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

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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.

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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)

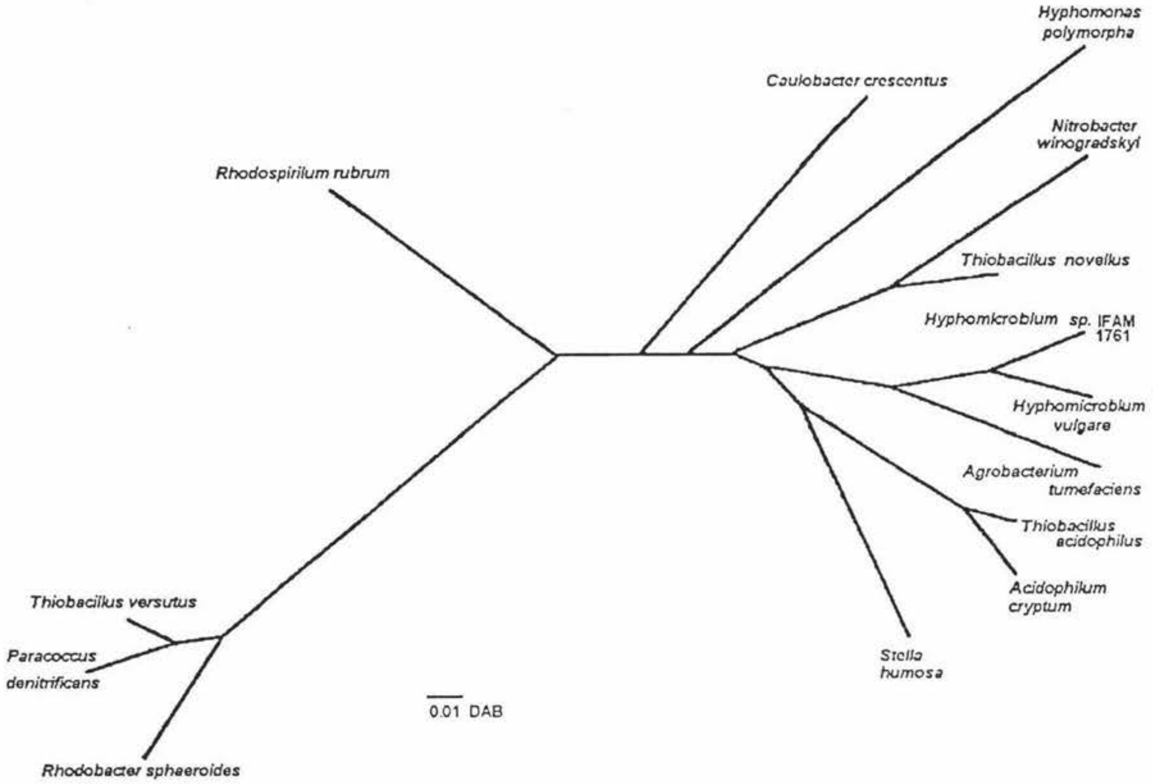
II. Cells not tapered

- 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 DAB values.


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

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°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 *Caulobactei* 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.