1981b). Accordingly stalk elongation is regarded as a morphological response to nutrient limitation and can be interpreted as a means of increasing the surface:volume ratio of the cell in dilute environments. A stalked cell whose appendage is ten times the cell length has a surface:volume ratio that is twice that of the cell alone. Even more important with respect to increasing the ratio of potential uptake sites to metabolically active cytoplasm, the *Caulobacter* appendages are composed almost entirely of membranes, which are generally inactive as sites of energy consuming biosynthesis and lack complete catabolic systems (Poindexter, 1981b). The cross walls peculiar to *Caulobacter* prosthecae may serve to restrict the entry of the cytoplasm into the stalk so that its contribution as an uptake organelle is not reduced by substrate consuming reactions.

Caulobacter are able to accumulate poly-β-hydroxybutyrate (PHB) and polyphosphate and can sometimes grow in anaerobic conditions. Under conditions of nitrogen or phosphate limitation, 26% of the dry cell weight can be attributed to PHB (Poindexter, 1981b). Cells provided with glucose but without a nitrogen source increased in dry weight by 21% in 12 hrs with 90% of the increase being accounted for by the synthesis of PHB and of poly-glucose (Poindexter, 1981b). Earlier cytological studies revealed that under conditions

of nitrogen starvation in a sugar phosphate medium, the cells also accumulated polyphosphate reserve granules (Poindexter, 1981b). It is concluded that *Caulobacter* has the capacity to form all three principal types of reserve polymers simultaneously and are able to survive during periods of nutrient exhaustion.

5. Taxonomy.

In the case of *Caulobacter*, what morphologically appears to be a *Caulobacter* will generally be called one without challenge. This is mainly due to a lack of other defining physiological or metabolic traits (Stahl *et al.*, 1992). The *Caulobacter* group has been well studied and in the past the taxonomy of this group has been based on morphological criteria and required growth factors (Poindexter, 1989). See figure 1.

16S rRNA analysis has shown members of *Caulobacter* to be members of the alpha subdivision of Proteobacteria (figure 2, Stackebrandt *et al.*, 1988). This group includes non-phototrophic and non-budding organisms (Albrecht *et al.*,1987). The budding and/or prosthecate non-phototrophic bacteria include the genera: *Hyphomicrobium, Hyphomonas, Pedomicrobium, Filomicrobium, Stella* and *Caulobacter*. Three large groups can be distinguished among this group: caulobacter-like, hyphomonas-like and hyphomicrobium-like bacteria (Nikitin *et al.*, 1990). Relatively little information is available concerning the genetic diversity of prosthecate bacteria. Early DNA hybridisation (Moore *et al.*,1978) and more recent 5S and 16S rDNA sequence comparisons (Lee and Fuhrman, 1980; Nikitin *et al.*, 1990; and Stackebrandt *et al.*,1988) suggest that there is considerable diversity among this group.

Figure 1. CLASSIFICATION OF *CAULOBACTER.*

Caulobacter Classification

I. Cells tapered

A. Long axis of cells curved

1. Organic growth factors required

a. Vit B_{12} necessary, but not sufficient.

C. vibrioides

(nearly ovoid cells)

b. Vit B_{12} necessary and sufficient

C. henricii

c. Biotin necessary, but not sufficient

C. intermedius

(vibrioid, short cells; colourless colonies)

d. Growth not stimulated by B vitamins

C. subvibrioides

(Straight to curved cells; orange or colourless colonies)

2. Organic growth factors not required

C. crescentus

(colourless colonies; not inhibited by penicillin G 1000units/ml)

B. Long axis of cell not curved

1. Organic growth factors required

C. fusiformis

(long straight cells; bright yellow colonies)

2. Organic growth factors not required

C. ledidyi

(short cells, short stalks; not inhibited by Streptomycin 0.1 mg/ml or Penicillin G 1000 units/ml)

II. Cells not tapered

A. NaCl not required for growth

C. bacteroides

B. NaCl required

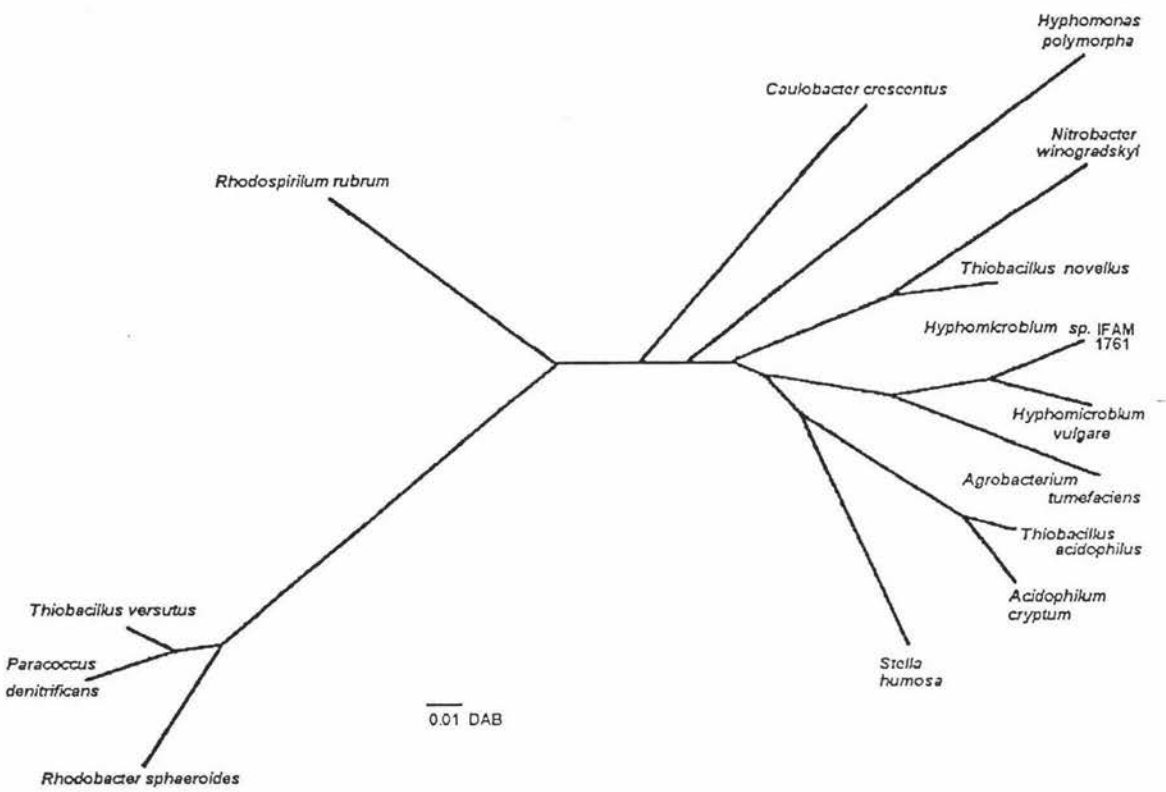
C. halobacteroides

C. maris

Figure 2. **UNROOTED 5S rRNA TREE OF MEMBERS OF THE ALPHA SUBDIVISION OF PROTEOBACTERIA.**

(Stackebrandt *et al.*, 1988)

This tree is derived from D_{AB} values.


Hyphomonas polymorpha
Caulobacter crescentus
Nitrobacter winogradskyi
Rhodospirilum rubrum
Thiobacillus novellus
Hyphomicrobium sp. IFAM 1761
Hyphomicrobium vulgare
Agrobacterium tumefaciens
Thiobacillus acidophilus
Acidophilum cryptum
Thiobacillus versutus
Paracoccus denitrificans
Stella humosa
0.01 DAB
Rhodobacter sphaeroides

16S rDNA analysis by comparative sequencing of 'typical' *Caulobacter* strains found them to be a relatively closely related subgroup of freshwater isolates while atypical strains were different from the typical cluster and from each other (Stahl *et al.,* 1992). Typical *Caulobacter* were still measurably dissimilar exhibiting rRNA similarity values of about 99% (DNA similarities of 50% generally correspond to rRNA similarity values of 98 to 99%, Stahl *et al.,* 1992). The most distantly related of the *Caulobacter* characterised were associated at approximately 88% 16S rDNA sequence similarity. Notably affiliation with either one of the two phylogenetically distinct lines of descent (88 to 90% similarity) generally corresponded to a marine or a freshwater habitat. One line of descent was composed exclusively of marine *Caulobacter.* The other line of descent included the freshwater *Caulobacter* and some marine isolates. Most *Caulobacter* isolated from waste water treatment systems belonged with the terrestrial or freshwater lineage (Stahl *et al.,* 1992*).* An apparent exception to this pattern was of *C. subvibrioides* which morphologically would be included in the genus *Caulobacter* but is phylogenetically distinct from both the terrestrial and the marine types (Stahl *et al.,* 1992).

The cloned paracrystalline surface (S) layer gene of *C. crescentus* CB15A hybridised to specific regions of the genome for most of the *Caulobacter* analysed under moderate stringency conditions (Walker *et al.,* 1992). Restriction fragment length polymorphism analysis with the S layer gene as the probe, failed to reveal patterns of close relatedness between the strains. This indicates a greater genetic diversity than is suggested by morphological similarities. This correlates with 16S rDNA comparative analysis that showed that these *Caulobacter* were a coherent group but still sufficiently different to have significant variation in their overall genomic DNA composition.

When a flagella filament protein gene was used to probe a group of non-*Caulobacter* isolates from waste water treatment systems, one strain in 150 isolates hybridized with the probe DNA (MacRae and Smit, 1991). This isolate was examined by the Biolog commercial identification scheme (which does not include *Caulobacter*) and a match to *Pseudomonas vesicularis* was obtained (Stahl *et at.,* 1992). This species is similar to *P. diminuta* on the basis of RNA homology and these two species form a highly distinctive branch of pseudomonads (Gilardi, 1985). Also, one of the freshwater *Caulobacter* when examined by the Biolog system, scored an acceptable match to *P. diminuta.* It is conceivable that these species are *Caulobacter* strains locked in the motile phase. By classical definition, a bacterium which does not posses a stalk, cannot be called a *Caulobacter.* A stalk-less *Caulobacter* might be identified as a pseudomonad since they are physiologically similar. A comparison of rDNA gene sequences is needed to confirm the relationship between *Caulobacter* and *Pseudomonas diminuta.*

6. Aims of this Investigation.

1. The enrichment of New Zealand *Caulobacter* strains from a sewage treatment plant and freshwater sources.

2. The isolation and identification of *Caulobacter* from the enrichments.

3. The comparison and characterisation of isolates by their morphology and physiological capabilities (Vit B_{12} requirement and tolerance to salt).

4. The characterisation of *Caulobacter* isolates by plasmid content and sensitivities to certain antibiotics.

5. To extract the DNA from all isolates and analyse restriction endonuclease total genomic digest patterns.

6. Determination of the taxonomic relationships between NZ isolates, recognised type strains and published data by comparative analysis of the 16S rDNA sequences using the neighbor-joining method.

MATERIALS AND METHODS

1. MICROBIOLOGICAL METHODS.

1.1 Strains Used.

The bacterial strains used in this study are listed in table 1.

1.2 Media Used.

1.2.1 Peptone Yeast Extract (PYE) (Poindexter, 1964) contained (g/l): Peptone (Difco), 2.0; Yeast Extract (Difco), 1.0; $MgSO_4.7H_2O$, 0.2; Riboflavin, 0.001(optional); in distilled water. The pH was adjusted to 7.0 followed by autoclaving. PYE agar (PYEA) was obtained by adding 15 g/l agar (Davis).

1.2.2 Low-Phosphate PYEA.

Inorganic phosphates were precipitated by a chemical method, or by raising the pH of the liquid media to 8.0, and removing the precipitate by filtration.

CHEMICAL PRECIPITATION of inorganic phosphates (Volkin *et al,* 1957):
2 x PYE medium, 100 ml; Solution A, 4 ml; NH_4OH (conc.), 2.5 ml.
Solution A: 0.5 M $MgCl_2$ (10.15 g/100 ml); 0.5 M NH_4Cl, (2.67 g/100 ml). The correct strength was obtained by making the volume up to 200 ml with a non-phosphate buffer. 15 g/l of Davis agar was added and then autoclaved.

Concentration of Inorganic Phosphate in PYEA Media.*

Untreated PYEA	58 mg/l
pH precipitation	22 mg/l
Chemical precipitation	7 mg/l

(* Analysed by The Department of Chemistry, Massey University)

1.2.3 Peptone Water.

For enrichment purposes, a 0.01% solution of Difco peptone in distilled water was used. For solid media, Agar (Davis), 15.0 g/l was added. For isolation purposes, a 0.05% solution can be used in solid media. Autoclave to sterilise.

1.2.4 Sarcosine Solution.

A 0.1% solution of sodium-n-laurylsarcosine was made up with Milli-Q water and autoclaved to sterilise.

1.2.5 Peptone supplemented with $CaCl_2$ (PCa) Medium (Poindexter, 1989) contains g/l: Peptone (Difco), 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_20$, 0.15; Agar (Davis), 15; in distilled water. Autoclave to sterilise.

1.2.6 Violet Red Bile Agar (VRBA) (Richardson, 1985) contains (g/l):
Yeast extract (Oxoid), 3.0; Peptone (Oxoid), 7.0; Bile salts No. 3 (Oxoid), 1.5; Lactose (Analar BDH), 10.0; NaCl (Analar BDH), 5.0; Neutral red, 0.03; Crystal violet, 0.002; Agar (Davis), 15.0; in distilled water. After adjusting the pH to 7.4, the solution was boiled for no longer than 2 minutes then dispensed into 15 ml sterile test tubes. The tubes of molten agar were held at 45 - 48 ^{o}C until they were poured into sterile petri plates.

1.2.7 Luria Broth (LB) (Miller,1972), contains (g/l):

Tryptone (Difco), 10.0; Yeast extract (Difco), 5.0; NaCl, 0.5; in distilled water. Adjust the pH to 7.0. Sterilise by autoclaving. Davis Agar can be added at 15 g/l to make solid.

1.3 Cultivation and Storage.

Typical conditions for the aerobic incubation of a purified isolate were 30°C for 16 hrs. Liquid cultures were gently agitated (x 100 rpm). For long term storage, the isolates were frozen at -70°C in 20% glycerol.

1.4 Environmental Samples Examined.

The sample of sewage was taken from the Palmerston North City Council Sewage Treatment Plant. Approximately 500ml was taken from an aerobic area of secondary treatment in a sterile bottle. A sample of approximately 500ml was collected from the Manawatu River, underneath the Fitzherbert Bridge, after rain (the river was brown and silty), using a sterile bottle. The Tiritea Stream sample was taken near the Massey University Ring Road. Approximately 500ml was collected using a sterile bottle, and the water was clear. Domestic supply tap water had been stored in a plastic 1.25 litre bottle, in a dark cupboard, for over a year before samples were taken for enrichment. A sample of rain water was taken from a farm water tank on Old West Road, Palmerston North. The water had been stored in the tank for approximately six months before being used for enrichment. The Taranaki Blood Bank water sample was taken from their routine water supply by blood bank staff in an unknown manner, and posted to the university.

Table 1: Bacterial Strains Used In This Investigation

Bacterial Strains	Source
Escherichia coli B	MU 113
Escherichia coli w	MU 109
Escherichia coli PN200	Scott and Ronson, 1982
Caulobacter crescentus	ATCC 15252
***Caulobacter* Isolates**	
CDF series (16 isolates)	Palmerston North City Sewage Treatment Plant
MCDF23	Rifampicin resistant derivative of CDF 23
MCDF100	MCDF23 X PN200
MR1	Manawatu River
TS1	Tiritea Stream, Manawatu
TW1, TW2	Storage Tank, Old West Road, Palmerston North
SW1	Domestic water supply, Palmerston North

ATCC - American Type Culture Collection

MU - Massey University Culture Collection

1.5 Coliform Count.

Upon receipt, all samples except for the Taranaki Blood Bank sample were tested for coliforms using an overlay technique and violet red bile agar (VRBA), (section 1.2.6). Undiluted, 10^{-1} and 10^{-2} dilutions were plated in triplicate.

1.6 Enrichment Procedures.

The following procedure is a version of the method outlined by Poindexter (1964) and MacRae and Smit (1991). It was used to enrich both the freshwater and the sewage samples. As a control, an un-inoculated sterile petri dish containing 0.01 % peptone water (section 1.2.3) was incubated with the enrichments.

1.6.1 Surface Film Method.

An environmental sample (0.1 ml) was inoculated into 20 mls of 0.01% peptone water (section 1.2.3). The enrichment cultures were set up in a sterile container which had a large surface-to-air interface, such as a petri dish. They were incubated undisturbed at room temperature (20-25^{o}C) until microscopic examination (section 2) of the liquid\air interface showed the presence of stalked cells. An outline of this procedure is shown in figure 3.

1.6.2 Attachment Method.

The enrichment culture was set up as for the above method, but a sterile glass microscope slide was submerged below the surface. Any sterile object that *Caulobacter* cells might adhere to could be used. Cottonwool was also tried.

1.7 Isolation Procedures.

The following is a modified version of the isolation method outlined by Poindexter (1964) and MacRae and Smit (1991). Sarcosine is used to aid the seperation of attached cells. This method can be used to isolate *Caulobacter* cells from both freshwater and sewage sources (Figure 3).

1.7.1 Surface Film Method.

Small surface samples (approximately 10 µl) of the enrichment were removed and diluted in 0.5 ml of a 0.1% sarcosine solution (section 1.2.4). The diluted sample was vortexed in an attempt to mechanically seperate adhering cells. A loopful is then streaked on peptone yeast extract agar (PYEA, section 1.2.1), and incubated for 3 days at 30°C. Fast growing colonies were ignored and the plates were examined with a binocular microscope to detect pin-point size colonies. These colonies were transferred by toothpick to a sterile PYEA plate and incubated at room temperature. After approximately 7 days, the toothpick colonies were examined microscopically to see if they contained stalked cells. Positive colonies were resuspended in 0.5 mls of 0.1% sarcosine solution, vortexed and re-streaked on PYEA.

1.7.2 Physical Isolation Methods (Schmid, 1981).

If the enrichment culture did not contain many stalked cells in its surface film, the following procedures were more likely to be successful at isolating *Caulobacter* sp. especially if the cells have long stalks. They take advantage of the fact that a long stalked *Caulobacter* is longer (filtration method) and more buoyant (centrifugation method) than other bacteria.

(1) Filtration.

A sample of the surface film (10 ml) was filtered through a Swinnex filter holder (millipore, 25 mm diameter) with a sterile membrane filter with a pore size of 10 µm. The filtrate was examined microscopically (section2) and if *Caulobacter* cells were still present, it was re-filtered through a 5 µm filter. The filters were removed and placed on solid media (peptone-water, section 1.2) to incubate at 30°C. The filtrates were also streaked out onto solid media. After incubation, the plates are then screened as in the isolation procedure (section 1.7).

(2) Centrifugation.

The surface film (5 ml) of an enrichment culture was centrifuged for 10 minutes at between 1500 and 3500 x g. The pellet and the supernatant were streaked on solid media, and then incubated and screened as described in the isolation procedure (section 1.7). Generally, the *Caulobacter* with long stalks were buoyant so were in the supernatant, or in the floculant layer above the pellet.

1.8 Purification.

Once a stalked cell isolate had been repeatedly streaked, a single colony was innoculated in PYE broth (section 1.2.1), incubated for 16 hrs at 30°C with gentle agitation, and spread (0.1 ml) on solid media. After incubation, the *Caulobacter* isolate usually appeared as a lawn, and contaminants that were adhered to the *Caulobacter* produced obvious colonies. Microscopic examination was used to confirm the presence of *Caulobacter*-like cells. Finally, the lawn was re-streaked on solid media.

2 MICROSCOPIC EXAMINATION.

2.1 Transmitted Light Microscopy.

To facilitate the detection of stalks, cells can be stained with crystal violet and viewed under an oil immersion lens at x1000 magnification. If the stalks are difficult to see, Gray's flagella stain (McGraw-Hill, 1957) or another mordant based stain such as Kirkpatrick's flagella stain can be used.

2.1.1 Materials

(1) Gray's mordant for flagella staining contains:

Tannic acid (20% w/v aq.), 2.0 ml; $KAl(SO_4)_2 \cdot 12H_20$, 5.0 ml; Mercuric chloride (sat. aq.), 2.0 ml; Basic fuschin (3% w/v in 95% w/v ethanol), 0.4 ml. Combine, then filter.

(2) Kirkpatrick's mordant for flagella staining contains:

Ferric chloride (5%), 40 ml; Tannic acid (20%), 120 ml. The tannic acid solution required 2 days at 37°C to dissolve. After dissolving, the two ingredients were mixed and stored in dark, screw top bottles. Before using, the mixture was diluted with an equal volume of deionised water.

(3) Carbol Fuschin contains:

Basic fuschin, 1g; Absolute alcohol, 10 ml; Phenol (5% aqueous), 100 ml. Basic fuschin was dissolved in alcohol and then the phenol was added. The mixture was shaken to mix thoroughly.

(4) Crystal Violet contains:

Solution A:crystal violet 10g; ethanol (95%) 100ml. *Solution B*:1% aqueous solution of ammonium oxalate. 20ml of solution A was mixed with 80ml of solution B.

Figure 3. PROCEDURE FOR THE ENRICHMENT AND ISOLATION OF *CAULOBACTER.*

Enrichment and Isolation Procedure

Enrichment

Environmental sample
diluted into 0.01% peptone water

Incubate undisturbed
room temperature

Examine liquid/air interface

Once stalked cells evident
remove small surface samples

Isolation

vortex in 0.1% sarcosine

streak onto PYEA

Incubate for 3 days, at 30°C

pin-point colonies transferred
toothpick to new plate

Incubate for 7 days at 30°C

Vortex in 0.1% sarcosine

Restreak PYEA

2.1.2 Method

The enrichment surface (10 µl) was removed and added to an equal volume of 10% formalin on a slide. After mixing briefly, the slide was tipped to allow the drop to flow to the other end, then air dried and heat fixed. Mordant (Kirkpatrick's or Gray's) stain was applied for 5 minutes and washed off. Blotting paper was placed over the specimen and soaked with carbol fuschin for 3 minutes. Once the paper was removed, the slide was washed and blotted dry.

2.2 Phase Contrast Microscopy.

Without moving the enrichment, a coverslip was touched to the surface using forceps. This was placed wet side down onto a clean, flamed and cooled slide. The excess water was absorbed with blotting paper. Stalked cells could be detected by their swaying motion and the prostheca viewed by focusing up and down past the cell body.

2.3 Electron Microscopy.

Colonies were taken directly from a PYEA plate (section 1.2.1). The cells were mixed with 1% phosphotunsic acid (pH 7.0), and a drop was spread onto a copper grid which had a thin support film of formvar. The drop was smeared into a thin film and then examined by a transmission electron microscope (Philips 201). The background around the cell was stained (negative staining).

3 PHYSIOLOGICAL EXAMINATION.

3.1 Salt Tolerance.

Single colonies were inoculated into PYE broths (section 1.2.1) amended with 1% NaCl or 2.5% NaCl, and incubated at 30°C with gentle agitation. Broths were examined at 4 and 6 days.

3.2 Riboflavin Requirement.

Single colonies were inoculated into PYE broths (section 1.2.1) with and without riboflavin (Vitamin B_{12}) and incubated at 30°C. Broths were examined at 24 hours and 4 days.

3.3 Antibiotic Resistance.

Stock solutions (5 mg/ml) of the antibiotics were prepared up and filter sterilised. Each antibiotic (10 µl) was aseptically added to sterile blank discs (Difco). Single colonies were inoculated in PYE broth (section 1.2.1) and incubated at 30°C for 16 hours. These cultures (0.1 ml) were spread plated on a PYE agar and antibiotic discs were placed on the lawn surface. Zones of inhibition were measured after 2 days of incubation.

4 PLASMID ANALYSIS.

4.1 Eckhardt Procedure (Eckhardt, 1978).

The analysis of the number of plasmids that the isolates contained was carried out by a modified Eckhardt procedure .

4.1.1 Materials

All solutions are prepared from analytical grade reagents in deionised (Milli-Q) water.

(1) TE Sarcosyl Buffer contains (g/l):

Tris(hydroxymethyl)methylamine (10 mM), pH 8.0, 1.21; Ethylenediaminetra-acetic acid:disodium salt (EDTA,1 mM), 0.372g; N-Laurylsarcosine, 1.0. The pH was adjusted to 8.0 and the buffer stored at 4°C until required.

(2) Tris Borate Electrophoresis Buffer (TBE) x 10 contains (g/l):

Tris(hydroxymethyl)methylamine base (MW 121.1), 108; Ethylenediaminetra-acetic acid:disodium salt (EDTA), 9.3; Boric acid, 55. After diluting to 1 x TBE with milli-Q water, the pH was adjusted to 8.2 with dilute HCl. The buffer was stored at room temperature.

(3) Lysozyme Solution contains:

Lysozyme, 4 mg; TBE x1, 2 ml. The lysozyme was dispensed into 0.1 ml aliquots and stored at -20°C until required.

(4) RNAse contains:

RNAse, 20 mg; Sodium acetate (0.4 M, pH 4), 2 ml. After boiling in a water bath for 2 minutes, the RNAse was stored at -20°C until required.

(5) Solution 1 contains:

Ficoll 400,000 (10%), 2.0 g; Bromophenol blue (0.05%), 0.01 g; Lysozyme powder, 0.004 g; RNAse (0.01%), 0.02 ml;TBE x 1 (pH 8.2), 20 ml. The solution was stored at 4°C until required.

(6) Solution 2 contains:

SDS (0.2%), 0.02 g; Ficoll 400,000 (10%), 1 g;Proteinase K, 0.04 mg; TBE x 1 (pH 8.2), 10 ml. The solution was dispensed into 0.5 ml aliquots and stored at -20°C until required.

(7) Solution 3 contains:

SDS (0.2%), 0.04 g; Ficoll 400,000 (5%), 1.0 g; TBE x 1 (pH 8.2), 20 ml. The solution was stored at 4°C until required.

(8) Agarose gel: 0.8% w/v of agarose gel (Sea Kem or Sea Plaque Agarose FMC) in TBE x 1 buffer.

(9) Ethidium Bromide: Stock solution of 10 mg/ml.

(10) Destaining Solution: 1M Magnesium sulphate.

4.1.2 Method.

A single colony was inoculated into 5 ml of PYE broth (section 1.2.1) and incubated at 30°C with shaking (200 rpm) until the cells were in a logarithmic growth (OD_{600} = 0.4) which was usually after 18 to 24 hours. Aliquots of the broths (0.1 ml) were dispensed into Eppendorf tubes and cells harvested by centrifugation (15600 x g for 5 minutes in a 5414 S Eppendorf centrifuge) and washed in TE sarcosyl. The pellet was resuspended into 1 ml of TE buffer and cells harvested by centrifugation. Lysozyme (50 µl) was added to 500 µl of solution 1 and mixed well. The pellet was resuspended in 20 µl of this solution and immediately loaded into a well of an agarose gel (0.8% w/v in TBE x 1 buffer) in a Horizon gel electrophoresis system (BRL Horizon 11.14). After incubating for 15 minutes at room temperature, 20 µl of solution 2 was added to the well, and gently mixed with a fine blunt glass rod by stirring once. After being left for 15 minutes, the wells were overlaid with 20 µl of solution 3. The gel was then flooded with cold 1 x TBE buffer and run at 4°C for 1 hour at 20 volts and then for 5 hours at 80 volts. When examining for the presence of pPN1 the gel was run at 20 volts for 1 hour and 80 volts for 16 hours. The gel was stained for 30 minutes in ethidium bromide solution (0.5 µg/ml) and destained in 1M magnesium sulphate for 10 to 15 minutes. The gel was viewed on a UV transilluminator (Chromato-Vue^{R}, Watson Victor, model TS-

15) and photographed (aperture, f11; shutter speed, 1 minute) with a MP-4 land camera using polaroid 667 or 665 or Kodak Tri-X pan professional film through a Wratten 23A (red) filter.

4.2 Plasmid Transfer by Conjugation.

The membrane filter method (Buchanan-Wollaston *et al,* 1980) and the stationary broth method was used for *in vitro* conjugations between isolates and known strains to detect the transfer of plasmids.

4.2.1 Growth of Bacteria.

The *Caulobacter* isolates were grown on PYE broth (section 1.2.1), for 16 hours at 30°C. The *E. coli* strains were grown in Luria broth (1.2.7) at 37°C for 16 hours. The overnight cultures were diluted approximately to 10^8 CFU/ml based on the optical density at 600 nm (OD_{600}).

The strains used are listed on table 2.

4.2.2 Selective Media

When the recipient was an *E. coli,* Luria agar with added antibiotics was used as selective medium. When the recipient was a *Caulobacter* isolate, PYEA medium (section 1.2.1) with added antibiotics was used as a selective medium (table 3). *Caulobacter* strains can not grow on Luria agar.

Stock solutions of the antibiotics used were made up and added aseptically to the medium (Luria or PYEA) and then poured to make plates. The antibiotic concentrations used were: ampicillin 50 µg/ml, tetracycline 50 µg/ml, rifampicin 50 µg/ml and streptomycin 30 µg/ml (Table 3).

4.2.3 Membrane Filter Method

A sterile cellulose nitrate filter (0.2 μm pore size) was aseptically placed onto the surface of a PYEA (section 1.2.1) plate. Donor cells (50 μl) were added and left to absorb into the filter for 2 minutes, and then the recipient cells (25 μl) were added. The plate was incubated inverted overnight at 30°C. The membrane filter was removed from the agar plate and shaken in 2 ml of PYE media to release the bacteria. To select for transconjugants, duplicate 0.1 ml samples were spread on selective media and incubated for 4 days at 30°C.

4.2.4 Stationary Broth Method

Donor cells (0.1 ml) and recipient cells (0.1 ml) were added to 2.5 ml of PYE broth. After briefly shaking to mix the contents, it was incubated overnight without shaking at 30°C. To select for transconjugants, duplicate 0.1 ml samples were spread on selective media and incubated for 4 days at 30°C.

Table 2: Conjugative Plasmid Transfer

List of Strains

Parent strains	Antibiotic Resistance	Source
Caulobacter sp.		
CDF 23	Str^{30}	Sewage
CDF 9	Amp^{50} Tet^{50}	Sewage
CDF 20a	Amp^{50}	Sewage
CDF 46b	$Amp^{50}Tet^{50}$	Sewage
MCDF23	Rif^{50}	Sewage*
MR 1	Amp^{50}	Freshwater
C. crescentus	Amp^{R}	ATCC 15252
E.coli B	Amp^{S} Tet^{S} Str^{S}	MU113
E.coli w	Amp^{S} Tet^{S} Str^{S}	MU109
E. coli PN200 (pPN1)	Neo^{50}	Scott and Ronson, 1982

* Spontaneous rifampicin resistant derivative of CDF 23

Str - streptomycin 30 μg/ml; Amp - ampicillin 50 μg/ml;

Tet - tetracycline 50 μg/ml; Neo - neomycin 50 μg/ml;

Rif - rifampicin 50 μg/ml.

Table 3: Conjugation Crosses

Donor Strains	Recipients	Selective Media
Sewage Isolate to *E.coli*		
CDF 23	*E. coli* B	LB Str^{30}
CDF 9	*E. coli* w	LB Tet^{50}/ Amp^{50}
CDF 20a		LB Amp^{50}
CDF 46b		LB Tet^{50}/ Amp^{50}
Freshwater Isolate to *E.coli*		
MR 1	*E. coli* B	LB Amp^{50}
	E. coli w	
***E. coli* to Sewage Isolate**		
E. coli PN200	MCDF 23	PYEA Neo^{50} Rif^{50}
Freshwater Isolate to sewage isolate		
C. crescentus (ATCC 15252)	CDF 23	PYEA Amp^{50}/Str^{30}
MR 1		
Sewage isolate to sewage isolate		
CDF 23	CDF 9	PYEA Amp^{50}/Str^{30}
Sewage isolate to freshwater isolate		
CDF 23	MR 1	PYEA Str^{30}/Amp^{50}

Note: Antibiotic concentrations in µg/ml.

All donor strains were crossed with all the recipient strains included in each category.

5 DNA METHODS.

5.1 DNA Extraction (Jarvis *et al.*, 1992).

5.1.1 Materials

(1) Tris Electrophoresis (TE) Buffer contains: Trizma base, 10 mM; Ethylenediaminetra-acetic acid:disodium salt (EDTA), 1 mM. The pH was adjusted to 8.0 by adding conc. HCl.

(2) TEL Buffer contains: Tris base, 50 mM; EDTA , 20 mM.

(3) Protease contains: Protease type XIV, 5 mg/ml in TEL buffer. This was prepared fresh and incubated for an hour at 37^{o}C.

(4) 10% SDS contains: Sodium-dodecyl-sulphate, 1g; TEL buffer, 10ml.

(5) Phenol contains: Phenol, 1 kg; Deionised water, 110 ml; m-Cresol, 140 ml; 8-hydroxyquinoline, 1 g. This was mixed overnight on a magnetic stirrer and saturated with 0.1 M Tris pH 8 (stored in a dark bottle).

(6) Sodium Acetate, 3M, pH 4.85.

(7) Diethyl ether.

5.1.2 Method

Bacterial cells grown on PYEA slopes were washed in 3 ml of TEL buffer and collected in a sterile Falcon tube. Protease (375 µl) and 10% SDS (375 µl) were added to the suspension and was left for 30 minutes on ice, and then 30 minutes at room temperature.

Phenol was added (10 to 15 ml), and the tube capped tightly and inverted 25 times. The lysate/phenol solution was centrifuged at 4500 x g to separate the two phases. The aqueous phase (top) was transferred to a clean, sterile glass bottle, washed twice with 3 ml of diethyl ether and then allowed to stand. The

aqueous phase (lower) was removed and 0.2 ml of sodium acetate was added and gently mixed. DNA was precipitated by adding cold (4°C) 100% ethanol and collected on a hooked glass rod. The DNA threads were washed in 70% ethanol, air dried, dissolved in 0.2 ml of TE buffer and stored at -70°C, or for short term storage, 4°C.

5.1.3 Determination of DNA Purity and Concentration

The purity of the DNA was determined by measuring the optical density at 230 nm, 258 nm and 280 nm using a PYE-Unicam SP1800 UV spectrophotometer. The following spectral ratio values indicated that the purity of the sample was satisfactory: absorbance (A) at A_{258}/ A_{230} = between 1.8 and 2.3, (no significant protein contamination); A_{258}/ A_{230} = between 1.8 and 2.0 (no significant phenol contamination) (Jarvis *et al.,* 1980).

DNA concentration of 1 mg/ml have extinction coefficients of 20 for unsheared DNA and 24 for sheared DNA. The final concentration was calculated using the following formula: DNA concentration mg/ml = $(A_{258} - A_{300})/20$ x dilution factor (Brenner and Falkow, 1971).

5.2 Restriction Endonuclease Digests (Maniatis *et al.,* 1982).

Genomic DNAs extracted from the New Zealand isolates and the ATCC strain were digested by the restriction endonucleases *Eco*RI and *Hind*III.

5.2.1 Materials

(1) Assay Buffer (Gibco, BRL,Promega, Biolabs, Boehringer Mannheim): Tris-HCl (pH 8), 50 mM; MgCl, 10 mM; NaCl, 100 mM. Purchased in the 10 X concentrated form.

(2) Restriction Endonucleases (Gibco, BRL, Promega, Biolabs, Boehringer Mannheim): One unit is the amount of enzyme required to cleave 1 µg of lambda DNA for one hour at 37°C in the appropriate buffer. This is usually purchased as 10 units/µl.

(3) Tris Acetate Electrophoresis (TAE) Buffer contains:

Tris, 40 mM; Sodium acetate, 5 mM. The pH was adjusted to 7.8 using glacial acetic acid.

(4) Loading Buffer x 10 contains:

Ficoll, 20 %; EDTA, 0.1 M; Sodium dodecyl sulphate, 1 %; Bromophenol blue, 0.2 % OR sucrose/dye marker 50 %.

(5) Dye marker contains:

Bromophenol blue, 0.05 %; Sucrose, 50 %; EDTA, 0.1 M.

(6) Agarose Gel: 1 % agarose (Sea Kem of Sea Plaque Agarose FMC).

(7) Ethidium Bromide: Stock solution of 10 mg/ml.

(8) Destaining Solution: 1M Magnesium sulphate.

5.2.2 Method

Assay buffer (2µl) was added to 15 µl of genomic DNA, 2 µl of sterile Milli-Q water and 1 µl of restriction endonuclease (EcoRI or HindIII). The tubes were pulsed for 2 - 3 seconds in a microcentrifuge and then incubated for 2 to 5 hours at 37°C. A 1% agarose gel was poured into a Horizon 11.14 (BRL) gel box and allowed to set for an hour before being flooded with buffer. Loading buffer (2 µl) was added to the DNA digest, and 10 to 15µl of the mixture was loaded into a well on the gel. The gel was run for an hour at 20 volts and then for 6 hours at 90 volts, stained in 0.5 µg/ml ethidium bromide for 20 minutes and destained in 1% magnesium sulphate for 20 minutes. The gel was viewed under uv light, using a transilluminator, and photographed with a polaroid MP-4 land camera using polaroid or tec-pan film (section 4.1.2).

5.3 Polymerase Chain Reaction (PCR)

A method of thermal cycling (Ausabel *et al.*,1991) was used to amplify a 260 bp 16S rRNA gene fragment corresponding to positions 44 to 337 in the *E. coli* 16S rRNA sequence (Young *et al.*, 1991) from the isolates derived from this investigation, and the ATCC type strain.

5.3.1 Materials

(1) Deoxynucleotide triphosphates (dNTPs) contains:

Stock solutions (2 mM) of each of the four bases; dATP, dCTP, dGTP and dTTP (Pharmacia).

(2) Primers (Young *et al.*, 1991):

Y1 5' - TGG CTC AGA ACG AAC GCT GGC GGC - 3'

Y2 5' - CCC ACT GCT GCC TCC CGT AGG AGT - 3'

Y1 and Y2 correspond to conserved positions 20-43 and 361-338 respectively in the *Escherichia coli* 16S RNA sequence. Before use, the primers were diluted 1:50 using sterile Milli-Q water.

(3) Taq Polymerase Buffer x 10 contains:

Kcl, 500 mM; Tris-HCl (pH 8.8); $MgCl_2$, 15 mM; and 1% Triton-X - 100 (Life Technologies).

(4) Taq Polymerase: is isolated from *Thermus aquaticus* YT1. The storage buffer contains: Tris-HCl (pH 8), 20 mM; EDTA, 0.1 mM; Dithiothreitol, 1 mM; 50 % (v/v) glcerol; stabilisers (Life Technologies, Gibco, BRL).

(5) Sterile parafin oil.

(6) Agarose gel: 2% agarose gel in TBE x 1(Sea Kem Agarose).

(7) Loading Buffer contains:

Bromophenol blue, 0.05 %; Sucrose, 50 %; EDTA, 0.1 M.

(8) Ethidium Bromide: Stock solution, 10 mg/ml.

(9) Destaining Solution: 1 M Magnesium sulphate.

(10) ART^R Tips: Aerosol resistant tips (Biotek).

5.3.2 Method

All dilutions were made on the same day and the reagents were kept on ice. Aerosol resistant tips were used at all times, and a negative control (sterile Milli-Q water instead of DNA) was always run along side the PCR products.

The following reaction mixture was prepared:

Taq Polymerase buffer X 10 ,2 µl; dNTPs , 4 µl; Primer Y1 (1:50), 1 µl; Primer Y2 (1:50), 1 µl; Taq Polymerase, 0.4 µl; Milli-Q water, 11.6 µl.

The DNA sample was diluted 1:10 in sterile water and 2 µl was added to 18 µl of the reaction mixture in a sterile 1.5 ml Eppendorf tube. Sterile paraffin oil 20 µl was added and the tube was centrifuged briefly and loaded in the thermal cycler (Techne type PHC-3) running the following programme:

Stage 1: 95^{o}C, 3:00 minutes (template denaturation); 65^{o}C, 30 seconds (primer annealing); 72^{o}C, 30 seconds (extension of the annealed primers); number of cycles, 1:

Stage 2: 95^{o}C, 45 seconds; 65^{o}C, 30 seconds; 72^{o}C, 30 seconds; Cycles 35:

Stage 3: 95^{o}C, 45 seconds; 65^{o}C, 45 seconds; 72^{o}C, 3:00 minutes; Cycles 1:

PCR product (2 µl) mixed with loading buffer (5 µl) was examined by electrophoresis in a 2% agarose gel (1 x TBE buffer) on a minigel apparatus (BRL Horizon 58). The gel was run at 90 -100 volts, until the blue dye was within one inch of the bottom of the gel, then it was removed, stained in 0.5 µg/ml ethidium bromide for 20 minutes, and destained in 1 % magnesium chloride for 20 minutes. The gel was viewed under uv light using a transilluminator and photographed as described previously (section 4.1.2).

5.4 Purification of DNA Fragments

The 16S rDNA fragment amplified by PCR was purified using Promega Magic PCR Preps^{TM}.

5.4.1 Materials

(1) Direct Purification Buffer contains:

KCl, 50 mM; Tris-HCl (pH 8.8), 10 mM; $MgCl_2$,1.5mM; Triton X-100, 0.1 %. Included in the Promega Magic PCR Preps kit.

(2) PCR Preps Resin: Before use, the resin included in the Promega Magic PCR Preps kit was incubated at 37^{o}C to dissolve crystals in the preparation.

(3) Mini-column

(4) Magic PCR Preps Column Wash Solution contains: 80% isopropanol.

(5) 3 ml Syringe.

5.4.2 Method

An aliquot of the PCR reaction (15 - 20 µl) was removed into a Eppendorf tube (avoiding the paraffin oil) and direct purification buffer (0.1 ml) was added and mixed. Magic PCR Preps resin (1 ml) was added to the Eppendorf tube and vortexed 3 times over 1 minute. This mix was then loaded into a 3 ml syringe barrel and gently forced through the mini-column. The mini-column was washed with 2 ml of Magic PCR Preps column wash solution, transferred to a 1.5 ml Eppendorf and centrifuged for 20 seconds at 14000 x g to dry the resin. The column was then left at room temperature for 5 to 10 minutes to allow residual isopropanol to evaporate, and removed to a new tube. DNA was eluted from the column by adding water or TE buffer (30 µl, section 4.1.1) to the column allowing 1 minute contact time and then centrifuging for 20 seconds in a 1.5 ml Eppendorf tube. The eluant containing purified DNA was stored at 4^{o}C or -20^{o}C.

5.5 16S rDNA Sequence Determination

Direct sequencing of 16S rRNA gene fragments was performed with a Promega *Fmol*™ DNA Sequencing System using the chain termination method (Sanger *et al.*, 1977).

5.5.1 Preparation of Acrylamide Gels for Sequencing.

5.5.1.1 Materials

(1) Tris Borate Buffer (TBB) x 10 contains:

Boric Acid, 55 g; EDTA, 19 g; Tris base, 324 g; Milli-Q water,2 litres. The pH was adjusted to 8.9 and the buffer stored at 4°C.

(2) Urea Solution contains:

Urea, 288 g; Milli-Q water, 210 ml; (450 ml total).

(3) 40 % Acrylamide contains:

Acrylamide, 38 g; bis-acrylamide, 2 g in 100 ml milli-Q water.

(4) Acrylamide Mix contains:

Urea solution, 450 ml; Acrylamide (40%), 90 ml; TBB x 10, 60 ml.

(5) TEMED: (N,N,N',N' - Tetramethylethylenediamine).

(6) Ammonium Persulphate AMPS (10%)

(7) Mixed Bed Resin (AG 501-X8): 20 -5 mesh.

5.5.1.2 Method

To make the acrylamide mix, the urea and acrylamide solutions were mixed and stirred for one hour with a tablespoon of mixed bed resin (AG 501-X8) to de-ionise them. The solution was then filtered through a No. 1 scintillation filter, then the TBB buffer is added. Acrylamide mix (80 ml), TEMED (50 µl), and 500 µl of AMPS (10%) was stirred thoroughly and used immediately.

The small and large glass plates (dimensions: 31 cm x 38.5 cm) were cleaned with acetone. Two films of silicone were applied to the small plate which was then placed on the spacers (0.4 mm thick) running down the length of the large plate (the siliconised edge inner most). The edges were sealed with tape and secured with bulldog clips. The plates were tilted and the acrylamide gel (section 5.5.1) was loaded from one side into the gap between the plates and filled to the top of the small glass plate, allowing a small overhang. The gel comb was inserted so that the base of the comb sat just above the edge of the small glass plate with the points of the teeth uppermost. After one hour the gel and the glass plates were wrapped in Gladwrap to prevent drying out..

5.5.2 Cycle Sequencing

5.5.2.1 Materials

(1) Polymerase Buffer: 5 X buffer: Tris-HCl (pH 9.0); $MgCl_2$, 10 mM.

(2) [α - S^{35}]dATP (> 1,000 Ci/mmol, 10 μCi/μl).

(3) Taq Polmerase: (sequencing grade) Isolated from *Thermus aquaticus* YT1. The storage buffer contains: Tris-HCl (pH 8), 20 mM; EDTA, 0.1 mM; Dithiothreitol, 1 mM; 50 % (v/v) glcerol; stabilisers (Life Technologies, Gibco, BRL).

(4) Deoxy/dideoxy bases (Promega).

(5) Stop Buffer.contains: NaOH, 10 mM; foramide, 95%; bromophenol blue, 0.05%; xylene cyanide, 0.05% (Promega).

(6) PCR grade Paraffin Oil.

5.5.2.2Method

Both strands of the 16S rDNA gene fragment (section 5.3) were amplified using Y1 and Y2. For each primer, this reaction mixture was set up and kept on ice: Primer (Y1 or Y2, section 5.3.2), 1 µl; Polymerase buffer, 5 µl; Sterile Milli-Q water, 8.5 µl; Undiluted DNA template (purified PCR product), 1 µl; S^{35}, 5 µl; Taq Polymerase, 1 µl. NOTE: tips used for the S^{35} were discarded separately and gloves worn at all times. Aerosol resistant tips were used at all times.

The reaction mixture was mixed and pulsed in a microcentrifuge, then 4 µl dispensed into each of 4 Eppendorf tubes containing one of the deoxy/dideoxy bases (d/ddA, d/ddG, d/ddA, d/ddT), mixed, and 10 µl of PCR grade paraffin oil added. The 4 tubes containing the reaction mixture and the deoxy/dideoxy bases were loaded on to the thermal cycler (Techne, type PHC-3) when it was between 80°C and 90°C and the following programme was run:
Stage 1: 93°C, 2:30 minutes; 53°C, 30 seconds; 70°C, 30 seconds, cycles 1
Stage 2: 93°C, 30 seconds; 53°C, 30 seconds; 70°C, 30 seconds; cycles 30
The tubes were stored at -20°C as soon as the cycle was finished or else 4 µl of stop buffer was added.

5.5.3 Separation of Cycle Sequencing Products

5.5.3.1Materials

(1) Tris Borate Buffer (TBB).

(2) Stop Reaction contains: NaOH, 10 mM; foramide, 95%; bromphenol blue, 0.05%; xylene cyanide, 0.05% (Promega).

5.5.3.2Method

The PCR products for sequencing were placed in a heating block at 75°C until the gel was ready to be loaded. The Gladwrap and the bulldog clips were removed from the gel. Excess gel from around the comb area was washed away with water and the comb removed. The sealing tape at the bottom of the gel was cut and the gel was placed in the electrophoresis chamber with the large plate facing outermost. The drains were closed and the gel clamped tightly in place. The buffer (TBB x 1) was added to the top and bottom buffer chambers. The comb was placed so the points of the teeth just sat on the edge of the gel to create wells for the samples. Stop reaction (4 µl) was loaded into a well and allowed to run for a few centimetres to check for leaks. Bubbles and excess urea were expelled from the wells area using a Pasteur pipette. PCR sample (4µl) per well were loaded onto one side of the gel (long run) through the buffer. The remainder of the PCR sample was stored at -20°C for later use. The power was then turned on and the gel run at 65 W until the blue dye band reached the bottom of the gel.

The power to the apparatus was turned off, and the remainder of the thawed samples was loaded in the same manner as before. Once this side had run to the bottom of the gel, the power was turned off and the buffer was drained away.

The spacers, comb and the small plate were removed over a sink by dissolving the excess gel from the top of the comb using a water bottle. The large plate with the gel sitting on it was placed in fixative (10% methanol, 10% acetic acid) for 30 minutes. Once the gel was removed, Whatman paper (3 MM) and then blotting paper was placed on top of the gel, after which the large plate was

carefully turned over. The large plate was raised from one end allowing the gel stuck to the Whatman paper to be peeled off and dried at 80°C for 2 hours under vacuum on a slab drier (model 483) or a gel drier (model 583, Bio-Rad) .

The dried gel adhered to the Whatman paper and was loaded into a cassette with X-ray film in direct contact with the gel surface. The film was exposed at room temperature for about 24 hours and developed in a Kodak (XY2) processor.

5.5.4 16S rDNA Sequence from the Autoradiograph.

The developed autoradiograph was placed on a light box (CustomLight, Christchurch) and the 16S rDNA sequence was read manually. The sequence obtained from the Y1 strand was checked against the complementary Y2 strand. Sequences were double checked against the original autoradiograph.

6 ANALYSIS BY COMPUTER SOFTWARE.

6.1 Identification of Bacterial Strains.

The consensus 16S rDNA sequence was entered into a computer as a text file. A BLAST search (Altschul *et al.*, 1990) was conducted by sending an E-mail request to the National Center for Biotechnology Information, National Centre for Medicine, NIH, Bethesda, USA: blast@ncbi.nlm.nih.gov.

The 16S rDNA sequences were compared to sequences lodged in the GenBank/EMBL data library.

6.2 Phylogenetic Analysis

The following GenBank sequences were used (accession numbers in brackets): *Rhizobium leguminosarum* biovar trifolii ATCC14480, (X67227); *Mycoplana bullata,* (D12785); *Pseudomonas diminuta,* (M59064), *Hyphomicrobium vulgare,* (X53182); *Caulobacter subvibrioides,* (M83797); *Caulobacter bacteroides*, (M83796); *Caulobacter* sp. MCS17, (M83808).

The 260 bp 16S rDNA nucleotide sequences were aligned by using the PILEUP programme of the Genetics Computer Group Sequence Analysis Package (Devereux *et al.* 1984), version 7, on a VAX computer.

J. Felsenstein's (1982) PHYLIP 3.5c SEQBOOT programme provided 100 data sets for bootstrap analysis and the DNADIST programme was used to determine the Jukes-Cantor distances of the aligned sequences (Jukes and Cantor, 1969).

The NEIGHBOR programme was used to construct a phylogenetic tree based on the Neighbor-Joining method of Saito and Nei (1987).

RESULTS

1 ENRICHMENT AND ISOLATION.

Stalked cells were found in all of the environments that were enriched. The surface film enrichment procedure (Materials and Methods, section 1.6.1) was effective for both the freshwater and the sewage samples, although the incubation times differed. In general, the enrichment cultures were incubated until the number of stalked cells were high enough to provide optimum conditons for the isolation of *Caulobacter*. If the enrichment culture did not have high numbers of *Caulobacter* in the surface film, isolation was difficult. The attachment methods (Materials and Methods, section 1.6.2), and the physical isolation methods (Materials and Methods, section 1.7.2) were then more successful if used in conjunction with low phosphate PYEA (Materials and Methods, section 1.22) or PCa medium (Materials and Methods, section 1.2.5).

1.1 Enrichment and Isolation of *Caulobacters* from Sewage.

After 3 weeks of incubation, direct microscopic examination of the surface film of the sewage enrichment culture showed the presence of cells with very long stalks sometimes up to 3 times the length of the cell body (plate 1). Even when direct microscopic examination of the surface film of the enrichment culture showed many stalked cells, a sample plated onto solid media was over-representative of other faster growing bacteria which were present in the enrichment (plate 2) Isolation however was achieved after the enrichment culture was 6 months old and the surface film was dominated by stalked or caulobacter-like cells.

The '*Caulobacter*' isolates derived from the sewage enrichment culture, were assigned a number representative of the order in which they were isolated (CDF series). Occasionally, an isolate appeared to be mixed, and so the separated colonies were given the same number, followed by a suffix letter to distinguish them.

1.2 Morphology of *Caulobacter* sp. Isolated from Sewage.

In general, *Caulobacter* isolated from sewage were non-pigmented and had a variety of cell shapes (table 4). The colony morphology was typically colourless and translucent. The exception to this was an isolate which had an orange pigment (CDF'o') and was taken from the original enrichment after one year of incubation.

The sewage isolates could be divided into three groups based on morphology. One group comprised isolates that were short, crescent shaped cells, and included isolates CDF# 6, 9, 28, 29, 30b, 45 and 46b (plate 3, 12, 13 and 14). Another group included CDF# 7, 20a, 42 and 47, all of which had a slightly longer crescent shaped cell. The remaining group (CDF# 5, 18, 20b 23 and 35) had straight cells which were relatively thin and long. This group included the only pigmented strain isolated, CDF'o'. (plate 4, 15 and 16).

Rosette formation (plate 5 and 15) among sewage isolates was variable. Isolates were incubated for the same amount of time, under the same conditions and only some strains characteristically formed rosettes in broth medium. However, the other isolates did form rosettes occasionally (table 4).

Table 4: Morphology of Stalked Bacteria Isolated from Sewage.

CDF#	Cell shape	Rosette formation[1]
5	straight	++
6	Short crescent	-
7	crescent	-
9	short crescent	++
18	straight	+
20a	crescent	++
20b	straight	+
23	straight	++
28	short crescent	+
29	short crescent	-
30b	short crescent	-
35	straight	-
42	crescent	++
45	short crescent	-
46b	short crescent	-
47	crescent	-
'O'	straight	++

1. Rosette formation (in liquid culture):
   - ++ : many rosettes
   - + : rosettes present
   - - : rosette formation was not common.

Plate 1. **A SAMPLE OF SURFACE FILM FROM THE SEWAGE ENRICHMENT CULTURE.**

After 5 weeks of incubation at room temperature.

(1) Cell body, (2) Stalk, of a *Caulobacter*-like cell.

Phase contrast under oil immersion (X1000) objective.

Bar = 1 μm.

Plate 2. **STREAK PLATE OF A SAMPLE OF SURFACE FILM TAKEN FROM THE SEWAGE ENRICHMENT.**

The enrichment culture had been incubated for 5 weeks.

This plate was incubated at room temperature, for 16 hour

(1) A typical colony type for *Caulobacter* isolates.

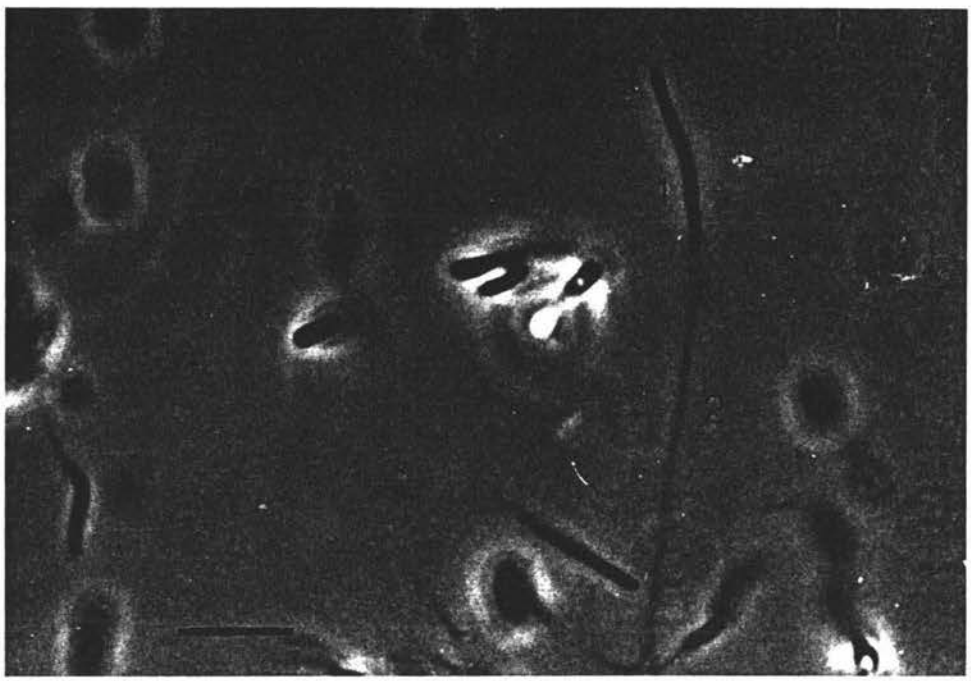


Plate 1.

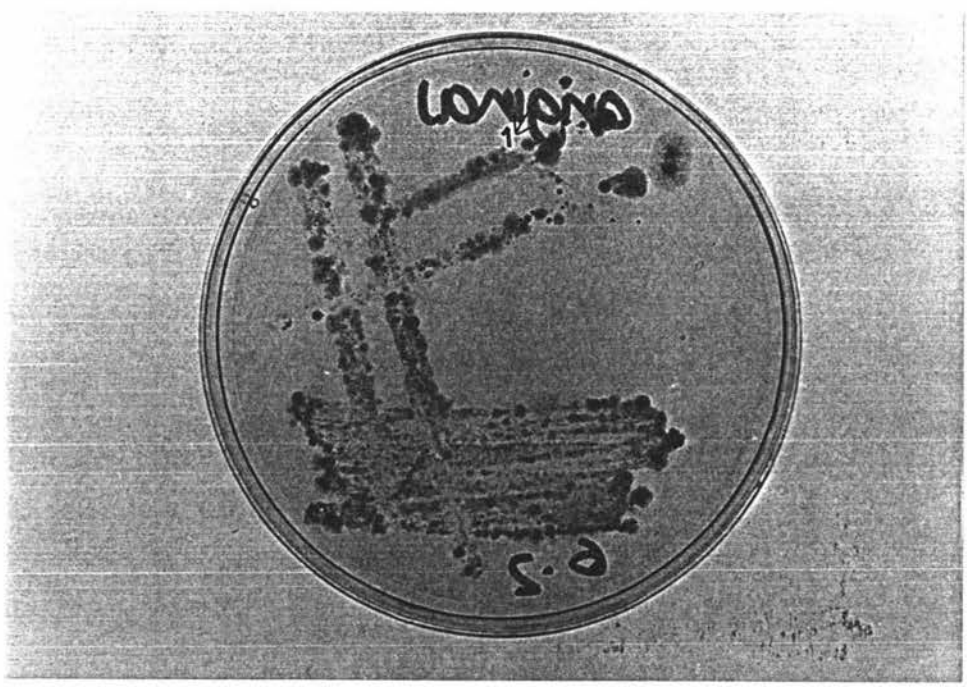


Plate 2.

Plate 3. SEWAGE ISOLATE CDF6.

This *Caulobacter* isolate exhibits:

(1) A short crescent shaped cell body, and (2) a stalk.

Phase contrast under oil immersion (x1000) objective.

Bar = 5 μm.

Plate 4. SEWAGE ISOLATE CDF35.

(1)Straight axis cell body type and a short stalk.

Phase contrast under oil immersion (x1000) objective.

Bar = 5 μm.

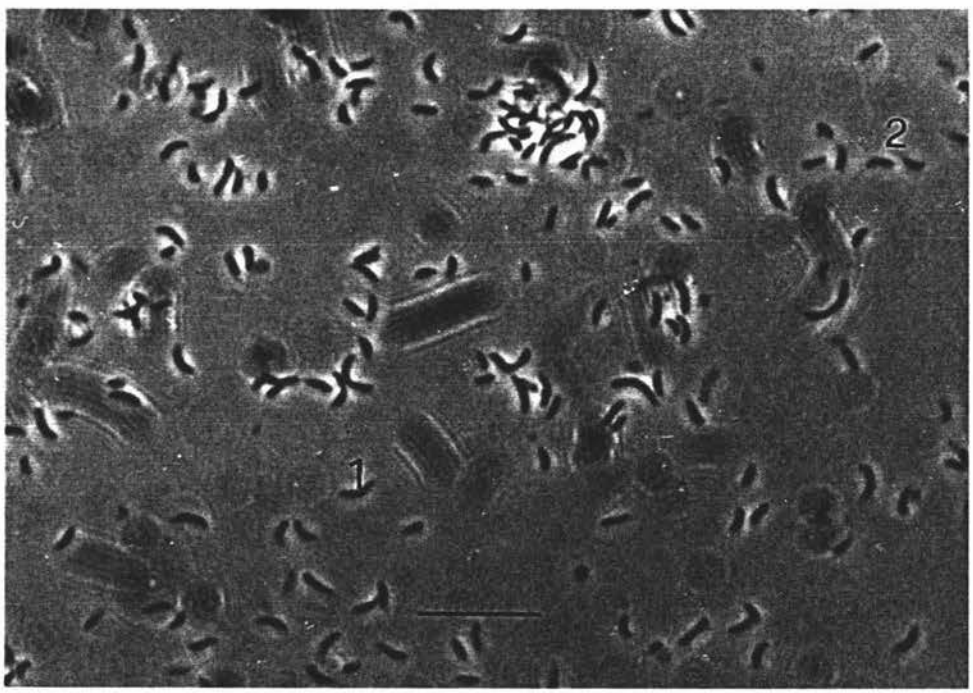


Plate 3.

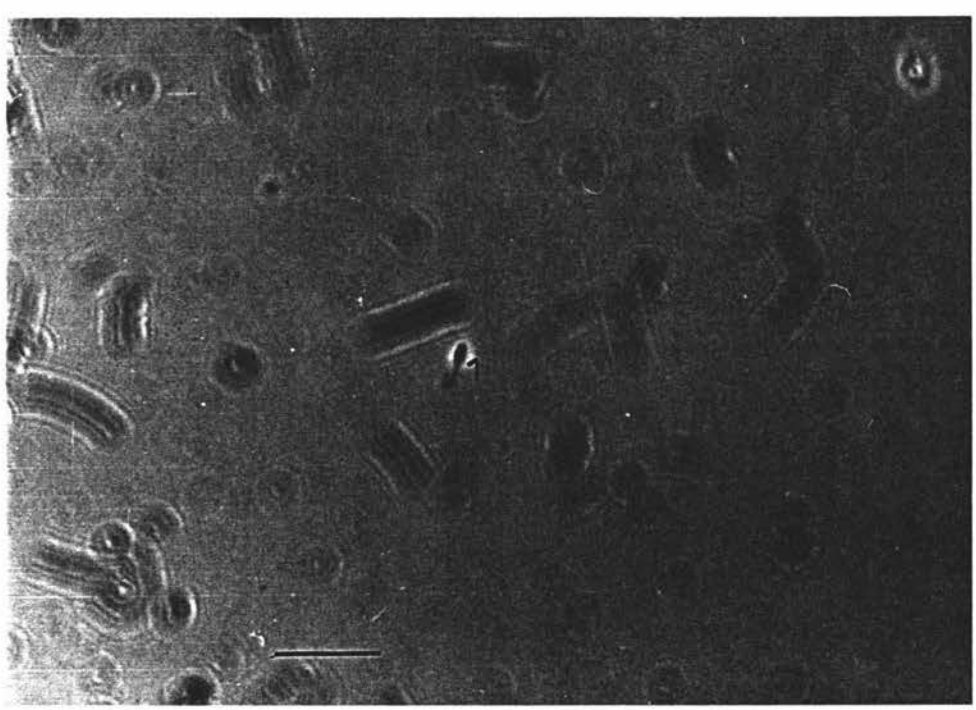


Plate 4.

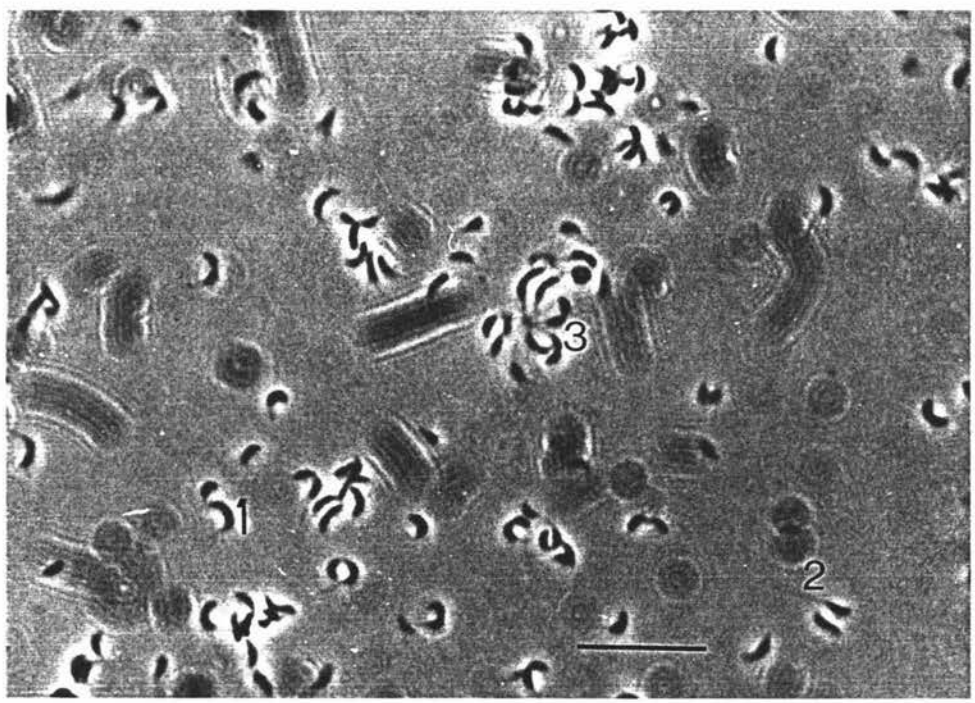


Plate 5.

1.3 Enrichment and Isolation of Freshwater *Caulobacter.*

*St*alked cells were present in all of the samples enriched after an incubation period of 10 weeks, although the first stalked cells were present in the stored tap water sample at 5 weeks. After almost 6 months of incubation, *Hyphomicrobium* cells formed the majority of the stalked cells in the Tiritea Stream enrichment (plates 6 and 7). After 9 months, *Caulobacter* cells were be isolated from the surface film of every enrichment (plates 8, 9, 10 and 11) except for the Taranaki Base Hospital sample where although *Caulobacter* were evident, they were not the dominant population.

1.3.1 Morphology of Stalked Bacteria Isolated From Freshwater.

All of the *Caulobacter* isolates from freshwater sources had a crescent cell shape, typical of most freshwater strains (table 5). On solid media, the colonies were colourless and translucent to white. The freshwater isolates had a similar cell morphology to the ATCC type strain of *Caulobacter crescentus* which is also a freshwater strain (plate 5).

1.3.2 Coliform Count.

See table 5. No coliforms were detected in the tank water and the stored tap water samples so the coliform count was deemed to be less than 1 CFU/ml.

Table 5: **Morphology of Stalked Bacteria Isolated from Freshwater.**

Isolate	Cell shape	Source	Coliform count (CFU/ml)[1]
MR1	crescent	Manawatu River	110
TS1	crescent	Tiritea Stream	44
TW1	crescent	Tank water	less than 1
TW2	crescent	Tank water	
SW1	crescent	Stored tap water	less than 1

1. CFU/ml : colony forming units per ml.

Plate 6. *HYPHOMICROBIUM* ISOLATE.

From the Tiritea Stream enrichment culture.

(1) Prosthecate.

Negative stain preparation. Transmission electron

(x 21,200). Bar = 1 μm.

Plate 7. BUDDING *HYPHOMICROBIUM* ISOLATE.

From the Tiritea Stream enrichment culture.

(1) A prosthecate, (2) a budding cell arising from the hyphal axis, and (3) flagella.

Negative stain preparation. Transmission electron

(x 21,200). Bar = 1 μm.

Plate 6.

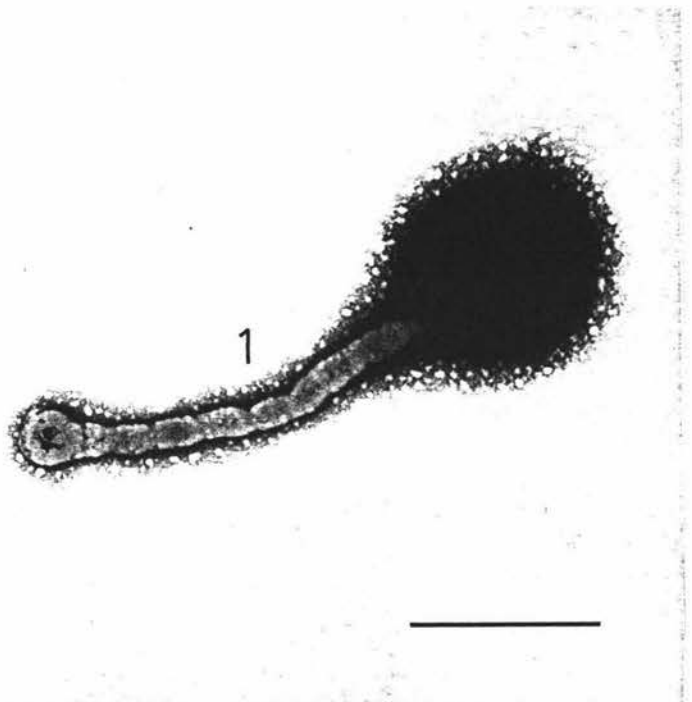


Plate 7.

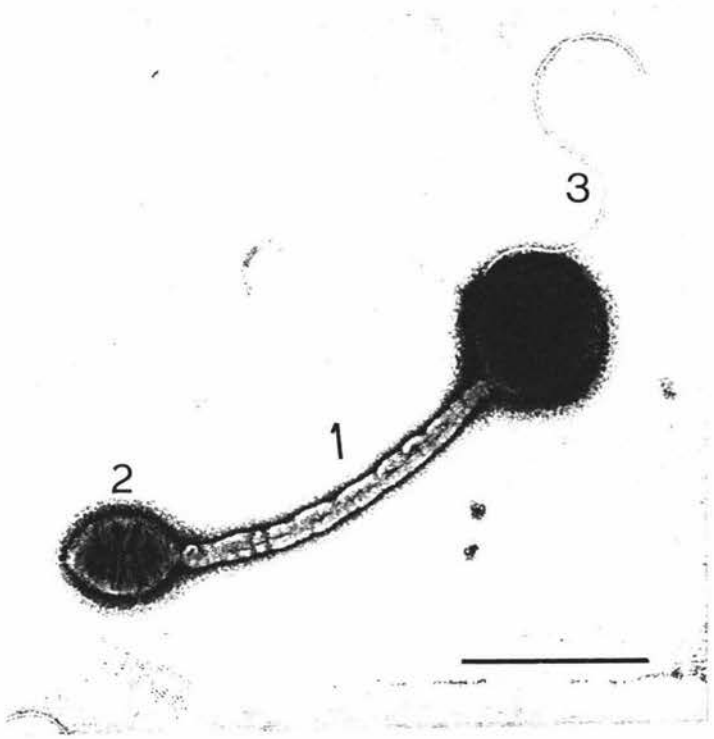


Plate 8. A SAMPLE OF SURFACE FILM FROM THE TIRITE STREAM ENRICHMENT CULTURE.

Prosthecate cells showing:

(1) The cell body, and (2) stalk.

Phase contrast under oil immersion (x1000) objective

Bar = 5 μm.

Plate 9. A SAMPLE OF SURFACE FILM FROM THE MANAV RIVER ENRICHMENT CULTURE.

Prosthecate cells showing:

(1) The cell body, and (2) stalked cells.

Phase contrast under oil immersion (x1000) objective.

Bar = 5 μm.

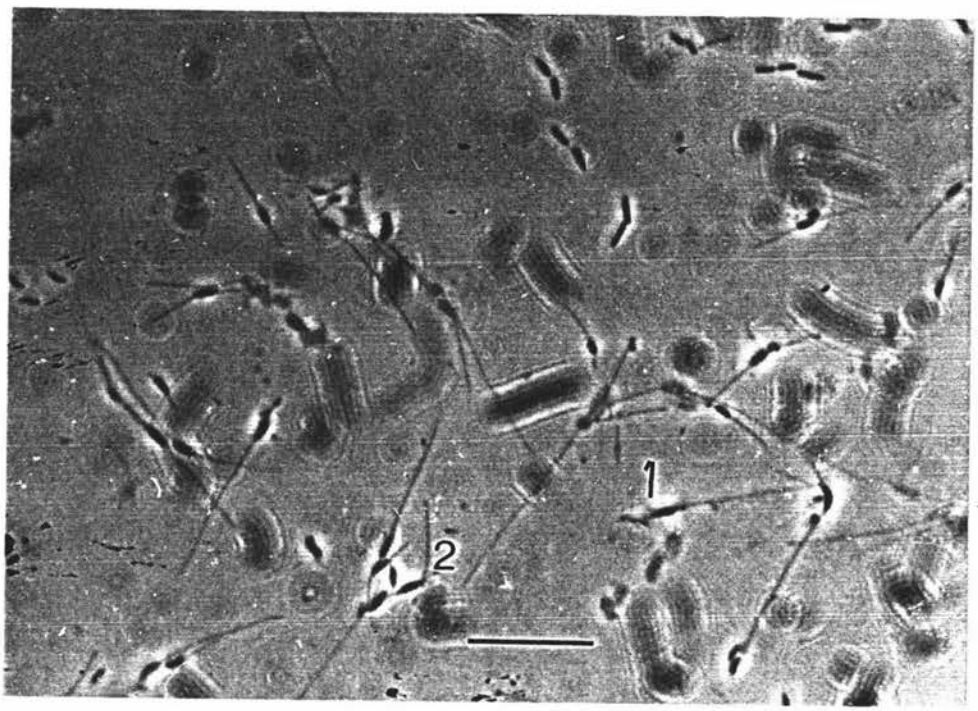


Plate 8.

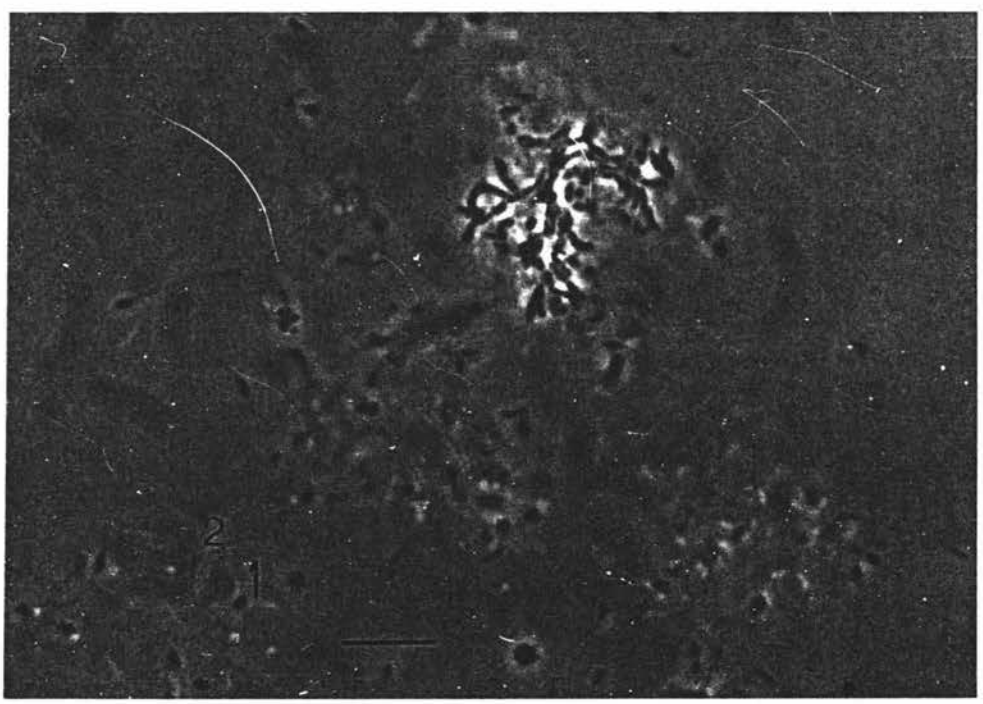


Plate 9.

Plate 10. A SAMPLE OF SURFACE FILM FROM THE WATER-TANK ENRICHMENT CULTURE.

Prosthecate cells showing:

(1) The cell body, and (2) stalked cells.

Phase contrast under oil immersion (X1000) objective.

Bar = 5 μm.

Plate 11. A SAMPLE OF SURFACE FILM FROM THE STORED-WATER ENRICHMENT CULTURE.

Prosthecate cells showing:

(1) The cell body, and (2) stalks.

Phase contrast under oil immersion (X1000) objective.

Bar = 5 μm.

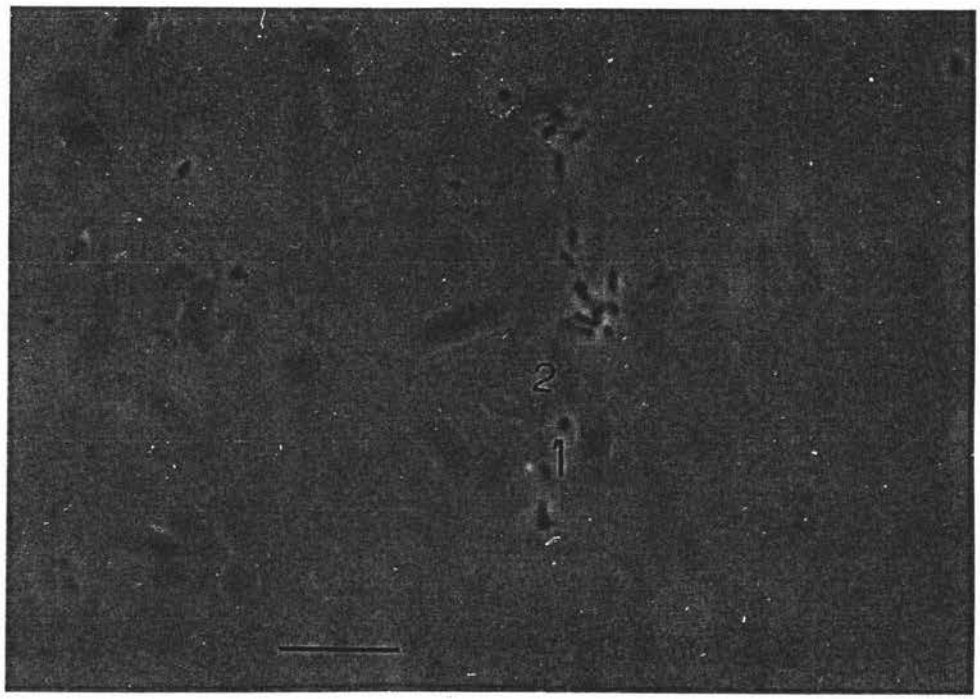


Plate 10.

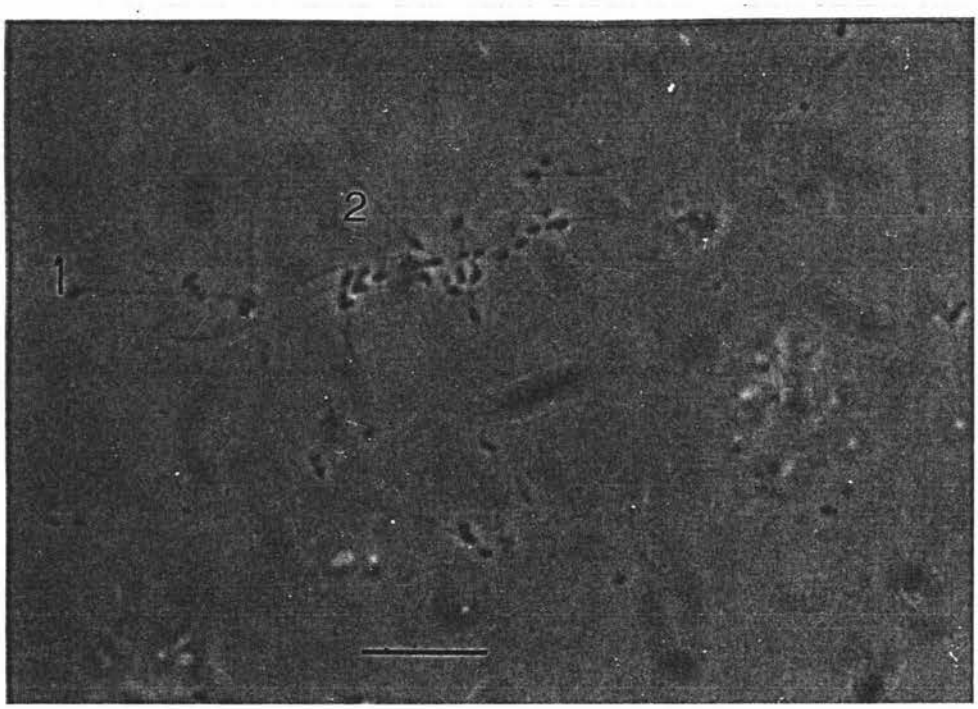


Plate 11.

Plate 12. SEWAGE ISOLATE CDF46b.

(1) Stalked parent cell, and (2) attached daughter cell with flagella.

Negative stain preparation viewed by tranmission electron microscope. (X11,200) Bar = 1 μm.

Plate 13. SEWAGE ISOLATE CDF46b.

Rosette formation.

Negative stain preparation viewed by transmission electron microscope. (X15,300) Bar = 1 μm.

Plate 12.

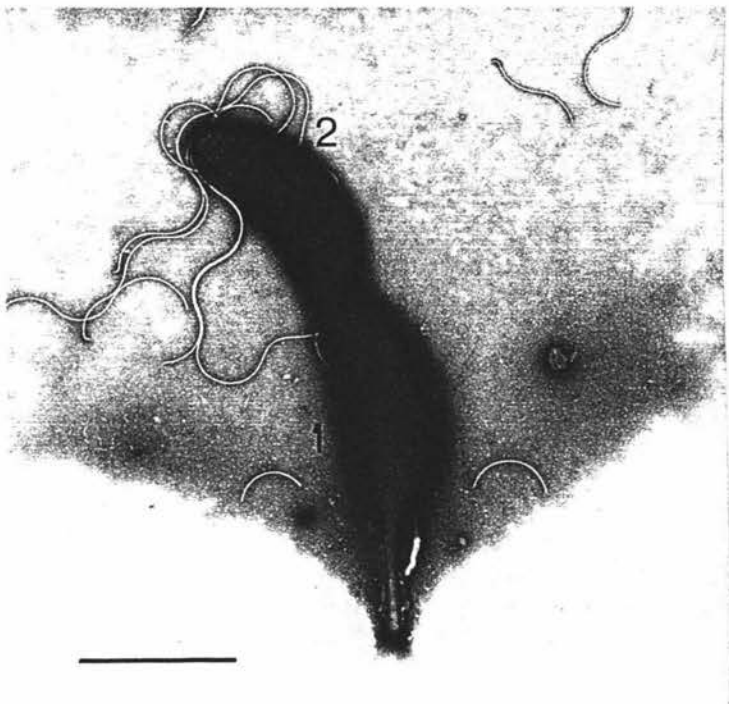


Plate 13.

Plate 14. SEWAGE ISOLATE CDF46b.

(1)The junction between the cell body and the stalk shows an inner layer of the cell wall. Negative stain preparation viewed by transmission electron microscope. (X31,800) Bar = 1 µm.

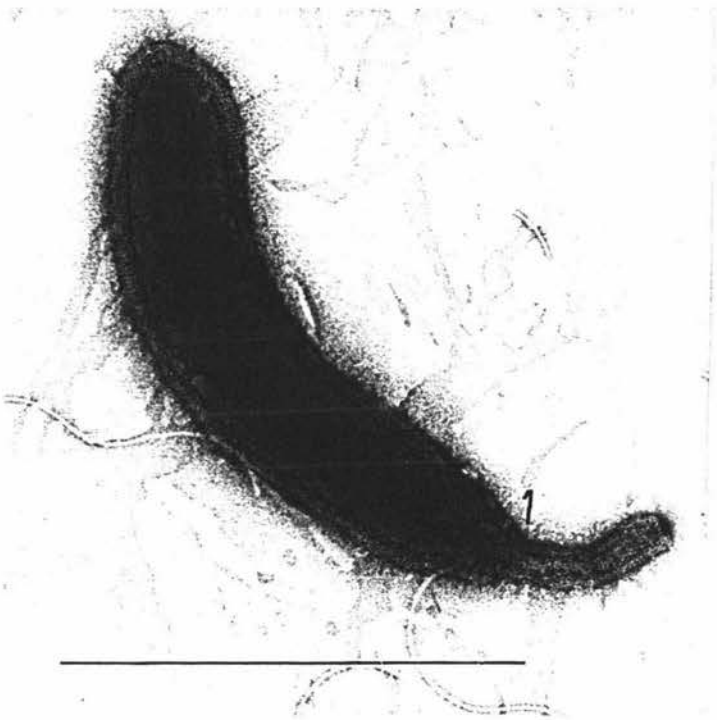


Plate 14.

Plate 15. SEWAGE ISOLATE CDF23.

(1) Rosette formation - cells attached at distal end of stalk.

(2) Straight cell type.

Negative stain preparation viewed by scanning electron microscope. (X11,200) Bar = 1 μm.

Plate 16. SEWAGE ISOLATE CDF'O'.

(1) Straight cell type.

Negative stain preparation. Scanning electron microscope (x 21,200). Bar = 1 μm.

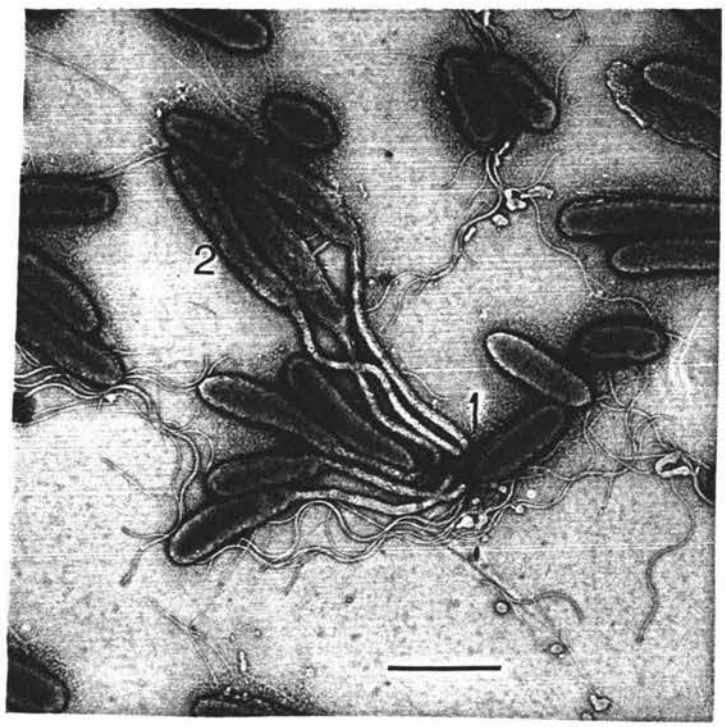


Plate 15.

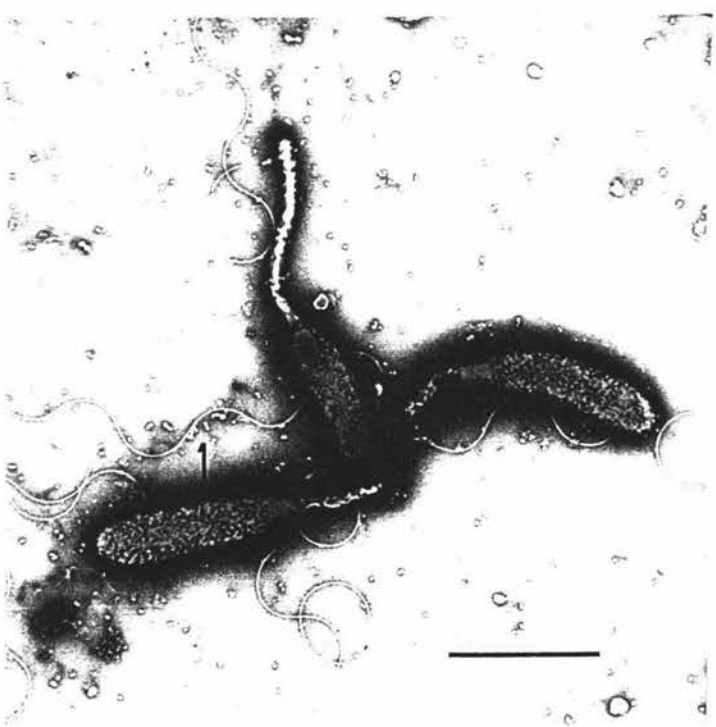


Plate 16.

2 PHYSIOLOGY

Results of physiological studies are recorded in table 6.

2.1 Riboflavin Requirement (Vit. B_{12})

All 22 sewage and freshwater isolates were capable of growth in PYE broth without riboflavin.

2.2 Salt tolerance.

2.2.1 Tolerance to 1% NaCl

Of the 22 strains tested for salt tolerance, 9 isolates did not grow in the 1% NaCl.PYE broth after 4 days (includes all of the freshwater strains): ATCC strain, TW1, TW2, MR1, TS1, SW1, CDF'o', CDF42, CDF5 and CDF29.

The following isolates did grow in 1% NaCl.PYE broth after 4 days: CDF30b, CDF46b, CDF45, CDF6, CDF20b, CDF47, CDF23, CDF20a and *Escherichia coli* B MU113.

Some isolates (CDF28, CDF35, CDF18, and CDF9) did grow in 1% NaCl.PYE broth but required 6 days of incubation.

2.2.2 Tolerance to 2.5% NaCl

Only CDF35, CDF18, CDF23 and CDF5 grew in 2.5%NaCl.PYE broth. All of these isolates had a straight cell shape.

2.3 Antibiotic Resistance.

Streptomycin, gentamycin, chloramphenicol, tetracycline, ampicillin, penicillin, vancomycin and kanomycin solutions (5 mg/ml) were used to prepare antibiotic discs (50μg/ml, section 3.3). Rifampicin 50 μg/ml, ampicillin 50μg/ml, tetracycline 50μg/ml, and streptomycin 30μg/ml were incorporated in agar.

All strains were sensitive to streptomycin, gentamycin, chloramphenicol, kanomycin and vancomycin (see figure 4) and to 5 μg/ml of rifampicin. The Tiritea Stream isolate was sensitive to all the antibiotics tested. The other freshwater isolates were resistant to penicillin and ampicillin (5 mg/ml) as were many of the sewage isolates (figure4). Some of the sewage isolates were resistant to tetracycline (5 mg/ml), where as all the freshwater isolates were sensitive. In general, the sewage isolates were less sensitive than the freshwater isolates to the same antibiotics (table 6).

Those strains that had a straight cell shape (CDF18, CDF5, CDF35, CDF23 and CDF'o') were sensitive to penicillin and ampicillin. However, these strains were more resistant to streptomycin (50 μg) and gentomycin (50 μg) when compared to the other isolates.

Table 6:

Average Zones of Inhibition by Antibiotics

Antibiotics (5 mg/ml)	Freshwater Isolates[1]	Sewage Isolates[1]
Streptomycin	13.0 mm	10.0 mm
Gentomycin	8.6 mm	6.6 mm
Chloramphenicol	14.4 mm	9.9 mm
Tetracycline	15.4 mm	9.4 mm
Vancomycin	10.5 mm	9.5 mm
Kanomycin	11.2 mm	9.6 mm

1. The average zone of inhibition was calculated using the following formula:

(the sum of the diameters) divided by (the number of isolates)

Figure 3. ANTIBIOTIC SENSITIVITY OF THE ENVIRONMENTAL ISOLATES.

The freshwater isolates are to the left of the graph (TS1, TW1, TW2, SW1, MR1) and the sewage isolates are to the right.

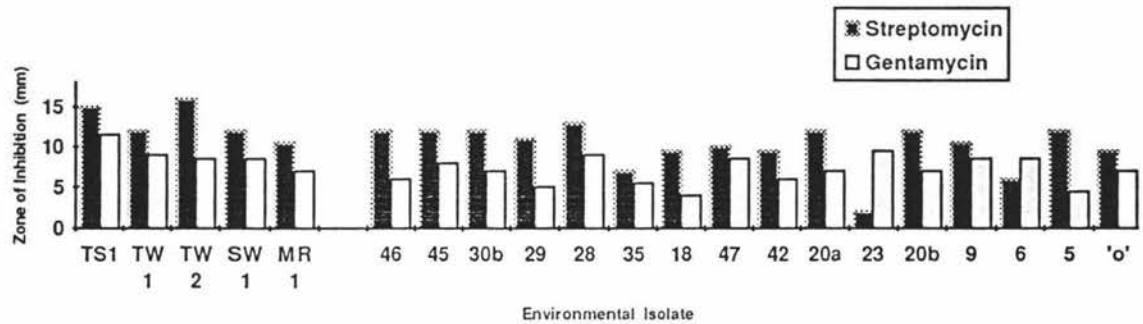
Zone of inhibition (mm) = the radius (taken from the edge of the disc to the beginning of the bacterial growth). The taller the column, the more sensitive the isolate to that particular antibiotic. The absence of a column denotes complete resistance.

A: Resistance of Environmental Isolates to Streptomycin and Gentomycin.

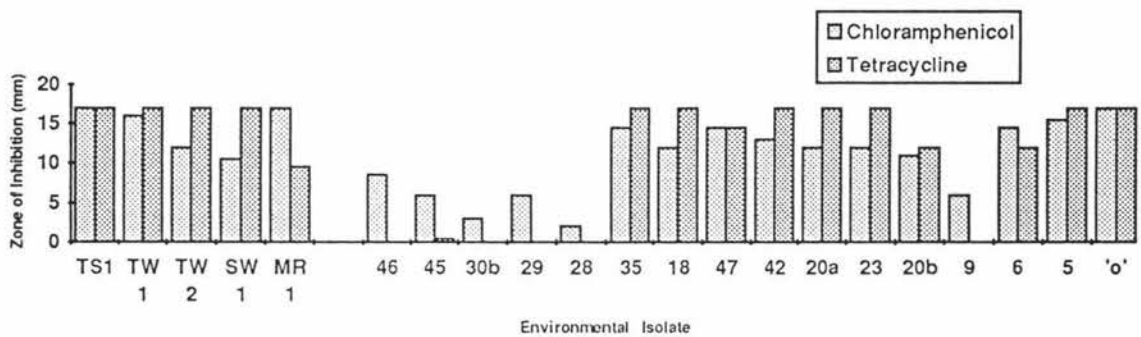
B: Resistance of Environmental Isolates to Chloramphenicol and Tetracycline.

C: Resistance of Environmental Isolates to Ampicillin and Penicillin.

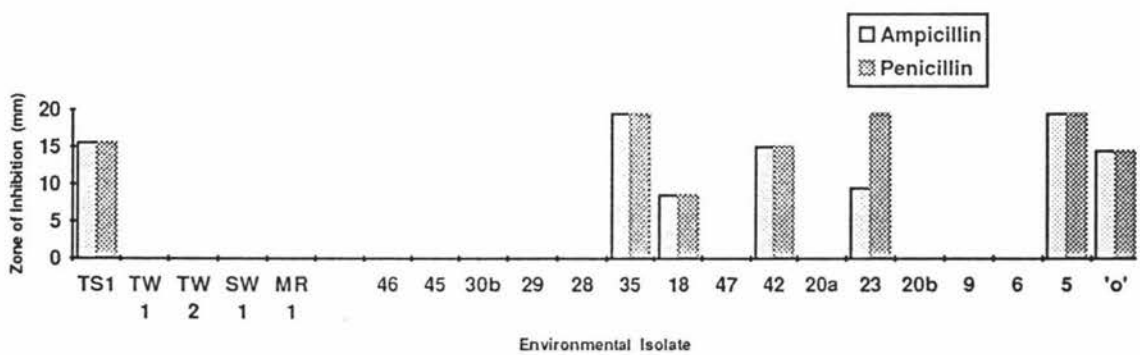
A.


Streptomycin
Gentamycin
Zone of Inhibition (mm)
0
5
10
15
TS1
TW 1
TW 2
SW 1
MR 1
46
45
30b
29
28
35
18
47
42
20a
23
20b
9
6
5
'o'
Environmental Isolate

B.


Chloramphenicol
Tetracycline
Zone of Inhibition (mm)
0
5
10
15
20
TS1
TW 1
TW 2
SW 1
MR 1
46
45
30b
29
28
35
18
47
42
20a
23
20b
9
6
5
'o'
Environmental Isolate

C.


Ampicillin
Penicillin
Zone of Inhibition (mm)
0
5
10
15
20
TS1
TW 1
TW 2
SW 1
MR 1
46
45
30b
29
28
35
18
47
42
20a
23
20b
9
6
5
'o'
Environmental Isolate

3 RESTRICTION ENDONUCLEASE DIGESTS.

Thirteen of the 23 isolates considered could be classidied into groups which had identical genomic digest profiles under digestion with both *Eco*RI and *Bam*HI as follows:

Group 1: CDF46b,CDF45, CDF28, CDF29 and CDF30b;

Group 2: CDF35 and CDF18 (plate 17 and 18);

Group 3: CDF47, CDF20a and CDF7 (plate 19 and 20);

Group 4: SW1, TW1 and TW2 (plate 19 and 20).

The rest of the isolates: CDF42, CDF20b, CDF9, CDF6, CDF'o', CDF23, CDF5, TS1, MR1, and SW2 all had different profiles so were treated as different strains (plate 21 and 22).

4 PLASMID ANALYSIS.

4.1 Eckhardt Gels.

The ATCC strain and the TS1 isolate (freshwater) had no detectable plasmids under the conditions used. Of the sewage isolates, most had at least one plasmid, with some of the isolates having multiple plasmids (plate 24).

The following isolates are grouped according to their plasmid profiles (based on the plasmids being of the same molecular weight):

CDF46b, CDF45b, CDF29 and CDF30b;

CDF28, CDF47, CDF20a and CDF42;

CDF35 and CDF18;

CDF23 and CDF'o'.

Plate 17. AGAROSE GEL ELECTROPHORESIS OF AN *Eco*RI DIGEST OF *CAULOBACTER* ISOLATES.

Lane 1.	Lambda *Hind*III size marker		
Lane 2.	CDF46b	}	
Lane 3.	CDF45	}	
Lane 4.	CDF28	}	group 1.
Lane 5.	CDF29	}	
Lane 6.	CDF30b	}	
Lane 7.	ATCC 15252		

Plate 18. AGAROSE GEL ELECTROPHORESIS OF A *Bam*HI DIGEST OF *CAULOBACTER* ISOLATES.

Lane 1.	Lambda *Hind*III size marker		
Lane 2.	CDF46b	}	
Lane 3.	CDF45	}	
Lane 4.	CDF28	}	group 1.
Lane 5.	CDF29	}	
Lane 6.	CDF30b	}	
Lane 7.	ATCC 15252		
Lane 8.	CDF35		
Lane 9.	CDF18		

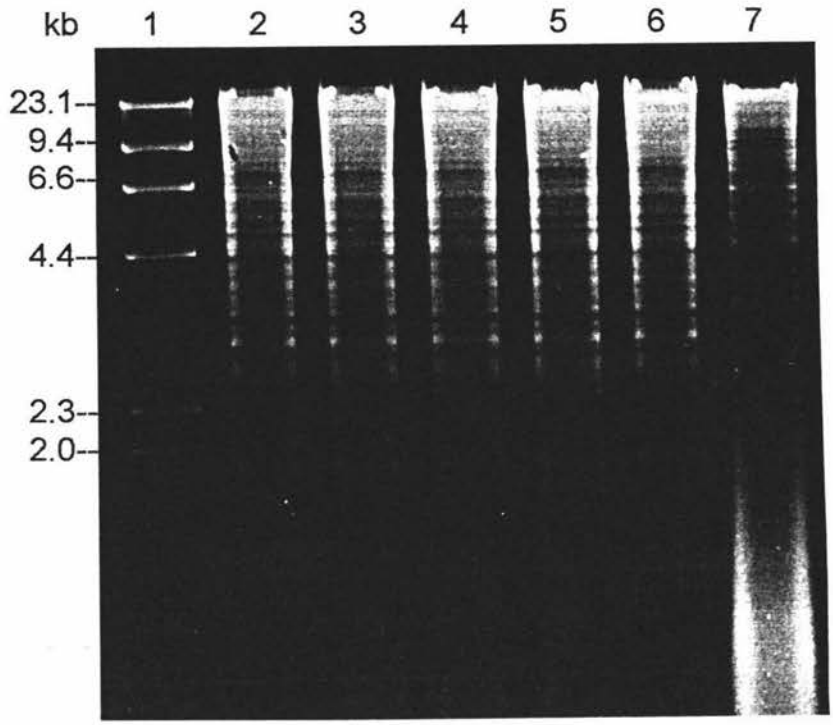


Plate 17.

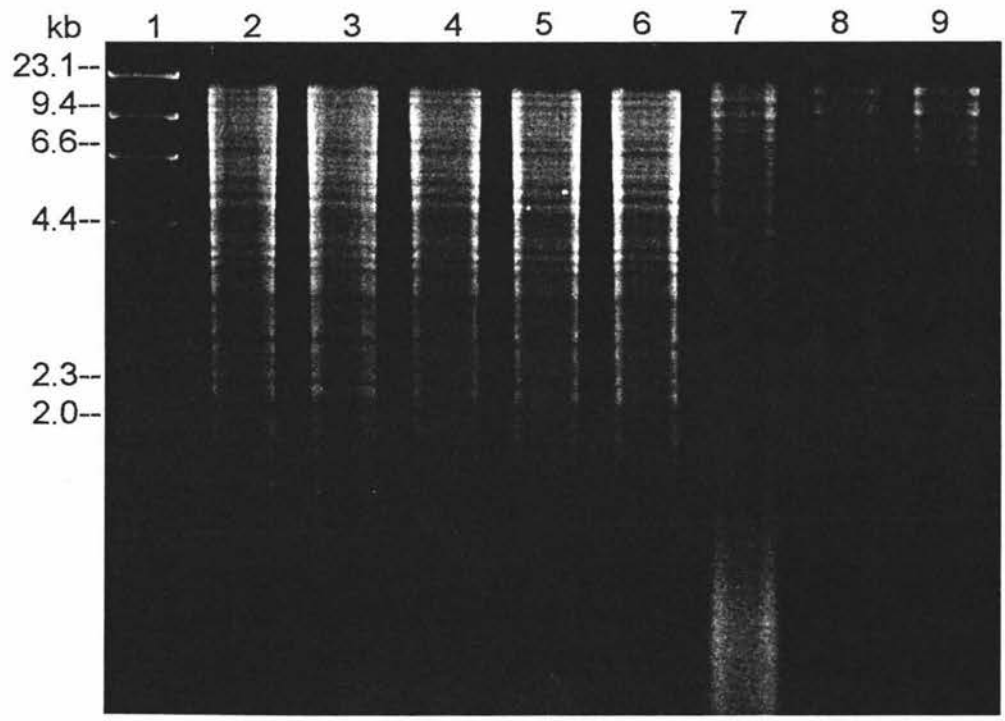


Plate 18.

Plate 19. AGAROSE GEL ELECTROPHORESIS OF AN *Eco*RI DIGEST OF *CAULOBACTER* ISOLATES.

Lane 1.	CDF47	}	
Lane 2.	CDF20a	}	group 2.
Lane 3.	CDF7	}	
Lane 4.	SW1	}	
Lane 5.	TW1	}	group 3.
Lane 6.	TW2	}	
Lane 7.	SW2		
Lane 8.	MR1		

Plate 20. AGAROSE GEL ELECTROPHORESIS OF A *Bam*HI DIGEST OF *CAULOBACTER* ISOLATES.

Lane 1.	Lambda *Hind*III size marker		
Lane 2.	CDF47	}	
Lane 3.	CDF20a	}	group 2.
Lane 4.	CDF7	}	
Lane 5.	SW1	}	
Lane 6.	TW1	}	group 3.
Lane 7.	TW2	}	
Lane 8.	SW2		
Lane 9.	MR1		

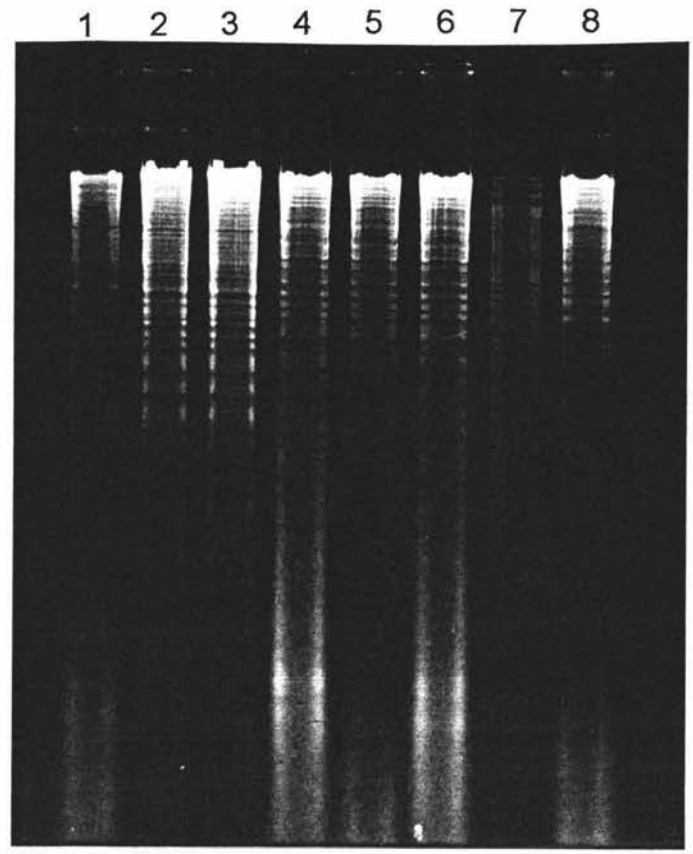


Plate 19.

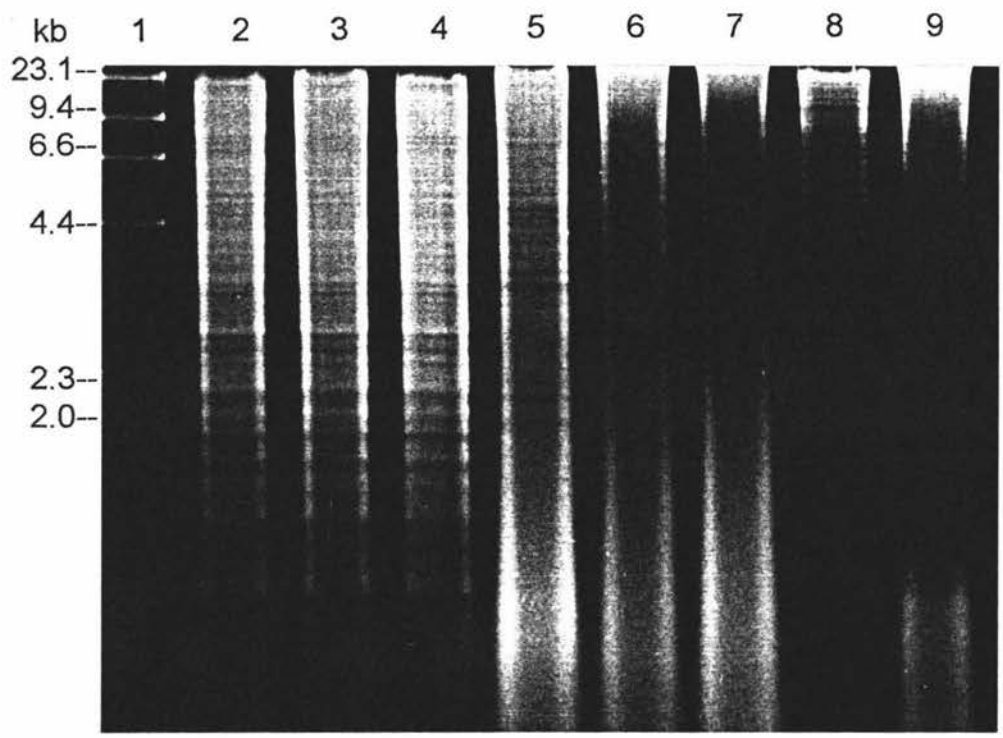


Plate 20.

Plate 21. AGAROSE GEL ELECTROPHORESIS OF AN *Eco*RI DIGEST OF *CAULOBACTER* ISOLATES.

Lane 1. Lambda *Hind*III size markers

Lane 2. CDF42

Lane 3. CDF20b

Lane 4. CDF9

Lane 5. CDF6

Plate 22. AGAROSE GEL ELECTROPHORESIS OF A *Bam*HI DIGEST OF *CAULOBACTER* ISOLATES.

Lane 1. Lambda *Hind*III size markers

Lane 2. CDF42

Lane 3. CDF20b

Lane 4. CDF9

Lane 5. CDF6

Lane 6. CDF'o'

Lane 7. CDF23

Lane 8. CDF5

Lane 9. TS1

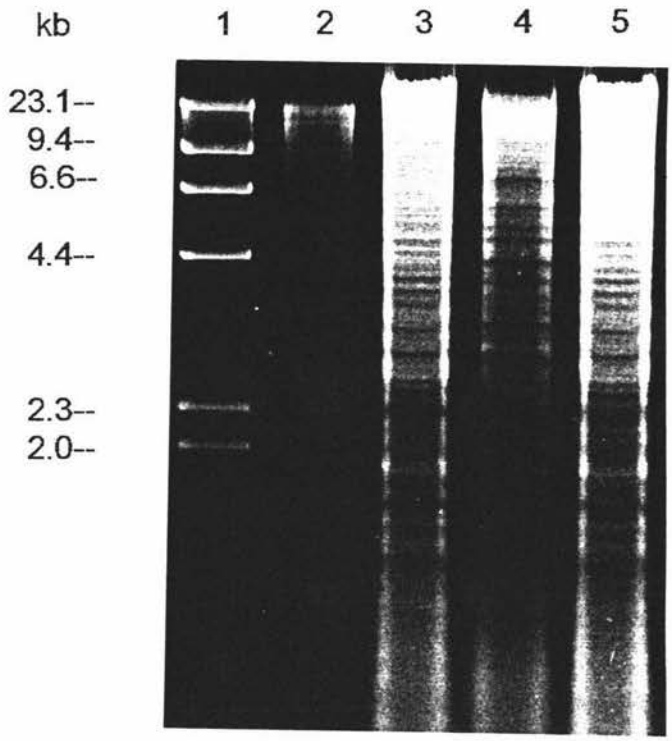


Plate 21.

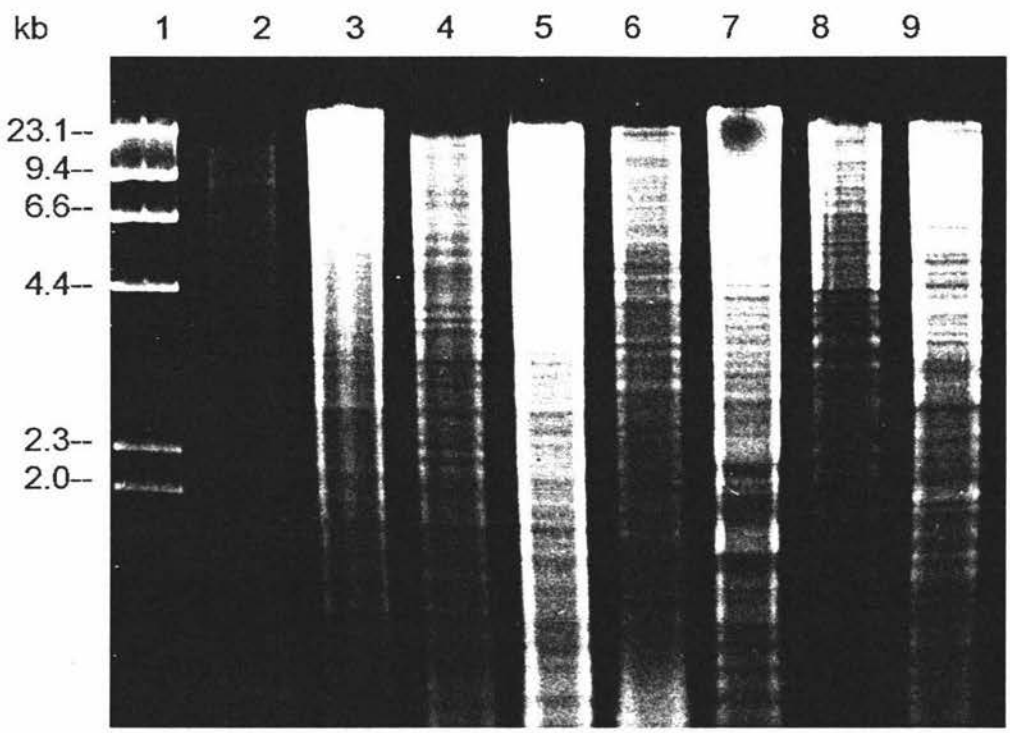


Plate 22.

Plate 23. ECKHARDT GEL ANALYSIS OF FRESHWATER *CAULOBACTER* ISOLATES.

Lane 1. MR1

Lane 2. TS1

Lane 3. SW1

Lane 4. TW1

Lane 5. TW2

Lane 6. ATCC 15252

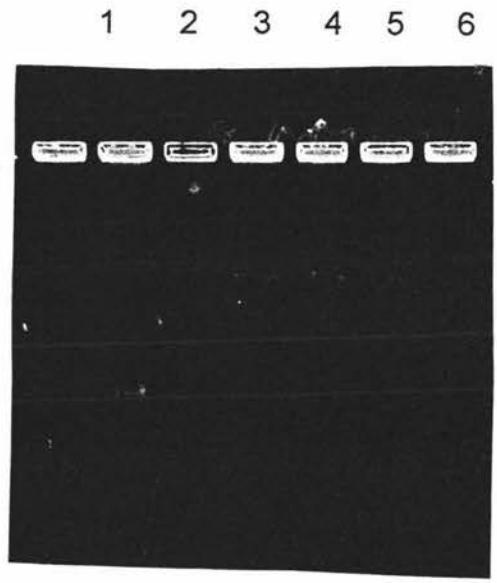


Plate 23.

Plate 24. ECKHARDT GEL ANALYSIS OF SEWAGE *CAULOBACTER* ISOLATES.

Lane 1.	CDF30b	
Lane 2.	CDF29	
Lane 3.	CDF28	
Lane 4.	CDF45	
Lane 5.	CDF46b	
Lane 6.	CDF18	
Lane 7.	CDF35	
Lane 8.	CDF7	
Lane 9.	CDF20a	
Lane 10.	CDF47	
Lane 11.	CDF23	
Lane 12.	MR1	} freshwater strains
Lane 13.	ATCC 15252	}

Plate 25. ECKHARDT GEL ANALYSIS OF RECOMBINANT *CAULOBACTER* CONTAINING pPN1.

Lane 1.	*E. coli* PN200
Lane 2.	MCDF100
Lane 3.	MCDF23

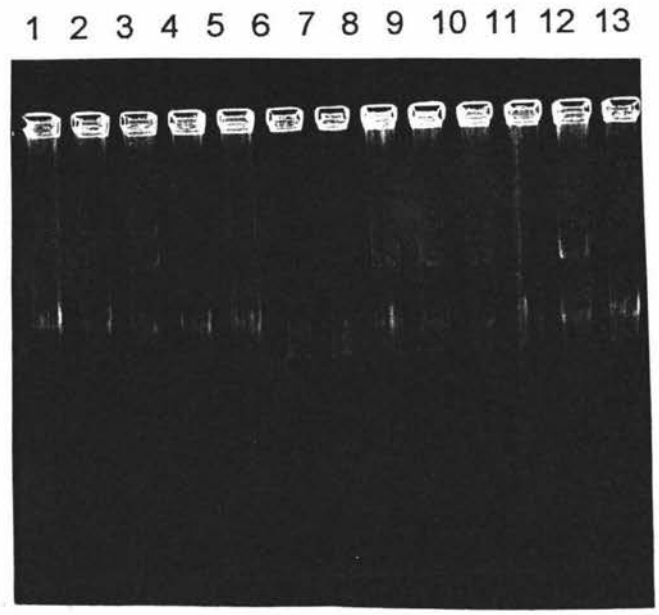


Plate 24.

Plate 25.

Out of the 18 isolates studied, similar plasmid profiles were observed in different host chromosomal backgrounds and different plasmids in similar host chromosomal backgrounds. For example, CDF28 had the same genomic digest pattern (plate 17 and 18) as CDF46b, CDF45b, CDF29 and CDF30b, yet their plasmid profiles are different.

4.2 Plasmid Transfer Experiments.

E. coli strain PN200 was used to transfer the co-integrate plasmid pPN1, containing symbiotic genes, to *Caulobacter* strain MCDF23 at frequencies of 1 in 10^{-5} to 1 in 10^{-6} (plate 25).

The transfer of antibiotic resistance plasmids by the other crosses was not detectable by either of the methods used (membrane filter method or the stationery broth method).

5. DNA ANALYSIS.

5.1 Polymerase Chain Reaction.

The polymerase chain reaction was successfully used to amplify a 260 bp 16S rDNA fragment from the environmental isolates used in this investigation (plate 26). Some of the isolates were grouped on the basis of having identical *EcoRI* and *BamHI* restriction endonuclease patterns. One isolate representing that group was sequenced.

CDF35 represents CDF18; CDF46b represents CDF45b, CDF28, CDF29 and CDF30b; SW1 represents TW1 and TW2.

The sequence data from this investigation is included in table 7.

Plate 26. **AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS.**

Lane 1. CDF35

Lane 2. CDF20b

Lane 3. CDF46b

Lane 4. TS1

Lane 5. MR1

Lane 6. SW1

Lane 7. ATCC

Lane 8. CDF42

Lane 9. Negative control

Lane 10. CDF'o'

Lane 12. Lambda *Hind*III size marker

Plate 27. **SEGMENT OF A DEVELOPED AUTORADIOGRAPH OF AN ACRYLAMIDE SEQUENCING GEL.**

Lanes 1 - 4 . Y1 primer direction

Lanes 5 - 8. Y2 primer direction

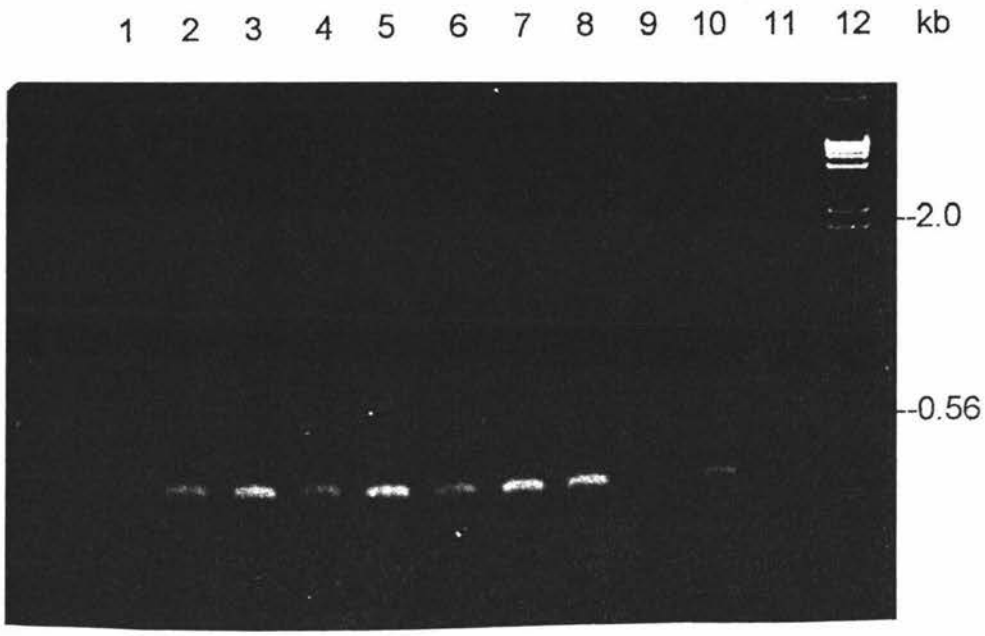


Plate 26.

Plate 27.

Table 7. **SEQUENCE DATA FROM ENVIRONMENTAL ISOLATES AND THE REFERENCE STRAIN.**

Aligned sequences of 16S rDNA genes from New Zealand *Caulobacter* sp. and reference strains from the α Proteobacteria corresponding to the *Caulobacter cresecentus* ATCC 15252 16S rDNA sequence between positions 50 -337 in the *E. coli* sequence.

Genus abbreviations used:

C	*Caulobacter*
P	*Pseudomonas*
M	*Mycoplana*
H	*Hyphomicrobium*
R	*Rhizobium.*

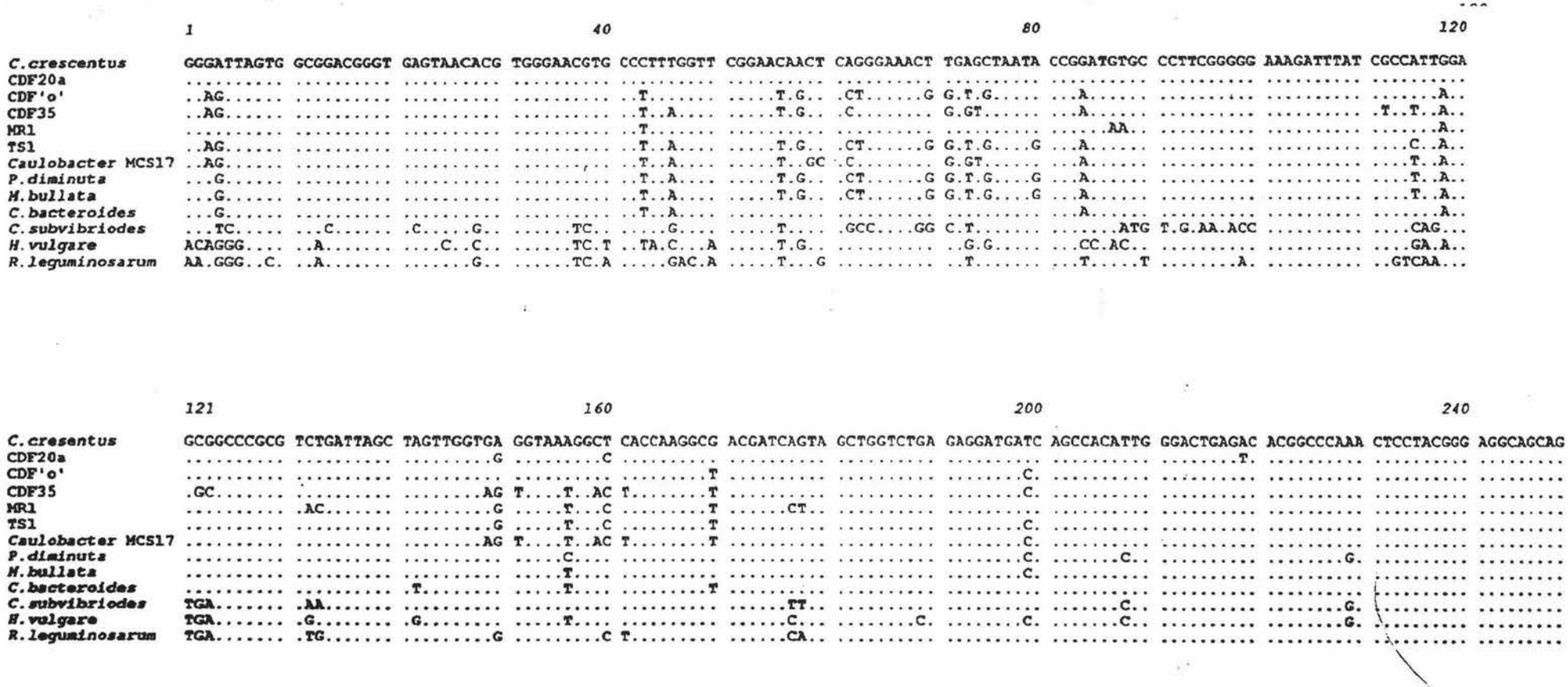

1
40
80
120
C.crescentus GGGATTAGTG GCGGACGGGT GAGTAACACG TGGGAACGTG CCCTTTGGTT CGGAACAACT CAGGGAAACT TGAGCTAATA CCGGATGTGC CCTTCGGGGG AAAGATTTAT CGCCATTGGA
CDF20a
CDF'o'
CDF35
MR1
TS1
Caulobacter MCS17
P.diminuta
M.bullata
C.bacteroides
C.subvibriodes
H.vulgare
R.leguminosarum
121
160
200
240
C.cresentus GCGGCCCGCG TCTGATTAGC TAGTTGGTGA GGTAAAGGCT CACCAAGGCG ACGATCAGTA GCTGGTCTGA GAGGATGATC AGCCACATTG GGACTGAGAC ACGGCCCAAA CTCCTACGGG AGGCAGCAG
CDF20a
CDF'o'
CDF35
MR1
TS1
Caulobacter MCS17
P.diminuta
M.bullata
C.bacteroides
C.subvibriodes
H.vulgare
R.leguminosarum

Figure 5. UNROOTED PHYLOGENETIC TREE CONSTRUCTED BY THE NEIGHBOR-JOINING METHOD.

The tree was constructed from a distance matrix with Jukes-Cantor corrections. Bootstrap probabilities are indicated at the branching points.

Genus abbreviations used:

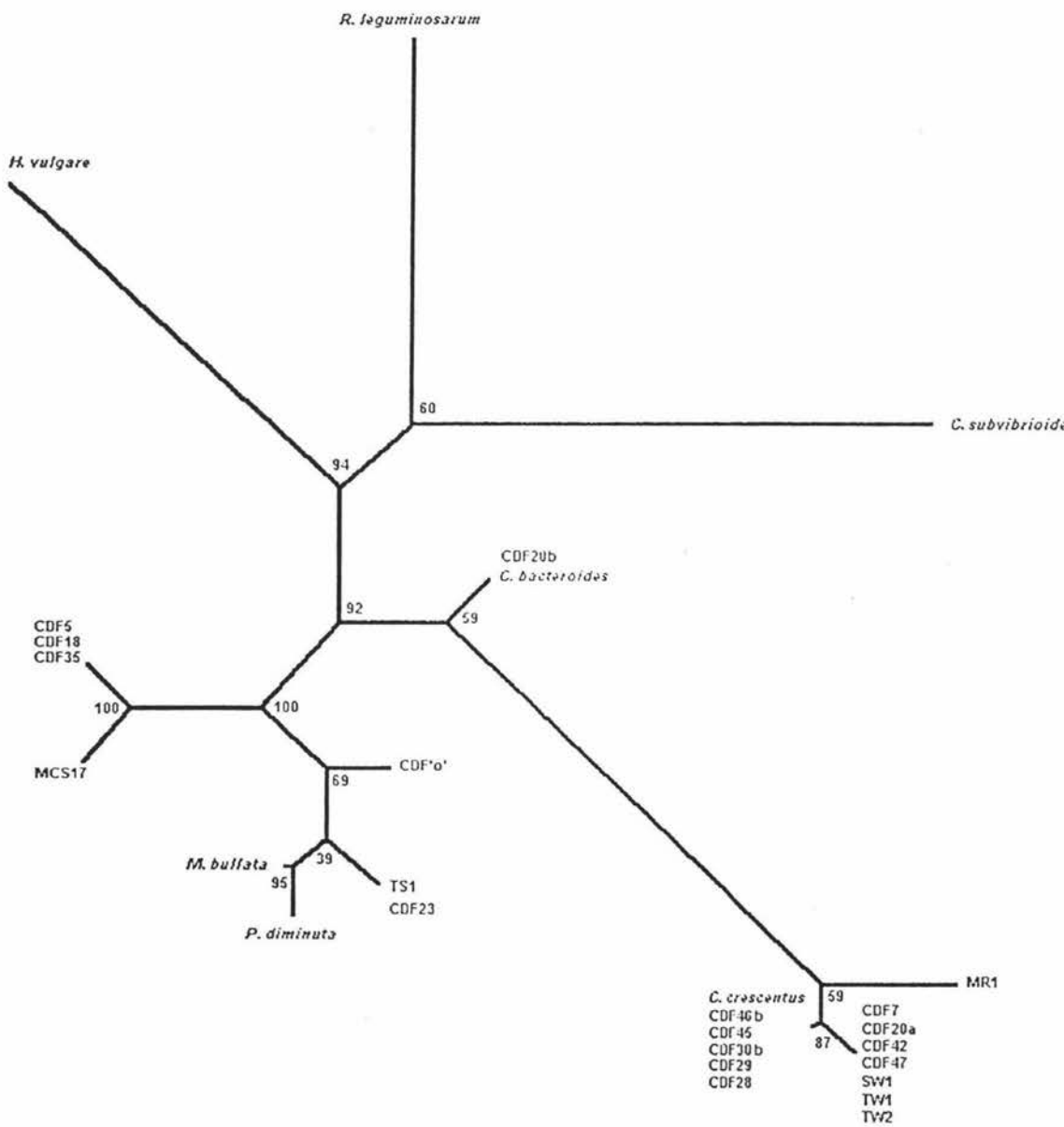
C *Caulobacter*

P *Pseudomonas*

M *Mycoplana*

H *Hyphomicrobium*

R *Rhizobium.*


R. leguminosarum
H. vulgare
C. subvibrioides
60
94
CDF20b
C. bacteroides
92
59
CDF5
CDF18
CDF35
100
100
MCS17
CDF'o'
69
M. bulfata
39
95
TS1
CDF23
P. diminuta
MR1
C. crescentus
CDF46b
CDF45
CDF30b
CDF29
CDF28
59
87
CDF7
CDF20a
CDF42
CDF47
SW1
TW1
TW2

DISCUSSION

1. Isolation and Enrichment

There are many publications on the genetics of *Caulobacter,* mainly because of its dimorphic life cycle, but very little on the microbiology and the ecology of it. Most of the studies were carried out on a few environmental isolates, some of which were isolated as early as the 1960's (Poindexter, 1964) and have been kept in laboratory culture ever since.

1.1 Identification of *Caulobacter* in Enrichment Cultures.

The literature which dealt with the enrichment and isolation of *Caulobacter* (Poindexter, 1964; Schmid, 1981; MacRae and Smit, 1991) failed to deal adequately with the problems associated with the isolation of *Caulobacter* from an enrichment culture. Most of the publications had photographs of isolates in a purified form which does not always represent the morphology of a *Caulobacter* in an enrichment culture. The length of the stalk, the formation of rosettes and the cell shape can appear different. Photographic evidence of the appearance of *Caulobacter* cells in an enrichment culture (as in this thesis) would have been useful. During the course of this investigation it was found that a wet mount was preferable to staining for the detection of *Caulobacter* cells. *Caulobacter* cells which had long stalks, as was usually the case, were detectable by their swaying movement. Focusing at different depths of field near the area of movement usually revealed a stalked bacterium.

1.2 Problems with the Isolation of *Caulobacter.*

The conditions under which an enrichment culture is incubated can influence the type of *Caulobacter* strains that dominate the population. The type of population present is influenced by the amount of illumination that the culture has, the amount of algae which was present, and the time of the year that the sample was taken (Schmid, 1981). Most of the strains mentioned in publications were isolated from Northern America. Based on information taken from the literature, it was decided that a pigmented *Caulobacter* would be the most common type present in the enrichments, under the conditions used in this investigation. Only one of the strains isolated from sewage was pigmented and the isolation of non-pigmented strains took longer than expected as they were initially over-looked. This is the first reported isolation of New Zealand *Caulobacter* species.

One of the enrichment cultures (Tiritea stream) contained a lot of another type of prosthecate bacteria (*Hyphomicrobium*, plates 6, 7 and 8). According to the literature reviewed, the media (PYEA, section 1.2.1) and procedure used to isolate *Caulobacter* strains should not have been suitable for the isolation of *Hyphomicrobium* (Poindexter, 1989). However, every initial attempt at the isolation of *Caulobacter* from the Tiritea stream resulted in the isolation of *Hyphomicrobium.*

The water from the Taranaki Base Hospital was the only sample taken where *Caulobacter* was not isolated. The hospital had been having a series of problems with contaminated water at the Blood Bank Unit. The contaminant appeared to be a 'webbed' bacteria (Dean Anderson, personal communication). The New Zealand Centre for Disease Control (Porirua, New Zealand), identified the contaminant as *Pseudomonas fluorescens.* As strains of

Caulobacter and some *Pseudomonas* species have been shown to be closely related, it was considered that the contaminant might have been a mis-identified *Caulobacter*. *Caulobacter* were present in the enrichment but not in sufficient numbers for it to be successfully isolated, nor to conclude that they were the mass contaminant.

In general, the best way to isolate *Caulobacter* is by the surface film method (Materials and Methods, section 1.7.1) using PYE medium and a long incubation period. The majority of the strains used in this study were isolated using this method but with a modification to published procedures in Poindexter, (1964) and MacRae and Smit, (1991). The surface film samples were washed repeatedly in 0.1% sarcosine to disperse clumps of bacteria and separate the cells before they were streaked on solid media. The length of time taken to isolate *Caulobacter* can sometimes be shortened by using the attachment and the physical isolation methods (Materials and Methods, section 1.6.1, 1.7.2) in conjunction with low phosphate PYE or PCa medium (Materials and Methods, section 1.2.2, 1.2.5). The low phosphate PYE medium helps in the detection of *Caulobacter* on solid media, because the stalks are elongated under low phosphate conditions (Poindexter, 1981b). For some *Caulobacter* isolates, the presence of yeast extract in the culturing medium can inhibit prosthecate development (Poindexter, 1989). PCa medium has no yeast extract. However, unless an enrichment culture has a high number of stalked bacteria in the surface film, isolation is still difficult.

None of the literature examined addressed the difficulties with purifying a bacterium that can adhere to other bacteria or debris. Normal streak plating methods often failed to completely disperse the *Caulobacter* cells even after they had been washed in 0.1% sarcosine and vortexed. As a final purity check,

each isolate was grown in PYE broth (inoculated from a single colony) and 0.1 ml was spread on solid media as outlined in Materials and Methods section 1.8. Colonies that had arisen from contaminating cells were obvious in the lawn.

2. Identification of Isolates.

2.1 16S rDNA Sequence Versus Phenotypic Characteristics.

The phylogenetic tree (figure 5) determined from a 260 bp sequence of 16S rDNA shows relationships comparable to those determined using complete 16S rDNA sequences (Stahl *et al.*, 1991).

Those isolates that phenotypically identified as *Caulobacter crescentus* (CDF# 46b, 45, 28, 29, 30b, 47, 20a, 42, TW1, TW2, SW1, and MR1) using the classification scheme in Bergey's Manual of Systematic Bacteriology (figure 1) had a 260 bp 16S rDNA sequence that showed high homology to the type strain *Caulobacter crescentus* ATCC 15252 (table 7). Other isolates (CDF# 20b, 23, 35, 5 and 18) would classically identify as *Caulobacter bacteroides* (figure 1) on the basis of phenotype. However, CDF20b was the only isolate that had the same 260 bp 16S rDNA sequence as *C. bacteroides*. The 260 bp 16S rDNA sequence from CDF35, CDF5 and CDF18 clustered closely with a marine *Caulobacter* sequence and these isolates can grow in NaCl concentrations which are normally inhibitory for freshwater isolates (Stahl *et al.*, 1992).

CDF23, TS1, and CDF'o', all branch off into a separate cluster of the phylogenetic tree (figure 5) and show high homology to the 260 bp 16S rDNA sequence of *Pseudomonas diminuta* (table 7). Using the phenotypic identification scheme outlined in figure 1, TS1 would be *Caulobacter*

crescentus, CDF23 would be *C. bacteroides* and CDF'o' would not be identifiable. CDF'o' has a straight cell shape, cannot tolerate NaCl, has an orange pigment and is sensitive to streptomycin and penicillin. Phenotypically this isolate would most likely be *C. fusiformis* except that it does not require complex growth factors other than those found in PYE medium.

3. Classification of *Pseudomonas* species.

The *Pseudomonas* family is a very heterogenous group. On the basis of rDNA/DNA homologies, *Pseudomonas diminuta* and *Pseudomonas vesicularis* should be excluded from the *Pseudomonas* family (Gilardi, 1985). They are retained at present due to uncertainty of their correct place in the phylogeny of the aerobic rods and cocci (Palleroni, 1984).

Caulobacter isolates CDF23, CDF 'o' and TS1 all showed high homology with a 260 bp fragment of the 16S rDNA sequence of *P. diminuta.* This supports studies done by MacRae and Smit, (1991) where a flagella filament protein gene probe was used to survey a group of non-*Caulobacter* isolates from waste water treatment systems. A single positive score was obtained from 150 isolates. This isolate was examined by the Biolog commercial identification scheme (which does not include *Caulobacter*) and a match to *P. vesicularis* was obtained (Stahl *et at.,* 1992). Also, one of their freshwater *Caulobacter,* when examined by the Biolog system, scored an acceptable match to *P. diminuta.* Like *Caulobacter,* both of these pseudomonads can be found in water systems, and can accumulate poly-β-hydroxybutyrate reserves (Palleroni, 1984). The DNA GC content is between 66.3-67.3% for *P. diminuta* and 65.8% for *P. vesicularis* compared to 62-67% for *Caulobacter.* By classical definition, a bacterium which does not posses a stalk, cannot be called a *Caulobacter.*

However, it appears that these pseudomonads would be more correctly identified as *Caulobacter* and it is conceivable that these species are *Caulobacter* strains locked in the motile phase (stalkless). Furthur studies on DNA/DNA relatedness to accepted reference strains will clarify the relationship.

4. Antibiotic Resistance.

In general, the antibiotic resistance of the sewage isolates seemed to be greater than the freshwater isolates (figure 4). The calculated mean of the zones of inhibition were greater for the population of sewage isolates compared to the freshwater strains (figure 6). A number of the sewage isolates from this investigation (CDF# 46, 45b, 30b, 29, 30b, 42) had identical partial 16S rDNA sequences to the freshwater *Caulobacter crescentus* ATCC 15252 (resistant to ampicillin), but were more resistant to chloramphenicol and completely resistant to penicillin and tetracycline (figure 4).

Classically, *Caulobacter crescentus* has no native plasmids (Ely, 1979). The sewage isolates had a number (2 or 3) of uncharacterised plasmids (plate 24), and the freshwater counterparts had one, or none (plate 23). During the course of this investigation, antibiotic resistance plasmids that were transferred by conjugation were not detectable under the conditions used. However, the evidence that this had occurred in other studies has been well documented (Ely, 1979; Anast, 1988) using broad-host range or mobilising plasmids.

A successful attempt was made to transfer the neomycin resistance carrying co-integrate plasmid pPN1 (Scott and Ronson, 1982) from *E. coli* PN200 to *Caulobacter* isolate MCDF23. The plasmid carries symbiotic genes from *Rhizobium leguminosarum* biovar trifolii and transconjugants were able to form

a tumour-like growth when inoculated onto white clover seedlings (Fenton, 1994).

Caulobacter are capable of taking part in the transfer of antibiotic resistance plasmids between human associated bacteria and other *Caulobacter* i.e, marine *Caulobacter* (Ely, 1979; Anast and Smit, 1988). Their widespread distribution coupled with their ability to survive in oligotrophic environments could play a role in reducing the life span of clinically important antibiotics. In the Palmerston North Sewage Treatment Plant the incubation times of the waste-water in the liquid aeration lagoons is 5 days and 30 days in the sludge digesters (Jones, 1994). This would be an adequate time for plasmid transfer to occur. When the treatment process is complete, the waste water is released into the Manawatu river, which flows to the Tasman sea.

5. Tolerance to NaCl.

Some of the sewage isolates (CDF 5, 18, 35) from this investigation had a 260 bp 16S rDNA sequence that showed high homolgy to a marine *Caulobacter* sequence and were also tolerant to salt (2.5%). Normally, freshwater *Caulobacter* can not tolerate NaCl, and marine *Caulobacter* require NaCl for growth. Stahl *et al.* (1992) carried out a phylogenetic study on the relationships between freshwater and marine *Caulobacter* and found that the two groups appeared to be separate. Affiliation with either one of the two phylogenetically distinct lines of descent generally corresponded to a marine or a freshwater habitat. The marine isolates that were freshwater tolerant (usually isolated from areas that had physical ties with freshwater environments) and the freshwater isolates that appeared to be genetically more similar to the marine *Caulobacter* than the freshwater strains, appeared to make up a distinct group within the freshwater line of descent. It was hypothesised by Stahl *et al.* (1992)

that some marine *Caulobacter* have terrestrial origins. The salt tolerant *Caulobacter* strains isolated from sewage during the course of this investigation have the potential to decrease the lifetime of antibiotics by increasing the resistance plasmids in the gene pool and aiding in the distribution of antibiotic resistant bacteria.

6. Direct Studies of the Environment.

Direct and determinative studies of the environmental distribution of *Caulobacter* will be facilitated by the use of phylogenetically based DNA probes. Probes complementary to regions that identify major groups within the species could be used to evaluate marine and freshwater environments directly, without the problems associated with the identification of *Caulobacter*. Hybridisation probing with genes for surface array proteins has been successful (Stahl *et al.*, 1992).

The amplification of a region of the 16S rDNA by the polymerase chain reaction has potential in the identification of *Caulobacter* species if a primer specific for each species could be used. From the sequence data shown in table 7, it may be possible to confirm the presence of marine *Caulobacter* strains in a sample by using an 8 bp primer (CTGGGTCT). The suitability of any particular sequence to act as a diagnostic aid and the practicalities involved with amplification directly from samples would have to be investigated further.

CONCLUSION

This investigation has shown the presence of *Caulobacter* in New Zealand sewage and freshwater environments.

Some isolates (TS1, CDF23 and CDF'o') appear to be more phylogenetically related to *Pseudomonas diminuta* than to other recognised *Caulobacter* species. It is suggested that *P. diminuta* is a stalkless *Caulobacter* and could be renamed *Caulobacter diminuta*.

Some of the sewage *Caulobacter* isolates are salt tolerant. The widespread distribution of *Caulobacter* coupled with their ability to survive in oligotrophic environments could play a role in reducing the life span of clinically important antibiotics.

The significance of *Caulobacter* in the environment has probably been overlooked due to the difficulties in correctly identifying their presence. Amplification of part of the 16S rRNA gene could aid in the identification of isolates without relying upon morphological characteristics.

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