Engineering new metabolic capabilities in bacteria: lessons from recombinant cellulolytic strategies.

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Abstract

Cellulose waste biomass is the most attractive substrate for "biorefinery strategies" producing high-value products (*e.g.* fuels, plastics) by fermentation. However, traditional biomass bioconversions are economically inefficient multistep processes. Thus far, no microorganisms able to perform single-step fermentation into products (consolidated bioprocessings, CBP), have been isolated. Metabolic engineering is currently employed to develop recombinant microorganisms suitable for CBP.

The heterologous expression of extracellular proteins (*e.g.* cellulases, hemicellulases) is the key feature of recombinant cellulolytic strategies, conferring cellulolytic ability to microorganisms exhibiting high product yields and titers. Although more and more molecular tools are becoming available, efficient heterologous expression of secreted proteins is still a challenge. The present review summarizes both bottlenecks and solutions of organism engineering for biomass biorefinery strategies.

Towards engineered microorganisms for biomass consolidated bioprocessing

Cellulose biomass is the largest waste produced by human activities and the most attractive substrate for "biorefinery strategies" to produce high-value products (*e.g.* fuels, bioplastics, enzymes) through fermentation processes [1-3]. However, so far, no natural microorganisms with the necessary metabolic features for single-step biomass fermentation, *i.e.* consolidated bioprocessings (CBP), have been isolated. Traditional biomass bioconversion processes are economically inefficient multistep processes that require dedicated cellulase production [4]. Research efforts have been aimed at developing recombinant microorganisms that have the characteristics required for CBP [5-7].

The heterologous expression of extracellular proteins (*e.g.* cellulases, hemicellulases) is the key feature of recombinant cellulolytic strategies (RCS), as they confer cellulolytic ability to microorganisms with high-value product formation properties [6,8-10]. Although more and more molecular tools and related literature are available, one of the main challenges of metabolic pathway engineering is to find an efficient heterologous protein secretion method. Efficient transformation protocols have been established for few model bacteria. Although the choice of a suitable constitutive or inducible promoter for efficient gene transcription is essential, the latter is only one of several mechanisms, at both mRNA (*i.e.* mRNA stability, translation efficiency) and protein (*i.e.* stability, transport and activity) levels, involved in gene expression in microorganisms [11-14]. Such mechanisms have been optimized in natural organisms through evolution. Those researchers who wish to engineer "new" (*i.e.* recombinant) organisms should modulate heterologous gene expression in order to mimic naturally occurring mechanisms, that evolved through mutation plus selection, or, at least, to obtain functional systems for the envisaged industrial application (Figure 1).

This is particularly difficult for RCS since they involve cloning and expression of multiple genes and gene product translocation across the cell envelope and possibly post-translational modifications and anchoring to the cell surface.

The present review is aimed at summarizing both the bottlenecks and innovative solutions employed in organism engineering for RCS. Such topics will be detailed in the subsequent sections, after a brief introduction on the native cellulase systems.

Natural cellulolytic systems: structure and regulation

Natural plant degrading microorganisms biosynthesize extracellular multiple enzyme systems. These systems consist of different substrate specificities (*e.g.* cellulases, xylanases, pectinases) and catalytic mechanisms, which can be either free or cell associated [15-18]. Aerobic microorganisms, such as filamentous fungi (*e.g. Trichoderma reesei*) and actinomycete bacteria, generally produce "free" cellulases that do not form stable complexes [15,17-18]. Anaerobic bacteria, such as *Clostridium spp.* and *Ruminococcus spp.*, and fungi (*i.e.* Chytridomycetes) have developed "complexed" cellulase systems called "cellulosomes" [16-17,19] (Box 1).

The genes encoding cellulases are either randomly distributed or clustered on the chromosome of cellulolytic microorganisms [15]. The mechanisms regulating cellulase gene expression remained obscure for many years since transcriptional promoters could not be found within large gene clusters. However, the existence of large polycistronic operons has recently been demonstrated in *Clostridium cellulolyticum* [20]. The *C. cellulolyticum* 26 kb *cip-cel* cluster of cellulosomal genes constists of at least a 14 kb operon and other smaller transcriptional units that include 1 to 5 genes. It has even been hypothesized that the entire *cip-cel* cluster could be a single operon transcribed as a whole primary mRNA that is then processed into various secondary transcripts, which display different stabilities [20]. Two further operons, *i.e. celC*, consisting of *celC-glyR3-licA*, and *manB-celT*, have been identified in *Clostridium thermocellum* [21]. The promoter of the *celC* operon is repressed by GlyR3, while it is activated when laminaribiose, a β-1,3 glucose dimer, is available. Moreover, a set of six putative alternative σ factors and membrane-associated anti-σ factors, which may play a role in cellulosomal gene regulation, has recently been identified in *C. thermocellum* [22] (Box 2).

Gene expression optimization

Choice of the promoter

Metabolic engineering by gene manipulation traditionally aims at generating many-fold overexpression of heterologous genes which are considered to be the rate determining step in a pathway [23]. RCS has been performed, in most cases, by cloning heterologous cellulase genes under the control of constitutive promoters in *Bacillus subtilis*, *Clostridium acetobutylicum*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Saccharomyces cerevisae*, and *Zymomonas mobilis* [24-29]. Such a strategy appears more appropriate for microorganisms aimed to biorefineries since it avoids the non negligeable supplemental cost of large amounts of specific inducers [5]. Nonetheless, constitutive "uncontrolled" heterologous cellulase biosynthesis may lead to saturation of transmembrane transport mechanisms with inhibitory effects on cell growth and viability [28-30]. Toxicity can therefore be diminished by weakening the promoter strength through rational or random mutagenesis [29-30]. Alternatively, inducible promoters could be used to delay protein biosynthesis in a growth phase (*e.g.* mid-log phase) which would be more suitable for both effective protein biosynthesis and reduced toxic effects [28]. Inducible promoters have also been employed to engineer *L. lactis* and *S. cerevisiae* strains with heterologous cellulases, in order to obtaining improved silage fermentation and digestibility of ensiled biomass and amorphous cellulose fermentation to ethanol, respectively [31-33]. As the understanding of cellulase system regulatory networks in natural microorganisms is increasing, it is tempting to mimic such models in recombinant hosts [22]. Furthermore, synthetic biology and metabolix flux analysis will problably play key roles in developing artificial promoters for the fine tuning of heterologous genes and gene networks [23,34].

Regulation of mRNA stability

mRNA concentration is a balance between gene transcription and mRNA degradation. The fine tuning of mRNA degradation is actually used by prokaryotes to modulate gene expression, *e.g.* the expression of cellulase genes [11,12,20].

The improvement of mRNA stability can be used as a further effective tool to increase the expression of heterologous cellulases, thus eliminating the need for time-consuming promoter screening procedure [35-36]. mRNA 5′-untranslated leader sequences (UTLS) have a 5′ stem–loop structure and a ribosome binding site (RBS), and have been reported to contribute to mRNA stabilization in *Bacillus subtilis*, *Escherichia coli* and *Lactobacillus acidophilus* [35,37-38]. Increased amounts of the α-amylase from *Streptococcus bovis* 148 could be biosynthesized in *L. casei* by fusing the UTLS (and the RBS) of the *slpA* gene from *Lactobacillus acidophilus* with the promoter of the gene encoding lactate dehydrogenase of *Lactobacillus casei* [35]. The same strategy has been used to optimize *C. thermocellum* CelA cellulase expression in *Lactobacillus plantarum* [26]. In some cases, the improvement in mRNA stability could be even more effective for the secretion of large amounts of heterologous proteins than using stronger promoters [36].

Modulation of translation efficiency

The genome GC content is the primary determinant of the codon and amino acid usage patterns observed in different bacterial groups [39]. The use of amino acids encoded by GC-rich codons increases by approximately 1% for each 10% increase in genomic GC content [39].

The GC content compatibility between donor and recipient strains should therefore be taken into account for an efficient heterologous protein translation. In this respect, the heterologous expression of pyruvate decarboxylases (PDC) for the construction of ethanol over-producing strains can be taken as a paradigm. Engineering gram-positive hosts for robust ethanol production has long been limited by the availability of a suitable pool of PDC encoding genes [40]. Since PDC is widespread in plants, yeasts and fungi, but rare in bacteria, the *Zymomonas mobilis pdc* gene has been the workhorse for prokaryote engineering, though with very limited success on gram-positive strains [40]. Talarico and co-workers [40] demonstrated that the levels of heterologous PDC in *B. subtilis* depended on the GC content, *i.e.* the codon usage, of the *pdc* donor strain, although mRNAs were present in similar concentrations. When “donor” strains with a suitable GC content are not available

for a given gene, two strategies can be adopted to optimize protein translation: 1) the introduction of accessory tRNA genes to complement the tRNA set of the recipient strain [41,42]; 2) the design of synthetic genes with optimized codon usage, which is obtained by replacing rare codons with optimal codons for the recombinant host without affecting the amino acid sequence of the gene product [25,43-44].

Multiple gene expression: clusters, operons,multiple strains or engineered enzymes ?

The ability of natural microorganisms to degrade plant biomass relies on multiple enzyme systems. Similarly, engineering cellulolytic capabilities in a host implies cloning and expressing multiple genes. In this perspective, two aspects need to be managed: i) the physical arrangement and the coordination of the regulation of such multiple genes (*i.e.* the construction of operons and/or clusters); ii) the carrying capacity of the recipient strain: the higher the number of the required genes, the harder it is to introduce and maintain such large sized heterologous DNA [45].

As far as the gene arrangement is concerned, artificial operons are probably the most suitable for industrial process requirements of simple and easily regulated protein systems [29,30]. However, an optimal activity of cellulase systems is obtained for non-equimolar ratios of the different components [10,20,21,27]. The simplest way to obtain non equimolar amounts of heterologous proteins in the same strain is by using different transcriptional promoters [33,46].

Furthermore, natural cellulase systems are highly dynamic structures that are able to rapidly adapt to environmental changes, *i.e.* substrate availability, by modifying the subunit composition of the complex. Differential proteomic analysis has proven to be a valuable tool to directly detect cellulase components that are biosynthesized in response to specific cellulosic materials [47-49]. The use of promoters with different regulatory mechanisms and strengths could optimize both the quantity of required subunits and complex composition flexibility.

An intriguing strategy to both prevent the cloning of large sized DNA fragments and to obtain flexible enzyme systems has recently been explored in *B. subtilis* and *S. cerevisiae* [24,27]. Designer

158 cellulosomes were assembled by co-culturing recombinant cells expressing different single
159 cellulosomal components (*i.e.* intercellular complementation). Here, the amorphous (*i.e.* phosphoric
160 acid-swollen) cellulose-ethanol bioconversion rate and yield (93% of the maximum theoretical yield)
161 were optimized by adjusting the ratio of each *S. cerevisiae* population [27]. However, it still has to
162 be demonstrated that such a strategy could be manageable once scaled-up to the size of an industrial
163 process.

164 Nature offers a further paradigm to avoid multiple cellulase expression, *i.e.* the multidomain
165 multicatalytic megazymes from the *Caldicellulosiruptor spp.* thermophilic anaerobic gram-positive
166 bacteria [49-50] (Box 3). Such a protein arrangement inspired the design of unconventional and
167 covalent cellulosomes [51]. A panel of enzymes and complex architectures was engineered by
168 combining family 48 and 9 GH domains with efficient CBMs and optional cohesin and/or dockerin
169 modules from *C. cellulolyticum* [51]. A "covalent cellulosome", consisting of both endoglucanase
170 and exoglucanase modules, two CBMs, a dockerin and a domain of unknown function, was twice
171 more active on crystalline cellulose than the parental free cellulases (Cel48F plus Cel9G). However,
172 this bifunctional protein was 36% less active than "conventional" designer cellulosomes containing
173 Cel48F plus Cel9G plus a miniscaffoldin [51]. Althought these results somehow contradict the
174 improved synergy of the megazyme paradigm of *Caldicellulosiruptor spp.*, optimized artificial
175 covalent cellulosomes could probably be designed by increasing catalytic module mobility. The
176 catalytic domains in bi-functional megazymes from hyperthermophylic bacteria are always very
177 distant from each other in the primary sequence, *i.e.* they are separated by at least one carbohydrate
178 binding module (CBM), suggesting that high catalytic domain mobility is essential for efficient
179 substrate degradation [49-50].

180 Detailed understanding of cellulase catalytic mechanisms, with particular regard to
181 interdomain (*i.e.* CBM-catalytic domain interactions) and intermolecular (*i.e.* cellulase mixtures)
182 synergistic interactions in enhancing crystalline cellulose hydrolysis, is essential to engineer enzymes
183 with superior activity on native substrates [52]. Efficient recombinant cellulolytic organisms could

be developed by introducing fewer optimized enzymes. Improved enzymatic activity could also compensate for low secretion yields (see next section). Both directed evolution and rational design have been employed to improve cellulase activity on crystalline cellulose, although, so far, these approaches have achieved only moderate success [52,53].

Heterologous protein secretion

The heterologous expression of cellulases is often affected by the bias against their secretion which causes a reduction in or loss of cell viability [28-30,54-55].

E. coli has been extensively used to express heterologous proteins, although such strategies have mainly been addressed to cytosolic or periplasmic polypeptides [56-58]. Protein secretion in gram-negative bacterial models actually deals with the challenge of translocation across a double membrane system, although a number of secretion pathways (*e.g.*, types I, II, III, IV, V, and VI) have been studied in detail [59]. However, a number of other bacterial models, especially gram positive bacteria (*e.g. B. subtilis* and *L. lactis*), have been optimized for heterologous protein (*e.g.* proteases, α-amylases) secretion [56,60].

Most secreted proteins are translocated across the cytosolic membrane by the Sec translocase machinery through a general mechanism that is probably shared by both Gram negative and Gram positive bacteria [for reviews see 57,59,60] (Box 4). The products of genes encoding cellulosomal components of cellulolytic clostridia, including their original signal peptide, could be efficiently secreted by *C. acetobutylicum* and *Lactobacillus plantarum* [26,29]. However, although the *B. subtilis* and *E. coli* SecYEG complex subunits exhibit a high sequence similarity, they do not seem to be functionally exchangeable: this indicates that secretory machines have species specificities [57]. Furthermore, additional components of the translocation machine (*e.g.* the *E. coli* SecDF/YajC and YidC proteins) are continously being identified, as well as paralogues of SecA, which are probably involved in the secretion of different protein subsets [57]. These specific factors can be limiting for heterologous protein expression, as was probably the case in the expression of some *C. cellulolyticum*

cellulosomal genes in *C. acetobutylicum* [29]. Original cellulase signal peptides have been replaced by signal peptides of efficiently secreted autologous proteins or synthetic sequences to improve secretion efficiency and lower cell toxicity in recombinant hosts. The engineered *sacB* levansucrase signal sequence and the Strep-Tactin octapeptide have been used to express *Clostridium cellulovorans* cellulosome components in *B. subtilis* [24,46]. The signal peptide of Usp45, the main secreted protein of *L. lactis*, has been extensively used for heterologous protein secretion in *L. lactis*, *e.g.* the *C. thermocellum* scaffolding protein CipA [28,56]. Other peptide sequences, located between the signal peptide and the mature protein sequence (propeptides), are essential to either keep the nascent polypeptide in a competent conformation for translocation across the cell membrane or for rapid post-translocation folding which increase secretion efficiency (Box 4) [28,56,60].

C. cellulolyticum cellulases, with respect to the possibility of being secreted by *C. acetobutylicum*, can be divided into two distinct groups: i) enzymes with small catalytic modules (and a dockerin), *e.g*. Cel5A, Cel8C and Cel9M, can be easily secreted in an active form; ii) more "bulky" cellulases characterized by large catalytic modules (*e.g.* Cel48F), or possessing additional modules (*e.g.* Cel9G and Cel9E), are toxic and have resulted in non viable clones [29]. As far as Cel48F is concerned, the unsuitable secretion machinery of *C. acetobutylicum* has been proven to cause cell toxicity, since the same protein could be synthesized in the *C. acetobutylicum* cytoplasm [29]. The secretion of family 48 of cellulosomal glucan hydrolases therefore seems to require specific components that are missing in *C. acetobutylicum* [29]. However, fusion of CBM3a and X2 domains to the Cel48F/Cel9G catalytic module, prevented toxic effects and triggered enzyme secretion [61].

Several membrane and periplasmic proteases contribute to the quality control of secreted proteins by removing misfolded or incompletely synthesized polypeptides [60]. Although these systems are essential for high quality protein biosynthesis in natural organisms, they can be among the major bottlenecks of heterolous protein expression. For this reason, *B. subtilis* WB800 and *L. lactis* HtrA mutants, which are defective of 8 surface/extracellular proteases of *B. subtilis* and the unique exported housekeeping protease HtrA of *L. lactis*, respectively, have been employed for the

efficient secretion of heterologous cellulases [24,28]. Given the high complexity and specificity of the secretion machineries, it is currently difficult to foresee whether a given translocation complex is adapted to secrete a protein of interest. In the case of inefficient protein secretion, the use of weaker or inducible promoters or engineered host secretory system (*e.g.* chaperones, translocation machinery, protein quality check) can diminish the toxic effects on cell growth [29,57].

Cell surface anchoring

The assembly and spatial organization of enzymes in naturally occurring cellulosomes constitutes the base of their synergistic activity. Several aspects in cellulosome self-assembly remain to be elucitaded with the goal of improving biomass conversion using cellulosomes [62]. Synergistic activity is further enhanced in cellulosomes that are anchored to the cell surface and thus form ternary cellulose-enzyme-microbe (CEM) complexes. CEM complexes benefit from the limited escape of hydrolysis products and enzymes, and minimal distance products must diffuse before the cellular uptake occurs [28]. Furthermore, surface anchoring probably protects enzymes from proteases and thermal degradation [28]. For all these reasons, the assembly of cell surface displayed designer cellulosomes in recombinant microbes is highly desirable.

Surface display techniques have been developed for Gram-negative bacteria, with autodisplay probably being the most efficacious technique [58,63]. As far as Gram-positive bacteria are concerned, at least four mechanisms can be exploited for protein surface display either through binding to the cell membrane, *via* trasmembrane domains or by covalent linkages to long-chain fatty acids (lipoproteins), or by anchoring to the cell wall through covalent (*via* sortase) or non-covalent (*via* cell wall binding domains) interactions [59,64].

Some of these strategies have been recently exploited for the surface display of cellulase components in recombinant microorganisms. Minicellulosomes have covalently been linked to the cell wall of the yeast *S. cerevisae* using the agglutinin/flocculin display system [27,33,65]. Such cell wall proteins, *e.g.* α-agglutinin and cell wall protein 2, contain a glycosyl phosphatidylinositol (GPI)

signal motif and are covalently linked to the cell wall β 1-6 glucan. Miniscaffoldins have been fused with either a GPI signal motif, in order to be covalently linked to the cell wall, or with the C-terminus of the AGA2 protein, which is tethered to the yeast surface *via* non-covalent bonds with the (surface covalently bound) α-agglutinin mating adhesion receptor [27,33,65]. Trifunctional minicellulosome displaying *S. cerevisiae* cells were able to convert amorphous cellulose to ethanol with 62% of the theoretical yield [33].

As far as bacteria are concerned, fragments of the scaffolding protein CipA of *C. thermocellum* have functionally been displayed on the cell surface of *Lactococcus lactis* by fusing them with the C-terminal anchor motif of the streptococcal M6 protein, a sortase substrate [28] (Box 4). Surface-anchored complexes were displayed with efficiencies approaching 10^4 complexes/cell, although significant differences in efficiency were observed among the constructs, depending on their structural characteristics (*i.e.* protein conformation and solubility, scaffold size, and the inclusion and exclusion of non-cohesin modules) [28]. Similarly, engineered scaffoldins and cellulases from *C. thermocellum* have covalently been anchored to the *B. subtilis* cell wall by fusing them with the C-terminal sortase sorting signal of *S. aureus* fibronectin binding protein B [66].

A non-covalent surface display system for lactic acid bacteria has been developed by fusing a target heterologous protein, *i.e.* the α-amylase, with the C-terminal cA peptidoglycan binding domain, which shows high homology with LysM repeats, of the major autolysin AcmA from *Lactococcus lactis* [67].

Post-translational modifications

Signal peptides of cellulases and cellulosome components, as of other secreted proteins, are generally cleaved by signal peptidases during or shortly after translocation across the cytoplasmic membrane [60]. A further post-translational modification, *i.e. O*-glycosylation, of cellulosome components has been reported in *C. thermocellum* and *Bacteroides cellulosolvens* (particularly on the scaffoldin moiety) and hypothesized for the ScaC and the CipA scaffoldins of *Acetivibrio*

cellulolyticus and *C. acetobutylicum*, respectively [15,54,68-70]. The glycosyl groups may protect the cellulosome against proteases, but may also play a role in cohesin-dockerin recognition and in adhesion to the substrate [71].

Concluding remarks

As far as recombinant cellulolytic strategies are concerned, efficient secretion of designer cellulase systems is still among the most challenging tasks. The high complexity and diversity of protein secretion mechanisms is far to be fully understood. Currently, we cannot predict if a specific cellulase will be secreted in high amounts in a recipient strain or it will result in cell toxicity.

However, remarkable progress is continuously being made and recombinant microorganisms that could directly ferment cellulosic substrates to ethanol have recently been reported [31,33]. Even in such cutting edge studies, amorphous, either carboxy-methylated or phosphoric acid-swollen, cellulose was used, while crystalline cellulose could not be metabolized with significant efficiencies by engineered strains. There are still some major gaps in our understanding of the mechanisms by which cellulase systems catalyze crystalline cellulose hydrolysis [52,53]. Synergistic interactions between CBM and catalytic domain and in cellulase mixtures likely play a key role for efficient native plant biomass degradation, but detailed molecular mechanisms need to be clarified. This information is crucial for designing improved enzymes and artificial complexes for biotechnological applications, with particular regard to recombinant strains that are intended for CBP.

Researches on natural microrganisms, whose metabolism has been shaped by evolution for cellulolytic lifestyle, indicate that cellulose depolymerization by cellulases is not the only bottleneck of cellulose metabolism [72]. From a metabolic standpoint cellulose cannot be considered as a simple sum of soluble carbohydrate units. Experimental evidences clearly show that the use of cellulose does not result in the same metabolism as soluble sugars, *e.g.* cellobiose. Metabolic flux analysis could be an essential tool to further improve recombinant cellulolytic strains by rational engineering of central metabolic pathways. *In vivo* directed evolution by continuous culture under selective pressure is a

very promising alternative approach to optimize cellulose overall metabolism in engineered microorganisms [72,73].

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Box 1. Cellulosomes: nanomachines for efficient cellulose degradation

Anaerobic cellulolytic bacteria (*e.g.* belonging to *Clostridium* and *Ruminococcus* genera) and fungi (*i.e.* Chytridomycetes), biosynthesize "complexed" cellulase systems called "cellulosomes" [15-17,19]. In *C. thermocellum*, *C. cellulovorans* and *R. flavifaciens*, it has been demonstrated that these complexes are bound to the cell surface [16]. However, this does not seem to be true for other cellulosome biosynthesizing microorganisms [16].

The cellulosome architecture consists of multiple enzyme subunits, with different substrate specificities (*e.g.* cellulases, xylanases, pectinases) and catalytic mechanisms, organized by scaffolding proteins [10,16-17] (Figure 2a). As far as catalytic mechanisms are concerned, glucan hydrolases (GH) can be divided into four classes: 1) endoglucanases, which cut at random internal sites of the polysaccharides and generate oligosaccharides of various lengths; 2) exoglucanases, which act in a processive manner on the reducing or non-reducing ends of polysaccharide chains, liberating either mono- or di-saccharides; 3) processive endoglucanases, that share properties of both endo- and exo-glucanases; 4) β-glucosidases, which hydrolyze soluble di/oligo saccharides to monosaccharides [15, 74]. Cellulosome GHs, apart from the catalytic module, always contain at least one supplementary domain, *i.e.* the dockerin module involved in enzyme interaction with the scaffolding proteins. Furthermore, single or multiple carbohydrate-binding modules (CBM) can be

attached to the N or C terminus of catalytic domains through flexible linker-rich regions. CBMs affect polysaccharide binding and hydrolysis, by bringing the catalytic domain into close proximity with the substrate and are particularly important for the initiation and processivity of exoglucanases [15]. Other additional modules, such as immunoglobulin-like domains (*e.g.*, for CelE of *C. cellulolyticum*), or fibronectin type III domains (*e.g.* in CbhA of *C. thermocellum*) can be found in GH [15].

"Scaffoldins" are large multidomain, multifunctional proteins deputed to: i) recruit catalytic proteins by means of multiple cohesin domains that interact with glucan-hydrolase dockerin domains; ii) improve complex affinity for the substrate and catalytic efficiency *via* carbohydrate binding domains (CBMs). Anchoring scaffoldins provide further function by binding the cellulosome to the cell wall through covalent (sortase mediated) or non-covalent (through surface layer homology domains) interactions [16] (Figure 2a). Generally, scaffoldins do not contain catalytic modules but an exception is ScaA from *Acetivibrio cellulolyticus* that includes a GH9 domain [16].

Such a complex architecture enables the enzymatic components to act in a synergistic and coordinated manner *via* intra- and inter-molecular interactions and makes the cellulosomes the most efficient biochemical systems for cellulose degradation [10,61].

Box 2. Cellulosomal genes are activated by alternative σ factors and anti-σ factor borne CBMs in Clostridium thermocellum

The mechanisms by which cellulase gene expression is regulated have long remained an enigma. A set of six putative operons encoding alternative σ factors (homologues to *B. subtilis* σI) and their cognate membrane-associated anti-σ factors has recently been identified in the *Clostridium thermocellum* genome [22]. These proteins likely play essential roles in regulating cellulosomal gene expression in this bacterial strain (Figure 2b, c). Such anti-σI factors are multimodular proteins that include a strongly predicted transmembrane helix, an intracellular anti-σ domain, and an extracellular module with polysaccharide-related functions, *i.e.* either a CBM, a sugar-binding element, *e.g.* PA14, or a glycoside hydrolase family 10 (GH10) module. Apart from such structural heterogeneity, a

unique extracellular carbohydrate sensing mechanism emerges: the presence of extracellular polysaccharides is detected by a corresponding anti-σ factor-borne CBM, GH or PA element (Figure 2c). This event triggers conformational changes in the intracellular domain of the anti-σI factor: this releases the alterative σ factor and enables it to interact with RNA polymerase and promote the transcription of selected cellulosomal genes [22].

A similar set of multiple σI and anti-σI factors has recently been discovered in another cellulosome-producing bacterium, *Acetivibrio cellulolyticus* CD2 (also belonging to Clostridia) and in the Gram-negative human gut bacterium *Bacteroides thetaiotaomicron* [22]. Apart from these studies, very few information about the molecular mechanisms that modulate cellulosomal gene expression is currently available. Only further researches on other bacterial models will be able to establish if common systems have been evolved by cellulolytic bacteria or if *species* specific solutions are prevalent.

Box 3. Multifunctional megazymes from Caldicellulosiruptor spp.: paradigms to engineer new designer cellulosomes with improved efficiency.

The engineering of cellulolytic capabilities in a heterologous host implies cloning and expressing multiple genes: this constitutes one of major obstacles to the development of efficient recombinant cellulolytic microorganisms. *Caldicellulosiruptor spp.* hyperthermophilic anaerobic gram-positive bacteria have bypassed multiple cellulase expression by synthesizing multidomain multicatalytic megazymes [49,50]. Unlike clostridial cellulosomes, which consist of multiple enzymes containing single glucan hydrolase (GH) domains, *Caldicellulosiruptor spp.* cellulase systems consist of large amounts of a few bifunctional glucan hydrolases with broad substrate specificities [49]. These latter enzymes consist of different permutations of a small set of catalytic modules (*i.e.* GH5, GH9, GH10, GH43, GH44, GH48, and GH74), together with highly conserved family 3 carbohydrate binding modules (CBM3), in a single polypeptide chain [49]. The most abundant enzymes in *Caldicellulosiruptor* supernatants are the bifunctional proteins Athe_1867 (COB47_1673), which

consists of a GH9 domain (encoding a endo- l,4-D-glucanase activity), three CBM3 domains, and a GH48 domain (encoding a processive exoglucanase activity), CelC-ManB (Athe_1865 COB47_1669), consisting of a GH9 domain, three CBM3 domains, and a GH5 domain (encoding a mannanase activity), Athe_1857, containing GH10 (likely coding for an endo-l,4-D-xylanase activity) and GH48 domains, and COB47_1671, where the GH10 domain is associated with another GH5 module [49]. Interestingly, when expressed separately, the GH5 and GH10 domains both independently exhibit the same broad substrate specificity, but at decreased hydrolysis rates. Mixing the single enzymes did not completely restore the activity of the full-length version, thus demonstrating the synergistic effects of multidomain proteins [49].

Such arrangements suggest an evolution *via* domain shuffling and they could also be interpreted as primitive alternatives to operons [15]. It is possible that the multidomain architecture of *Caldicellulosiruptor* enzymes is an adaptation to high-temperature environments that is characterized by increased enzyme/substrate diffusion rates. This arrangement actually provides an improved synergistic effect due to a closer intramolecular spatial proximity in hyperthermophilic environments that would likely prevent subunit assembly by cohesin-dockerin interactions. Furthermore, multiple CBMs enable stronger binding to the substrate[49]. Such an architecture could inspire protein engineers and lead to advantages associated with designer cellulosomes in recombinant microorganisms through an improved synergism between different catalytic domains.

Box 4. Cellulosomes: from translation into the cytoplasm to surface display

A general mechanism for protein translocation across the cytosolic membrane, which is mainly based on the Sec translocase machinery, is probably shared by both Gram negative and Gram positive bacteria (for reviews see [57,59,60]). The signal peptide of nascent proteins is bound by cytoplasmic rybozymes (homologues to the signal recognition particle, SRP) and transferred to the SecYEG complex *via* membrane-bound SRP receptors. General molecular chaperones (*e.g.* GroEL/GroES and DnaK/DnaJ which are also involved in cytoplasmic protein folding) maintain the nascent polypeptide

chain in an “unfolded” translocation-competent conformation and prevent protein aggregation. Other chaperones with more dedicated roles in the secretion of specific proteins (*i.e. B. subtilis* CsaA and ClpX) have also been identified [57]. Polypeptide translocation occurs through the aqueous transmembrane channel that is formed by the integral membrane SecYEG complex and is driven by ATP hydrolysis catalyzed by the peripheral motor domain SecA. The polypeptides that emerge from the Sec translocase are unfolded. It has recently become clear that the rate at which proteins are post-translocationally folded by pro-peptides, peptidyl-prolyl cis/trans isomerases, disulfide isomerases, and metal ions is a key element of their productivity [75]. Class I propeptides are essential for the rapid post-translocational folding of their cognate mature protein, while class II propeptides appear to decrease the rate of intracellular folding, thereby facilitating interactions with chaperones that maintain secretion competence.

Cellulosomal complexes can then be anchored to the bacterial cell surface (mainly through non-catalytic scaffolding proteins) by either non-covalent, *via* surface layer homology (SLH) domains, or sortase-catalyzed covalent interactions [16]. Sortases are widely distributed in Gram positive bacteria and recognize proteins that contain a sortase recognition motif (*e.g.* LPXTG, where X is any amino acid). The target protein C-terminal domain usually includes a positively charged tail, a hydrophobic region, which is inserted into the cytosolic membrane, and an LPXTG motif. Cytoplasmic membrane anchored sortases cleave the peptidic bond between T and G of the LPXTG motif and then transfer the N-terminal part of the precursor surface proteins to lipid II, a cell wall precursor that is subsequently incorporated in the peptidoglycan [76].

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609

610 ***Figure legends***

611

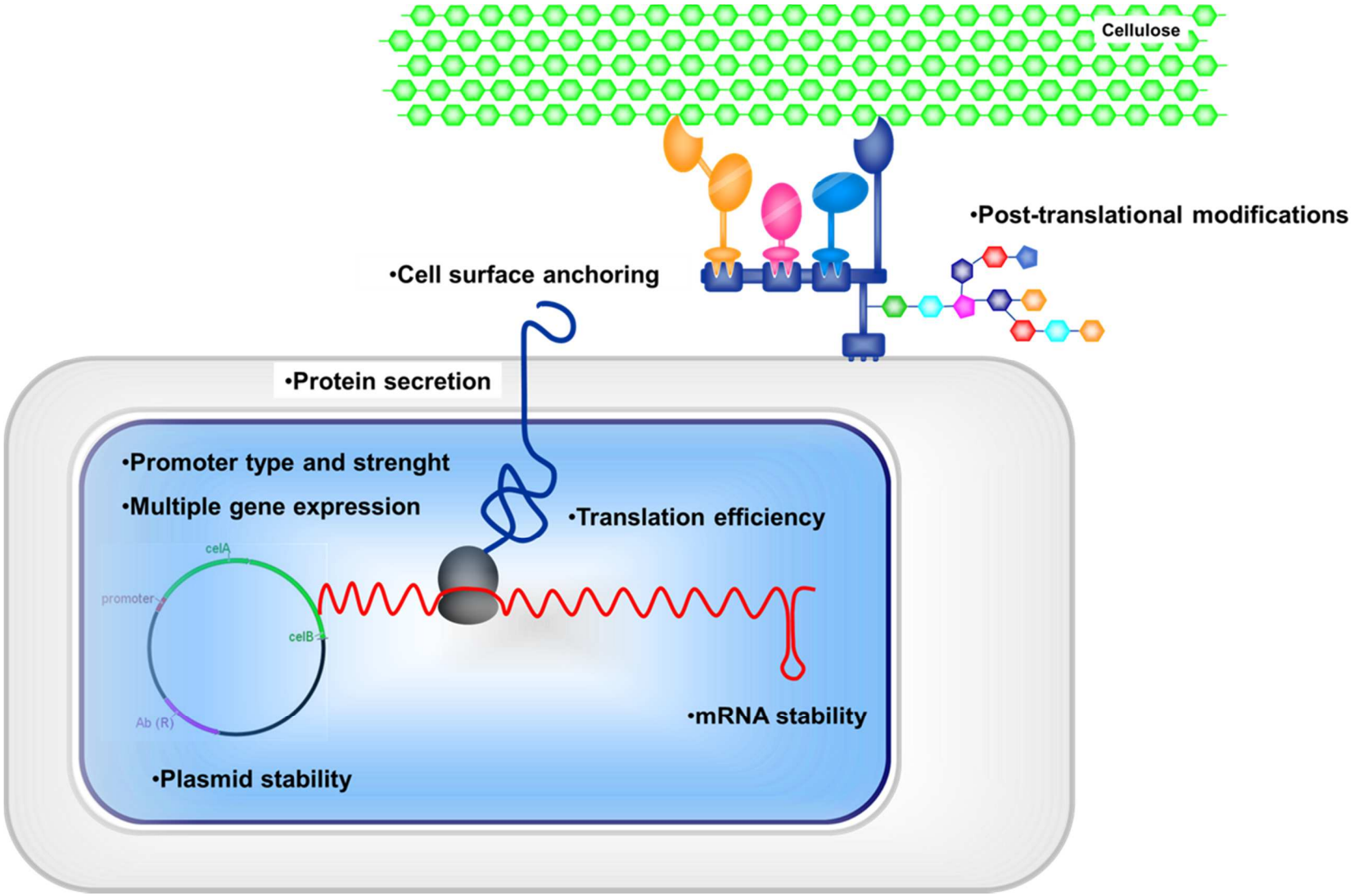
612 Figure 1. **The efficient expression of heterologous cellulase genes is a key feature to**
613 **engineer performant recombinant cellulolytic microorganisms.** Recombinant cellulolytic
614 strategies deal with the problem of biosynthesizing and secreting sufficient amounts of
615 heterologous designer cellulase systems for efficient cellulose degradation. The choice of
616 suitable transcriptional promoters and the improvement of mRNA stability and translation
617 efficiency are essential to optimize gene expression. Furthermore, suitable strategies should
618 be adopted to coordinate the expression of the multiple genes required. Nascent proteins need
619 to be maintained in an unfolded conformation so as to be translocated across the cytoplasmic
620 membrane. After translocation, proteins undergo further modifications that include folding,
621 surface anchoring and, possibly, glycosylation. Genetic stability is a further essential
622 requirement for engineered strains that are intended to industrial applications.

623

624 Figure 2. **Simplistic model of a cellulosome that includes only one anchoring scaffoldin**
625 **(a) and proposed mechanism for the cellulosomal gene transcription activation in**
626 ***Clostridium thermocellum* (b, c adapted from [22]).** a) The scaffolding protein (blue) binds
627 the enzymatic components through cohesin-dockerin interactions, enhances the cellulosome
628 affinity for cellulose through the carbohydrate binding modules (CBM), and anchors the
629 cellulosome complex to the cell surface through either non-covalent (by means of multiple S-
630 layer homology domains) or covalent (mediated by sortases) bonds. Apart from the catalytic
631 domains, cellulosomal enzymes include dockerin modules and, possibly, additional domains
632 (*e.g.* CBM, SLH). b) Extracellular polysaccharides are sensed by a system that consists of
633 alternative σ factors and integral membrane anti-σ factors (pink). The latter proteins include
634 an extracellular carbohydrate binding domain (CBM), a transmembrane helix and an
635 intracellular anti-σ module. c) When the extracellular carbohydrate binding domain interacts

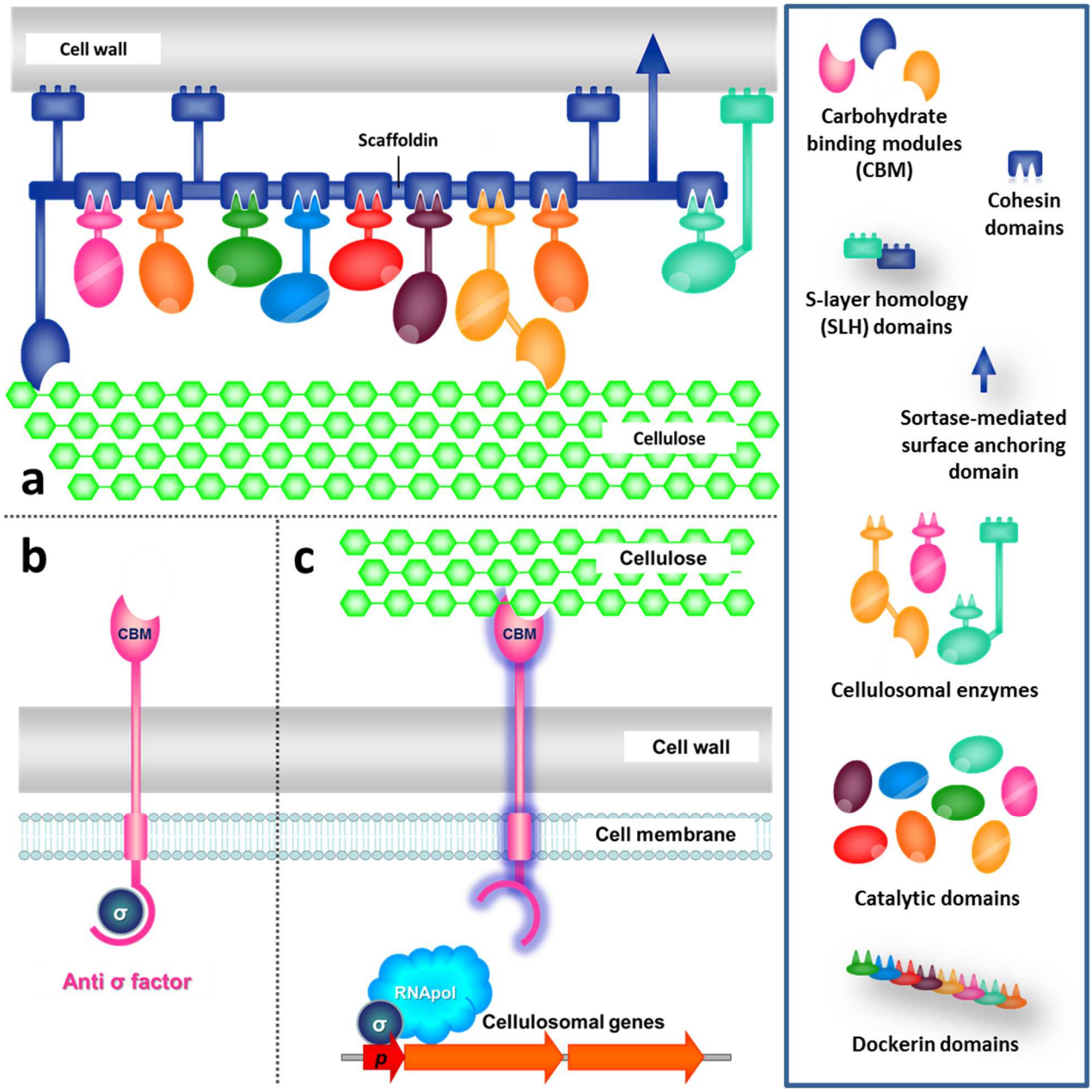
with polysaccharides (*e.g.* cellulose), it induces a conformational change in the intracellular anti-σ domain that releases the alternative σ factor. The latter is then able to bind specific promoters (*p*) and trigger cellulosomal gene transcription by RNA polymerase (RNApol).

640 Figure 1



641

642 Figure 2



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