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13. ABSTRACT *(Maximum 200 words)*

This project was focussed on issues related to the adhesion device used by marine and freshwater biofouling Caulobacter bacteria. The longterm goal of these studies was to improve our understanding of how fouling bacteria attach to surfaces and to consider potential strategies to prevent bacterial attachment to engineered surfaces. Progress on the four following OBJECTIVES during the course of the grant are reviewed:
1) Determination of the chemical composition and structural arrangement of monosaccharides and other substituents within the holdfasts of selected marine and freshwater Caulobacters.
2) Characterization of the types of surfaces to which holdfasts will adhere.
3) Cloning and analysis of the genes specifying the holdfasts of selected marine and freshwater Caulobacters.
4) Continuation of the development of capabilities for molecular genetic experimentation in a marine Caulobacter strain.
5) Evaluation of the occurrence, stability and behavior of Caulobacters on surfaces and in complex biofilms.

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FINAL TECHNICAL REPORT

GRANT # N00014-89-J-1749 R&T CODE: 441h006

PRINCIPLE INVESTIGATOR: John Smit

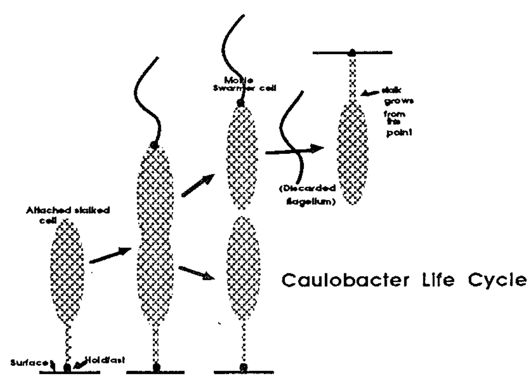
INSTITUTION: University of British Columbia, Department of Microbiology

GRANT TITLE: Chemical, structural and genetic analysis of the adhesive holdfast of biofouling Caulobacters.

REPORTING PERIOD: 1 March 1989 to 28 February 1994.

OBJECTIVES:

1) Determine the chemical composition and structural arrangement of monosaccharides and other substituents within the holdfasts of selected marine and freshwater Caulobacters.
2) Characterize the types of surfaces to which holdfasts will adhere.
3) Clone and analyze the genes specifying the holdfasts of selected marine and freshwater Caulobacters.
4) Continue developing and evaluating the capabilities of selected marine Caulobacters for molecular genetic experimentation.
5) To evaluate the occurrence, stability and behavior of Caulobacters on surfaces and in complex biofilms.



Caulobacter Life Cycle

ACCOMPLISHMENTS

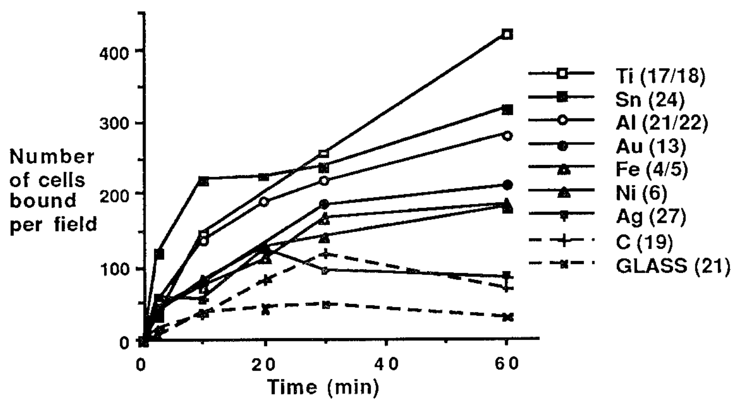
1) Chemical analysis of the adhesive holdfast of *C. crescentus* CB2A

We have refined an isolation procedure based on the observation that holdfasts bind very tightly to colloidal gold particles. Once bound, the holdfast is no longer adhesive and the complex can be isolated by density centrifugation. We isolated overproducing, holdfast-"shedding" mutants that greatly assisted this process. We still have problems getting the holdfast sufficiently purified from other surface polysaccharides, but by selecting mutants that are missing offending polysaccharides, characterizing a major source of contamination (LPS) and modifying the isolation technique by using both sucrose and CsCl density gradients we have greatly improved the overall procedure. In the end we worked out media and growth conditions for large scale fermentor runs (ie, 60-100 liters) and engineered a triple mutant that was a holdfast shedder, holdfast overproducer and in the absence of calcium showed repressed synthesis of the offending LPS species. We are still analyzing polysaccharide that resulted from a last large scale run. The difficulty is not in identifying major sugars (which we already know about), but to find some new aspect of the polysaccharide that may lead to an understanding of why it is adhesive.

2) On the adhesive properties of the holdfast.

As part of studies aimed at discerning what types of surfaces to which the Caulobacters will attach, glass surfaces were covalently modified with a variety of chemical substituents (provided by Dan Rittschoff, Duke University Marine Labs), resulting in surfaces ranging from highly charged to very hydrophobic. Using a quantitative static flow attachment assay we learned was that Caulobacters will attach to virtually all surfaces at at least moderate frequency, but appear to prefer substrates that are moderately hydrophobic. Freshwater Caulobacters attach better to very hydrophobic surfaces than do marine Caulobacters. By growing marine strains that tolerate low ionic strength media in a freshwater medium, we learned that the salts in seawater are apparently responsible for lowered adhesiveness to hydrophobic surfaces.

We also used the same assay to determine the preference of the Caulobacter adhesive for various metal surfaces, by evaporating metals onto glass surfaces. We knew the holdfast binds tightly to metals such as gold and silver, but wish to learn if there is a preference for these metals over other types of surfaces. Some of the results are shown in this figure.



It is clear that Caulobacters attach very efficiently to most metals and have a particular preference for titanium.

3) Identification and analysis of Caulobacter holdfast related genes

A-Genes that specify the holdfast structure of a freshwater Caulobacter

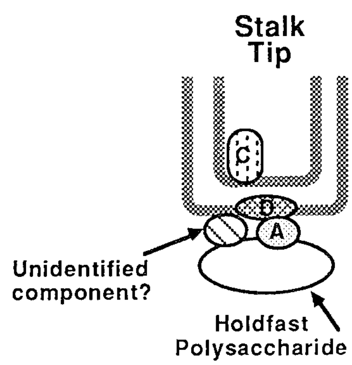
We prepared a library of 16,000 independent transposon Tn5 insertions in *Caulobacter crescentus* CB2A, isolated 78 holdfast-defective mutants and began genetic analysis of the relevant gene regions. We have learned that the mutants cluster in 4 regions throughout the genome and have a number of phenotypes. We also cloned a segment of DNA from each of the 4 regions. Our results are summarized in the table on the following page.

We placed emphasis of the shedder phenotype, because we believe the genes in question are responsible for anchoring the holdfast to the cell surface. Thus the stalk tip is a "perfect" substrate and therefore

TABLE. Holdfast related genes of the freshwater *Caulobacter crescentus*

Cluster	Phenotypes	Size of Cloned Region
A	Reduced level or altered holdfast .	9.2 Kb (Eco R1)
B	Holdfast shedder	12 Kb (SstI/EcoR1)
C	No Holdfast	14.3 Kb (SstI)
D	a)No Holdfast	12.2 Kb (ClaI/KpnI)
	b)Reduced level or altered holdfast	

molecular genetic methods can ultimately be used to analyze the molecular details of adhesion. We have defined 4 genes; one (B) is a transcriptional activator for one other gene (C), whose role is probably the transport of holdfast out of the cell (it is an ATP-binding protein). A and B are transcribed from a σ54-type promoter, associated with developmental regulation in Caulobacter. We believe D is a transmembrane anchor, while A is a linker protein, bridging the holdfast and D. A model for our current view of the region follows:



We have cloned uninterrupted genome segments from the other 3 regions as well and are defining the minimum size of DNA that will complement the holdfast-defective phenotype produced by the Tn5 insertions, in preparation for DNA sequencing of these regions. At the conclusion of funding we are focussing on the Cluster D region, which appears to contain multiple structure-related holdfast genes and DNA sequencing of this region is occurring at this writing

B-Genes that specify the holdfast of marine Caulobacter MCS6

From a library of 20,000 Tn5 insertion mutants we isolated holdfast-defective mutants. Southern analysis defined 4 regions of the genome that have holdfast-related genes; mutants in 3 regions produce little or no holdfast. The fourth group exhibit a holdfast that appears normal by visual techniques but is poorly adhesive to polystyrene. Regions of DNA have been identified in a cosmid library prepared for MCS6 DNA for all groups, complementation has been confirmed for all but one. As chemical analysis information becomes available for the marine holdfast, learning the exact chemical defect will be important.

4) Developing the capabilities of marine Caulobacter MCS6 for molecular genetic experimentation. We published a series of plasmid vectors, designed for gene expression in Caulobacters, including the marine MCS6. These will become important for future studies on holdfast gene regulation. We also developed a procedure for introducing plasmids into marine Caulobacters by electroporation, greatly increasing the ease of introducing genes into this bacterium.

5) <u>The occurrence, stability and behavior of Caulobacters on surfaces and in complex biofilms, in collaboration with ONR-funded researchers and others</u>.

In collaboration with David Stahl (presently at Northwestern) we published a study comparing the 16S rRNA sequences of marine and freshwater Caulobacters. In it we theorized that all Caulobacters are at least distantly related and that the marine Caulobacters may be the ancestral source of present-day freshwater Caulobacters. Another consequence of the work was the identification of nucleotide sequences within the 16S mRNA that are apparently unique to the marine and freshwater Caulobacter groups and which can now be used to generate probes to directly assess abundance of marine or freshwater Caulobacters in complex biofilms.

At the very conclusion of the funding period a study identifying signature lipids for the Caulobacters and the effects of typical environmental variables on the lipid profiles was begun in two laboratories: with Herb Frederickson and Wolf-Rainer Abraham at the German Biotechnology Institute in Braunschweig, Germany and with David White at the University of Tennessee. Data is still being analyzed but one result will be a phylogenetic analysis which will be compared to that obtained by 16S mRNA studies.

We are also midcourse in studies aimed at defining the stability of Caulobacters in engineered biofilms, monitoring survival characteristics under various types of stress and their response to a variety of nutrient loading rates. This is being done with Robin Turner (Biotechnology Lab, University of British Columbia) and with James Atwater (Engineering Department, UBC) and involves flow cells coupled to image analysis computers and bench scale bioreactors.

<u>PUBLICATIONS AND REPORTS RELEVANT TO THIS GRANT</u>

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•Mitchell, D., and J. Smit. 1990. Identification of the genes affecting production of the adhesion organelle of *Caulobacter crescentus* CB2. J. Bacteriol. 172, 5425-5431.

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•Gilchrist, A., and J. Smit. 1991. Transformation of freshwater and marine Caulobacters by electroporation. J. Bacteriol. <u>173</u>, 921-925.

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•Merker, R.I., D. Rittschoff, and J. Smit. Adhesive properties of marine and freshwater caulobacter holdfasts. For Appl. Environ. Microbiol.

•Ravenscroft, N., N. Kurunaratne, P. Valentine, and J. Smit. Isolation and chemical characterization of the adhesive holdfast of Caulobacter crescentus. For J. Bacteriol.

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•Smit, J. 1989. Characterization of surface adhesion in marine and freshwater Caulobacters. Office of Naval Research, Marine Biosurfaces Program, Hopkins Marine Station, Pacific Grove, California.

•Walker, S.G., N. Ravenscroft, G.G.S. Dutton, and J. Smit. 1990. *Caulobacter crescentus* surface polysaccharides and surface layer assembly. Abstracts of the Annual Meeting, Canadian Society of Microbiologists.

•Gilchrist, A., and J. Smit. 1990. Electrotransformation of Caulobacters. Abstracts of the Annual Meeting, Canadian Society of Microbiologists.

•Kurtz, Jr., H.D., and J. Smit. 1990. Preliminary characterization of the Caulobacter holdfast attachment protein. Annual Meeting, Northwest Branch, American Society for Microbiology.

•Ravenscroft, N., S.G. Walker, and J. Smit. 1990. The chemistry of adhesion in Caulobacters. International Conference: "Bioadhesion-A physico-chemical approach of biological adhesion in dentistry, medicine and industry", Groningen, Netherlands.

•Yun, C., and J. Smit. 1991. Identification of genes required for production of the adhesive holdfast organelle of marine Caulobacter

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N0014-89-J-1749
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