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Designer cellulosomes and bioenergy

The cellulosome

The cellulosome is an intricate multi-enzyme machine produced by many cellulolytic microorganisms. It is designed for efficient degradation of plant cell wall polysaccharides, notably cellulose — the most abundant organic polymer on Earth. The cellulosomes are composed of a conglomerate of subunits, each of which comprises a set of discrete interacting functional modules. A multi-functional integrating subunit (called scaffoldin) is responsible for organizing the cellulolytic subunits into the multi-enzyme complex. This is accomplished by the interaction of two complementary classes of domain, located on the two separate types of interacting subunits, i.e., a cohesin domain on scaffoldin and a dockerin domain on each enzymatic subunit. The high-affinity cohesin-dockerin interaction defines the cellulosome structure (Fig. 1). Attachment of the cellulosome to its substrate is mediated by a cellulose-binding module (CBM) that comprises part of the scaffoldin subunit.

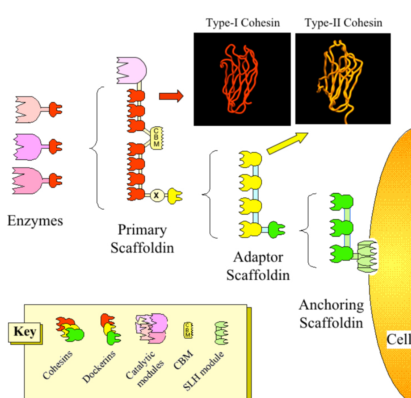

Fig. 1 Schematic representation of the supramolecular architecture and disposition on the bacterial cell surface of a representative cellulosome system. Respective specificities of the different cohesin-dockerin pairs are color-coded. A CBM in the primary scaffoldin targets the complex and the entire cell to the cellulose substrate. Crystal structures for type-I and II cohesins were determined in collaborative studies. The figure implies the Lego-like strategy of the cellulosome system.

The cohesin-dockerin interaction

We have cloned and expressed individual cellulosomal domains and have analyzed their structure-function relationship via biochemical, molecular, and structural studies. One approach has been to determine the crystal structures of the important domains. In an alternative, complementary bioinformatics-based approach, we have analyzed the cohesin-dockerin interaction by site-directed mutagenesis and "progressive gene swapping". The native interaction itself was found to be one of the strongest protein-protein interactions in nature. By comparing the cohesin-dockerin interaction between two different dockerin species, we were able to identify the important residues on both the cohesin and dockerin modules that contribute to interspecies specificity.

Cohesin-dockerin microarray

Most recently, increasing numbers of cellulosomal and non-cellulosomal cohesins and dockerins are in the process of being discovered, owing to genome sequencing projects. In order to accommodate this tremendous wealth of new cohesin-dockerin interactions, we have developed a microarray system for broad examination of cohesin-dockerin specificity (Fig. 2). For this purpose, a matching recombinant fusion protein system was designed. The cohesins were thus fused to a CBM, and the resultant fusion proteins were immobilized onto cellulose-coated glass slides. Subsequently, xylanase-fused dockerin samples were applied to the slides. This microarray system allows global comparison among the interactions between various members of these two complementary families of interacting protein modules. Knowledge of the specificity characteristics of native and mutated members of the cohesin and dockerin families provides insight into cellulosome architecture and allows selection of suitable cohesin-dockein pairs for future biotechnological and nanotechnological application.

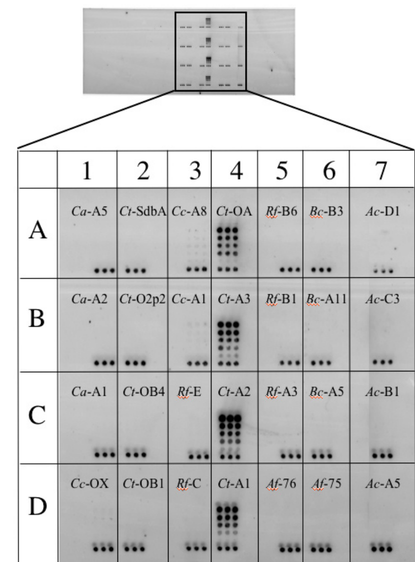

Fig. 2 Design of a cohesin-dockerin microarray. CBM-Coh fusion proteins were printed and XynDoc fusion proteins were then applied. The amount of dockerin bound to the cohesin samples was visualized by immunofluorscence. As expected, the Cel48A dockerin interacts specifically and exclusively with all type-I C. thermocellum CipA cohesins.

Characteristics of the dockerin modules

The molecular basis of the interaction between the binding domains of the cellulosome was studied in our group using biochemical and molecular techniques. One aspect of our work, focused specifically on the biophysical properties of the dockerin module. For this purpose, we employed progressive deletion and alanine-scanning techniques on the distinctive duplicated segment of the type-I dockerin module from *Clostridium thermocellum*. The results demonstrated that only one

of the repeated motifs is critical for high-affinity cohesin binding, which suggested that the near-perfect symmetry in sequence and structure of the repeated elements of the dockerin is not essential for cohesin binding per se. These biochemical findings support the structure-based hypothesis for the dual-mode of binding between the cohesin and dockerin modules.

We characterized a unique, atypical type-III dockerin from the ScaA scaffoldin of the important cellulosome-producing rumen bacterium *Ruminococcus flavefaciens*. By using spectroscopic measurements we discovered that the presence of calcium promotes folding of the atypical dockerin into its native secondary structure, by which hydrophobic sites are exposed on the surface of the dockerin module.

Sequence alignment of the type-III dockerin with a representative type-I dockerin suggested that its putative species specificity residues are small and hydrophobic. By replacing them with nucleophilic residues, derived from the type-I dockerin of *C. thermocellum*, we succeeded in switching the specificity of the type-III dockerin to emulate a type-I dockerin. We hypothesize that the type-III dockerin may bind to its type-III cohesin in a different mode than that of type-I, possibly in a non-symmetrical manner, in which both segments contribute to complex formation equally. Since the mode of interaction of the type-III cohesin-dockerin complex is still unknown, future research will also focus on the structure of the complex by using X-ray crystallography and NMR spectroscopy, in order to further understand the forces that drive this unique and tenacious interaction.

Crystal structures of cellulosomal modules

In order to understand the nature of the intermodular contacts formed between the cellulosomal subunits, or how polycellulosome assembly occurs, we employed crystallographic techniques. We thus cloned, expressed, purified, crystallized and solved several novel structures of type-II and type-III cohesin modules from different cellulosomal species.

The type-II cohesin structure shares the same jelly-roll topology with the type-I cohesin structures, in which a flattened 9-stranded β-sandwich is formed. However, several additional structural elements (an α-helix and two "β-flaps") were observed that were lacking in the type-I structures (Fig. 3A). These structural differences have been suggested to play a role in the type-specificity observed between the type-I and type-II cohesin-dockerin interactions.

The crystal structure of a type-III anchoring cohesin module from *R. flavefaciens* also revealed additional composite structural elements that represent a dramatic divergence from the other known cohesin structures (Fig. 3B). In this context, an elaborate α-helix is located between β-strands 8 and 9. The α-helix is enveloped by an extensive N-terminal loop, unseen in any other known cohesin, which embraces the helix, thereby enhancing its stability.

The structure of a different type-III "adaptor" cohesin (Fig. 3C) exhibited the standard jelly-roll topology without additional secondary structures. Despite the similarity with the type-I cohesins, the type-III adaptor cohesin is characterized by differences in the lengths of the β-strands and loops, which might reflect the differences in specificity that are inherent in its dockerin-recognition properties.

Finally, the crystal structure of a bi-modular type-II cohesin dyad revealed a novel arrangement of these cohesins on the scaffoldin subunit. The two cohesins are oriented in anti-parallel fashion, yet their dockerin-interacting surfaces (β-strands 8, 3, 6, 5) face the same direction — i.e., aligned on the same plane (Fig. 3D).

In several cases, the cohesin structures solved in our work represent precedent structures – the first of their class to be solved. The above structures provide new insight into the structural diversity and arrangement of the various cohesin types within the parent scaffoldin subunits. Further work in this direction will be performed to solve cohesin-dockerin heterodimers and higher order scaffoldin structures.

Designer cellulosomes

Designer cellulosomes comprise recombinant chimaeric scaffoldin

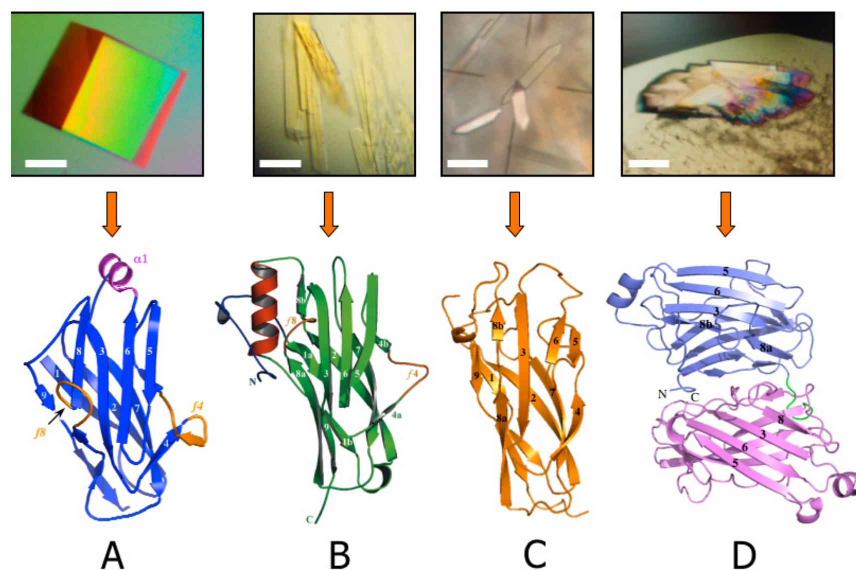

***Fig. 3** Crystals and structures of different cohesin modules. **A.** The structure of a type-II ScaB cohesin of the A. cellulolyticus cellulosome. **B.** The type-III ScaE anchoring cohesin structure from R. flavefaciens. **C.** The type-III ScaB adaptor cohesin structure from R. flavefaciens. **D.** The type-II bi-modular ScaB cohesin structure from A. cellulolyticus. The white bar corresponds to approximately 0.1 mm.*

constructs and selected dockerin-containing enzyme hybrids as a conceptual platform for promoting synergistic action among enzyme components (Fig. 4). This approach enables the precise incorporation of complementary dockerin-containing components into a designer cellulosome by simply mixing them in solution together with the chimaeric scaffoldin, thus controlling the composition and architecture of the resultant complexes. This approach will eventually be appropriate for general use as a molecular Lego for application in biotechnology and nanotechnology. In future studies, the genes for chimaeric cellulosome components will be introduced to host cell systems, and the expressed proteins will self-assemble to produce viable cellulosomes.

Conversion of cellulose to biofuels

The depletion of fossil fuels has generated world need for cheap and clean alternative energy sources. Cellulose and related plant cell wall polysaccharides (biomass) can be potentially utilized as a low-cost renewable source of sugars for conversion to biofuels like ethanol. Since cellulose is pure glucose, its conversion to fuels has remained a romantic and popular notion for weaning ourselves away from our dependence on fossil fuels. Thus by reducing the amount of agricultural waste or by growing appropriate "energy crops", we can create an environmentally friendly fuel.

Perhaps the major bottleneck for conversion of biomass to ethanol is the combined high cost and low efficiency of the cellulases and related enzymes that degrade such polysaccharides to simple sugars. Future research must thus focus on overcoming the natural recalcitrance of biomass. Rational bioengineering of cellulosomal components for production of tailor-made "designer cellulosomes" is now being developed for improved cellulose degradation. We are trying to pursue this objective by harnessing the unique, thermostable cellulose-degrading systems from *C. thermocellum* and from other bacteria. For this purpose, selected cellulosomal components (the CBM, cohesins, dockerins, catalytic modules and linker segment) are reassembled into desired interacting nanostructures using recombinant genetic techniques. Unlike native cellulosomes, designer cellulosomes can be produced in large amounts in host cell systems and their enzymatic content can be strictly controlled. The combination of designer cellulosomes with novel production concepts may provide future breakthroughs necessary for economical conversion of cellulosic biomass to biofuels.

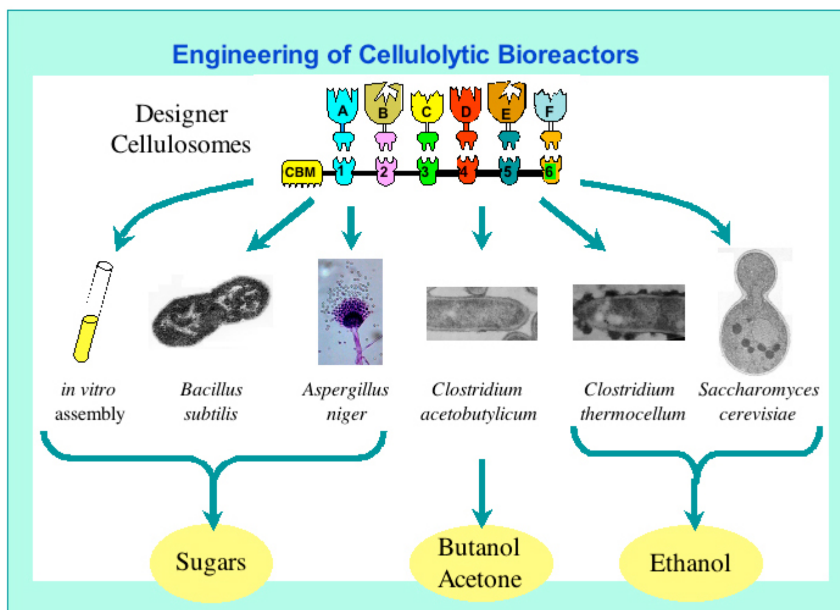

Fig. 4 *"Designer cellulosome" - an engineered system, designed to convert a free cellulase system into a cellulosome.*
Genes encoding for cellulases and/or designer cellulosome components can be cloned into a desired bacterial or fungal host cell, and the secreted proteins can be overexpressed for the degradation of cellulosic biomass in an industrial reactor (in vitro assembly). Alternatively, the genes can be cloned into a suitable bacterial, fungal or yeast host, and the transformed cell, with either de novo or improved cellulose-degrading capacity, can be grown directly on cellulosic biomass to produce a desired end product.

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