Clostridium thermocellum cellulosomal genes are regulated by extracytoplasmic polysaccharides via alternative sigma factors

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Clostridium thermocellum produces a highly efficient cellulolytic extracellular complex, termed the cellulosome, for hydrolyzing plant cell wall biomass. The composition of the cellulosome is affected by the presence of extracellular polysaccharides; however, the regulatory mechanism is unknown. Recently, we have identified in *C. thermocellum* a set of putative σ and anti- σ factors that include extracellular polysaccharide-sensing components [Kahel-Raifer et al. (2010) *FEMS Microbiol Lett* 308:84–93]. These factor-encoding genes are homologous to the *Bacillus subtilis* bicistronic operon *sigI-rsgI*, which encodes for an alternative σ^I factor and its cognate anti- σ^I regulator RsgI that is functionally regulated by an extracytoplasmic signal. In this study, the binding of *C. thermocellum* putative anti- σ^I factors to their corresponding σ factors was measured, demonstrating binding specificity and dissociation constants in the range of 0.02 to 1 μ M. Quantitative real-time RT-PCR measurements revealed three- to 30-fold up-expression of the alternative σ factor genes in the presence of cellulose and xylan, thus connecting their expression to direct detection of their extracellular polysaccharide substrates. Cellulosomal genes that are putatively regulated by two of these σ factors, σ^{11} or σ^{16} , were identified based on the sequence similarity of their promoters. The ability of σ^{11} to direct transcription from the *sigI* promoter and from the promoter of *celS* (encodes the family 48 cellulase) was demonstrated in vitro by runoff transcription assays. Taken together, the results reveal a regulatory mechanism in which alternative σ factors are involved in regulating the cellulosomal genes via an external carbohydrate-sensing mechanism.

biomass | carbohydrate binding modules | *Clostridium* regulation | glycoside hydrolases | anti-sigma factors

Gram-positive thermophilic *Clostridium thermocellum* is an anaerobic bacterium with a highly efficient cellulolytic system. The hallmark of the system is an extracellular multienzyme complex, termed the cellulosome (1–4). As the bacterium is also capable of producing ethanol, it potentially could be integrated into a consolidated bioprocessing system for the production of cellulosic ethanol as a renewable source of energy (5–7). The cellulosome complex consists of a noncatalytic polypeptide, the scaffoldin, that mediates the attachment of nine catalytic subunits and the binding to cellulose via an internal family 3 cellulose-binding module (CBM3). The cellulosomal enzymes possess a dockerin module that binds tenaciously to the nine scaffoldin-borne cohesin modules, thus forming the complex (7–9). The scaffoldin subunit also includes a special type of dockerin module (type II dockerin) for the attachment of the cellulosome to a complementary cohesin (type II) that is positioned on the cell surface via cell surface anchoring proteins (10). Thus, the scaffoldin mediates the attachment of the catalytic units, as well as the binding of the complex and the entire cell to insoluble crystalline cellulose.

The known number of dockerin-bearing enzymes in *C. thermocellum* is approximately eight times more than the number of cohesins in the scaffoldin subunit. Consequently, the composition of the cellulosome is governed by the relative amounts of the available dockerin-containing polypeptides that presumably are incorporated randomly into the complex (2). Individual cellulosome complexes would therefore differ in their exact content and distribution of subunits (11). The various cellulosomal genes in *C. thermocellum*, for the most part, are monocistronic, scattered throughout the chromosome (12), and their expression was shown to be affected by the carbon source and the growth rate (13–23). Several general regulatory mechanisms were proposed to be involved, including carbon catabolite repression (2, 21) and alternative σ factors (14). Surprisingly, the only regulator that has been characterized so far is GlyR3, which negatively regulates *celC*, a noncellulosomal cellulase gene (24). Although *C. thermocellum* can utilize mainly celodextrins and possesses specific ABC sugar transporters for their selective uptake (25, 26), the bacterium encodes and differentially expresses numerous cellulosomal glycoside hydrolases that act on hemicellulose and other cellulose-associated polysaccharides (23). These enzymes are required for unmasking the cellulose fibers from the surrounding hemicellulose fibers. Thus, the bacterium must possess a regulatory system that allows it to sense and react to the presence of high molecular weight polysaccharides in the extracellular surroundings without importing their low molecular weight soluble components intracellularly.

Recently, we have identified in *C. thermocellum* a set of six putative operons encoding alternative σ factors and their cognate membrane-associated anti- σ factors that may play a role in regulating cellulosomal genes (Table 1) (27). Deduced amino acid sequences of these σ factors share homology to the well characterized *Bacillus subtilis* alternative σ factor, σ^I (28–30). The second gene in these operons encodes for a multimodular protein that contains one strongly predicted transmembrane helix. The approximate 165-residue N-termini of these transmembrane proteins are homologous to the N-terminal segment of the *B. subtilis* anti- σ^I factor RsgI. The extracellular modules of these RsgI-like proteins appear to have polysaccharide-related functions, and include carbohydrate-binding modules (e.g., CBM3, CBM42), sugar-binding elements (e.g., PA14), and a glycoside hydrolase family 10 (GH10) module. In fact, two CBM3s from

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Table 1. σ /anti- σ pairs proposed to participate in regulating cellulosomal genes

RsgI1 (Cthe_0059) and RsgI4 (Cthe_0404) were found to bind cellulose, the PA14 dyad domains of RsgI3 (Cthe_0315) interact strongly with pectin (27) and the xylanase GH10 module of RsgI6 (Cthe_2119) interacts with xylans and cellulose (31). In addition, *C. thermocellum* encodes another transmembrane protein (Rsi24C; Table 1) with a carbohydrate-related function (glycoside hydrolase family 5) (31); in this case, the σ factor gene (*sig24C*) located upstream is weakly homologous to the *B. subtilis* extracytoplasmic function (ECF) σ factor σ^W (32). These findings strongly suggest that alternative σ factors are involved in regulating the cellulosomal genes via an external carbohydrate-sensing mechanism.

Results

operon in *B. subtilis* and various operons encoding ECF σ factors. This arrangement suggests an extracellular sensing mechanism that regulates the activity of the σ^I -like σ factor via its interactions with a cognate anti- σ peptide. To demonstrate the binding specificity of the putative σ^I -like factors for their corresponding anti- σ factors we have cloned, expressed, and purified representative recombinant σ 's and anti- σ domains of their cognate RsgIs, which are predicted to be on the N terminus (termed RsgIN; Table 1). The entire *sigI*-like structural genes were cloned fused to His-tags, whereas the anti- σ domains were designed to contain segments of only 51 to 90 aa residues of the N-terminal RsgI-like (27) or Rsi24C domain, again fused to His-tags at their N terminus. Of the seven protein pairs (6 σ^I -RsgIN pairs and σ^{24C} -Rsi24CN) tested, three σ^I -RsgIN pairs (pairs 1, 2, and 6) were efficiently expressed in *Escherichia coli* BL21, resulting in soluble proteins that were readily purified. The interaction between the σ factors and their anti- σ cognates was studied using isothermal titration calorimetry (ITC). By using this technique, multiple injections of the RsgI anti- σ domain into the calorimeter cell containing σ^I resulted in measurable heat changes from protein-protein interactions until saturation occurred. This analysis provides direct measurement of the binding enthalpy and allows simultaneous determination of several parameters, including the binding constant, free energy for binding, entropy of binding, and the binding stoichiometry. Representative titrations are presented in Fig. 1A–C, and the thermodynamic parameters together with the binding

Fig. 1. Isothermal calorimetric titration curves of the interaction of the N-terminal RsgI domains with the corresponding σ^I s at 30 °C: (A) σ^{11} with RsgI1N, (B) σ^{12} with RsgI2N, (C) σ^{16} with RsgI6N, and (D) σ^{16} with RsgI1N. The top panels show the calorimetric titrations and the bottom panels display the integrated injection heats derived from the titrations, corrected for control dilution heat. The solid lines are best-fit curves and were used to derive the binding parameters.

Table 2. Binding of σ^I -RsgIN proteins: thermodynamic parameters and binding constants

σ^I -RsgIN	K_d , μ M	ΔH_{ar} , kcal/mole	$T\Delta S_{ar}$, kcal/mole	ΔG_{ar} , kcal/mole
1	0.022 ± 0.013	-24.9 ± 0.3	-13.9	-10.7
2	1.0 ± 0.1	-11.3 ± 0.3	-3.0	-8.3
6	0.052 ± 0.008	-17.9 ± 0.2	-7.7	-10.1

ΔH_{ar} , binding enthalpy; ΔS_{ar} , entropy of binding; ΔG_{ar} , free energy for binding.

constants are summarized in Table 2. All the titration curves fit very well into a single binding site model with a calculated molar ratio close to one and binding constants of $5.4 \cdot 10^7$, $1.0 \cdot 10^6$, and $1.8 \cdot 10^7$ M $^{-1}$ for σ^{11} , σ^{12} , and σ^{16} with their corresponding RsgIN, respectively. All the binding interactions were enthalpy-driven with a negative entropy contribution and appeared to be specific within each pair, as no binding was observed with any other protein combination (Fig. 1D). These results confirm that (i) the binding between the σ factors and their cognate RsgIN (anti- σ domains) is highly specific and (ii) they are connected functionally.

Expression of *sigI*-like and *sig24C* σ Factors Is Influenced by the Composition of Polysaccharides in the Growth Media. Most studied ECF σ genes as well as the *B. subtilis sigI* are up-regulated by their specific external stimuli (29, 33). If the *C. thermocellum* putative σ^I -like and σ^{24C} factors are indeed regulating the cellulosomal genes, it is likely that their expression will be influenced by the presence of various polysaccharides in the medium. Real-time RT-PCR analysis was therefore used to monitor the expression of the related σ factor genes in the presence of cellulose and xylan in the growth medium. It is worth noting that xylan cannot be utilized by *C. thermocellum* although the bacterium codes for five xylanase proteins as well as numerous other related hemicellulases and carbohydrate esterases. Briefly, total RNA was extracted from logarithmic-phase batch cultures of *C. thermocellum* that had been grown in the presence of cellobiose, cellulose, or cellulose together with xylan. The cDNA was amplified with primers specific to the *sigI* and *sig24C* genes, as well as to the 16S rRNA gene that was used for normalization. The relative expression of the σ genes is presented in Fig. 2. The expression of *sigI1* and *sigI2* was up-regulated three- to sixfold in the presence of crystalline cellulose, and birchwood xylan did not appear to affect expression significantly in these two cases. These results are consistent with the fact that the sensing domains of the corresponding anti- σ factors (RsgI1 and RsgI2) are CBM3s, which are mainly specific for cellulose (Table 1). In this regard, the CBM3 of RsgI1 was tested experimentally and indeed found to bind cellulose substrates (27). Three σ genes, *sigI3*, *sigI5*, and *sigI6*, were significantly up-regulated (9- to 10-fold) only in the presence of xylan. The corresponding extracytoplasmic sensing modules for these genes can interact with hemicellulose components and include a dyad PA14 pectin-binding module (RsgI3), an arabinoxylan-binding module, CBM42 (RsgI5), and an active xylanase family 10 glycoside hydrolase (RsgI6). Two genes, *sigI4* and *sig24C*, were up-regulated (2- to 10-fold) in the presence of cellulose and further up-regulated (7- to 30-fold) in the presence of both cellulose and xylan. The corresponding extracytoplasmic sensing modules for these genes are, respectively, a cellulose-binding CBM3 and a family 5 glycoside hydrolase that appears to lack its general acid/base catalytic residue (31). Both modules were found to interact mostly with cellulose (27, 31). Taken together, the expression of all of the σ factors is up-regulated in the presence of cellulose and xylan in good agreement with the functions of their cognate extracytoplasmic sensing modules.

Fig. 2. Real-time RT-PCR analysis of gene expression. Relative expression of σ -factors in batch cultures of *C. thermocellum*, with cellobiose, cellulose, or cellulose and xylan as carbon sources. Samples were taken from midlogarithmic phase of growth. Normalization was performed using 16S rRNA.

Identifying Cellulosomal Genes Regulated by σ^I -Like Factors. To identify putative cellulose-related genes that are regulated by the σ^I -like factors, we took advantage of the fact that many σ factors positively autoregulate their own expression, and, therefore, their own promoter sequences should resemble those of their regulated target genes. The rapid amplification of cDNA ends (RACE) technique was applied to identify the 5'-ends of *sigI1* and *sigI6* mRNAs (Fig. 3). Two transcriptional start sites were identified for *sigI1*: P2, which corresponds to the putative vegetative σ^A binding site consensus sequences defined for *B. subtilis* [TTGACA(-35) and TATAAT(-10)], and P1, corresponding to a putative *sigI1* promoter with the sequence ACACAA(-35)-N19-AGTAAT(-10) (Fig. 3A). Close inspection of upstream regions of previously identified cellulosomal genes revealed sequences almost identical to P1 near a major transcriptional start site (P4) of *celS* (17). CelS is a critically important family 48 exoglucanase and the most abundant enzyme in the cellulosome complex. To identify additional σ^{11} -controlled promoters, we examined the upstream regions (250 bp) of all the cellulosomal genes. In the first round of our search, we looked for promoter sequences that maintain the -10 GTA sequence allowing up to two mismatches in each of the -10 and -35 sequences, but not more than a total of three. This search revealed similar promoter sequences in the upstream regions of *celA* and *sdhA* with the consensus sequences, ACANAA-N(17-19)-WGTAWW. Using this consensus sequence, a second search cycle was performed, allowing one mismatch in each sequence but not in the -10 GTA triad, and not more than two mismatches in each sequence compared with the original *sigI1* promoter sequences. This cycle revealed three more putative promoters (Fig. 3A). In all cases, the space between the -10 and -35 sequences was 17 to 19 bp.

For *sigI6*, only one transcriptional start site was identified (Fig. 3B). As *sigI6* was shown to be up-regulated in the presence of xylan, and its cognate anti- σ factor extracellular module is an apparent GH10 xylanase, we first examined the upstream regions of hemicellulolytic genes. Indeed, the upstream region of four of the five *C. thermocellum* xylanase genes contain highly similar sequences to the *sigI6* promoter, providing the consensus sequence, GCNACN-N(17-19)-CGAAWN. This consensus sequence was used for another search, allowing one mismatch in each of the -10 and -35 sequences, but not in the -10 GA, and not more than two in each sequence compared with the *sigI6* promoter sequence. This search provided five additional putative promoters (Fig. 3B). All nine of the predicted promoters are positioned upstream of genes encoding hemicellulases and/or carbohydrate esterases, consistent with the function of the cognate extracellular module (GH10 xylanase) of RsgI6.

Fig. 3. Promoter analysis of *sigI1-rsgI1* and *sigI6-rsgI6* operons. The top panels show the mapping of the 5' ends of *sigI1-rsgI1* (A) and *sigI6-rsgI6* (B) transcripts determined by the 5' RACE technique. Arrows indicate the transcriptional start site. Framed letters are the suggested -35 and -10 sequences of the σ -factor binding site. The bottom panels present the identified *sigI-rsgI* and *celS* promoters (asterisk), as well as other putative promoter sequences of several cellosome-related genes. Nucleotides similar to the *sigI-rsgI* promoters are shown in bold. Numbers indicate both the distance in nucleotides between the -35 and the -10 promoter regions and between the -10 promoter region and the start of the ORF. GH, glycosyl hydrolase; CE, carbohydrate esterase; CBM, carbohydrate-binding module; Doc1, dockerin type I; Coh2, cohesin type II; SLH, 5-layer homology module.

C. thermocellum σ^I Promotes Transcription from *sigI1* and *celS* Promoters. To demonstrate the ability of σ^I to promote transcription from its putative promoters, we conducted in vitro runoff transcription assays. For this purpose, *C. thermocellum* RNA polymerase was purified following the procedure of Mani and coworkers (34) with minor modifications. The purification procedure included ammonium sulfate precipitation, anion exchange chromatography, and a cellulose phosphate column resulting in 740-fold purification with 25% total yield. The purified protein was analyzed by 7% to 15% gradient SDS/PAGE, which revealed the expected mobilities for *C. thermocellum* β , β' , and α subunits (predicted molecular masses of 130, 140, and 35 kDa, respectively; Fig. S1). An additional band, with a mobility corresponding to a 50-kDa protein could represent the 41-kDa σ^A subunit; the 43-kDa *B. subtilis* σ^A protein has the mobility in SDS/PAGE consistent with a polypeptide of 57 kDa (35). For the runoff transcription assays, linear DNA containing the putative σ^I promoter sequences of *sigI1* (P1) and *celS* (P4) (17) were used together with the purified *C. thermocellum* RNA polymerase and recombinant σ^I . DNA with the promoter sequence of *sigI6* was used as a negative control. The addition of σ^I to *C. thermocellum* RNA polymerase resulted in transcription from the P1 promoter of *sigI1* (approximately 216 nt) and from the P4 promoter of *celS* (approximately 540 nt; Fig. 4). Moreover, the presence of the anti- σ domain of RsgI1 in the transcription reactions abolished transcription. No transcription was observed when the *sigI6* promoter-containing DNA template was used (i.e., negative control). These results confirm that σ^I interacts with *C. thermocellum* RNA polymerases and activates transcription from the *sigI* and *celS* promoters. Furthermore, the binding of the anti- σ domain of RsgI1 with σ^I served to arrest transcription, thereby demonstrating the function of the RsgI anti- σ factor.

Discussion

C. thermocellum uses a highly specialized system for the hydrolysis and utilization of crystalline cellulose of the plant cell wall. The system is based on its high molecular weight enzymatic complex, the cellosome, which provides the bacterium with the ability to hydrolyze cellulose efficiently together with other plant cell wall-associated polysaccharides, such as hemicellulose and pectin.

Fig. 4. In vitro transcription from the *sigI1* and *celS* promoters. Runoff transcription reactions were performed using DNA fragments containing the *sigI1* or *celS* promoters and *C. thermocellum* RNA polymerase (RNAP) pre-incubated in the presence or absence of σ^I or σ^I and RsgI1N. The asterisk indicates an in vitro transcription reaction in which the amount of the *celS* promoter-containing DNA added was increased from 0.017 to 0.068 pmol. The size markers (M) are labeled single-strand PCR products with the indicated nucleotide length.

Intriguingly, the bacterium utilizes only β -1,4 and β -1,3 glucan (glucose-based polysaccharides), yet encodes many hemicellulase genes, encoding enzymes that act on five-carbon sugars that do not appear to be metabolized or capable of entering the cell. Furthermore, the enzymatic composition of the cellulosome appears to be highly variable and affected by the composition of the extracellular medium. The mechanism by which the bacterium regulates the expression of its cellulosomal genes has remained an enigma for many years, whereas only a single negative regulator has been identified so far for the noncellulosomal β -glucanase gene *celC* (24). The discovery of carbohydrate-related modules associated with alternative σ systems, such as σ^I -RsgI, immediately suggested a unique extracellular carbohydrate-sensing mechanism, whereby the presence of polysaccharides is detected extracellularly by a corresponding RsgI-borne CBM or GH element resulting in the release of σ^I and promoting transcription of selected cellulosomal genes (27). In *B. subtilis* the activation of the σ factors σ^I and σ^W require auxiliary proteins (29, 32); however, such candidates were not identified in *C. thermocellum*. Interestingly, the recently released genomic data of another cellulosome-producing bacterium, *Acetivibrio cellulolyticus* CD2 (also belonging to the *Clostridia*), reveal a similar set of multiple σ^I - and RsgI-like factors. At least seven of 12 RsgI-like proteins contain C-terminal CBM3-, CBM42-, and PA14-like modules (genome analysis was performed via BLAST; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Another comparable system was characterized in the Gram-negative human gut bacterium *Bacteroides thetaiotaomicron*, in which ECF σ factors are involved in the activation of polysaccharide utilization loci (36).

Binding measurements demonstrated the specific interaction between four σ^I factors and their corresponding anti- σ domains and confirmed that they function together. In this regard, although RsgI1 and RsgI2 share similar sensing modules (i.e., CBM3), no cross-interaction was observed between these two systems. To the best of our knowledge, σ /anti- σ interactions of *B. subtilis* σ^I or ECF σ factors were never measured quantitatively and have been demonstrated only functionally by yeast two-hybrid analyses (29, 37). The dissociation constants (K_d) of σ^{II} -RsgI1N, σ^{II} -RsgI2N, and σ^{II} -RsgI6N are 0.022, 1.0, and 0.053 μ M, respectively. K_d values obtained for other alternative σ /anti- σ systems were within the range of 0.0001 to 0.1 μ M and were usually determined using surface plasmon resonance (38–40).

All seven investigated σ factor genes (*sigI* to *sigI6* and *sig24C*) were found to be up-regulated in the presence of extracellular polysaccharides (cellulose and xylan). The results are consistent with previous studies demonstrating that, in *B. subtilis*, σ^I and σ^W autoregulate their own expression following the appropriate extracytoplasmic signal (29, 33). The prospect that the *sigI* promoter should also resemble its target gene promoter allowed us to identify cellulosomal genes that are potentially regulated by the specific σ^I factors. Several upstream regions of the cellulosomal genes revealed homologous sequences to the -10 and -35 promoter sequences of *sigI1* and *sigI6* with good correlation to their cognate RsgI-sensing module (CBM3 and GH10 xylanase, respectively). The ability of σ^{II} to promote transcription from both *sigI1* and *celS* promoters further supports the identification of *celS* as a σ^{II} -regulated cellulosomal gene. As expected, the presence of RsgI1N abolished transcription, thus implementing its role as an anti- σ element. In the σ^{II} -RsgI1 system, the sensing module is a cellulose-binding module (i.e., CBM3), consistent with previous studies showing that *celS* expression is up-regulated during growth on cellulose versus cellobiose (17, 20, 22, 23).

The proposed regulatory mechanism of the cellulosomal genes, by which alternative σ factors are activated in response to the polysaccharides in the extracellular surroundings (Fig. 5), coincides well with the physiology of *C. thermocellum* in its natural

Fig. 5. Proposed mechanism for the activation of alternate σ factors by extracellular polysaccharides. The RsgI/Rsi24C transmembrane proteins (red) contain an extracellular carbohydrate-active module (CBM3, CBM42, PA14, GH10, or GH5) and an intracellular anti- σ peptide domain. In the OFF state, the anti- σ domain interacts strongly with the alternative σ factor (blue), thereby inactivating it. In the ON state, extracellular polysaccharides (green) interact with the CBM, which, in turn, induces a conformational change on the intracellular anti- σ domain, resulting in the release of the alternative σ factor. The σ factor is now free to interact with RNA polymerase (RNAP) and promote transcription of the σ -dependent promoters. Note that the σ factor also promotes transcription of its own bicistronic operon, which includes the cognate *rsgI/rsi24C* gene.

niche and further elaborates the cellulosome concept. First, this mechanism allows adjustment of cellulosome composition for hydrolyzing polysaccharides such as hemicellulose that are not consumed by the bacterium. Second, the RsgI-associated extracellular CBMs can also play a role in allowing the cells to adhere to the insoluble plant cell wall matrix. In this regard, many of the cellulosome catalytic subunits harbor additional CBMs. These features complement the inherent advantages of the cellulosome system, which include enzymatic synergism in the hydrolysis of the complex plant cell wall matrix, control of hydrolysis rate by cellobiose feedback inhibition, and enzyme localization at the interface between the cell and the insoluble substrate, thereby avoiding cell density-dependent growth.

The identification of a general regulatory mechanism for the cellulosomal genes in *C. thermocellum* should pave the way for the construction of mutants that overproduce components of the cellulosome complex. This can be readily achieved, for example, by inactivating the anti- σ domains of the *rsgI* and *rsi24C* genes.

Materials and Methods

A list of bacterial strains, plasmids, and chemicals; and details on cloning, protein purification, microcalorimetry titration, *C. thermocellum* growth conditions, RNA polymerase purification, RNA extraction, quantitative real-time RT-PCR, 5' RACE, and in vitro runoff transcription procedures are outlined in *SI Materials and Methods* and *Table S1*.

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