CHARACTERIZATION OF CAULOBACTERS ISOLATED FROM WASTEWATER TREATMENT SYSTEMS AND ASSAY DEVELOPMENT FOR THEIR ENUMERATION

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ABSTRACT

Caulobacters are gram-negative bacteria that have a biphasic life cycle consisting of a swarmer and a stalked stage. As a result they have elicited interest as a simple developmental model. Less attention has focussed on their role in the environment, although they have been found in almost every aquatic environment as well as in many soils. Caulobacters are often described as oligotrophic bacteria because of their prevalence in pristine waters but have now been isolated from the relatively nutrient-rich wastewater environment. In order to learn more about this population some basic characterization was carried out and an assay system to determine their prevalence in sewage plants was designed.

Most of the organisms isolated from sewage treatment facilities had similar gross morphological features, but differed in holdfast composition, total protein profile, antibiotic resistance and restriction fragment length polymorphism, thereby indicating a greater diversity than originally assumed. Most of the organisms hybridized with flagellin and surface array genes that had previously been cloned, and only one of 155 non-Caulobacter sewage isolates hybridized with the flagellin gene probe; consequently these were used in a DNA-based enumeration strategy.

DNA was isolated directly from sewage and probed with the flagellin and the surface array gene probes. The signals obtained were compared to standards made up of pooled Caulobacter DNA from the sewage isolates and non-Caulobacter DNA from organisms also present in sewage. Using this assay Caulobacters could only be detected above the 1% level, which was higher than their proportion

in the wastewater environment. It appears that this approach will not be useful in monitoring Caulobacters in treatment plants unless a more highly conserved or higher copy number probe is found.

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ABBREVIATIONS

AS	activated sludge
BSA	bovine serum albumin
CGY	casitone glycerol yeast extract
DNA	deoxyribonucleic acid
EDTA	ethylendiaminetetraacetic acid
EtBr	ethidium bromide
FA	fluorescent-antibody
FWC	freshwater Caulobacter
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Kb	kilobase
PAGE	polyacrylamide gel electrophoresis
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
PYE	peptone yeast extract
RNA	ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SSC	standard sodium citrate
SSPE	standard sodium phosphate EDTA
TAE	tris acetate EDTA
TBE	tris borate EDTA
TE	tris EDTA
TF	trickling filter
UV	ultraviolet

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INTRODUCTION

Water, vital to the survival of every life form, is the most important resource on earth. It is clearly important that safe and clean water supplies be maintained. Since human population densities have increased to the point that the ability of water systems and land to recycle our wastes has been surpassed, solutions to the problem of dealing with the excess have had to be addressed. Untreated wastewaters produce malodorous gases, favour the growth of undesirable marine organisms and harbour pathogenic organisms if they are allowed to accumulate. Decisions have to be made about which compounds must be removed from the waste, what the environment can endure and how to deal with the solid residual or sludge after the treatment process (72).

Primary treatment removes solids from the water which is then discharged into the receiving water. This approach is only viable when the receiving water is large enough to prevent overgrowing by the resident organisms or eutrophication. In most urban settings the organic load of domestic sewage exceeds the capacity of the receiving water and must be further treated to reduce that load. Engineers have designed treatment systems which are either biological or chemical processes that remove most of the organic carbonaceous material (or other materials in tertiary treatmant plants) from the used water, which can then be returned to the environment with less negative effect. Biological treatment systems are favoured because they are relatively inexpensive and produce sludges that are generally less offensive than those created by chemical treatment. These processes exploit the ability of

microorganisms, especially bacteria, to remove soluble organic material from water and convert it into cell components and energy for their growth (72).

Many advances have been made in the engineering field to effect the removal of other components such as phosphates and nitrates from the effluent. The Bio-P design, for example, was based on the observation that sludges that were exposed alternately to anaerobic and aerobic conditions tended to remove more phosphate from the water than sludges under constant aeration. Further study showed that phosphate is released from the organisms in the anaerobic zone while fermentation products are taken up and used to produce poly-ß-hydroxybutyrate (PHB) granules. These "primed" organisms are then more efficient at phosphate uptake and polyphosphate accumulation in the aerobic zone. This is an important advance because one of the leading causes of eutrophication of receiving waters is an excess of phosphate in the effluent (66).

Despite the fact that wastewater treatment is absolutely essential to protect our water supply, very little is known about the microbiology of the sewage environment (46). The design of sewage plants is therefore based on empirical data rather than a complete understanding of the ecosystem. As a result, most treatment facilities are probably operating sub-optimally. It seems likely that with the increasing world population, cheaper and more efficient systems will have to be designed to ensure the integrity of safe water sources. This can only be achieved through a better understanding of the underlying principles involved.

Research into the microbiology of sewage treatment has been hampered by the lack of appropriate tools. The classical microbiological technique of culturing organisms in order to

characterize the population is inadequate since only a small fraction of the organisms can be successfully cultured. In one study of marine planktonic bacteria it was shown that less than one percent of the bacteria observed in total counts were cultivable, although by microautoradiography 40-90% were shown to be metabolically active (19). The selective process of culturing may also allow some bacteria to grow that are usually held in check by environmental conditions or other organisms. Better methods will be required in studies on the populations of most natural habitats.

Clinically important bacteria like Salmonella have received a lot of attention since quick, accurate and inexpensive tests are needed to check foods that may be at risk of contamination. A number of antibody and gene probes are now available for the detection of Salmonellae so that samples may be enriched and probed without having to rely on obtaining pure cultures. One such probe was also used by Knight et al. (30) to directly enumerate Salmonellae in estuaries. They found that they could detect the organisms in many samples including some from which they were unable to obtain cultures. Another recent and interesting method has been developed that uses transducing phage containing the ice nucleation gene to enumerate Salmonallae (74). This assay is sensitive enough to obviate the need for enrichment or amplification steps in the protocol.

Vibrio cholerae is another organism for which alternatives to culturing have been found for its detection in waters. Brayton et al. (9) found that higher counts were obtained in all samples when using fluorescent-antibody (FA) direct counts than viable counts by most probable number. Huq et al. (26) used the FA technique as well as conventional culturing techniques to detect *Vibrio cholerae* in

water sources and made similar observations. They were able to show that viable but non-culturable cells exist in the waters of Bangladesh and postulated that the water may act as a reservoir for these organisms, especially when they are attached to aquatic plankton.

A DNA-based method was recently used to compare the populations from several aquatic sources (35). The DNAs isolated from each source were spotted onto filters and reciprocally probed with a labelled sample from the other source. This allowed the investigators to determine the degree of similarity among populations, which they found to be quite low despite the widely held view that aquatic bacterial populations are not very diverse. However, little information about the identities of the organisms present could be established.

Another group labelled the test sample and probed filters spotted with genes specific to a variety of organisms of interest (13). This enabled them to screen samples for several organisms simultaneously. This approach could be of interest if waters, for example, needed to be tested for the presence of a number of different pathogenic organisms.

DNA probes have been used by a number of groups for the detection of specific genes found in organisms present in the environment. Mercury resistance genes (7), toluene and naphthalene catabolic genes (52), polio virus (51), and PCB degrading genotypes (69) are among the genes that have been monitored in this way. The polymerase chain reaction (PCR) technique has been used to increase sensitivity when dealing with genetically engineered organisms (64, 11).

Immunobinding assays have also been used to detect and enumerate organisms from environmental samples. In one such study *Thiobacillus ferrooxidans* was targetted (3), ammonia and nitrite oxidizing organisms in another (71), but this approach has received most attention from workers interested in anaerobic bacteria since they are more difficult to culture. Macario (36, 37) and others have established the specificity of a large number of monoclonal antibodies as well as polyclonal antisera to methanogens. The binding "fingerprints" of many methanogens are now known and have been used to identify the populations in anaerobic sludge digesters by immunofluorescence and slide immunoassays. Antibody techniques have also been used to identify and enumerate anaerobic bacterial species in rumen (10).

Because of the culturing problems encountered when dealing with anaerobes and the difficulty of distinguishing between different Bacteroides species, it is not surprising that workers in this area have developed gene probing techniques. In 1983, Salyers et al. (53) produced a bank of randomly cloned fragments of *Bacteroides thetaiotaomicron* from which they were able to obtain a probe that was specific to that organism. Later Kuritza et al. used a similar approach to obtain probes for several other Bacteroides species and strains (31,32,33) which were used to identify and enumerate Bacteroides strains in fecal samples. Attwood et al. (4) found they could monitor the fate of *Bacteroides ruminicola* introduced into the rumen of sheep using a similar approach.

A different method was adopted by Stahl et al. (63) where studies were carried out using rRNA based probes to label total nucleic acids (mostly rRNA) extracted from rumen samples. In this case they were looking at changes in the proportions of two organisms

under different conditions and did not relate their results back into absolute numbers of organisms present. They did argue that since the number of ribosomes in an organism reflects its metabolic activity, the proportion of its ribosomes in the sample should reflect the organism's metabolic significance.

Phylogenetically-based probes like 16S rRNA probes can be used to detect strains, species, classes or kingdoms of organisms. Probes which recognize all Archaebacteria, eubacteria and eukaryotes but do not cross the primary divisional lines have been constructed, and a "universal" probe that recognises all ribosomes (that have been sequenced) has also been defined (22). At the other extreme, probes generated to more variable domains of the rRNA can be highly specific. For example, virulent strains of *Yersinia enterocolitica* can be distinguished from non-virulent strains (28). This kind of probe has now been used for *in situ* hybridization as well as for probing RNA extracts (14). Recently a fluorescent oligonucleotide was used to label cells from aquatic sources which were then enumerated either by phase contrast-fluorescence microscopy or by flow cytometry (1), and in a similar manner *Nitrosomonas* species were enumerated in sewage plants (68).

Caulobacters are gram-negative bacteria that have a biphasic life cycle consisting of a sedentary stalked stage and a motile swarmer stage. Stalks appear to be extensions of the cell envelope and do not contain cytoplasmic materials. At the end of the stalk or the base of the flagellum in the motile stage, is an adhesive, probably polysaccharide, holdfast material which serves to anchor the cell to surfaces. The stalked cell gives rise to one stalked and one swarmer cell at each division and the swarmer cell can then move about the medium until it loses motility and stalk initiation begins. It has

been postulated that attachment to surfaces via the holdfast occurs during the swarmer cell stage since the probability of collision with the surface is much higher and the mechanical force required to overcome electrostatic repulsion from the surface is provided. The cell would then eject the flagellum and initiate stalk development for the next round of division (49, 50).

In nature Caulobacters have been found in most aquatic environments (barring samples from Antarctica) and many soils. They are chemoorganotrophic and are often cited as classical examples of oligotrophs being most numerous and proportionally significant in pristine, clear waters. In soils they appear to be most prevalent when the pH is near neutrality and the moisture content is high (48, 49, 50).

Because of their presumed preference for environments with low suspended particulate concentrations and heterotroph counts, it was not anticipated that they could be isolated from wastewater treatment facilities. They were, however, successfully isolated from almost every type of treatment system that was analysed. The only exception was an anaerobic sludge digester. This was also interesting because Caulobacters can produce polyhydroxybutyrate granules as well as polyphosphate granules (48, 56), and there was some indication that the prevalence of Caulobacters in the enrichment cultures from Bio-P systems was somewhat higher than in conventional activated sludge systems. The perceived increase in numbers could simply be an artifact of the enrichment procedure or it could be due to the longer cell residence time typically seen in these plants, which might allow more Caulobacters to be maintained in the system. Alternately, it could indicate that Caulobacters are

involved in the phosphate uptake process and are selected for in this system.

To determine if Caulobacters play a role in the important process of biological phosphate removal, and to monitor their responses to different sewage plant operating schemes, a method of enumeration is required. Some of the basic characteristics of our collection of sewage isolates were studied to see what unified and distinguished the isolates. It was noted that flagellin and surface array gene probes were capable of hybridizing with chromosomal DNA from most strains, so these were used in a DNA hybridization enumeration strategy.

METHODS AND MATERIALS

1. Characterization:

1.1 Isolation of strains:

1.1.1 Freshwater Caulobacters (FWCs):

These strains were isolated by J. Smit (Department of Microbiology, U.B.C.) by diluting sewage samples 1/1000 in 0.01% (w/v) peptone. The samples were allowed to incubate at room temperature without shaking for 3-7 days after which a loopful of the surface film was diluted and plated onto peptone yeast extract (PYE) plates (47). The colonies were examined by phase contrast microscopy and Caulobacters were purified and stored at -70°C in 10% dimethyl sulfoxide.

1.1.2 Non-Caulobacter sewage isolates:

Strains from A. Chu (Department of Microbiology, U.B.C.) were isolated by dilution and plating on casitone glycerol yeast extract (CGY) (72) and were identified using the Biolog identification system (Biolog Inc. Hayward, California). Otherwise, strains were isolated by dilution and plating on PYE and examined by phase contrast microscopy to confirm they were not Caulobacters.

1.2 Cultures:

Freshwater Caulobacters isolated from sewage were grown at 30°C in PYE media supplemented with 0.1 g/l $CaCl_2.2H_2O$, 0.2 g/l $MgSO_4.7H_2O$ and vitamin B_2 (1 µg/ml). Laboratory strains were grown on media lacking the vitamin supplement.

1.3 Lectin binding assay:

This assay was carried out as previously described (40). In short, fluorescein isothiocyanate-conjugated lectins (Vector Labs, Inc. kit I containing concanavalin A, *Dolichos biflorus* agglutinin, peanut agglutinin, *Ricinus communis* agglutinin, soybean agglutinin, *Ulex europaeus* agglutinin and wheat germ agglutinin) were incubated on ice with mid-log cultures for 15-30 minutes, washed twice with distilled water and the cell pellets suspended in 20 mM potassium phosphate, 50% glycerol, 2% N-propyl gallate. Labelling was then observed by fluorescence microscopy.

1.4 Antibiotic sensitivity:

Difco Dispenso-discs were placed on PYE plates overlaid with soft agar containing an inoculum of log phase cells. The plates were then incubated at 30^{o}C for 24-48 hours and the zones of inhibition measured in mm.

1.5 Isolation of high molecular weight plasmids:

Plasmids were isolated by the method of Kado and Liu (27). A 5 ml stationary culture was pelleted and the cells suspended in 1 ml TAE (40 mM Tris, 2 mM EDTA pH 7.9 with acetic acid). Two volumes of lysing solution (3% SDS, 50 mM Tris pH 12.6) were added and the mixture briefly agitated before incubation at 65^{o}C for 20 minutes. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was mixed with the lysed cell material and the tube centrifuged to separate the phases. Fifty µl of the aqueous phase was electrophoresed on a 0.7% agarose/TBE gel (38).

1.6 Total protein profiles:

A 5 ml late log phase culture was centrifuged and the cell pellet was suspended in distilled water and centrifuged again. This pellet was frozen, thawed and suspended in 250 µl TE (10 mM tris, 1mM EDTA pH 8) with 1 µl 0.5 mg/mL DNAse, 20 µl 10 mg/ml lysozyme and 3 µl 1 M $MgCl_2$, mixed and incubated at room temperature for 10-15 minutes before storing at -20°C. Protein concentrations were determined by a modified Lowry assay (39) and approximately 40 µg of each sample were electrophoresed on a 10-17% gradient sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) (34, 73). The gels were stained with Coomassie blue and destained with 40% methanol/10% acetic acid.

1.7 Surface array extracts:

Twenty-five ml cultures were centrifuged and the cell pellets suspended in 1 ml 0.1M HEPES pH 2 buffer. This was allowed to stand at room temperature for five minutes, centrifuged twice to remove the cells and the supernatant was neutralized with 10 M NaOH.

1.8 Western blot analysis:

Total proteins and surface array extracts were electrophoresed on a 12% polyacrylamide gel using a Bio-Rad Mini-protean II dual slab cell (Bio-Rad Laboratories. Richmond, California), blotted onto nitrocellulose (58) and labelled with antiserum raised against *E. coli* expressing the major surface array protein of *C. crescentus* CB15 (59).

1.9 Colony hybridizations:

Caulobacter isolates were patched onto PYE plates and allowed to grow at 30°C for 24-48 hours. Gelman Bio-Trace RP filters were pressed onto the plates then floated colony side up in puddles of the following solutions: twice in 0.5 M NaOH/0.5 M NaCl, twice in 1 M Tris-HCl (pH 7), and twice in 1.5 M NaCl/0.5 M Tris-HCl (pH 7). The air dried filters were soaked in 2x SSC (20 xSSC= 3 M NaCl, 0.33 M sodium citrate) for three minutes, air dried and baked at 80°C under vacuum for two hours. The fixed filters were then soaked for 24 hours in 3x SSC/0.1% SDS at 65°C with several buffer changes. Any cell debris left on the filters at this time was removed by spraying with buffer from a pasteur pipette.

Prehybridization was carried out at 65°C for 2 hours in 5x SSC, 0.1% sarcosyl and 2 mg boiled, sonicated salmon sperm DNA. $1x10^6$ cpm nick-translated (38) probe [either the 0.9 kb flagellin gene from pCA161 (41) or the 4.4 kb surface array gene from pUC13A19 (59, 17)] was boiled for three minutes, chilled on ice and added to the prehybridization mix. Hybridization was allowed to continue overnight at 65°C. The filters were then washed twice in 3x SSC, 5 mM EDTA, 0.02% sarcosyl and twice in 0.3x SSC, 0.5 mM EDTA, 0.002% sarcosyl at 65°C for 30 minutes [allows approximately 20% mismatch (55)]. Autoradiography of the dried filters was carried out at -70°C overnight with Kodak X-OMAT AR film (Eastman Kodak Co. Rochester, NewYork) (38.).

1.10 Southern blotting:

DNA was extracted from each isolate (38) and 1 µg was digested with *Bam*HI, *Eco*RI, and *Hind*III. These were electrophoresed on 0.7% Agarose/TBE gels at 50-75 V overnight and blotted onto

Hybond-N nylon filter paper (Amersham) The blots were prehybridized in 6x SSPE (20x SSPE= 3 M NaCl, 200 mM EDTA, 200 mM sodium phosphate, pH 7.4), 5x Denhardt's solution [100x Denhardt's= 2% bovine serum albumin (BSA), 2% Ficoll 400, 2% polyvinylpyrollidone (PVP)], 0.5% SDS and 0.02 mg/ml single stranded salmon sperm DNA for 2 hrs. then hybridized with 2-5x10^{6}cpm nick-translated surface array or flagellin probe overnight. Washes were carried out at 55^{o}C in 1x SSPE/0.1% SDS (allows approximately 43% mismatch) and the blots exposed to autoradiographic film at -70^{o}C for 48 hrs (38).

2. Enumeration

2.1 Disinfection of samples:

Samples of trickling filter (TF) scrapings or sludge were added to a 50 mL tube containing 2 ml of Environ D (1/25 dilution; the recommended strength for disinfection of surfaces etc. is 1/200). Environ D (Vestal laboratories, St. Louis) contains o-benzyl-p-chlorophenol, 5%; o-phenylphenol, 4.6%; p-tertiary amyl-phenol, 4.5%; 2,2'-methylene bis(3,4,6-trichlorophenol), 0.4%.

2.2 Extraction of DNA from sewage samples:

2.2.1 Cell disruption:

Twenty ml samples that had been frozen and thawed, were sonicated with three 60 second bursts with a Braun-Sonic 2000 ultrasonic homogenizer (B. Braun Instruments, Burlingame, California) using the intermediate probe at maximum output in the presence of 125 mM EDTA and 0.2 g acid washed insoluble

polyvinylpolypyrollidone (PVPP). The samples were then incubated for 10 minutes with 5 mg lysozyme at 37^{0}C. SDS was added to a final concentration of 1% and the samples transferred to a 65^{0}C incubator. After 10 minutes, 2 mg proteinase K was added and digestion was allowed to continue for 1-2 hours.

2.2.2 Precipitation of DNA:

The cell lysate was centrifuged for 15 minutes at 17,000xg and DNA was precipitated from the supernatant with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol or 0.6 volumes isopropanol at -20^{0}C overnight. The supernatant fluid was removed after centrifugation for 15 minutes at 17,000xg.

2.2.3 Purification of DNA:

The pellets were dissolved in 4 ml TE, the resultant volume in mL was determined and an equal number of grams of cesium chloride was added. The solution was transferred to Quickseal tubes, mixed with 100 µl 10 mg/ml ethidium bromide (EtBr) and centrifuged in a Beckman VTi65.2 rotor for 5 hours at 62,000rpm or for 18 hours at 55,000 rpm in a Beckman L8-70 ultracentrifuge (Beckman Instruments, Inc. Fullerton, California). The DNA bands were removed with a syringe and transferred to a new tube, topped up with 1 g/ml CsCl and 50 µl 10 mg/ml EtBr and centrifuged as above. The EtBr was repeatedly extracted with TE saturated butanol until the butanol phase was colourless. The DNA was diluted three fold with sterile water and precipitated with 1/10 volume 3 M sodium acetate and 2.5 volumes ethanol at -20^{0}C overnight. The pellet, after centrifugation for 15 minutes at 17,000xg, was dissolved in 0.7 ml TE, reprecipitated, washed with 70% ethanol, and made up to a final volume of 100 µl.

The concentration of DNA was determined by absorption at 260nm

using a LKB Biochrom Ultrospec II (LKB Biochrom Ltd. Cambridge, England) and by comparison to double stranded salmon sperm DNA standards on agarose/EtBr plates viewed under UV light. Samples whose A_{260}/A_{280} ratios were greater than or equal to 1.8 were considered pure enough to use. If the ratio was too low, the DNA was again precipitated with isopropanol and washed with 70% ethanol, and if the low ratio persisted the sample was further CsCl purified.

2.3 Determination of probe specificity:

The specificity of the probes was determined by colony hybridization of Caulobacter and non-Caulobacter isolates as described previously.

2.4 Standard DNAs:

DNA was extracted from both Caulobacter and non-Caulobacter isolates as follows: stationary phase Caulobacter cultures were combined to form one pool and the non-Caulobacter sewage isolates formed another. Cell pellets from 50 mL pooled cultures were suspended in 10 ml TE and incubated first at 37°C with 0.5 mg lysozyme, then at 65°C with 1% SDS and 0.2 mg proteinase K. DNA was precipitated from the cleared supernatant and purified on CsCl gradients as described above. The final concentration of each was adjusted to 0.4 mg/ml and the two were combined in various proportions to give 100%, 10%, 1%, 0.1%, 0.01%, 0.001%, and 0% Caulobacter DNA in a background of non-Caulobacter DNA.

2.5 Dot blots:

One volume of 0.4 M NaOH/4 mM EDTA was added to the DNA samples to be blotted and allowed to sit at room temperature for ten minutes. The samples were then neutralized by the addition of 1/10 volume of 2 M ammonium acetate and immediately placed on ice until being applied to the Hybond-N filters using a Hybri-dot manifold. The filters were fixed at 80°C for 30 min. and prehybridized at 65°C in 5x SSC, 0.1% sarcosyl, 0.02% SDS and 0.1 g Boehringer-Mannheim blocking reagent for 1.5 hours. Hybridization with 2-24x10^6 cpm nick translated probe (38) or probe generated by random primer extension (15) was carried out overnight at 65°C. The blots were washed at room temperature with 2x SSC/0.1% SDS, then 1x SSC/0.1% SDS and twice at 65°C with 1x SSC/0.1% SDS (allows approximately 33% mismatch). X-ray films were exposed at -70°C overnight. Alternately, the blots were probed and developed with the Boehringer-Mannheim non-radioactive probing kit (Boehringer Mannheim GmbH, Mannheim, West Germany) according to the manufacturer's instructions using the washing regime described above.

When counts were to be determined, the dots were cut out of the filter using a hole puncher. Merit liquid scintillation fluid (Isolab, Akron, Ohio) was added to the vials and ^{32}P counts were taken for 2 or 10 minutes in a Beckman LS6000IC Liquid Scintillation System (Beckman Instruments, Inc. Fullerton, California).

RESULTS

1. Characterization:

Table I shows a list of the sources of the freshwater Caulobacters (FWCs) isolated by J. Smit. Most of the Caulobacters seen at the air-water interface of the enrichment media and subsequently most of the isolates preserved had a number of characteristics that identified them as the "typical" Caulobacter detected in wastewater treatment systems (Table II). The cells were crescent-shaped, produced relatively few "rosettes" (several cells held together by the holdfast material at the end of their stalks) and, via electron microscopy of samples stained with ammonium molybdate (carried out by P. Edwards, Department of Microbiology, U.B.C.), nearly all showed the presence of a hexagonally-packed, paracrystalline S layer.

Sewage isolates FWC 14, 21, 30, 38, 40 and 43 did not fit the above pattern. They had longer, more visible stalks, and had long, thin rod or ovoid cell bodies. They produced rosettes more readily and lacked a visible S layer. FWC23, which did have the typical crescent shape, lacked a surface array and formed yellow colonies on PYE where the typical colony colour was tan. To minimize duplication of strains in the collection, usually only one "typical" Caulobacter from a particular source was retained, although numerous typical Caulobacter colonies might be noted. The "atypical" strains, however, were always kept; therefore the atypical isolates are over-represented in the collection of 33 strains as compared to their abundance in the samples that were examined.

Table I. Sources of Caulobacter isolates

Strain	Type of facility	Location
Caulobacters from wastewater treatment sources		
FWC8, 14, 19, 20	Secondary treatment, activated sludge process, Bio-P	University of British Columbia Dept. of Civil Engineering, test plant
FWC9, 12	Bench-scale secondary treatment system, Bio-P fed with landfill leachate	University of British Columbia Department of Civil Engineering
FWC11	Holding tank for raw influent sewage	University of British Columbia
FWC21	Bench-scale rotating biological contactor	University of British Columbia Department of Civil Engineering
FWC15, 16, 18	Primary treatment facility for greater Vancouver, BC area	Iona Island, BC
FWC17	Secondary treatment facility, rotating biological contantor	Langley, BC
FWC22	Secondary treatment facility, aerobic digestor	Langley, BC
FWC23, 24, 27, 28, 31	Contour trench wastewater treatment system	Takla, BC
FWC25	Secondary treatment facility, primary treatment region	Edmonton, Alberta, Gold Bar facility
FWC26	Secondary treatment facility, activated sludge	Edmonton, Alberta, Gold Bar facility
FWC30	Untreated influent sewage	Edmonton, Alberta, Gold Bar facility
FWC29	Secondary treatment facility, activated sludge system	Bozeman, Montana
FWC32, 33	Trickle filter secondary treatment facility	Coeur d'Alene, Idaho
FWC34	Secondary treatment facility, activated sludge system	Pullman, WA
FWC35	Trickle filter secondary treatment facility	Las Vegas, Nevada
FWC38	Secondary treatment facility, activated sludge system	Las Vegas, Nevada
FWC37	Secondary treatment facility, activated sludge system	Post Falls, Idaho
FWC39, 40	Secondary treatment facility, activated sludge system, Bio-P	Kelowna, BC
FWC41, 42, 43	Secondary treatment facility, activated sludge system	Calgary, Alta, Bonnybrook facility
Caulobacters from non wastewater treatment sources		
FWC1	Lake Washington	Seattle, WA
FWC4, 5	Surface water in waterlogged area	Bothell, WA
FWC6, 7	Tap water	Oakland, CA
FWC13	Surface water from a peat bog	Richmond, BC
FWC45	stream water	Burnaby, BC

Table II. Characteristics of Caulobacters isolated from wastewater treatment facilities

Strain	Plasmids	S Layer	Colony colour	Cell shape	Degree of Rosetting	Anti-130K	Hybridization with probes: FLA	SA
FWC8	+	+	tan	crescent	+	+	+++	+++
FWC9	+	+	tan	crescent	+	-	+++	+++
FWC11	+	+	tan	crescent	+	+	+++	+++
FWC12	+	+	tan	crescent	+	-	+++	+++
FWC14	-	-	white	rod	+++	-	+	+
FWC15	+	+	tan	crescent	+	+	+++	+++
FWC16	+	+	tan	crescent	+	-	+++	+++
FWC17	+	+	tan	crescent	+	-	+++	+++
FWC18	+	+	tan	crescent	++	+	+++	+++
FWC19	+	+	tan	crescent	+	+	+++	+++
FWC20	+	+	lt yellow	crescent	++	+	+++	+++
FWC21	+	-	tan	rod	+++	-	+	-
FWC22	-	+	tan	crescent	+	+	++	++
FWC23	+	-	yellow	crescent	+	-	++	+
FWC24	+	+	tan	crescent	-	-	+++	+++
FWC25	+	+	tan	crescent	+	-	+++	+++
FWC26	+	+	tan	crescent	+	+	+++	+++
FWC27	+	+	tan	crescent	+	+	+++	+++
FWC28	+	+	tan	crescent	+	+	+++	+++
FWC29	-	-	tan	crescent	+	+	+++	+++
FWC30	+	-	red	ovoid	+	-	+	-
FWC31	+	+	tan	crescent	+	+	+++	+++
FWC32	+	+	tan	crescent	-	-	+++	+++
FWC33	-	+	tan	crescent	++	+	+++	+++
FWC34	+	+	tan	crescent	++	+	+++	+++
FWC35	+	+	tan	crescent	+	+	+++	+++
FWC37	-	+	tan	crescent	+	+	+++	+++
FWC38	-	-	br yellow	rod	-	-	++	-
FWC39	+	+	tan	crescent	+	+	+++	+++
FWC40	-	-	tan	rod	+++	-	++	+
FWC41	+	+	tan	crescent	++	+	+++	+++
FWC42	+	+	tan	crescent	++	-	+++	+++
FWC43	+	-	br yellow	rod	+++	-	++	+

Legend: Plasmids were detected by the method of Kado and Liu (27). S Layers were observed by EM on cells negatively stained with ammonium molybdate by Patti Edwards. Colony colour refers to colonies on PYE. Cell shape and degree of rosetting were determined by phase contrast microscopy. Anti-130K refers to cross reactivity of low pH surface array extracts on Westerm blots to the antiserum prepared against the major surface array protein of Caulobacter crescentus CB15. (59). Hybridization with probes (FLA= flagellin; SA= surface array) scores are based on both colony lift and Southern blotting data.

Although the typical Caulobacters were morphologically similar, there were characteristics that distinguished them. Protein band profiles obtained by SDS-PAGE were used as an initial screening procedure. Figure 1 shows the total protein profiles of all the "typical" isolates in the collection. Some of the isolates do show similar profiles to others in this figure, notably FWCs 9 and 12 and FWCs 16 and 17. These pairs do have other features that distinguish them, however, so they were retained in the collection. Two other strains, FWC10 and FWC36, were dropped from the original collection of 35 due to similarities to FWC8 and FWC33 respectively.

Western blot analysis was carried out on total protein preparations of all isolates using a polyclonal antiserum against the "130K" major surface array protein of *Caulobacter crescentus* CB15. The cross reactivity of the antiserum with other proteins made it impossible to determine if the isolates did have antigenically related surface array proteins, so low pH surface array extracts were performed and western blots carried out on these. Despite the similarity in appearance of the S layers to that of *Caulobacter crescentus* CB15A, not all the "typical" isolates' extracts cross reacted with the anti-130K antiserum (Table II and Figure 2).

The holdfasts of the isolates also showed a good deal of heterogeneity as determined by lectin binding assays (Table III). The binding specificities of the lectins that were used are shown in Table IV. There did not seem to be any relationship between lectin binding profile and the ability to form rosettes (Table II).

Colony lift hybridization using both gene probes gave a positive

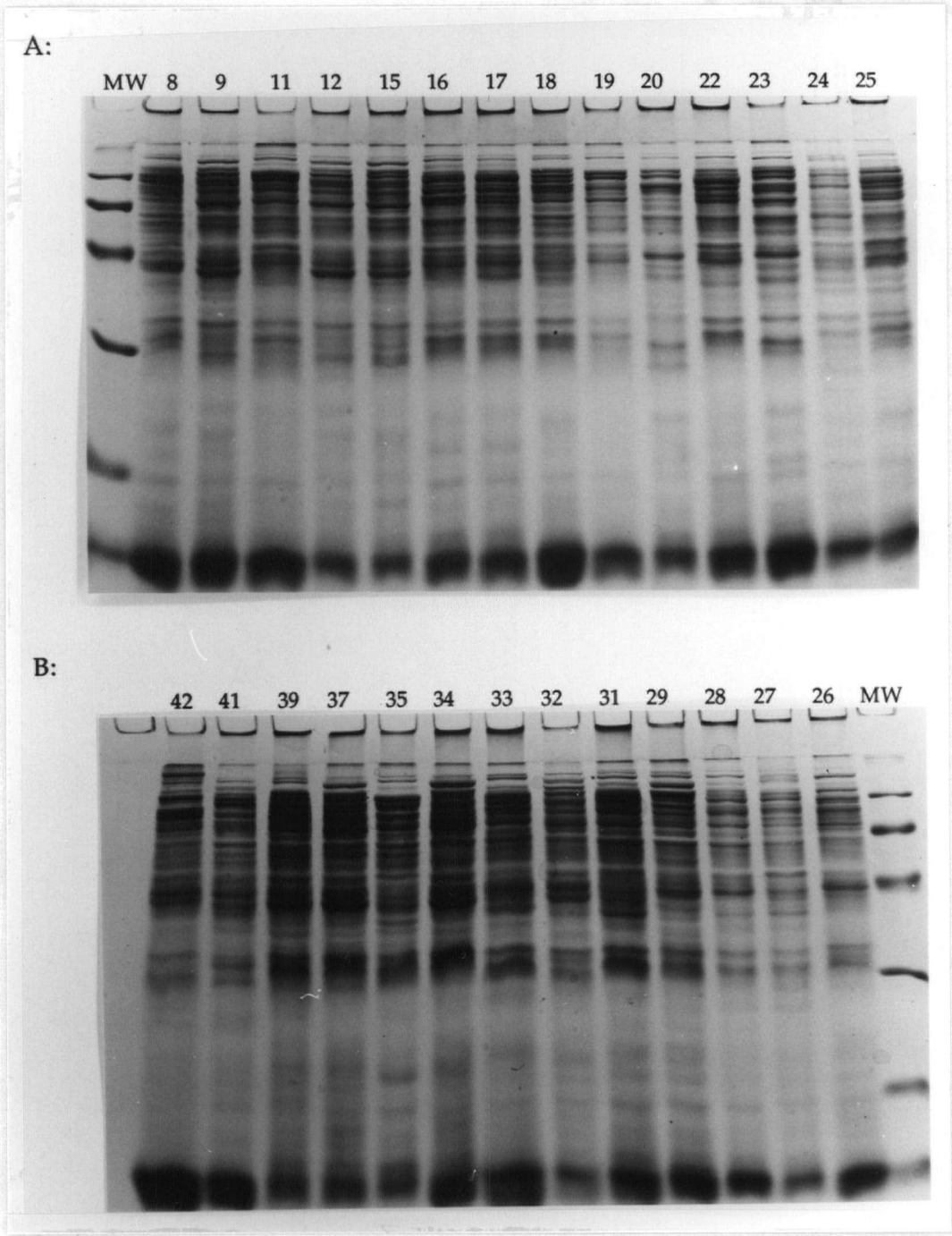


Figure 1: SDS-PAGE of "typical" FWC total proteins. In A and B the samples were loaded as indicated where MW= molecular weight standards and the numbers represent FWC strains (i.e. 8= FWC8). BioRad low molecular weight range standards: rabbit muscle phosphorylase 97.4kDa, bovine serum albumin 66.2kDa, hen egg white albumin 42,699Da, bovine carbonic anhydrase 31kDa, soybean trypsin inhibitor 21.5kDa, hen egg white lysozyme 14.4kDa.

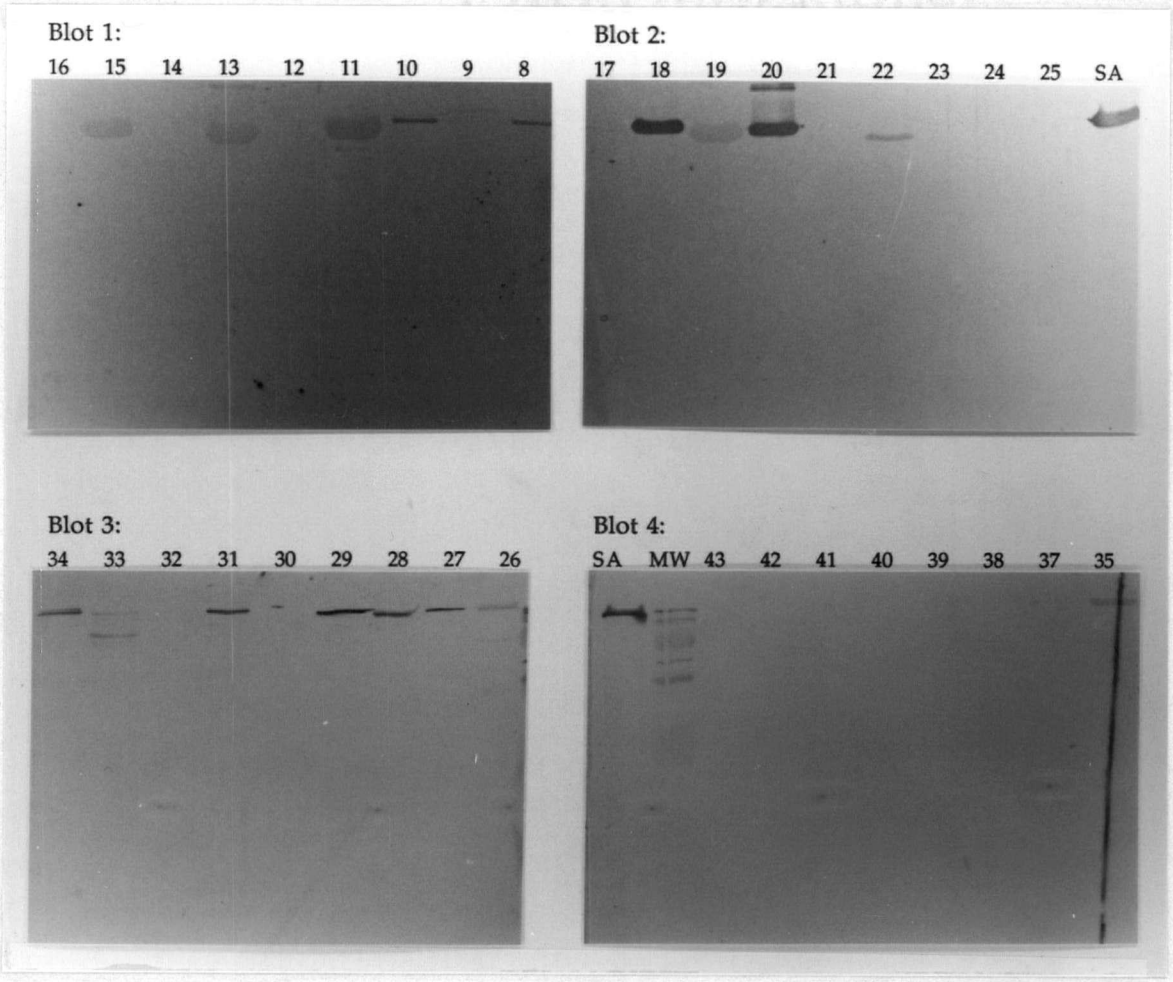


Figure 2: Western blots of low pH surface array extracts of FWC isolates. The samples are indicated above the blots where 8=FWC8 etc., SA= standard low pH surface array extract from Caulobacter crescentus CB15A and MW=BRL prestained molecular weight standards (Bethesda Research Laboratories, Gaithersberg, MD).

Table III. Holdfast composition as determined by lectin binding

	Lectin:						
Strain:	Peanut	DBA	Soybean	ConA	UEA1	WGA	Ricinus
FWC8	-	-	-	-	-	+	-
FWC9	-	-	-	-	-	-	-
FWC11	-	-	-	-	-	+	-
FWC12	-	+	-	-	+	+	-
FWC14	-	-	-	-	-	+	-
FWC15	-	-	-	+	-	+	-
FWC16	-	+	+	-	+	+	+
FWC17	-	-	-	-	-	+	-
FWC18	-	-	-	+	-	+	-
FWC19	-	-	-	-	-	+	-
FWC20	-	+	+	-	+	+	+
FWC21	-	-	-	+	-	+	-
FWC22	-	-	-	-	-	+	-
FWC23	-	-	-	-	-	+	-
FWC24	-	-	-	-	-	-	-
FWC25	-	-	-	-	-	+	-
FWC26	-	-	-	-	-	-	-
FWC27	-	-	-	-	-	-	-
FWC28	-	-	-	-	-	-	-
FWC29	-	-	-	-	-	-	-
FWC30	-	-	-	-	-	+	-
FWC31	-	-	-	-	-	-	-
FWC32	-	-	-	-	-	+	-
FWC33	-	-	-	-	-	-	-
FWC34	-	-	-	-	-	-	-
FWC35	-	-	-	-	-	-	-
FWC37	-	-	+	-	+	+	-
FWC38	-	-	-	-	-	-	-
FWC39	-	+	+	+	+	+	+
FWC40	-	+	-	-	+	+	-
FWC41	-	-	-	-	-	-	-
FWC42	-	+	-	-	+	+	-
FWC43	-	+	+	+	+	+	+

Legend: The assay was performed as previously described (40) using fluorescein isothiocyanate-conjugated lectins. Peanut, peanut agglutinin; DBA, *Dolichos biflorus* agglutinin; soybean, soybean agglutinin; ConA, concanavalin A; UAE1, *Ulex europaeus* agglutinin 1; WGA, wheat germ agglutinin; Ricinus, *Ricinus communis* agglutinin.

Table IV. Lectin Specificity:

Lectin	Sugar specificity
Soybean agglutinin	D-galNAc
Peanut agglutinin	ß-D-gal(1-3)-D-galNAc
Dolichos biflorus agglutinin	α-D-galNAc
Ulex europaeus I agglutinin	α-L-fuc
Wheat germ agglutinin	(D-glcNAc)2, NeuNAc
Ricinus communis agglutinin	ß-D-gal
Concanavalin A	α-D-man, α-D-glc

Legend: galNAc= N-acetyl galactosamine; fuc= fucose; glcNAc= N-acetyl glucosamine; NeuNAc= N-acetyl sialic acid; gal= galactose; man= mannose and glc= glucose.

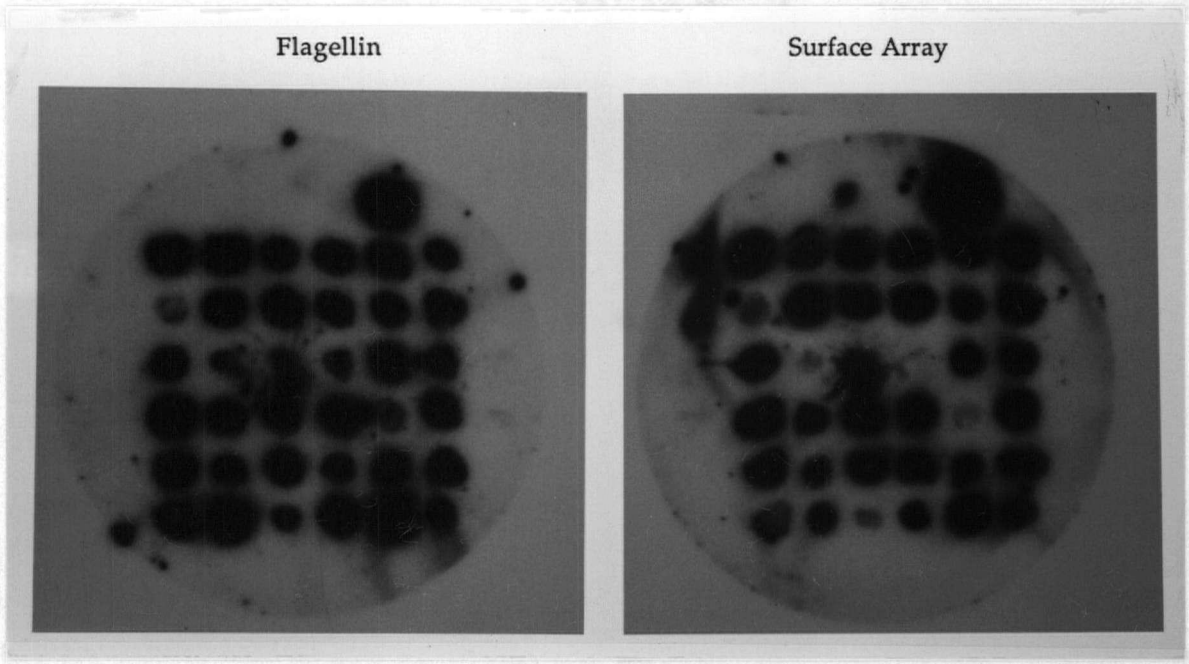


Figure 3: Colony Hybridization of FWCs. Autoradiograms of colony lifts probed with the flagellin and surface array probes. Row 1: Caulobacter crescentus CB15A; Row 2: FWC8, 9, 10, 11, 12, 13; Row 3: FWC14, 15, 16, 17, 18, 19; Row 4: FWC20, 21, 22, 23, 24, 25; Row 5: FWC26, 27, 28, 29, 30, 31; Row 6: FWC32, 33, 34, 35, 36, 37; Row 7: FWC38, 39, 40, 41, 42, 43.

signal for most of the Caulobacter strains (Fig. 3). The exceptions with the surface array gene probe were FWC14, 21, 23, 30, 38 and 40; none of these have a visible surface array and all are "atypical" in some way.

Table II shows that two other strains also lack a surface array. FWC43, which has an atypical appearance, gave a very weak signal on a Southern blot despite repeated strong signals using the same probe on colony lifts. No surface array was observed by electron microscopy on FWC29 but in western blot analysis, this was one of the isolates that had a band similar to that of the CB15A surface array protein that cross-reacted with the anti-130K antiserum. This, taken with the fact that it hybridizes strongly with the surface array probe in both colony blots and southern blots, probably indicates that it does have a surface array.

Using the flagellin gene probe for colony hybridizations, the same isolates gave decreased signals as for the surface array gene probe. In Southern blotting experiments at least one band was seen for each isolate (2-3 bands were typical) but FWC38 and FWC43 gave lighter bands. The combined colony hybridization and southern blotting results are summarized in Table II.

Similar patterns on Southern blots confirmed that FWCs 8 and 10 as well as FWCs 33 and 36 are at least closely related if not identical. FWCs 9 and 12 also gave similar restriction patterns on southern blots but neither was discarded because their antibiotic resistance patterns and holdfast compositions differed. There was no overall restriction pattern that unified the group or could be used to subdivide the collection of wastewater isolates beyond the similarities mentioned above, so restriction fragment length

polymorphism (RFLP) analysis was not pursued.

Colony hybridization was also carried out on 155 non-Caulobacter wastewater isolates. None gave a strong signal when probed with the surface array gene probe and a single positive score was observed using the flagellin gene probe. Most of the isolates tested were not identified but the strains used in the lifts shown in Figure 4 were isolated and given tentative identifications by A. Chu using the Biolog identification system. The colony that probed positive was identified as *Pseudomonas vesicularis* (acceptable identification: fair species match; good species separation).

Because the antibiotic resistance assays were carried out on PYE and not the medium recommended by Difco, organisms that fell into the "intermediate" resistance category were called sensitive. Most of the strains from wastewater and freshwater Caulobacter strains from other sources were resistant to ß-lactam antibiotics (ampicillin and penicillin), nalidixic acid and polymyxin B (Table V). Nearly all were sensitive to streptomycin. Resistance to chloramphenicol, tetracycline, erythromycin and, to a lesser extent, tobramycin, however, was elevated among the wastewater Caulobacters compared to those isolated from non-wastewater sources. The one exception was FWC13, which was isolated from a municipal nature park.

Large plasmids were observed in many of the isolates (Table II). Negative assignments in this table should be regarded as tentative since the method used did not always yield plasmids when they were present.

2. Enumeration:

The method described in the methods and materials section for

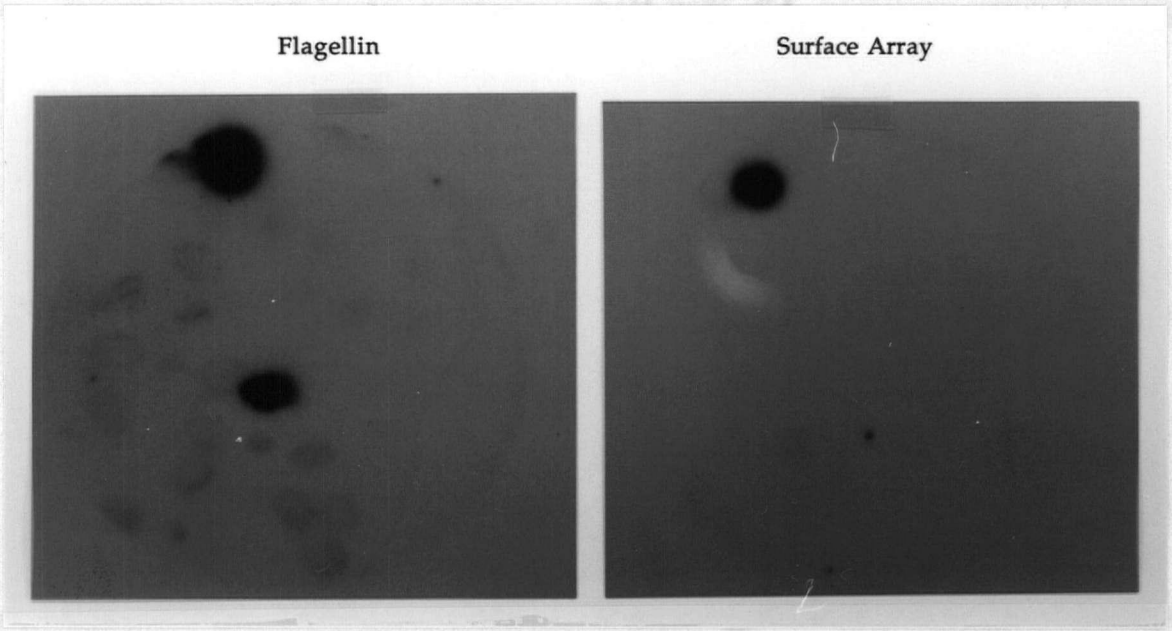


Figure 4: Colony hybridization of 44 non-Caulobacter sewage isolates. Autoradiograms of colony lifts of strains isolated and identified by Angus Chu using the Biolog identification system and probed with the flagellin and surface array probes. The positive control was Caulobacter crescentus CB15A and the single positive signal was obtained for an isolate identified as *Pseudomonas vesicularis*.

Table V. Antibiotic resistance in Caulobacter isolates

Strain	Antibiotic Cm	Tet	Sm	Em	PB	Tm	Nal	Amp	Pen
Wastewater treatment Caulobacters									
FWC8	R	R	s	R	R	s	R	R	R
FWC9	s	R	s	R	R	R	R	R	R
FWC11	R	R	s	R	R	s	R	R	R
FWC12	s	s	s	s	R	R	R	R	R
FWC14	s	R	s	R	R	s	R	s	R
FWC15	R	R	s	R	R	s	s	R	R
FWC16	s	s	s	s	R	R	R	R	R
FWC17	R	R	s	R	R	s	R	R	R
FWC18	R	R	s	R	R	s	R	R	R
FWC19	R	R	s	R	R	s	R	R	R
FWC20	R	s	s	R	R	s	R	R	R
FWC21	R	s	s	R	R	s	R	R	R
FWC22	R	R	s	R	R	s	R	R	R
FWC23	s	R	s	R	R	s	R	R	R
FWC24	s	s	s	R	R	s	R	R	R
FWC25	s	R	s	s	R	s	R	R	R
FWC26	s	s	s	R	R	s	R	R	R
FWC27	s	R	s	R	R	s	R	R	R
FWC28	s	R	s	s	R	s	R	R	R
FWC29	s	R	s	s	R	s	R	R	R
FWC30	R	R	s	R	R	s	R	R	R
FWC31	s	R	s	s	R	s	R	R	R
FWC32	s	s	s	s	R	s	R	R	R
FWC33	s	R	s	R	R	s	R	R	R
FWC34	s	R	s	s	R	s	R	R	R
FWC35	R	R	s	R	R	s	R	R	R
FWC37	s	R	s	s	R	R	R	R	R
FWC38	R	R	R	R	R	R	R	R	R
FWC39	s	s	s	R	R	R	R	R	R
FWC40	s	R	s	s	R	s	R	R	R
FWC41	s	s	s	s	R	s	R	R	R
FWC42	s	s	s	s	R	s	R	R	R
FWC43	s	s	s	s	R	s	R	s	s
Non-sewage Caulobacters									
C. crescentus CB15A	s	s	s	s	s	s	R	R	R
C. crescentus CB2A	s	s	s	s	R	R	R	R	R
C. vibrioides	s	s	R	s	R	s	R	R	R
C. bacteroides	s	s	s	s	R	s	s	R	R
FWC1	s	s	s	s	R	s	R	R	R
FWC4	s	s	s	s	s	s	R	s	s
FWC5	s	s	s	s	s	s	R	s	s
FWC6	s	s	s	s	R	s	R	R	R
FWC7	s	s	s	s	R	s	R	R	R
FWC13	R	R	s	R	R	s	R	R	R
FWC45	s	s	s	s	R	s	R	R	R

Legend: Cm is Chloramphenicol, resistant (R) when the diameter of the zone of clearance is <13mm; Tet is Tetracycline, R<15mm; Sm is Streptomycin, R<12mm; Em is Erythromycin, R<14mm; PB is Polymyxin B, R<9mm; Tm is Tobramycin, R<13mm; Nal is Nalidixic Acid, R<14mm; Pen is Penicillin, R<12mm. Larger clear zones indicated sensitivity (s).

the extraction of DNA from sewage was arrived at after attempting several different procedures.

After plating full strength sewage treated with various disinfectants, Environ-D was determined to be the most effective method for stabilization of samples taken from the UBC Bio-P pilot plant (aerobic section). Other treatments evaluated include: 50mM EDTA and 0.1% sodium azide, 5% phenol, 5% p-phenylphenol, ammonia, bleach and MikroQuat (a quaternary cationic detergent). Samples treated with EDTA/azide, phenol, phenylphenol, ammonia and MikroQuat yielded colonies on PYE plates after 24 hours. No colonies appeared on the plates containing sewage treated with Environ-D (1/40 and 1/80) or bleach (full strength). DNA could not be satisfactorily isolated from the bleach-treated samples so the Environ-D treatment was used in subsequent experiments.

Several methods of cell disruption were tried. Sonication of the samples for 3x60 seconds followed by treatment with lysozyme and proteinase K proved to be the easiest and most reliable procedure. DNA from cells disrupted by boiling, with and without enzyme treatment, was highly sheared, and sonication or enzyme treatment alone gave decreased yields of DNA.

Purification of the DNA from the complex lysate was best accomplished by subjecting the mixture to two successive CsCl/EtBr gradients. DNA isolated by phenol and phenol:chloroform extractions was more highly sheared and this method was much more time consuming. Attempts to purify DNA by passing a solution of the first precipitate in 4 M $NaClO_4$ over a glass fibre filter, followed by elution of the DNA with low ionic strength buffer failed to satisfactorily remove contaminants as indicated by low A_{260}/A_{230}

and/or A_{260}/A_{280} ratios.

The specificities of the flagellin and surface array probes were tested by colony hybridization with Caulobacters isolated from sewage and non-Caulobacter sewage isolates as described above. Because the probes could hybridize with all of the "typical" FWCs and only one of the non-Caulobacter isolates gave a positive signal with the flagellin probe they were judged to be quite specific for the target group. They were not, however, completely specific so the problem of distinguishing target signal from background had to be addressed.

A series of standard DNAs were made up by mixing pooled DNA extracted from FWCs with DNA extracted from the non-Caulobacter sewage isolates in various proportions. The background signal obtained when samples were dotted onto membranes was such that Caulobacter DNA could only be detected at a >1% level (Fig. 5). That is, the resolution, or my ability to distinguish signal derived from Caulobacter DNA from background signal, was quite limited. This level was not improved by adding more DNA per dot in the undiluted samples or by adding more probe. When the standards were made up with laboratory strain *Caulobacter crescentus* CB2A DNA, which is more highly homologous to the probe fragments, however, the resolution was improved. Caulobacter DNA could be detected above the 0.1% level (Fig. 6). In this figure the bottom two rows are samples of DNA extracted from sewage to which 10% and 1% (v/v) FWC18 culture had been added. Only the 10% sample gives a signal above background.

Figure 7 shows another blot of DNA extracted from sewage samples to which 10%, 1%, 0.1% and 0% (v/v) FWC18 culture had been added. Again, when the culture accounted for 10% of the total

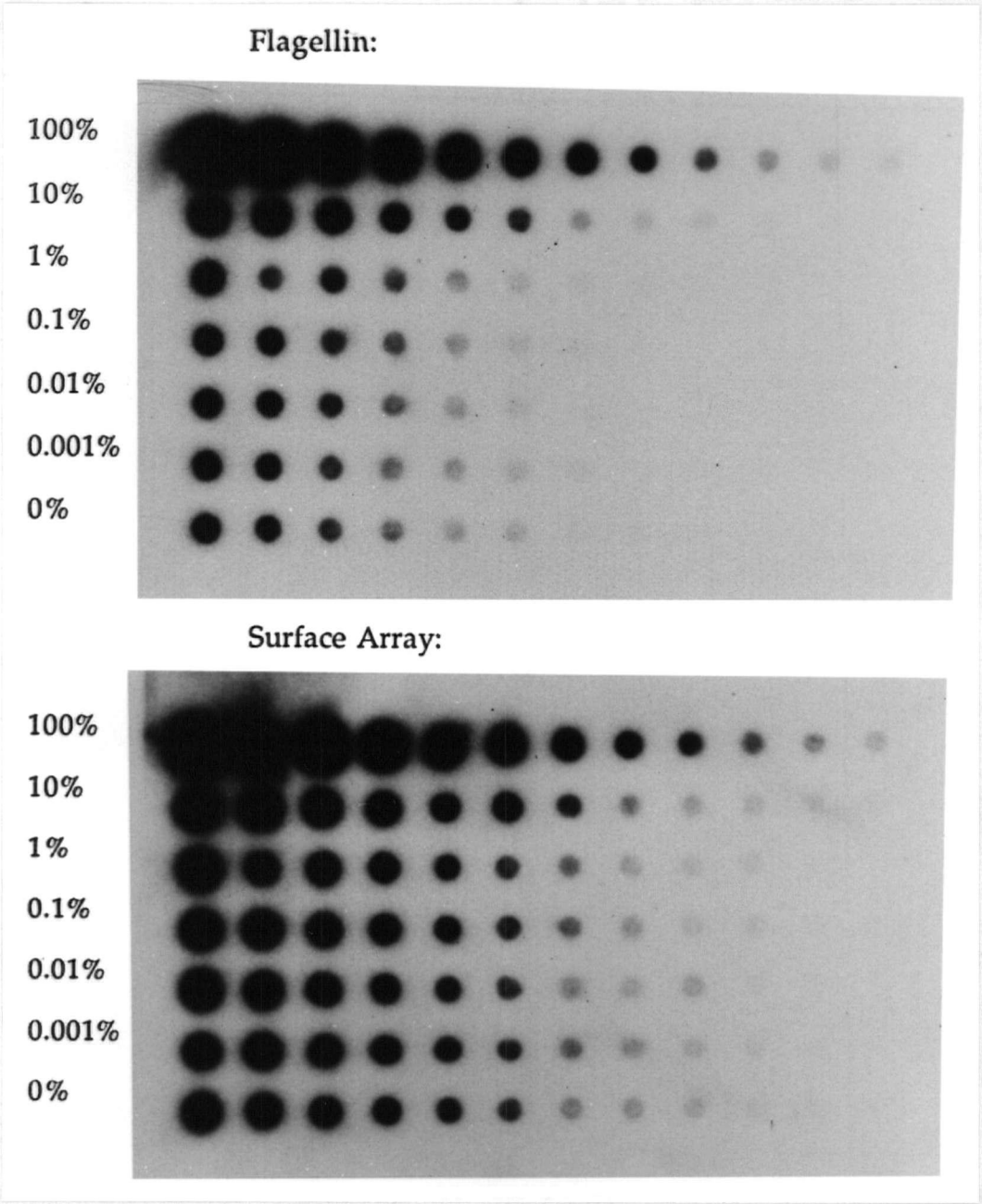


Figure 5: FWC Pool standards. Autoradiogram of standard DNA dot blots probed with flagellin and surface array probes. A two-fold dilution series was performed on each sample with the undiluted sample on the left (1.1 ug DNA) and the far right dot is a 1/2048 dilution. The standards from top to bottom are: 100%, 10%, 1%, 0.1%, 0.01%, 0.001% and 0% Caulobacter DNA in a background of non-Caulobacter DNA.

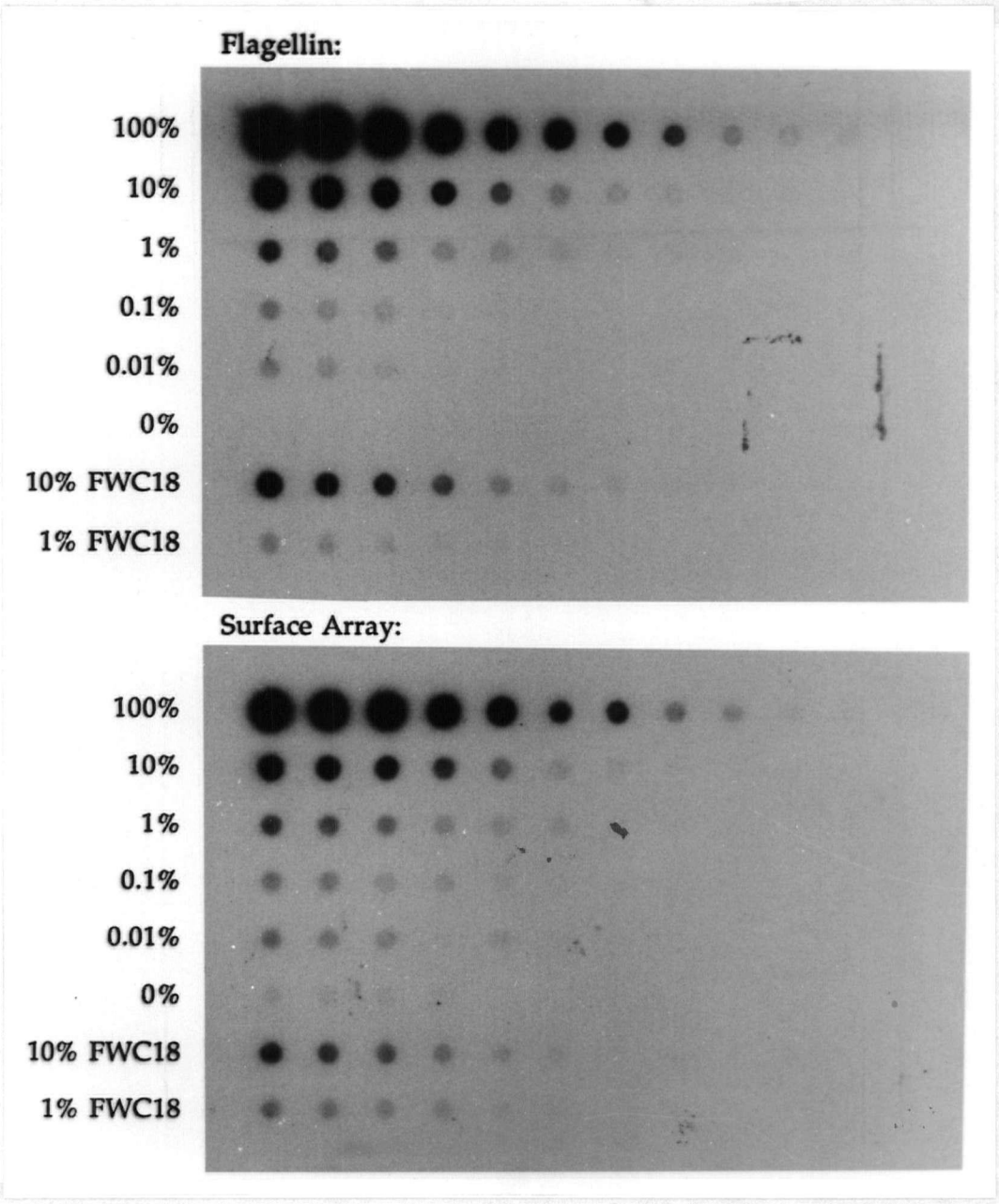


Figure 6: Standards made up with Caulobacter crescentus CB2A DNA. Autoradiogram of dot blots of standard DNAs made up with CB2A DNA. The undiluted samples are 1.1ug DNA and the samples from top to bottom are: 100%, 10%, 1%, 0.1%, 0.01%, 0% CB2A DNA in a background of non-Caulobacter DNA followed by sewage spiked with 10% (v/v) FWC18 culture and sewage spiked with 1% (v/v) FWC18 culture.

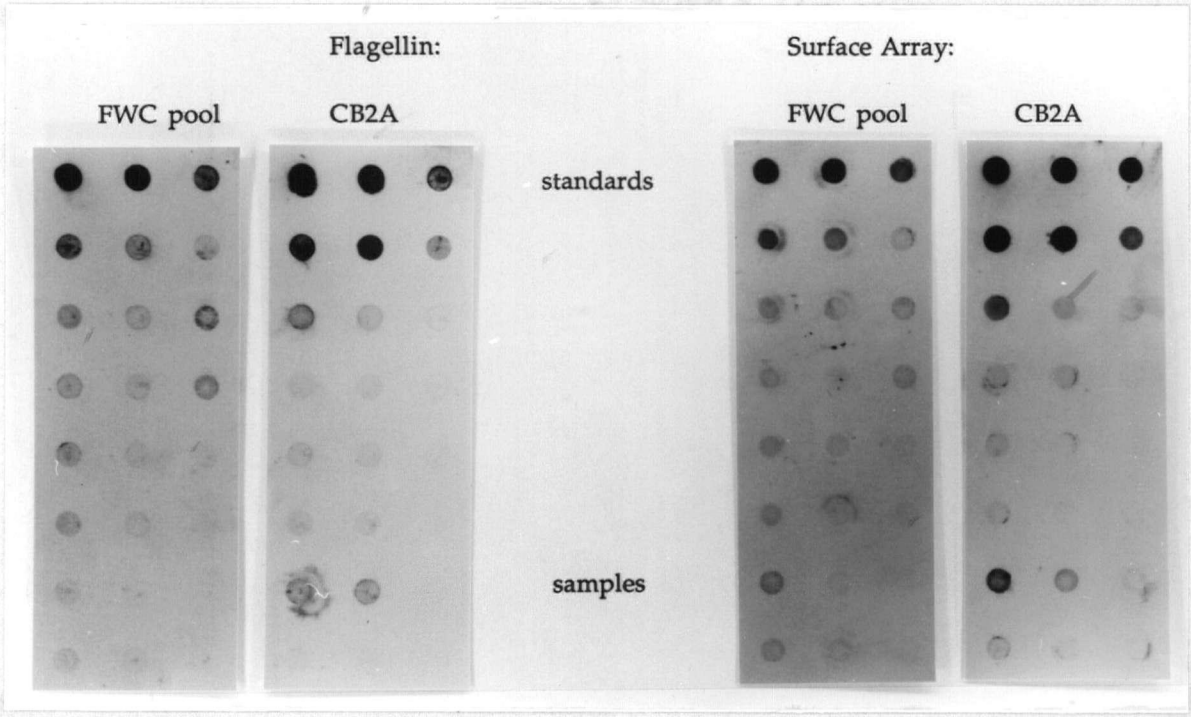


Figure 7: Sewage with FWC18 culture: Dot blots probed with non-radioactive flagellin and surface array probes. On the left in each panel are the FWC pool standards (800ng in the first column, 400ng in the second and 200ng in the third) and on the right are the CB2A standards. From top to bottom both standards are 100%, 10%, 1%, 0.1%, 0.01% Caulobacter DNA in a non-Caulobacter DNA background, and then 0.001% in the FWC pool column and 0% in the CB2A column. Below the FWC standards column is DNA isolated from sewage and DNA isolated from sewage with 1% (v/v) FWC18 culture. Below the CB2A standards column are DNA isolated from sewage with 10% and 0.1% (v/v) FWC18 culture.

volume the signal was well above background but at the 1% and 0.1% levels the signal obtained was essentially equal to that of sewage alone.

A known amount of Caulobacter DNA was added to the final DNA extracted from sewage to be certain that there was nothing in the purified preparation that would inhibit binding with the probe. In Figure 8, FWC pool DNA or FWC18 DNA accounts for 10% of the DNA in the samples and the other 90% was DNA extracted from sewage. The resultant mixtures were blotted as usual. The FWC Pool sample looked like the 10% standard and the FWC18 enriched sample gave a somewhat more intense signal indicating that hybridization was not blocked.

In an attempt to increase the resolution of the assay, a new sewage Caulobacter isolate DNA pool was made excluding some of the "atypical" isolates since they were over-represented in our sample collection and since the degree of homology was a limiting factor in the sensitivity of the assay (as demonstrated by the results of the experiment using CB2A DNA as the target DNA). The pool was made up without FWC14, 21, 30, 38, 40 and 43, however the change in positive control DNA did not improve the resolution appreciably.

In order to establish the quantitative limits of this approach, the dots were cut out of the filter with a single hole punch and counted by liquid scintillation. The autoradiograms and standard curves obtained with the undiluted samples (550ng) when the "typical" FWC pool standards were assayed are shown in Figure 9. This confirmed that the resolution of the assay is limited to the 1% level.

Despite the low resolution of the assay, it was used to try to

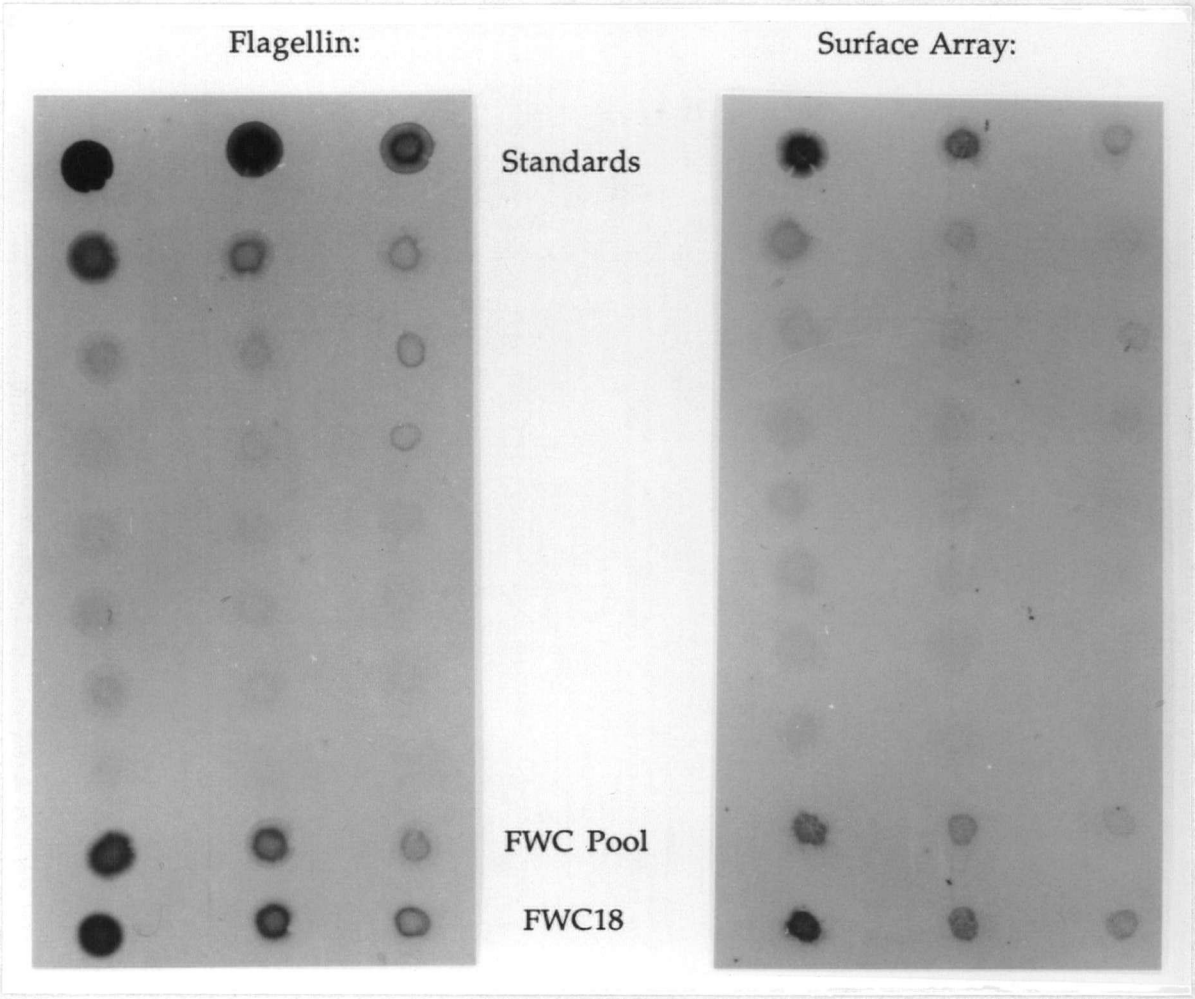


Figure 8: Sewage DNA samples with added Caulobacter DNA. Dot blots of FWC pool standards and mixed sewage and Caulobacter DNA samples probed with non-radioactive flagellin and surface array probes. 800ng, 400ng and 200ng of each sample was added to the filter and the samples from top to bottom are: 100%, 10%, 1%, 0.1%, 0.01%, 0.001% and 0% FWC Pool standards followed by sewage DNA with 10% (w/w) FWC Pool DNA and sewage DNA with 10% (w/w) FWC18 DNA.

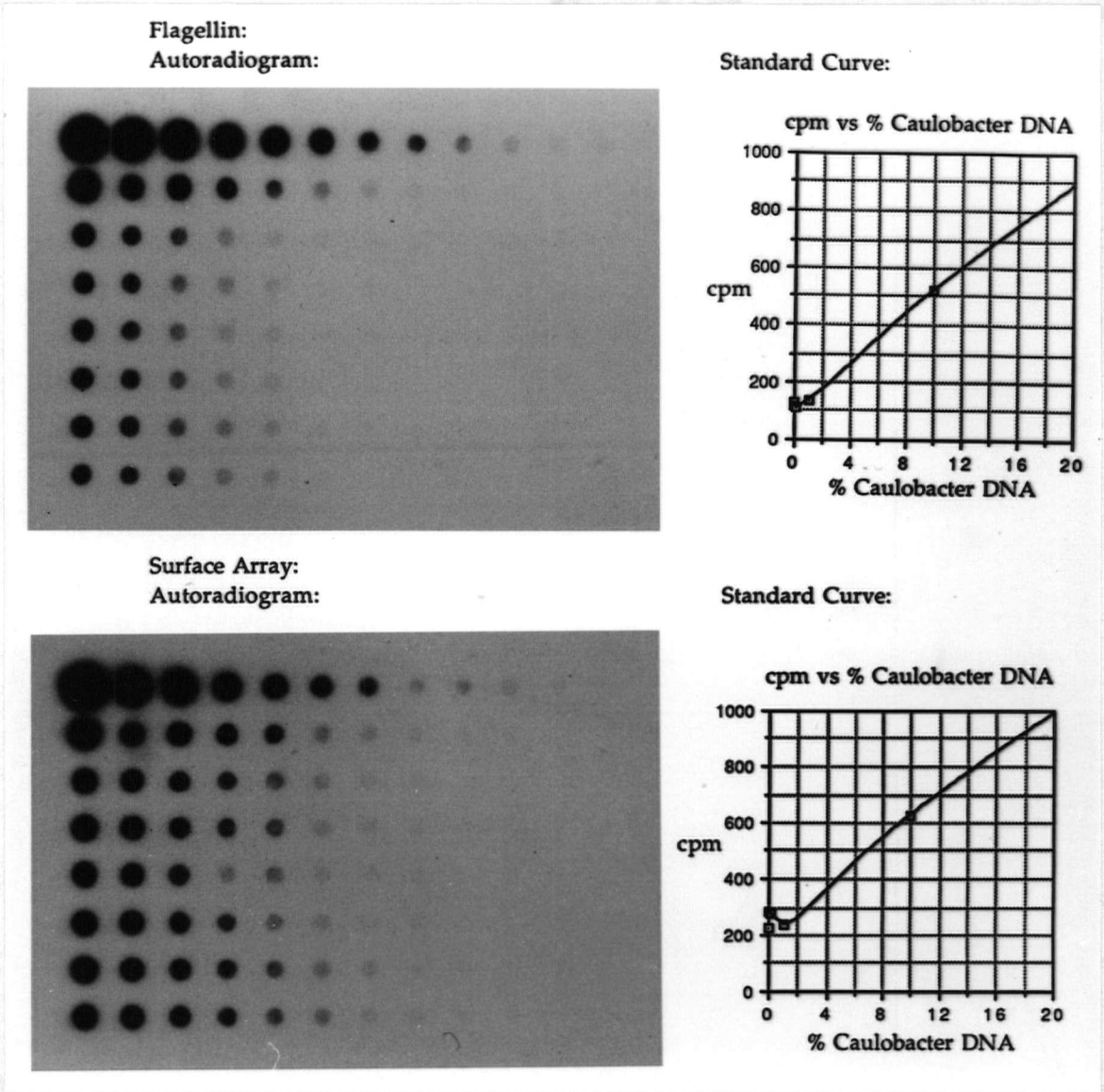


Figure 9: Standards: autoradiograms and standard curves generated by scintillation counting. The left panel shows autoradiograms obtained when dot blots of the standards made up with "typical" FWC DNA were probed with the flagellin and surface array probes. The graphs on the right were obtained by plotting counts per minutes vs percent Caulobacter DNA in the sample. Shown is the portion of the curve with up to 20% Caulobacter DNA which reveals the loss of resolution below 1% Caulobacter DNA.

determine the proportion of Caulobacters in several sewage treatment systems. Eight samples were collected from different sites and treatment facility designs. The locations and plant types are listed in Table VI. DNA was extracted from each sample, dotted along with the standards and compared to them. Looking at the autoradiograms of the standards and samples for both probes, the proportion of Caulobacters in the samples was not obvious (Figures 10 and 11) so the amount of probe bound to each dot was quantitated by liquid scintillation counting. A standard curve was then generated for each dilution. The smoothest curves were observed for the 1/8 dilutions and these are seen in Figure 12. All the samples yielded signals below the background threshold when probed with the flagellin gene probe (Figure 12). With the surface array gene probe, however, the results were not as clear. In Figure 12 some of the samples appear to have Caulobacter DNA present at above background levels. In fact, it appears from the Post Falls sample that Caulobacters may account for over 10% of the population. At other dilutions, however, that does not appear to be the case. Figure 13 shows the standard curves of the undiluted, 1/2, 1/4 and 1/8 dilutions and the position of the Post Falls sample on each one. The amount of Caulobacter DNA present in this sample as predicted by these data ranges from 0% using the undiluted standards to approximately 16% with the 1/8 dilution.

Table VI. Sample Sources and Plant Types

Location	Plant Type
Kelowna, B.C.	Activated sludge Bio-P
Edmonton, Alta	Activated sludge
Calgary, Alta	Activated sludge
Calgary, Alta	Activated sludge Bio-P
Coeur d'Alene, Idaho	Trickling filter
Post Falls, Idaho	Activated sludge
Priest River, Idaho	Activated sludge
Sandpoint, Idaho	Activated sludge

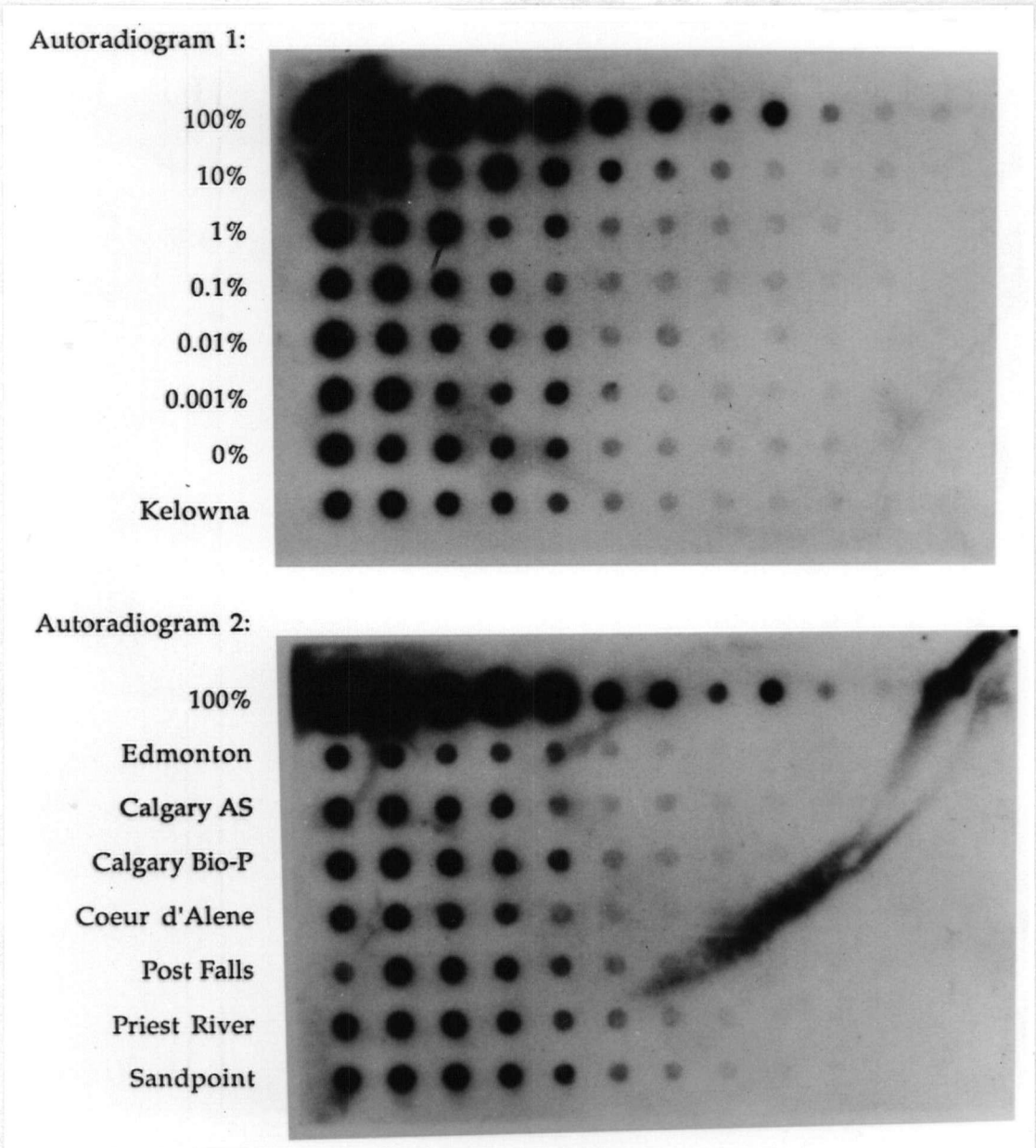


Figure 10: Standards and samples from eight treatment systems hybridized with the flagellin probe. The top autoradiogram shows the standards from 100% to 0.001% and 0% "typical" FWC pool DNA plus the sample from Kelowna. The lower autoradiogram shows from top to bottom: 100% standard, samples from Edmonton, Calgary activated sludge unit, Calgary Bio-P plant, Coeur d'Alene, Post Falls, Priest River and Sandpoint.

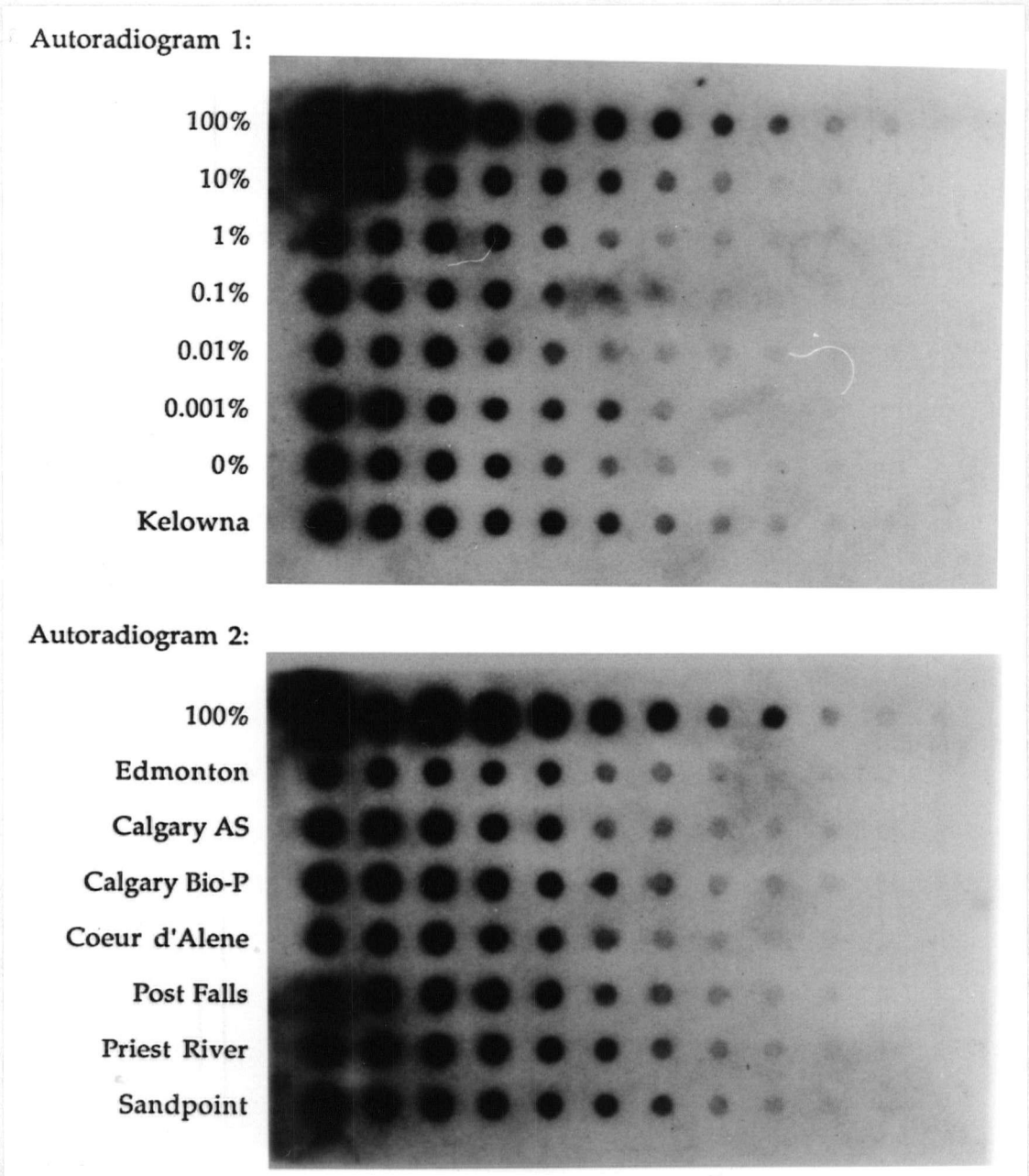
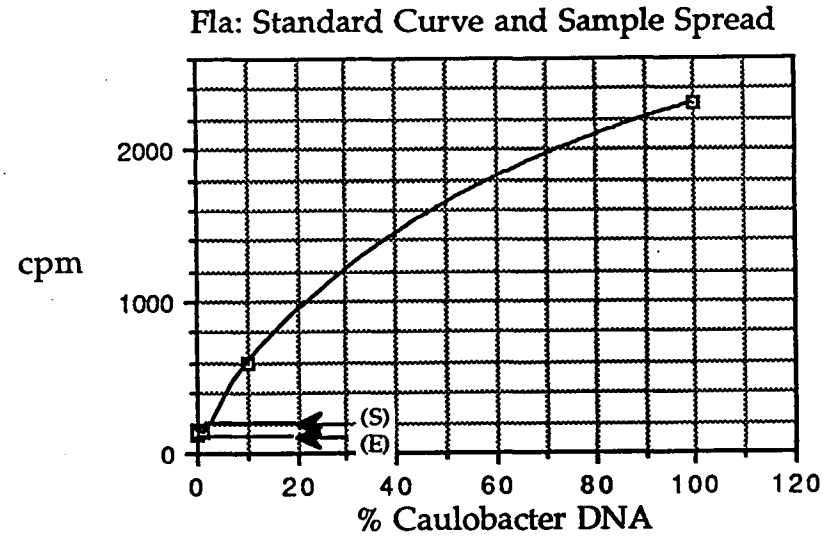


Figure 11: Standards and samples from eight treatment systems hybridized with the surface array probe. As in figure 10, the top autoradiogram has the standards and the sample from Kelowna and the lower autoradogram shows the 100% standard followed by the rest of the samples.

A:



B:

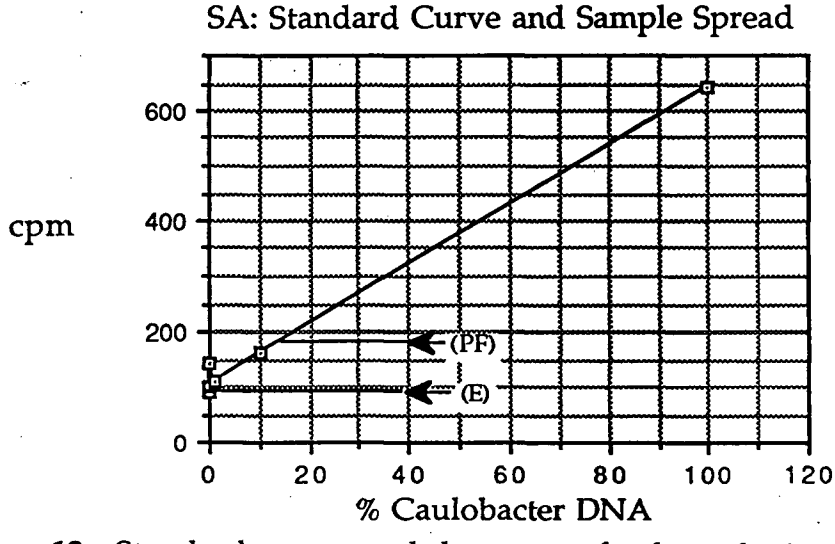


Figure 12: Standard curves and the range of values obtained for the samples. The graphs were generated by plotting the counts per minute obtained for the 1/8 dilutions of each of the standards versus the percent of Caulobacter DNA. The arrows show the spread of values obtained for the samples. The counts obtained for each of the samples probed with the flagellin gene (A) are as follows: Kelowna=144; Edmonton=105; Calgary (Activated Sludge)=138; Calgary (Bio-P)=164; Coeur d'Alene=120; Post Falls=167; Priest River=155; Sandpoint=187. Using the surface array probe (B) the counts obtained were: Kelowna=118, Edmonton=94; Calgary (Activated Sludge)=142; Calgary (Bio-P)=176; Coeur d'Alene=125; Post Falls=186; Priest River=174; Sandpoint=178.

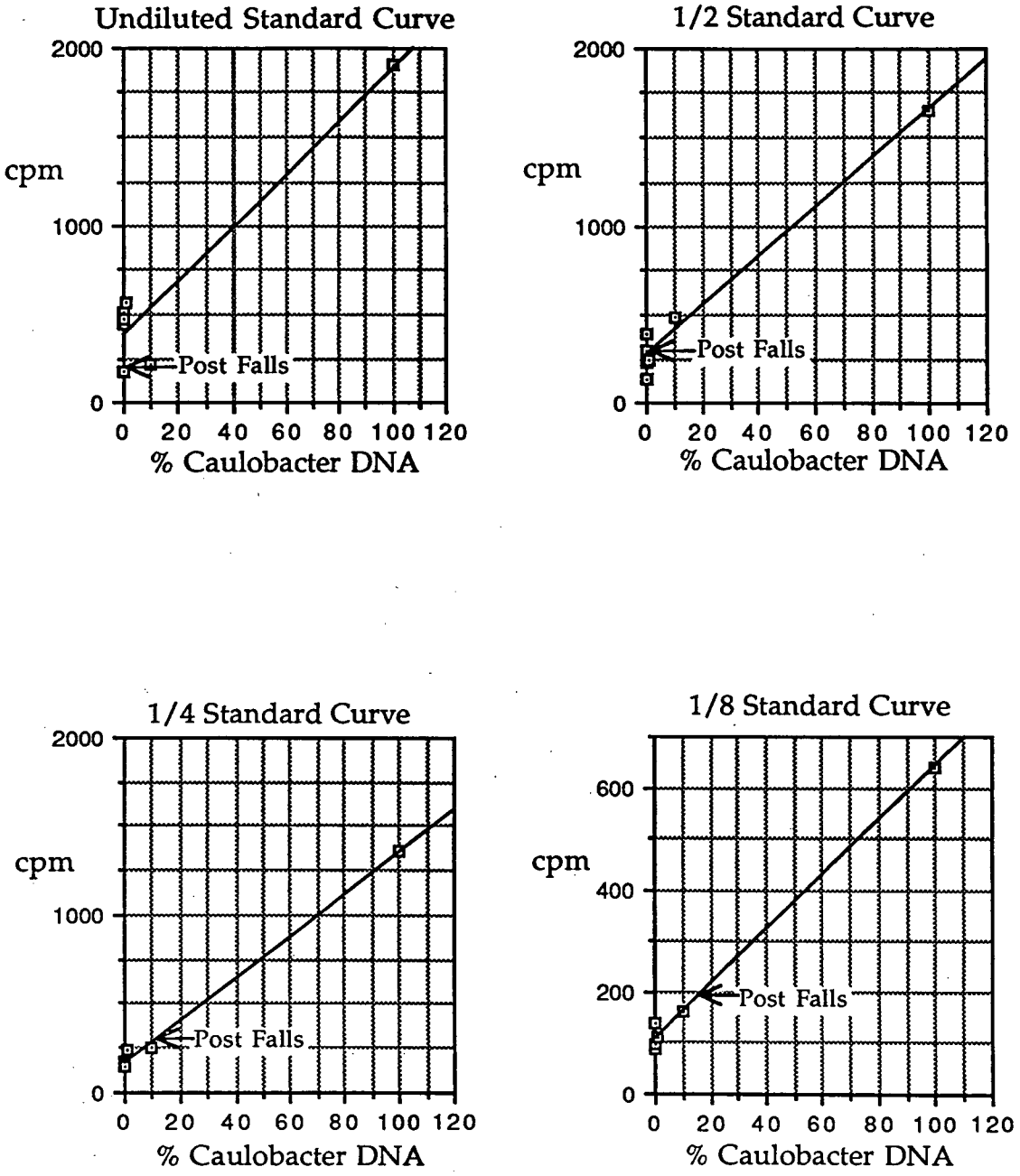


Figure 13: Position of Post Falls sample on undiluted, 1/2, 1/4 and 1/8 standard curves. This shows the variability of position of the sample on the standard curves using the surface array probe. The proportion of Caulobacter DNA in this sample taken from these data ranges from 0% to near 16%.

DISCUSSION

1. Characterization:

As stated in the Results section, the majority of Caulobacters isolated from wastewater sources had crescent-shaped cell bodies, formed few rosettes, produced tan colonies on PYE media and had a surface array. Apart from the obvious "atypical" ones, isolates had to be distinguished by their total protein profiles, holdfast compositions, antibiotic resistance profiles and restriction fragment length polymorphism. Two strains were excluded from the collection of 35 because the similarity of their characteristics indicated that they were probably duplicates of two other strains. FWC12 may be a duplicate of FWC9 but it was retained in the collection due to the differences between their antibiotic resistance and lectin binding profiles.

Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates. The pattern of lectin binding to the holdfast material should give information about the sugar composition of the holdfast since lectins bind to specific sugars (23). The sugar specificities of the lectins used in this study are given in Table IV. Interestingly, there are seven different lectin binding profiles in this group of bacteria and there is no apparent correlation between any of the patterns and the "stickiness" of the holdfast if the ability to form many and large rosettes is indicative of sticky holdfast (Table II). It is also interesting that a group that is so morphologically similar should display such a diversity in holdfast compositions.

Despite the fact that the isolates, by and large, have some homology to the surface array and flagellin gene probes, their RFLP patterns do not indicate any close relatedness or degree of conservation among them. That is, there was no common pattern of restriction fragments among the isolates. There was some antigenic similarity to the laboratory strain CB15A surface array as determined by western blot analysis but not all the strains that hybridized with the surface array gene probe yielded low pH extracts that cross reacted with the anti-130K antiserum. This could either be a result of antigenic diversity or failure to extract the surface arrays of some isolates by this method. These data, together with the lectin binding data, seem to imply a greater heterogeneity in the population than originally assumed given the morphological similarity of the isolates.

It was observed that the isolates that fit the "typical" wastewater isolate profile also hybridized best with the two gene probes. The source of the probes, *Caulobacter crescentus* CB15A, also shares many of the gross characteristics of the "typical" sewage isolate. [The fact that growth of many of the isolates was stimulated by the addition of riboflavin to the media might suggest that they belong to the *Caulobacter vibrioides* group which also shares these morphological characteristics (50)]. The "atypical" strains may be more distant relatives of *Caulobacter crescentus*. Preliminary 16S rRNA sequencing data derived from five of these isolates by D. Stahl (University of Illinois) seems to bear this out (in progress). He found that while the 16S rRNAs of FWC17, 18 and 26 were highly homologous and very similar to the laboratory strains *C. crescentus* CB15A and CB2A, FWC14 and 38 did not fit into that cluster.

Because only one isolate of 155 non-Caulobacters hybridized with the flagellin gene and none with the surface array it seemed that the probes were almost specific for Caulobacters. The isolate that gave a positive signal with the flagellin probe was identified by the Biolog system as *Pseudomonas vesicularis* (Acceptable i.d.: fair species match, good species separation). There are no data on Caulobacter strains in the databank of this identification system, however, and it happened that one of the sewage Caulobacters, FWC30, also keyed to *Ps. vesicularis* (Acceptable i.d.: fair species match, good species separation). Most of the other Caulobacters that were tested in this system did not key to any strain.

Most Caulobacters, regardless of their source, are resistant to ß-lactam antibiotics, such as ampicillin and penicillin, as well as to nalidixic acid and Polymyxin B. Caulobacters isolated from wastewater treatment facilities, however, were more frequently resistant to chloramphenicol, tetracycline, erythromycin and, to a lesser extent, tobramycin compared to those isolated from other sources. It has been reported that resistance to antibiotics is transferred among residents of treatment plants (20) and that many bacteria in such situations contain high molecular weight plasmids. The latter is also true of many of the sewage isolates characterized in this study, contrary to the general lack of plasmids observed in Caulobacters isolated from other sources. The implications of this kind of transfer in terms of the release of genetically engineered organisms into the environment and the length of time an antibiotic remains medically useful is not clear. The increased antibiotic resistance observed among the wastewater Caulobacters tends to support more research into this question. Access of engineered organisms to the sewage system, transfer of resistance from plant

residents to pathogens and many other problems will have to be addressed.

2. Enumeration:

The major biological problem being addressed in this work is the study of organisms in a complex environment about which very little is known. It has been estimated that less than 1% of organisms in the environment can be cultured in the laboratory (19, 43). Nevertheless, the classical method of studying the diversity of organisms in nature has been by culturing. This method has been shown to give a biased view of populations because many organisms' nutritional requirements will not be met even by so-called non-selective media, and other organisms may escape selective pressures that normally keep them in check (21, 71, 30, 63). Even when two media give similar plate counts for a particular environment, the diversity of the populations obtained may be quite different. (62). Increasingly, microbiologists have been attempting other methods of study.

Direct microscopic examination of samples can give absolute numbers of organisms (dead and alive) but until recently could not yield much information about the organisms themselves. This approach is complicated in some environments by the interfering presence of silt, other particulate matter, decaying organic material, and the tendency of organisms to flocculate or form clumps (18). This is a problem with samples from wastewater.

Another approach in determining the absolute numbers of cells is to extract and quantify some cellular constituent such as total phospholipids, cell wall material, ATP, DNA or RNA. (18, 29, 6, 16).

Because this is an indirect approach, the efficacy of the procedure used to extract the cell constituent of interest will have a large impact on the success and accuracy of the assay as will the conversion factor used to relate the result back to total number of cells. Further complications include the presence of eukaryotic organisms and dead organic material which may contribute to the pool of that constituent. Unless this approach is coupled with a probing step (as in the case with DNA or RNA) it can only be related back to numbers of organisms and does not give any information about which organisms are present. If the cellular constituent can be probed, however, a fixed (theoretical) conversion factor is not necessarily required because the signal obtained may be compared to standards. It is also possible to bypass the conversion of signal to absolute numbers of cells if the proportion of the organism with respect to the whole population is all that is required.

Researchers who have tried to detect specific organisms in the environment have adopted a number of different approaches. Since comparative studies have underlined the inadequacy of culture techniques in many environments, the use of DNA, RNA and antibody probes has been favoured. In most of these studies a single species or strain was targetted and researchers probed for prominent members of the community. As a result background signals were not a problem. If the target was present in low quantities but was important for safety reasons (such as pathogens in food or water or genetically engineered organisms in the environment) simple detection was sufficient and amplification of the target by methods such as PCR could be employed.

In this assay, the aim was to determine the proportion of Caulobacters relative to other bacteria present in the sewage

environment with a view to using this as a tool to determine if Caulobacters play an important role in wastewater treatment, and to monitor their responses to various operational changes. The use of DNA probing was the method of choice because gene probes were already available and the method did not require special equipment. A number of different methods of DNA isolation from the environment have been reported (67, 24, 25, 5, 61, 65, 42). Most of the early methods require the separation of cells from other materials in the environment. It was reasoned that repeated washing would not be an effective method of extracting cells from wastewater because of their tendency to flocculate in large masses containing cells, carbohydrate polymers and trapped organic material from the water. Initial separation of the cells also represented a step in which certain members of the population might be lost. Steffan et al. (65) and Ogram et al. (42) in their studies on sediments and soils adopted the approach of lysing cells directly and purifying the DNA from the complex mixture. Insoluble polyvinylpolypyrollidone (PVPP) was added to bind humic acids which might otherwise remain associated with the DNA. This approach was adopted and various methods of lysis and DNA purification were tried to determine which method would be easiest and most effective with sewage samples.

It was important to recognize that in adopting the use of a cellular component, DNA, to quantify Caulobacters, certain assumptions were made about the population which were not completely true. Firstly, it was assumed that the disinfection procedure used to stabilize the samples for transport to the laboratory had an equal effect on all bacteria such that no more DNA was produced, or at least the overall proportions of bacteria did not

change. It was also assumed that the phenol present in the solution would effectively destroy all DNAse activity so that the DNA from cells that lysed was not lost. It is unlikely that any feasible disinfection method could maintain the population in a completely static condition.

Secondly, the efficiency with which DNA can be extracted from cells will vary from strain to strain. Some bacteria are extremely resistant to disintegration where others are very easily lysed. The recalcitrant bacteria will therefore be under-represented when the DNA is ultimately purified such that the conversion of proportion of DNA to proportion of cells will not be not entirely accurate.

Implicit in the use of DNA as an indicator is the assumption that all bacteria in the sample contain equal quantities of DNA and that all of their genomes are equally susceptible to degradation in the isolation procedure. This, taken with the fact that DNA from non-bacterial sources will also be extracted with the procedure, will also limit the accuracy of the assay as a whole.

There are other steps in the Caulobacter assay procedure where inaccuracies will be introduced. Firstly, the specificity of the probes could present problems because a relatively small number of non-Caulobacters was tested and not all Caulobacters hybridize with the probes to the same degree. The surface array probe also contained the promoter region for that gene which could cross react with promoters from other organisms in the environment when the blots are washed at the test stringency. Secondly, both standard DNAs were made up with the isolates that were available and the proportions and the makeup of both pools are not likely to be truly representative of the population in the environment. These factors

taken together should indicate that a certain degree of caution be used when interpretting results. This assay should, however, be of some considerable use in comparative studies where changes in the population are to be monitored under different operating conditions .

The decision to use this method despite the number of assumptions required was based on the lack of alternatives readily available to us at the time. Culturing was not feasible for all the reasons stated in the Introduction and because enumeration of Caulobacters on plates is complicated by their tendency to form multicellular rosettes and to attach to other cells (49, 50). It was also hoped that, because the population of organisms in sewage is so large, many of the differences in genome size and stability and cell fragility, etc. between the individual members would effectively cancel out.

The wash stringency used in the assay to pick up the target DNAs also gave a fairly high background signal when probing purified non-target DNA. This meant that a series of standards that included non-target DNA had to be used to determine detection limits and to distinguish signal that can be attributed to the presence of Caulobacter DNA from non-specific binding. A negative control DNA was therefore made up by pooling DNA isolated from several non-Caulobacter sewage isolates. Also, because the signal strength obtained when probing individual Caulobacter isolates from wastewaters was not constant (and was consistently lower than that obtained using either of two laboratory strains), a pool of these Caulobacters was used as the positive control.

Both standard DNAs were made up of a limited number of organisms and were therefore not likely to be truly representative of the population in the wastewater environment. Our collection of

Caulobacters came from a number of treatment system types and locations. In early experiments, DNA from the entire collection was used to make up the standards despite the fact that the collection is biased toward the "atypical" strains. Later a new standard DNA was made up excluding those strains from the pool. Clearly, the standard cannot accurately represent every test population, but this seems a more realistic approach than using the laboratory strain as a positive control. Again, the results obtained from this approach would be most valuable in the context of comparative studies on a single population over time rather than between systems.

The signals obtained from the samples were not above background levels, therefore it was important to establish that the DNA isolated from the sewage samples did not contain any substances that would inhibit binding of the probes to the target. This was carried out by adding 10% (w/w) FWC pool and FWC18 DNA to DNA isolated from the UBC pilot plant. This experiment not only showed that the probes do hybridize with Caulobacter DNA when it is present but that the specific organisms present do make a substantial difference to the signal obtained in the assay. The sample that was spiked with FWC18 DNA, which was chosen at random, gave a significantly darker spot than the sample spiked with the FWC pool DNA although equal amounts of DNA were added (Fig. 8). This was presumably because it had greater homology with the probes than the average FWC pool isolate.

Experiments were carried out on sewage to which a pure culture of FWC18 was added to show that Caulobacter DNA would be extracted in the isolation procedure if Caulobacters were present in the sample. The samples containing 1% and 0.1% FWC18 gave a signal equivalent to that of sewage alone, however, when one tenth

of the volume was FWC18 culture, a signal above background was observed. This indicated that the DNA was extracted but that a large number of Caulobacters is required to be seen in this assay.

Given the high background signals obtained using the moderate stringency wash regime employed in this method and the narrow range of linearity in X-ray film it was thought that visual examination of the autoradiograms might not yield sufficient information about the samples. Dots were cut out of the blots with a hole punch to ensure that the area of all of the filters was equal and these were counted by liquid scintillation. In the first instance only the undiluted samples were counted and as seen in Figure 9, the utility of the standard curve broke down at the 1% level. Later when all the dots were counted it could be seen that some of the more dilute samples gave standard curves (again above the 1% level) that were more linear. It could be that more dilute samples give more linear responses because binding to the target DNA is blocked in the more concentrated samples by the presence of large quantities of non-target DNA; however, in the study by Attwood et al. (4), it was determined that there was a threshold amount of target DNA required to give a signal. If the proportion of target cells is low, the total amount of DNA bound to the filter will have to be quite high to provide enough target to be detected.

Two different probes were used in this system in order to verify the results. If both probes were specific for the target group then the results using both probes should have been identical. In this case the results did not agree. The surface array probe gave counts above background in some samples while the counts obtained using the flagellin gene were consistently at or below background. The

elevated counts observed in the samples probed with the surface array gene could be a result of cross reactivity with the promoter region (the flagellin probe does not contain the promoter sequence) but that does not account for the variability at different dilutions of the same sample. It is likely that the wash stringency used simply allowed too much non-specific binding of the probes.

Unfortunately the assay system described here does not have the sensitivity required to quantitate and monitor the Caulobacter population in sewage because their proportion to the whole is too low. There is no reason to believe that a similar approach using different probes cannot work. If a ten-fold increase is all that is required, a more highly conserved probe could be used. In Caulobacters it is conceivable that a developmental gene involved in the shift from one phase of the life-cycle to the other would be a good candidate since it might be conserved among Caulobacters and limited to them.

Sensitivity would also be increased with the use of a higher copy number target. This would decrease the number of organisms needed to give a signal that can be detected. 16S rRNA-based probes are good candidates in this respect as well for reasons of specificity. They are also attractive because of the wide range of probes that can be generated. Since there are highly variable regions and highly conserved regions (44), it is possible to generate probes that recognize all bacteria or probes that hybridize only with members of a single species. A group has recently developed a method of generating probes to a variable region of the 16s rRNA genes by using PCR and primers complementary to the conserved regions that flank it (8). In this way it is possible to obtain probes for organisms about which very little is known. This method also has

the advantage of versatility. These probes are small enough to label whole cells so that they may be examined by fluorescence microscopy or counted by flow cytometry. If the sensitivity of the assay is of importance, the complexity of the mixture (and therefore the background) can be reduced by isolating total RNA or ribosomal RNA.

The method of choice for an organism or group of organisms to be investigated in the environment will depend on the nature of the environment, time, equipment and cost, as well as the kind of probes available. In the case of Caulobacters, the approach adopted in this work does not give the required sensitivity. 16S rRNA sequence data on five FWCs from wastewater suggests that a probe can be made that recognizes at least the numerically more significant "typical" Caulobacters. One such probe is now available in our laboratory and could be used to probe total RNA from wastewater or whole, fixed samples. In either case, a new protocol will have to be developed to address the specific problems encountered using the RNA approach in this environment.

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