Using the high level Type I secretion apparatus of *Caulobacter crescentus* for recombinant protein secretion or surface display

John F. Nomellini and John Smit* Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, BC V6T1Z3 Canada

ABSTRACT

Secretion of recombinant proteins is often desirable since it offers lower downstream processing costs. However many proteins of interest in biotechnology cannot be secreted via the universal "Sec-dependent" pathway, which requires export in an unfolded state. Type I secretion, present in some in bacteria, offers the only practical alternative. Yet few bacteria with Type I systems are non-pathogenic and capable of high level secretion via this mechanism. *Caulobacter crescentus* is a notable exception; it is a harmless Gram bacterium that produces a two-dimensional crystalline assembly on its outer surface, composed of a single highly expressed protein exported by Type I secretion. We have adapted the export system to enable secretion of a wide variety of proteins (using the C-terminal secretion signal) or for display of peptides and proteins on the cell surface, using the entire S-layer gene. Display of peptides of 10-300 amino acids is commonly accomplished. Because of the crystalline nature of the S-layer successful presentation can occur at densities of 40,000 copies/cell. In one application we display a complete Protein G binding capability (involving 3 IgG binding domains and spacer peptides), enabling use of the bacteria for pull-down immunoassays. Other applications of the S-layer display in development include whole cell vaccines for veterinary or anti-cancer applications, and infection blocking agents. Cloning and expression is based on small, high copy number plasmids that readily shuttle from *E. coli*. Protein secretion by fusion to the C-terminal secretion signal can also take advantage of a built-in purification mechanism; though normally soluble, under certain conditions aggregate forming properties of the S-layer monomer secretion signal enable rapid purification by simple filtration. This system is available to the research and commercial community, formulated as protein secretion and protein display kits available through an internet portal.

Background

***Caulobacter crescentus* and its S-layer** *C. crescentus* is a gram-negative, non-pathogenic bacterium found in many soil and freshwater microbial biofilms. It is a dimorphic bacterium, alternating between a motile swarmer cell and a nonmotile stalked cell. Because of its readily identified life cycle, it has been studied for nearly 50 years and the main laboratory strain (CB15) is well characterized genetically and biochemically. This includes a sequenced genome. *C. crescentus* is readily grown using standard laboratory equipment. They can also be easily grown in commercial fermenters to at least 30 ODs in defined minimal media, free of animal protein.

C. crescentus elaborates on its outer surface the hexagonal crystalline protein lattice surface layer (S-layer). This S-layer is composed a single protein, RsaA. It is thought that the function of the S-layer is to act as a physical barrier to parasites and lytic enzymes. The RsaA protein is naturally expressed to high levels in *C. crescentus* cells, accounting for 10-12% of the total cell protein; some of our recombinant constructs produce much more. The RsaA protein is high in hydroxyl-containing residues (25% of the amino acids are serine or threonine) and small neutral residues. It contains few charged amino acids and there are no cysteines.

Structural Features of RsaA

- The N-terminus (approximately amino acids 1-220) is required for attachment of at least some of the protein monomers to the cell surface
- Glycine-Aspartate rich "RTX" motifs in the C-terminal portion of the protein (amino acids 860-985) are required for calcium binding, which may, in turn, be needed for proper folding and secretion of the native protein.
- The C-terminal (minimally amino acids 944-1026) is required for secretion of the protein. In practice, the last 120 amino acids or more is needed for useful secretion levels for heterologous fusion proteins.

Type I Secretion. Because RsaA is the protein that forms the S-layer it must be produced inside the cell and exported to the cell surface. Export is mediated by a secretion mechanism categorized as a Type I transporter. It is a bacterial secretion system that uses ATP to generate the energy needed for protein export. In Type I-secreted proteins the secretion signal resides in the C-terminus of the protein and remains on the exported protein. The Type I secretion pathway is very different from the more typical "General Secretory Pathway" (also called the sec-dependent pathway). In the latter, an N-terminal signal leader peptide is recognized by the secretion pathway and later is cleaved. Because the Type I secretion mechanism creates a water-filled pathway to the exterior, proteins that might not be suited for secretion by the general secretory pathway might be successfully secreted by such a system. Indeed, it is really the only known alternative secretion mechanism that is adaptable for recombinant protein secretion. The *C. crescentus* version is one of few that expresses at high levels and is the only one we are aware of in a host with low levels of endotoxin.

Of particular interest for protein secretion or display is that the export pathway is tolerant of many foreign proteins, either when fused to the *rsa*A secretion signal or inserted into a "permissive site" in the complete gene.

The point of this poster:

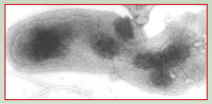
The abilities of the Caulobacter S-layer secretion apparatus to export recombinant proteins has two major applications:

1) Secretion of Recombinant protein via fusion with the C-terminal secretion signal.

2) Display of proteins on the surface of the bacteria.

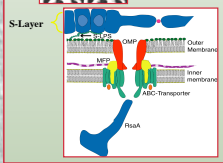
Some examples of what we are currently working on in both arenas are shown in this poster. Many projects are collaborations with other laboratories in Academia, Government or Commercial enterprises.

The Caulobacter S-layer and the Type I Transporter



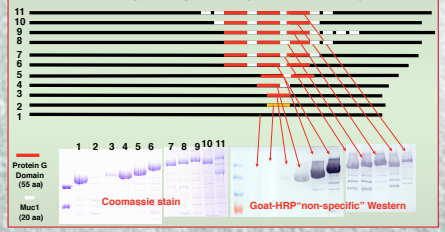
Negative stain electron microscopy of lysed Caulobacter cell

Image reconstruction by the Baumeister group- MPI Munich



Protein G Display

The Protein G IgG binding domain expressed with Muc-1 spacers.



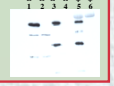
IgG binding capacity of Caulobacter constructs - comparison with Staph A (Pansorbin) cells

Cell type	IgG binding Capacity*
Pansorbin®	500 +/- 100
S-layer control	90 +/- 30
"old" Pro G	400 +/- 90
"new" Pro G (Spe1/Nhe1)	600 +/- 200
G-Muc1B (M)	200 +/- 50
G-M-G	600 +/- 300
G-M-G-M-G	800 +/- 400
M-G-M-G-M-G-M	1000 +/- 400

*ng rabbit IgG/mg dry weight of cells

Immuno-precipitation using Caulobacter cells with the Protein G-Muc-1 multimer

FLAG-tagged lysate	+	--	+	+	+	--
Mock lysate	--	+	--	--	--	--
IM PPT Ab anti FLAG	--	--	+	+	+	--
Protein G beads	--	--	+	--	--	--
S-layer control cells	--	--	--	+	--	--
MGMGMGM cells	--	--	--	--	+	+
	1	2	3	4	5	6



Display Applications

Secretion examples

Two ways to produce secreted S-layer fusion proteins

1 - Don't do anything unusual. Shake cultures at normal speeds or grow in any standard fermenter and the protein will be soluble in the culture supernatant. Often it is present at a high enough level to be seen on Coomassie stained gels by sampling 10-20 µl of culture fluid.

2 - With slow shaking recombinant/S-layer fusions generally form a gel-like aggregate that is readily filtered. It is ~97% water. The protein is 90.99+% pure and can be solubilized with urea followed by dialysis. This is useful for quick assessment of success or yield and if your protein can tolerate the unfolding/ refolding due to urea treatment, it is a great way to get pure protein in high quantities quickly.

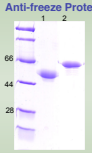
Single Chain Fv antibody secretion

Coomassie Blue stained SDS-PAGE of urea treated aggregates.
1) Secretion signal only
2) p136C F240, a 251 AA single chain F240 fused to the C-term S-layer.

This fusion protein will tested in Pseudotyped and HIV-1 virus inhibition assays to see if can be formulated with display constructs (see panel on right) as part of a topical microbicide.

A collaboration with Lisa Cavacini- Harvard

Anti-freeze Proteins



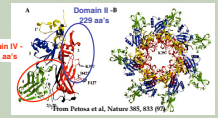
Coomassie Blue stained SDS-PAGE of urea treated aggregates (LEFT) or 20 µl sampling of culture fluids (RIGHT)

1) Secretion signal only
2) p136C
WFAF31R - a 31 AA Type I antifreeze protein

A collaboration with AF Proteins, Inc.

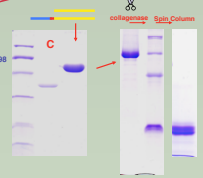
Anthrax Protective antigen - Vaccine and diagnostics dev't

The current Anthrax vaccine uses a partially purified whole proteative antigen. There is growing evidence that domain IV is the key region to develop an immune response. There is also some data for domain II. We have found that both regions express well in the Caulobacter S-layer secretion system and the protein produced may be come part of a subunit vaccine and will be part of Elisa-based diagnostics tools.



Anthrax Vaccines - a collaboration with Brad Berger (Defence Res. & Dev't Canada) and Cangene Corporation

Protein prepared from the "aggregates" method:



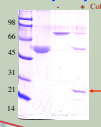
Secretion signal only

Domain IV/Secretion signal

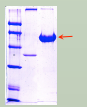
PLGP

+ Collagenase

Direct sampling of culture fluid: (15 µl)



Domain II fusion protein



You can also do this:

Two Kits have been formulated, one for secretion and one for display and are available for purchase at: www.flintbox.com/caulobacter.

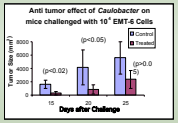
A host strain (sent as aliquots of electrocompetent cells), cloning vector (that readily shuttles from *E. coli* to Caulobacter, growth medium, collagenase enzyme, DNA primers for sequence confirmation, instruction manual, etc are all included. Other materials, such as S-layer specific antiserum and additional vectors are also available.

This is a licensed technology and the kits include a license agreement. For long term use in commercial applications, non-exclusive Research and Production licenses are available. Short-term contract research with the Smit lab, especially for proof of concept projects is also available.

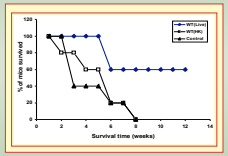
Anti-Tumor therapy - a collaboration with Mav Suresh and Babita Agrawal - U. Alberta

Caulobacter cells have a natural anti-tumor effect - can we make it better?

Therapeutic mode studies with the EMT-6 Mammary Carcinoma model



Prophylactic efficacy of injected Caulobacters in Lewis Lung Carcinoma Model

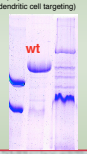


Lungs of protected mice

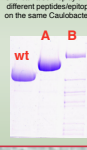
Lungs of unprotected mice

Some current examples of cancer-related peptides displayed in the Caulobacter S-layer

Display of OEC205 SCFv (dendritic cell targeting)



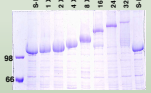
Mouse GM-CSF

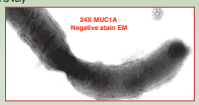


One can also display two different peptides/epitopes on the same Caulobacter

Display of the MUC1 Cancer antigen

Tandem multimers of the 20 AA Muc-1 epitope is very well tolerated when expressed in the S-layer.

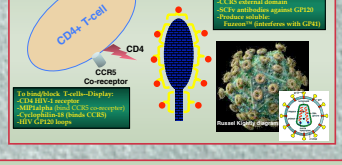




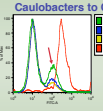
S-layer was extracted with low pH, separated by SDS-PAGE and stained with Coomassie. Mol Wt markers are indicated and the number of extra amino acids from Muc1 multimers are in ( ). All epitopes were inserted at AA 723 in the S-layer.

Developing a microbicide for HIV-1 - a collaboration with Mark Horwitz (U. Br. Columbia)and Lisa Cavacini (Harvard U.)

Microbicide development: What should one display on the S-layer to block HIV infection?



Using Fluorescence Activated Cell Sorting (FACS) to evaluate attachment of MIP1Alpha-displaying Caulobacters to CCR5 displaying glial cells

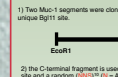


Binding of Caulobacter expressing MIP1alpha on the surface to CCR5 receptor on U87 cells. The Caulobacter and U87 cells were mixed at a 500:1 ratio for 30 minutes on ice (green). Binding of control Caulobacters to CCR5 on U87 cells at a 500:1 ratio for 30 minutes on ice (blue), binding of CCR5 expressing U87 cells alone without Caulobacter (yellow), incubation of MIP1a expressing Caulobacter alone without cells (red).

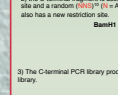
RESULTS: The green line peak (arrow) shows a clear increase indicating the binding of MIP1alpha displaying Caulobacters. Further work work, especially with additional cell lines displaying CCR5 is currently in progress.

In progress: Random 10-mer peptide display library

1) Two Muc-1 segments were cloned into the S-layer gene at AA 723, separated by a unique Bgl11 site.

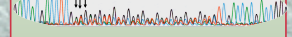


2) the C-terminal fragment is used as PCR target. The forward oligo has a new unique site and a random (NNS)^10 (N = A, C, G or T; S = C or G) segment. The reverse oligo also has a new restriction site.

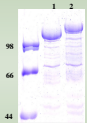


3) The C-terminal PCR library products are ligated into the S-layer gene creating a display library.

4) The Library is evaluated by sequencing and FACS.



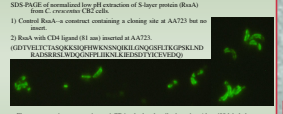
Display of CD4 domain 1



SDS-PAGE of normalized low pH extraction of S-layer protein (RsaA) from *C. crescentus* CB2 cells.

1) Control RsaA--a construct containing a cloning site at AA723 but no insert

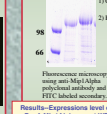
2) RsaA with CD4 ligand (81 aas) inserted at AA723.
(GDTVELICTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKLND
RADSRRESLWDQGNFPLIIKNLKIEDSDTYICEVEDQ)



Fluorescence microscopy using anti-CD4 polyclonal antibody and an Alexa488 labeled secondary. The lower images are combined phase contrast and fluorescence microscopy.

Results--Expression levels of RsaA-CD4 are somewhat below WT levels and this seems to be reflected in a degree of patchiness in the surface label, but the ligand is readily recognized by the CD4-specific antiserum.

MIP1α display

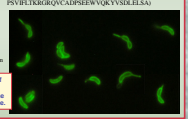


SDS-PAGE of normalized low pH extraction of S-layer protein (RsaA) from *C. crescentus* CB2 cells.

1) Control RsaA--a construct containing a cloning site at AA723 but no insert

2) RsaA with Mip1alpha (70 aa) inserted at AA723
(APLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSK
PSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA)

Fluorescence microscopy using anti-Mip1Alpha polyclonal antibody and an FITC labeled secondary.



Results--Expressions level of RsaA-Mip1Alpha are at WT levels and this results in dense uniform labeling of the surface.