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The Issue of Secretion in Heterologous Expression of *Clostridium cellulolyticum* Cellulase-Encoding Genes in *Clostridium acetobutylicum* ATCC 824▽

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The genes encoding the cellulases Cel5A, Cel8C, Cel9E, Cel48F, Cel9G, and Cel9M from *Clostridium cellulolyticum* were cloned in the *C. acetobutylicum* expression vector pSOS952 under the control of a Gram-positive constitutive promoter. The DNA encoding the native leader peptide of the heterologous cellulases was maintained. The transformation of the solventogenic bacterium with the corresponding vectors generated clones in the cases of Cel5A, Cel8C, and Cel9M. Analyses of the recombinant strains indicated that the three cellulases are secreted in an active form to the medium. A large fraction of the secreted cellulases, however, lost the C-terminal dockerin module. In contrast, with the plasmids pSOS952-cel9E, pSOS952-cel48F, and pSOS952-cel9G no colonies were obtained, suggesting that the expression of these genes has an inhibitory effect on growth. The deletion of the DNA encoding the leader peptide of Cel48F in pSOS952-cel48F, however, generated strains of *C. acetobutylicum* in which mature Cel48F accumulates in the cytoplasm. Thus, the growth inhibition observed when the wild-type *cel48F* gene is expressed seems related to the secretion of the cellulase. The weakening of the promoter, the coexpression of miniscaffoldin-encoding genes, or the replacement of the native signal sequence of Cel48F by that of secreted heterologous or endogenous proteins failed to generate strains secreting Cel48F. Taken together, our data suggest that a specific chaperone(s) involved in the secretion of the key family 48 cellulase, and probably Cel9G and Cel9E, is missing or insufficiently synthesized in *C. acetobutylicum*.

The biological conversion of plant biomass such as agricultural by-products to biofuels has become a major economic and environmental challenge during the past decades. The main polymers composing this biomass are lignin, hemicellulose, and cellulose. The principal issue still remains the complete breakdown of cellulose, the most abundant polysaccharide, into fermentable glucose, and this has motivated a strong interest in cellulolytic microorganisms. One of the most studied is the aerobic fungus *Trichoderma reesei*, which secretes large amounts of several cellulases and related enzymes (hemicellulases) in the free state (17). These enzymes act synergistically to degrade the cellulose and the other plant cell wall polysaccharides (7). *T. reesei* cellulase-rich culture supernatants currently are assayed in separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) bioprocesses, using yeast strain(s) as the fermenting microorganism(s) (20).

In addition to SHF and SSF, consolidated bioprocessing (CBP), in which a single organism catalyzes the conversion of plant biomass into biofuel, represents another attractive process. Although some cellulolytic bacteria, like *Clostridium phytofermentans*, were shown to produce ethanol (34), a microorganism combining both fast growth on cellulosic substrate and the substantial production of biofuel has not yet been isolated. Another option to achieve an economically viable CBP therefore involves the use of engineered microorganism(s). Several strategies currently are pursued and include heterologous cellulase production combined with the metabolic engineering of well-characterized bacteria, the modification of a cellulolytic microorganism to produce biofuel, and enabling a biofuel producer to utilize plant biomass as the carbon and energy source.

With respect to the latter strategy, several attempts to introduce heterologous cellulases in *Saccharomyces cerevisiae* were reported (15, 36, 38). Nevertheless, the complete conversion of the plant biomass to ethanol by recombinant *S. cerevisiae* also requires the introduction of various hemicellulases and the modification of its metabolism for xylose and arabinose uptake (20).

Our group currently is exploring another microorganism, *Clostridium acetobutylicum*, as a putative candidate for CBP. This anaerobic bacterium, which produces substantial amounts of butanol, acetone, and ethanol (ABE fermentation) (23), naturally secretes hemicellulose-degrading enzymes (1, 18). Furthermore, the solventogenic *Clostridium* species grow on most monosaccharides released by the enzymatic depolymerization of plant biomass (glucose, mannose, xylose, and arabinose) (32). *C. acetobutylicum*, however, is unable to grow on cellulose, although its genome contains a large cluster of genes

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TABLE 1. Sequence of the primers used

Primer	Sequence[a] (5′→3′)	Site
A1	GGGATCCAGAATTTAAAAGGAGGGATTAAAATGAAGAAAACAACAGCTTTTTTATTATG	BamHI
A4	AAGGCGCCTTAGTGGTGGTGGTGGTGGTGGTTGCTTGGAAGCTTACTTACCAT	NarI
C1	GGGATCCAGAATTTAAAAGGAGGGATTAAAATGATCAAAGGTTCAAGCTTAAAG	BamHI
C4	AAGGCGCCTTAGTGGTGGTGGTGGTGGTGGTTAAGCAGTTTAACTTTTAGCTGAGC	NarI
M1	GGGATCCAGAATTTAAAAGGAGGGATTAAAATGAAAAGTAAATTGATAAAATTAAGTGC	BamHI
M4	AAGGCGCCTTAGTGGTGGTGGTGGTGGTGACCTAAGATAGCCTTCTTTAAAAGAG	NarI
E1	AGGATCCAGAATTTAAAAGGAGGGATTAAAATGAAAAAAAGGTTAGTGAAGA	BamHI
E4	AAGGCGCCTTAGTGGTGGTGGTGGTGGTGCAGTGTGATTTTTCCTAACAAG	NarI
F1	GGGGATCCAGAATTTAAAAGGAGGGATTAAAATGAAGTAAGAATTTTAAAAGAGTAGG	BamHI
F4	CCCGGCGCCCTATTGGATAGAAAGAAGTGCT	NarI
G1	AGGATCCAGAATTTAAAAAGGAGGGATTAAAATGCTTAAGACTAAAAGAAAATTGACAA	BamHI
G4	CCGGCGCCTTAGCCTTGAGGTAATTGGG	NarI
Fmat	GGGGATCCAGAATTTAAAAGGAGGGATTAAAATGGCTTCAAGTCCTGCAAACAAGGTGTA	BamHI
sosdir	ACTATTGGTTGGAATGGCGTG	
rSF	ACGATCCTGGTACACCTTGTTCGCGCCAGTACCTGCTGCAAAAGCTGT	
fSF	ACAGCTTTTGCAGCAGGTACTGGCGCGAACAAGGTGTACCAGGATCGT	
F3R	ACGTCAAGTATCCAGTGCATTC	
48A1	AGGATCCAGAATTTAAAAGGAGGGATTAAAATGTTAAAGATAAGTAAGAATTTTAAAAAAATAATGG	BamHI
48A2	AATAAAGTTGCAGCTGCTACAACTACAGCAAACAAGGTGTACCAGGATCG	
48A3	CGATCCTGGTACACCTTGTTTGCTGTAGTTGTAGCAGCTGCAACTTTATT	
48A4	AAGGCGCCCTATTTTGCAATTAATTTAGTAAGTTCCATAATATCTC	NarI
cip1r	AAGGATCCTTCGAACTACTCGAGTTCCTTTGTAG	BamHI
Fox	AAGGCGCCACGCGTTAAAAGGAGGGATTAAAATGAAAAAAGTATTATTGGCAA	NarI
Rox	TTGGCGCCTTATTTAACTGTTATCTCACCCTC	NarI

[a] Underlined sequences symbolize introduced restriction sites.

that encode a cellulolytic complex called the cellulosome (24). These large assemblies are produced by several anaerobic bacteria and generally are composed of a scaffolding protein exhibiting several cohesin modules that tightly bind to the dockerin modules hosted by the catalytic subunits (for a review, see references 2 and 3). The bacterial cellulosomes usually are characterized by an elevated specific activity on crystalline cellulose, but it was shown formerly that the complex secreted by *C. acetobutylicum* is inactive toward this substrate (30). The solvent productivity and capacity to degrade hemicellulose of *C. acetobutylicum* make this bacterium an attractive microorganism for the production of heterologous (mini)cellulosomes and the generation of a suitable strain for CBP.

Despite a high homology between cohesin and dockerin domains from different bacteria, their interaction is species specific. Thus, cohesins from one microorganism cannot bind to dockerins from another microorganism (11). This property was used to build a library of 75 hybrid minicellulosomes (9). These complexes were composed of two *C. cellulolyticum* cellulases appended with divergent dockerins and bound to a hybrid scaffoldin displaying the complementary cohesins and an optional carbohydrate binding module (CBM). Analyses of their activity *in vitro* revealed that the minicellulosomes, composed of the enzyme pair Cel48F and Cel9G or the pair Cel9E and Cel9G, bound onto a hybrid scaffoldin containing a single family 3a CBM and were the most active on crystalline cellulose (9).

We formerly cloned in *C. acetobutylicum* the genes encoding two miniscaffoldins, which were successfully secreted to the supernatant (27), and we constructed a strain that secretes a two-component minicellulosome (one miniscaffoldin and one mannanase) (22). To explore further the ability to convert *C. acetobutylicum* into a genuine cellulolytic microorganism, six genes encoding the most characterized cellulosomal cellulases from *C. cellulolyticum* were cloned in the solventogenic bacterium. The enzymes Cel5A (10), Cel8C (8), and Cel9M (4) were successfully produced and secreted by *C. acetobutylicum*, but recombinant strains secreting Cel48F (29), Cel9G (12), and Cel9E (13) could not be obtained. Our data suggest that the synthesis of the latter cellulases has a deleterious effect that is related to their interaction with the secretory system of the heterologous host.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* SG-13009, harboring the pREP4 repressor plasmid (Qiagen, Venlo, The Netherlands), was used as the host for recombinant expression vectors, whereas the strain *E. coli* ER-2275, carrying the pAN1 methylating plasmid, was used to *in vivo* methylate (21) the recombinant plasmids prior to the transformation of *C. acetobutylicum* ATCC 824. *E. coli* strains were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin (SG-13009 [pREP4, derivative of pSOS952]), or with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol (ER-2275 [pAN1, derivative of pSOS952]). *C. acetobutylicum* was grown routinely anaerobically at 37°C in 2YT medium (containing 16 g/liter of Bacto tryptone, 10 g/liter of yeast extract, 4 g/liter of NaCl, 1 mM $CaCl_2$) supplemented with cellobiose (5 g/liter). Recombinant *C. acetobutylicum* strains carrying derivatives of pSOS952 (27) were grown in 2YT-cellobiose supplemented with 40 μg/ml of erythromycin. To prepare spore suspensions, *C. acetobutylicum* was grown at 37°C for a week in 10 ml of synthetic medium supplemented with erythromycin (40 μg/ml) for recombinant strains. The culture then was aliquoted and frozen at −20°C.

Expression vectors and cloning of cellulase genes. The various primers used for the amplification of wild-type and engineered cellulase genes are listed in Table 1. The expression vectors pSOS952 (containing the wild-type thiolase promoter and *adc* gene transcriptional terminator) and pSOS954 (containing a mutated thiolase promoter and *adc* gene transcriptional terminator) were described previously (22, 27).

Construction of the plasmids containing wild-type *C. cellulolyticum* cellulase genes. The genes were amplified from the genomic DNA of *C. cellulolyticum* using the primer pairs A1/A4 (for *cel5A*; Table 1), C1/C4 (for *cel8C*), M1/M4 (for *cel9M*), E1/E4 (for *cel9E*), G1/G4 (for *cel9G*), and F1/F4 (for *cel48F*), introducing a BamHI site at the 5′ extremity and an NarI site at the 3′ extremity of each gene. The amplicons were cloned into BamHI-NarI-linearized pSOS952 (all

amplicons) or BamHI-NarI-linearized pSOS954 (in the case of *cel9E*, *cel9G*, and *cel48F*).

The vector pSOS952-cel48Fmat was constructed similarly using the primer pair Fmat/F4.

Construction of pSOS952-cel48A. The gene encoding the family 48 cellulase (24, 30) was amplified from the genomic DNA of *C. acetobutylicum* using the primers 48A1/48A4 and cloned into BamHI-NarI-linearized pSOS952.

Construction of pSOS952-Scip-cel48F and pSOS952-S48a-Cel48F. The DNA encoding the signal sequence of CipC (25) was amplified from pSOS952-cipC1 (27) using the primer pair sosdir and rSF (Table 1), and the DNA coding for the N-terminal extremity of the mature Cel48F was amplified from pSOS952-cel48F using the primers fSF and F3R. The resultant overlapping fragments were mixed, and a combined fragment was synthesized using the external primers sosdir and F3R. The fragment subsequently was cloned into BamHI-SphI-linearized pSOS952-cel48F. The plasmid pSOS952-S48a-cel48F was constructed similarly using the primer pair 48A1/48A3 for the amplification of the DNA encoding the leader peptide of the endogenous cellulase Cel48A (30) and the primers 48A2/F3R to amplify the DNA coding for the N-terminal extremity of the mature Cel48F.

Cloning of the operons *cipC1-cel48F* and *cipC1-cel48F-orfX*. The vector carrying the two-gene operon was prepared by the amplification of the gene encoding the miniscaffoldin miniCipC1 from pSOS952-cipC1 (27) using the primers sosdir and Cip1r. The amplicon subsequently was cloned into BamHI-linearized pSOS952-cel48F. The vector containing the operon *cipC1-cel48F-orfX* was obtained by the amplification of the gene *orfX* using the primer pair Fox/Rox and genomic DNA from *C. cellulolyticum*. The amplicon was cloned in NarI-linearized pSOS952-cipC1-cel48F.

The plasmids were verified by DNA sequencing, and *C. acetobutylicum* was electrotransformed as previously described (27).

Analysis of recombinant *C. acetobutylicum* strains. The recombinant clones were grown at 37°C anaerobically in 10 ml of 2YT medium supplemented with cellobiose and erythromycin. The cultures were stopped at the late exponential phase (the optical density at 620 nm [OD_{620}] ranged from 2.8 to 3.2) and centrifuged at 4°C for 30 min at 12,000 × *g*.

The cells (corresponding to 1 ml at an OD_{620} of 1) were washed three times with 25 mM Tris-maleate (pH 6.0) and resuspended in 100 μl of denaturing loading buffer for SDS-PAGE and boiled for 10 min.

The supernatants (9 ml) were dialyzed against 25 mM Tris-maleate (pH 6.0) and concentrated on vivaspins (cutoff, 10 kDa; Vivascience, Littleton, MA) to a final volume of 90 μl. Forty μl of the concentrated supernatants was mixed with 10 μl of denaturing loading buffer and boiled for 10 min. The remaining supernatants were frozen at −20°C.

SDS-PAGE (12% polyacrylamide) was performed using a vertical electrophoresis apparatus (GE Healthcare, Uppsala, Sweden). Gels were stained by Coomassie blue or electrotransferred on nitrocellulose BA83 membrane (Schleicher and Schuell, Dassel, Germany). After saturation, membranes were probed with polyclonal antibodies raised against Cel5A, Cel8C, Cel48F, or Cel9M purified from *E. coli*-overproducing strains. Antibodies were detected using anti-rabbit horseradish peroxidase conjugate and a chemiluminescent substrate (GE Healthcare).

Alternatively, dockerin-containing cellulases also were detected by far-Western blot analysis using a biotinylated miniscaffoldin (miniCipC1) as formerly described (22).

The detection of cellulase activity in the supernatants was performed on agar plates using the Congo red staining method of Teather and Wood (33), with some modifications. The medium was composed of 1.5% (wt/vol) agar, 0.5% (wt/vol) carboxymethyl cellulose (CMC), and 0.1 M potassium phosphate, pH 7.0. Twenty μl of supernatants was loaded in 5-mm-diameter wells and incubated for 14 h at 37°C. The plates were stained afterwards for 15 min with 1% (wt/vol) Congo red and washed several times with 1 M NaCl.

RESULTS

Cloning of wild-type cellulase genes from *C. cellulolyticum* in *C. acetobutylicum* ATCC 824. It was shown formerly that recombinant strains of *C. acetobutylicum* secreting miniscaffoldins or a complex composed of a miniscaffoldin and one mannanase from *C. cellulolyticum* could be obtained (22, 27). In the present study, we have investigated the capacity of the solvent-producing bacterium to produce and secrete the most characterized cellulosomal cellulases from *C. cellulolyticum*. Since the transformation of *C. acetobutylicum* with pSOS952-cipC1 (27) generated recombinant strains that secrete the miniscaffoldin at 15 mg/liter, the same strategy was employed. Thus, the genes encoding the wild-type Cel5A (10), Cel8C (8), Cel9E (13), Cel48F (29), Cel9G (12), and Cel9M (4) also were cloned in this expression vector downstream of the strong and constitutive promoter P_{thl} of the thiolase-encoding gene.

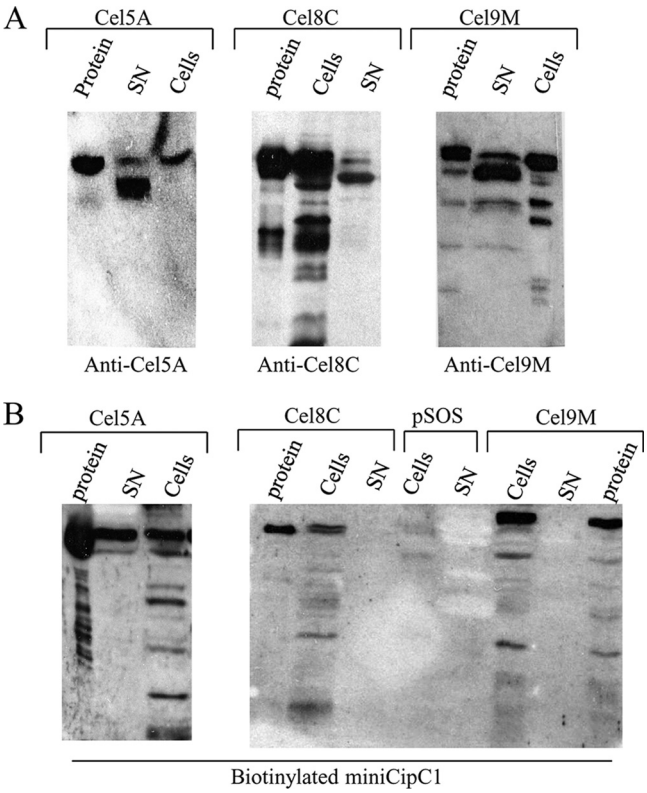

FIG. 1. Production and secretion of Cel5A, Cel8C, and Cel9M by *C. acetobutylicum*. Western blot analyses of SDS-PAGE using antiserum raised against purified Cel5A, Cel8C, or Cel9M (A) and biotinylated scaffoldin miniCipC1 (B). Lanes: protein, whole form of Cel5A, Cel8C, or Cel9M purified from overproducing *E. coli* strains; SN, concentrated supernatant of recombinant strains; cells, whole-cell extracts. pSOS designates the control strain containing pSOS952.

Between 20 and 100 colonies were obtained on erythromycin-containing medium after transformation with pSOS952-cel5A, pSOS952-cel8C, and pSOS952-cel9M, whereas electrotransformations performed simultaneously with the control vector pSOS952 generated 50 to 400 clones. For each vector, several transformants were grown in 10 ml of rich medium (2YT) supplemented with cellobiose and erythromycin. The cultures were stopped during the late exponential phase at an OD_{620} of 3.0 ± 0.2. After centrifugation, the supernatants were concentrated approximately 100 times and analyzed with the cellular fractions by SDS-PAGE followed by Western blot analysis, using either polyclonal antisera raised against purified Cel5A, Cel8C, and Cel9M or the biotinylated miniscaffoldin miniCipC1.

As shown in Fig. 1A, in all cases the heterologous cellulases were detected in the concentrated supernatants but as a truncated form displaying a molecular mass reduced by 5 to 10 kDa compared to that of the control proteins. A minor band cor-

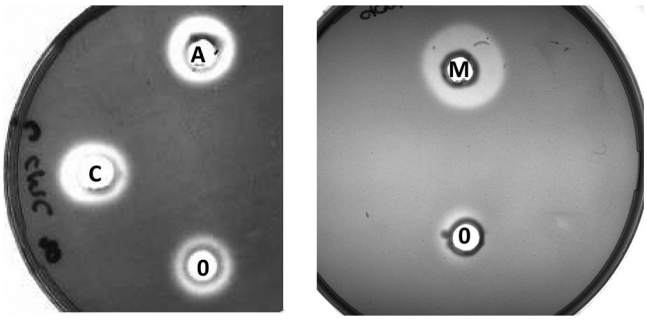

FIG. 2. Activity of supernatants on CMC plate. Twenty μl of supernatant of recombinant strains producing Cel5A (A), Cel8C (C), Cel9M (M), and the control strain containing pSOS952 (0) were loaded in the corresponding wells. After 14 h of incubation at 37°C, the CMCase activity (clear halos) was detected by the Congo red method.

responding to the full-length protein, however, was observed for the strain producing Cel5A. In contrast, the band corresponding to full-length Cel5A, Cel8C, or Cel9M was preponderant in the cell extracts, thus indicating that the proteolysis of the cellulases occurred mostly in the extracellular medium. A similar phenomenon already was observed in the case of the recombinant strain secreting the mannanase Man5K from *C. cellulolyticum*, and analyses showed that the truncated form of the hemicellulase was lacking the N terminus dockerin module (22). Although the dockerin is located at the C terminus of the enzymes described here, its presence in the secreted forms of the cellulases also was investigated by the far-Western blotting technique using a biotinylated scaffoldin containing the complementary cohesin module from *C. cellulolyticum* (22). As shown in Fig. 1B, the bands corresponding to full-length cellulases were labeled (cell extracts), but the bands corresponding to the truncated forms of the cellulases in the supernatants failed to react with the miniscaffoldin. This result confirmed that the proteolysed forms of the enzymes lack the C-terminal dockerin module, as previously observed for the heterologous mannanase (22). Some full-length cellulase, however, was detected in the supernatant of the strain producing Cel5A (Fig. 1B).

The dockerin module mediates the binding to the cognate cohesin module and is not essential for enzymatic activity (8). Thus, despite the proteolysis of the docking module, the supernatants of the recombinant strains secreting Cel5A, Cel8C, and Cel9M exhibited enhanced endoglucanase activity on CMC agar plates (Fig. 2) compared to that of the supernatant of the control strain.

In contrast, despite several attempts, the transformation of *C. acetobutylicum* with pSOS952-cel9E, pSOS952-cel48F, or pSOS952-cel9G failed to generate any erythromycin-resistant clone.

Cloning the cellulase genes downstream of the mutated P_{thl} promoter. Since high levels of expression of the heterologous genes induced by the strong and constitutive P_{thl} promoter of pSOS952 may be responsible for the deleterious effect on cells, the *cel48F*, *cel9E*, and *cel9G* genes were cloned in the plasmid pSOS954 (22). In this vector, P_{thl} is mutated in the −35 box (TGATAA→TGAT*T*A; the mutation site is in italics), presumably leading to lower expression levels in *C. acetobutylicum*. This strategy was employed formerly with the *man5K* gene, and transformation with pSOS952-K failed to generate recombinant strains of *Clostridium* (22). The same study showed that the use of the mutated promoter (pSOS954-K) generated recombinant clones secreting the heterologous mannanase.

This approach, however, was found to be unsuccessful in the case of the genes encoding wild-type Cel48F, Cel9E, and Cel9G. Despite several electrotransformations with pSOS954-cel48F, pSOS954-cel9G, and pSOS954-cel9E, no colony appeared on erythromycin-containing medium, suggesting a more deleterious effect of the cellulases encoded by these genes.

To further investigate this phenomenon, we assumed that the expression of the three heterologous genes induced similar effects on the cells, and we selected the *cel48F* gene to clarify the causes of the toxicity and determine alternative approaches to circumvent this issue. The various strategies developed are summarized in Fig. 3.

Construction of the *cipC1-cel48F* and *cipC1-cel48F-orfX* operons. We hypothesized that the impact of the three large cellulases was related to the lack of complementary scaffoldin or cohesin in *C. acetobutylicum*. To assess this hypothesis, the gene encoding the miniscaffoldin miniCipC1 (27) was cloned upstream of the *cel48F* gene (Fig. 3). The transformation of *C. acetobutylicum* with the resulting vector, however, did not generate any recombinant colony on selective medium.

Earlier studies also pointed out the possible role of the cohesin-containing protein OrfXp, whose gene is located in the central part of the large *cip-cel* cluster of *C. cellulolyticum* (25). Compared to the typical CipC cohesins, the receptor module found in OrfXp exhibits a 20-times-lower affinity constant for the enzyme dockerin, and its location in the membrane fraction suggested a function in the cellulosome assembly (25). Nevertheless, the transformation of the solventogenic bacterium with the vector pSOS952-cipC1-cel48F-orfX (Fig. 3) failed to generate any recombinant clone, thus showing that the negative impact of the synthesis of the cellulase is not diminished by the coexpression of both scaffoldin-encoding genes.

Removal of the leader peptide of Cel48F. To determine whether *cel48F* is intrinsically toxic for *C. acetobutylicum* or if the deleterious effect is related only to the secretion of the heterologous cellulase, the DNA encoding the native signal sequence of Cel48F was deleted in pSOS952-cel48F, thereby generating pSOS952-cel48Fmat (Fig. 3). The electrotransformation of *C. acetobutylicum* with the latter generated a number of clones in the same range of that obtained with the control vector pSOS952. One transformant was selected and grown up to an OD_{620} of 3.0 in rich medium 2YT supplemented with cellobiose and erythromycin. The concentrated supernatant and the cellular fraction were analyzed by Western blotting using a polyclonal antiserum raised against purified Cel48F. As expected (Fig. 4B), the heterologous cellulase was detected in the cells but not in the culture supernatant. Furthermore, the concentration of Cel48F in the cell extract was high enough to generate a band that is detectable using Coomassie blue staining (Fig. 4A). These data therefore indicate that the accumulation of the heterologous cellulase in the cytoplasm is not harmful for the solventogenic bacterium. Taken together,

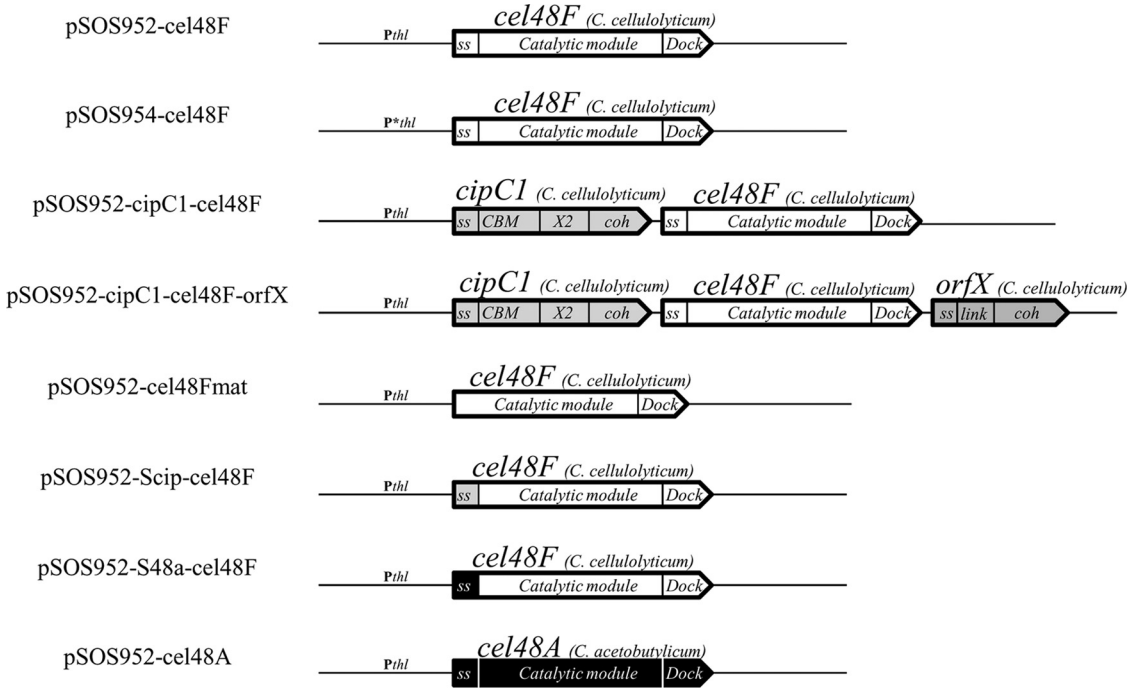

FIG. 3. Schematic representation of the various vectors containing either the *cel48F* or the *cel48A* gene. White and black boxes symbolize the *cel48F* (from *C. cellulolyticum*) and *cel48A* (from *C. acetobutylicum*) genes, respectively. Light and dark gray boxes symbolize the *cipC1* and *orfX* genes from *C. cellulolyticum*, respectively. P_{thl}, wild-type promoter of the thiolase gene; P^*_{thl}, mutated promoter of the thiolase gene; *ss*, DNA encoding the signal sequence; *catalytic module*, DNA encoding the catalytic module; *Dock*, DNA encoding the dockerin module; *CBM*, DNA encoding the CBM3a module of CipC; *X2*, DNA encoding the first X2 module of CipC; *coh*, DNA encoding the first cohesin module of CipC; *link*, DNA encoding the linker sequence of OrfXp.

these results strongly suggest that the deleterious effect is due to the enzyme precursor and its interactions with the secretion machinery of *C. acetobutylicum*, whereas the latter is not affected by other *C. cellulolyticum* enzymes, like Cel5A, Cel8C, and Cel9M.

Replacement of the leader peptide of Cel48F. The *C. acetobutylicum* genome contains the genes that putatively encode the major proteins of the Sec machinery. Although it is currently very difficult to predict which signal sequence will improve the secretion of a specific protein (5), we assessed the hypothesis that the native leader peptide of Cel48F was not the most suitable for secretion by the solventogenic bacterium and provoked toxicity. The DNA encoding the leader peptide therefore was replaced by that encoding the heterologous signal sequence of the scaffoldin CipC (Fig. 3). This sequence was shown formerly to enable the secretion of two miniscaffoldins and the mannanase Man5K by *C. acetobutylicum* at yields ranging from 1 to 15 mg/liter (22). Nevertheless, transformation with pSOS952-Scip-cel48F did not generate any recombinant clone on selective medium.

A *C. acetobutylicum* leader peptide also was assayed. The signal sequence of Cel48F was replaced by that of the endogenous cellulosomal Cel48A. The latter GH48 enzyme was shown previously to be secreted in detectable amounts by *C. acetobutylicum* on cellobiose-containing medium supplemented with cellulose (30). The cellulosomes produced by *C. acetobutylicum* are secreted in small amounts, but Cel48A represents the major component of the endogenous complexes. This cellulase shares 52% sequence identity with Cel48F from *C. cellulolyticum*, but the leader peptide of Cel48A is slightly longer. Quite unexpectedly, transformations of *C. acetobutylicum* with pSOS952-S48a-cel48F (Fig. 3) also failed to generate any recombinant colony, thus indicating that the Cel48A endogenous signal sequence does not alleviate the negative impact of Cel48F precursor on cell viability.

Cloning of *cel48A* in pSOS952. The result described above prompted us to clone the wild-type *cel48A* gene downstream of the constitutive promoter P_{thl} in the vector pSOS952 (Fig. 3) for homologous overexpression in *C. acetobutylicum*. Despite several attempts, electrotransformation with pSOS952-cel48A did not generate any recombinant colonies on erythromycin-containing medium. This observation suggests that high expression levels of *cel48A* are as harmful as the heterologous overexpression of *cel48F*.

Transformation with pSOS952-cel48F and selection at 30°C. We performed another electrotransformation of the solventogenic *Clostridium* with pSOS952-cel48F using the same general procedure, except that all steps were carried out at 30°C instead of 37°C. The purpose was to investigate if slowing the translation of the heterologous gene and the folding of the corresponding cellulase precursor circumvents the secretion issue. Several colonies appeared after 4 days of incubation at 30°C on erythromycin-containing selective medium, and two clones subsequently were grown at the same temperature in 10 ml of rich medium (2YT) supplemented with cellobiose. The cultures were harvested at an OD_{620} of 3.0, and the concentrated supernatants as well as the cell extracts were analyzed by Western blotting using an antiserum raised against Cel48F

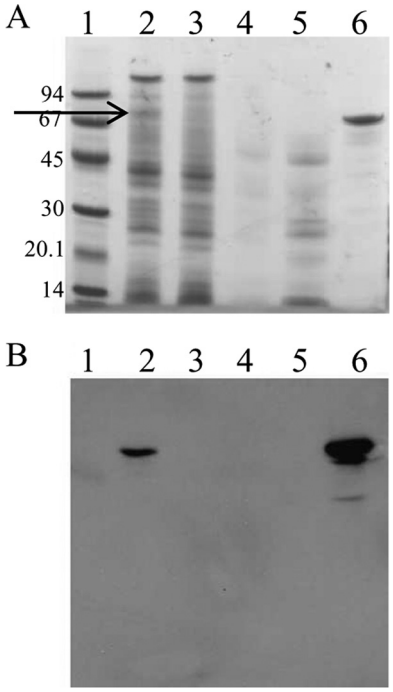

FIG. 4. Cytoplasmic production of Cel48F by *C. acetobutylicum*. Coomassie blue-stained SDS-PAGE (A) and Western blot analysis using antiserum raised against purified Cel48F (B). Lane 1, molecular mass markers; lane 2, cell extract of *C. acetobutylicum*(pSOS952-cel48F); lane 3, whole-cell lysate of *C. acetobutylicum*(pSOS952); lane 4, concentrated supernatant of *C. acetobutylicum*(pSOS952-cel48F); lane 5, concentrated supernatant of *C. acetobutylicum*(pSOS952); lane 6, purified Cel48F from an *E. coli*-overproducing strain. Numbers in panel A indicate the molecular mass of markers in kDa, and the arrow designates the band corresponding to mature Cel48F.

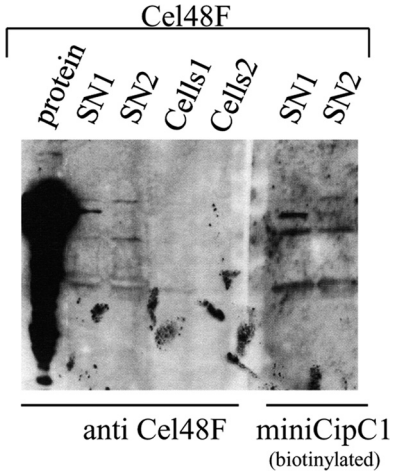

FIG. 5. Production of Cel48F by *C. acetobutylicum* at 30°C. Western blot analysis of SDS-PAGE using antiserum against Cel48F or biotinylated miniCipC1. Lane protein, purified Cel48F from an *E. coli*-overproducing strain; lane SN1, concentrated supernatant of *C. acetobutylicum*(pSOS952-cel48F) clone 1 grown at 30°C; lane SN2, concentrated supernatant of *C. acetobutylicum*(pSOS952-cel48F) clone 2 grown at 30°C; lanes cells1 and cells2, whole-cell lysate of clones 1 and 2, respectively, grown at 30°C.

(Fig. 5). The putative presence of Cel48F in the supernatants also was investigated with biotinylated miniscaffoldin as previously described. The results indicated that reducing the temperature of growth notably decreased the toxicity related to Cel48F secretion, since one of the selected clones was capable of secreting Cel48F. Detection with either antiserum or the biotinylated probe required long exposure, thus indicating that the cellulase was secreted in very small amounts. In contrast, Cel48F was undetectable in the cell extracts. Unfortunately, upon storage as a spore suspension and the subsequent germination of the most interesting clone, the recombinant strain lost the phenotype, thus indicating it was not stable.

DISCUSSION

C. acetobutylicum produces significant amounts of butanol, acetone, and ethanol and therefore could be a relevant candidate for the conversion of cellulosic material using a consolidated bioprocess. In this respect, this microorganism was expected to be a suitable solventogenic host for the production and secretion of *C. cellulolyticum* cellulosome components. The two clostridia share a similar GC content. Furthermore, *C. acetobutylicum* produces an extracellular cellulosome that resembles that of *C. cellulolyticum*, although it is inactive toward crystalline cellulose and is secreted in smaller amounts (30). Our previous studies confirmed that the choice of *C. acetobutylicum* was pertinent, since recombinant strains secreting two miniscaffoldins or a mannanase-miniscaffoldin complex were obtained (22, 27).

The next step involved the cloning and expression of the genes encoding the most characterized cellulases from *C. cellulolyticum* in the solventogenic bacterium. The present study clearly shows that the selected *C. cellulolyticum* cellulases can be divided into two distinct groups. The cellulases Cel5A, Cel8C, and Cel9M, which are composed of a rather small catalytic module and a dockerin, can be produced easily and secreted in an active form by *C. acetobutylicum*. In contrast, the expression of the genes encoding the cellulases Cel48F, Cel9G, and Cel9E, which possess additional modules (Cel9G and Cel9E) or are characterized by a large catalytic module (Cel48F), prevented the formation of colonies on selective medium. The case of Cel48F was further investigated, and it was demonstrated that this effect is related to secretion, since the deletion of the native signal sequence of Cel48F generated many clones producing the heterologous cellulase in the cytoplasm. In contrast, other strategies, like the replacement of the native signal sequence of Cel48F or coexpression with a scaffoldin gene(s), failed to lower the toxicity and prevent the secretion issue.

One possible explanation is that Cel48F precursor, and possibly unprocessed Cel9G and Cel9E, induces a blockade of the secretory system, which in turn inhibits the growth of *C. acetobutylicum*. The molecular mechanism leading to the breakdown of the secretion machinery remains unidentified, but one may hypothesize that at the optimal growth temperature of 37°C, Cel48F precursor (as well as the two other "toxic" cellulases) rapidly adopts a conformation incompatible with secretion. The cellulase precursor thus would induce an obstruction of the Sec complex, perhaps similar to that observed in *E. coli* with β-galactosidase hybrids (37). This hypothesis is consistent with the fact that cooling the temperature to 30°C

during transformation and growth, which is known to slow down protein folding, reduced sufficiently the negative impact of Cel48F to generate a recombinant strain secreting small amounts of the cellulase. In contrast, the fact that Cel5A, Cel8C, and Cel9M, as well as the scaffoldins miniCipC1 and Scaf3, are efficiently secreted by *C. acetobutylicum* suggests that their precursors are easily maintained in a competent state for translocation, presumably because these proteins do not rapidly fold at 37°C. It is worth noting that the mature forms of Cel5A (10), Cel8C (8), Cel9M (4), miniCipC1 (26), and Scaf3 (11) are produced in a soluble form in *E. coli* cytoplasm at 37°C. In contrast, the overproduction of Cel48F (28, 29), Cel9G (12), and Cel9E (13) generates inclusion bodies unless the expression of their genes is induced at a lower temperature (15 to 18°C). Thus, the translation and folding of the large cellulases need to be slowed down to prevent aggregation and obtain soluble and active forms of these enzymes when produced by *E. coli*. It should be noted that at 37°C, even an engineered form of Cel48F fused with PelB signal sequence, which was successfully translocated across the cytoplasmic membrane, generated inclusion bodies in the periplasmic space of *E. coli* (28). The formation of inclusion bodies in *E. coli* at 37°C also seems to be a general rule for cellulosomal family 48 enzymes, since similar observations were made in the case of Cel48S from *C. thermocellum* (16) and ExgS from *C. cellulovorans* (19). In contrast, noncellulosomal GH48 appended with other modules, such as CBM, appear to behave differently when they are produced in heterologous hosts. For instance, Cel48C from *Paenibacillus* sp. BP-23 was synthesized in a soluble form in *E. coli* cytoplasm at 37°C (31), and CpCel48 from *C. phytofermentans* was successfully secreted as a soluble form by *Bacillus subtilis* (39).

With respect to the deleterious effect related to the secretion of Cel48F, one may hypothesize that a specific chaperone(s) that maintains the key family 48 cellulase (and probably Cel9G and Cel9E) in a competent state for translocation is missing or is not sufficiently produced in *C. acetobutylicum*. The fact that the overexpression of the endogenous wild-type *cel48A* gene also was harmful, although the wild-type strain does secrete small amounts of the corresponding cellulase (30), suggests that *C. acetobutylicum* is not equipped for the larger-scale secretion of key cellulosome components. The deficiency of the secretory system with respect to cellulosome proteins thus may have contributed to the loss of the cellulolytic phenotype by *C. acetobutylicum*. The cloning of genes encoding these cellulases downstream of an inducible promoter would indeed be an attractive strategy to control and further investigate this phenomenon. Unfortunately, a suitable promoter system fully repressed in the absence of inducer, like the *E. coli* arabinose operon P_{BAD} promoter, still needs to be discovered for tightly controlled expression in *C. acetobutylicum*. For instance, the P_{xyl} promoter from *Staphylococcus aureus*, which was shown previously to be functional in *C. acetobutylicum* (14), probably generates deleterious levels of expression of the *cel48F* gene even in the absence of the inducer (xylose), since no colonies were obtained in these experimental conditions.

One of the most active minicellulosomes on crystalline cellulose contains the enzyme Cel48F (or Cel9E), displaying a *C. thermocellum* dockerin combined with Cel9G (containing its native *C. cellulolyticum* dockerin) and bound to a miniscaffoldin harboring the cognate cohesins. Engineering a cellulolytic strain of *C. acetobutylicum* therefore implies the modification of the secretory system and enables the bacterium to secrete enough efficient hybrid minicellulosome containing these latter cellulases.

Comparison between *C. cellulolyticum* and *C. acetobutylicum* genomes indicates that both bacteria lack a twin arginine translocation system but exhibit a Sec pathway (6). The gene encoding the general chaperone SecB (*E. coli*) or CsaA (*Bacillus subtilis*), which prevent folding and target the proteins to the Sec translocon, are missing from both genomes (6). In contrast, ORFs coding for other common chaperones that can play a similar role, such as GroES/GroEL (GI:15895959/GI:15895960 and GI:220927847/GI:220927848 for *C. acetobutylicum* and *C. cellulolyticum*, respectively) (35), HtpG (GI:15896558 for *C. acetobutylicum* and GI:220927945 for *C. cellulolyticum*) (6), and GrpE/DnaK/DnaJ (GI:15894563/GI:15894564/GI:15894565 and GI:220929220/GI:220929219/GI:220929218 for *C. acetobutylicum* and *C. cellulolyticum*, respectively) are found in both bacterial chromosomes; the genome of *C. acetobutylicum* also contains an additional GrpE/DnaK/DnaK′-encoding gene cluster (GI:15893762/GI:15893763/GI:15893764). The inability of *C. acetobutylicum* to efficiently secrete key cellulosomal components, however, suggests that their secretion requires specific chaperones that are present (or adequately synthesized) only in *C. cellulolyticum*. Future prospects therefore will include the identification of the protein(s) that prevent(s) the folding and promote(s) the targeting of critical cellulases precursors to the Sec translocon in *C. cellulolyticum.* The corresponding gene(s) will be coexpressed afterwards with *cel48F* and *cel9G* in the solventogenic bacterium.

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