Heads Up S-Layer Display: The Power of Many

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Many microbes produce surface protein layers (S-layers) that are organized as geometric arrays. These S-layers offer unique applications in nanotechnology, but are a challenge for atomic structure analysis. Pavkov et al. (2008) have made inroads, however, with an S-layer of *Geobacillus stearothermophilus*.

Imagine displaying useful proteins (antibodies, cytokines, antigens, enzymes) at very high density (>20,000 copies/μm^2) on a surface, robustly but without covalent linkages. Imagine that every copy of the displayed protein is oriented so that it can function correctly and is displayed in exactly the same manner as all the other copies, exhibiting activity that is not compromised by chemical crosslinking and that any influence of nearby protein sequence is the same for each copy. Additionally, effects of the underlying surface (plastic, glass, metals, biological polymers, or membranes) are also minimized because the surface is coated with ~10 nm of protein.

If one could do all this then dense affinity matrices with precise binding constants (for "lab on a chip" applications) could be produced as well as very precise ELISA assays (with high sensitivity, large protein loading range, low background). One could envision capturing viruses from dilute environments, retaining them with a binding constant greater than a covalent bond (ensured by multiple additive interactions) or concentrating toxic ions or macromolecules for disposal. If the "targeting" ligands or antibodies were displayed on a bacterium it could be used to deliver toxic cargo to tumors or stimulate localized antitumor immune responses.

Imagine further that two or more proteins could be simultaneously displayed in a dense manner, interacting with each other in a useful way or binding a target in two independent ways. On a bacterial surface two ligands could mimic a receptor/coreceptor complex normally present on a host cell and thereby entrap a virus so that it cannot bind its host and induce disease (producing "microbicides"). Then imagine that the capability for all of the above can be built into the genomes of bacteria that are safe and inexpensive to produce, can be stored for any length of time, and the components can be produced in any quantity needed, often using only corn sugar and fertilizer as feedstocks.

Perhaps you have already guessed (or knew) that at least early versions of everything described above are presently available using the capabilities of proteins that form microbial surface S-layers (see Nomellini et al., 2004; Pleschberger et al., 2004; Sleytr et al., 2007; Tschiggerl et al., 2008). These are structures present in a wide range of bacteria and archaea. They are composed of a secreted protein that self-assembles to form a two-dimensional crystalline array on the cell surface. The tetragonal, hexagonal, or oblique patterns can be seen with several electron microscopic techniques and have fascinated a generation of microscopists. Indeed, S-layers are considered as a group because of the surface associated geometric arrays, not because they are a cohesive group of similar proteins. Indeed they are not—most share little sequence homology.

The function of S-layers has never really been satisfactorily addressed, probably because they are needed in the complex milieu of natural environments but not in laboratory isolation. Most working with the structures agree that a prime function is probably protection from predators (viruses, parasitic bacteria) and enzymatic attack. Some S-layers have further evolved to be virulence determinants or to attach enzymes that are useful to the cell; some may attract scarce ions such as iron. For protection the microbe presumably must make significant amounts of protein, enough to coat the entire cell. Often 10%–15% of total cell protein is suggested, but our recent studies with the *Caulobacter crescentus* S-layer have indicated that ~25% of cell protein synthesis is devoted to this one protein (Figure 1). Many bacteria with S-layers live in nutritionally limited environments so the necessity for protection must be paramount to devote scarce resources to such an extent. On the other hand, natural prodigious levels of expression are a boon for biotechnology applications.

To be useful in the applications alluded to above, the protein must be capable of tolerating insertions or fusions with foreign proteins, remaining able to assemble the geometrically arranged layers, and of incorporating a segment that has never participated in such a venture. Ideally, the chimeric genes should be capable of being translated and secreted by the cells. When that latter bar cannot be achieved the proteins are produced inside a host such as *E. coli*, extracted, and purified. But then they must have an extraordinary propensity to refold correctly after complete denaturation, self-assembling, and attaching to a surface even with the foreign segment inserted.

Such challenging requirements in part define why only a small number of S-layers are currently being developed in significant ways for display applications. This preview is not exhaustive (and so my apologies to many workers in this area), but most structure analysis and development for foreign protein display has been done in the Gram-positive bacteria *Geobacillus stearothermophilus* and *Bacillus sphaericus* (by Uwe Sleytr and a range of collaborators). For Gram-negative bacteria, *Caulobacter crescentus* is the major example in development. They are a study in contrasts. For the former, the S-layer monomers have a remarkable ability to refold and crystallize after denaturation, attaching readily to the peptidoglycan or

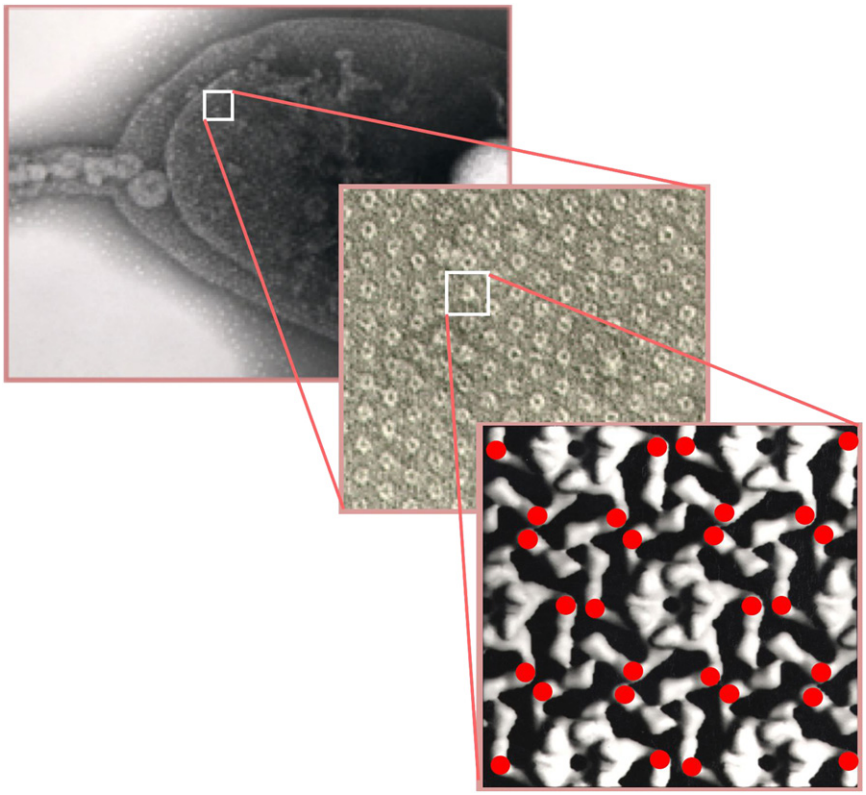

Figure 1. The *Caulobacter crescentus* S-Layer
The structure can be seen on lysed cells prepared for negative stain electron microscopy. An enlargement shows the structure in more detail and the further enlargement is the result of tomography and image filtration methods (Smit et al., 1992). The red dots suggest the position of foreign insertions into the structured layer.

secondary cell wall polymers contained within peptidoglycan sacculi isolated from bacteria. However, S-layers from Gram-positive bacteria are secreted across the cell membrane via the standard ''sec-dependent'' pathway and then must traverse a relatively thick peptidoglycan layer. Native S-layer proteins are adapted to this tortuous pathway in the natural host, but it appears that recombinant chimeric forms are often not secreted efficiently. Thus most are made as heterologous proteins in *E. coli* and most applications are oriented to display on surfaces other than the bacterium they came from. For the latter *Caulobacter crescentus*, secretion is accomplished with Type I secretion, an ATP-driven protein pump that extrudes proteins through a large, gated aqueous channel from cytoplasm directly through two membranes to the outside. We have experienced significant levels of success in secreting numerous recombinant S-layer proteins containing up to 300 amino acids at selected sites (Nomellini et al., 2004); it seems the pump is quite tolerant to addition of many types of nonnative protein sequence to the S-layer monomer. Moreover, assembly and surface attachment of the S-layer is nearly always unimpeded, a characteristic shared with the Gram-positive S-layers. In contrast, however, attachment is mediated by a variant of the bacterium's lipopolysaccharide, such that assembly onto artificial lipid vesicles is readily accomplished (Nomellini et al., 1997) but extensive crystallization on solid surfaces is more challenging and still in development. Thus most current applications of display in this case are oriented to display on the bacterium. One pointed example of the two different approaches might be that display of immunoglobulin binding has been engineered in both systems (via Protein A or G); in each case a somewhat different range of potential applications are suggested as a raison d'être (Nomellini et al., 2007; Völlenkle et al., 2004).

However, the performance standard for foreign protein display within S-layers is rising. It is increasingly important to move from if a protein can be displayed to where within the crystallized structures. Many candidate proteins are functional as dimers (e.g., cytokine IL10) or trimers (e.g., the CD40 receptor ligand, which activates immune cell function in T cells); F_{AB} versions of artificial antibodies are composed of two interacting chains. Two enzymes with related activities (e.g., one processing the product of another) might function more efficiently if spaced an appropriate distance from each other. Some inserted proteins will require their N and C termini to remain close to each other for proper functioning. Thus juxtaposition and molecular distances often matter and solved atomic resolution structures could greatly help discern why certain sites are found to be tolerant and which should be used for a given protein(s).

And there is the rub. For all the potential utility of spontaneous two-dimensional S-layer array formation, the penalty is that it is a serious impediment to producing three-dimensional crystals for X-ray crystallography. I am not a structure specialist, but the analogy put to me has been that of the difficulty in reassembling a ream of paper into its former precise block shape after being dispersed into individual sheets: the tendency for two dimensional array formation impedes high quality crystallization in the third dimension. Over the years remarkable work has been done using tomography methods to discern S-layer structure, notably by Wolfgang Baumeister and colleagues (see Smit et al., 1992), reconstructing tilt series of Fourier transformed and filtered transmission electron micrograph images. But atomic resolution is not achieved by this method and precise positioning of inserts remains elusive.

So this is the challenge taken up by Pavkov et al. (2008). Atomic resolution has been attempted only a couple of times previously in S-layers of far different composition. The approach is to crystallize only portions of the S-layer monomer and in this case much of the emphasis is on understanding the attachment domain—the region that is not directly involved in self-assembly of the array. But attachment is as important as self-assembly for display applications and the report represents a significant advance for one of the *Geobacillus* S-layer proteins (there are several expressed by the same

bacterium). Regions of the monomer are now more accessible for designing positioning of foreign inserts. Out of such studies will also come a definition of structural domains that cannot be easily deciphered from primary sequence. Alas, the domains responsible for self-assembly still defy high-resolution analysis; the struggle continues.

The display applications mentioned earlier by no means exhaust the technology areas presently in development by at least one of the S-layer systems. Enzyme bioreactors, ultrafilters (Sleytr et al., 2007), whole cell vaccines, cancer therapeutics (Bhatnagar et al., 2006), cytokine display (to modify immune responses), allergy treatment (Gerstmayr et al., 2007), and peptide or antibody display libraries (rapidly screened for "hits" by fluorescence activated cell sorting) can also be added to the list. There will be more; not all the potential of S-layers has yet been exploited.

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Exchange We Can Believe in

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Newly published papers by Polier et al. in *Cell* and by Schuermann et al. in *Molecular Cell* present structures of Hsp110:Hsp70 complexes that reveal a compelling picture for the mechanism of nucleotide exchange in Hsp70s of the eukaryotic cytosol.

Diverse molecular chaperones participate in protein folding and other processes controlling polypeptide conformation, and the 70 kDa heat-shock proteins (Hsp70s) are the most universal of chaperones. Hsp70 chaperones function in ATP-driven cycles of polypeptide binding and release: ATP binding elicits polypeptide substrate release, substrate rebinding stimulates ATP hydrolysis, and replacement of the ADP and inorganic phosphate (P_i) products by ATP completes the cycle. Typically, nucleotide exchange in Hsp70s is affected by nucleotide exchange factors (NEFs). Various Hsp70 NEFs have been characterized and crystal structures have been described for a few Hsp70:NEF complexes. Recent studies show that Hsp110 proteins are the most effective NEFs for Hsp70s of the eukaryotic cytosol (Raviol et al., 2006; Dragovic et al., 2006; Shaner et al., 2006). Now, from the structures of two Hsp110:Hsp70 complexes (Polier et al., 2008; Schuermann et al., 2008), we can see in atomic detail the elegant mechanism by which Hsp110s elicit nucleotide exchange in Hsp70s.

Characteristics of Hsp70 chaperone activity are determined by the lifetimes of its states, and these lifetimes are regulated by cochaperones as well as intrinsic Hsp70 activities and affinities. The situation is analogous as for small G proteins, where guanine exchange factors (GEFs) promote GTP for GDP exchange and guanine activating proteins (GAPs) accelerate GTPase activity. For G proteins, the active state is G(GTP), initiated by the GEF and terminated by the GAP. For Hsp70s in protein folding, Hsp70(ADP, polypeptide) can be considered to be the "active" state, wherein an aggregation-prone substrate is captured with high affinity and given the chance to disentangle. The time constant for folding depends on the frequency of Hsp70 release and rebinding events. Intrinsic ATPase activity is low in Hsp70s, but polypeptide and cochaperone Hsp40 bindings promote hydrolysis synergistically for productive substrate entrapment. Although Hsp70s have greater affinity for ATP than for ADP, and cellular concentrations are many-fold higher for