

Production of Heterologous and Chimeric Scaffoldins by *Clostridium acetobutylicum* ATCC 824

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***Clostridium acetobutylicum* ATCC 824 converts sugars and various polysaccharides into acids and solvents. This bacterium, however, is unable to utilize cellulosic substrates, since it is able to secrete very small amounts of cellulosomes. To promote the utilization of crystalline cellulose, the strategy we chose aims at producing heterologous minicellulosomes, containing two different cellulases bound to a miniscaffoldin, in *C. acetobutylicum*. A first step toward this goal describes the production of miniCipC1, a truncated form of CipC from *Clostridium cellulolyticum*, and the hybrid scaffoldin Scaf 3, which bears an additional cohesin domain derived from CipA from *Clostridium thermocellum*. Both proteins were correctly matured and secreted in the medium, and their various domains were found to be functional.**

Clostridium acetobutylicum ATCC 824 is one of the best-known solventogenic bacteria that convert sugars and various polysaccharides into acids and solvents (4, 5, 11, 13, 15, 27, 28). Unfortunately, this bacterium is unable to grow on crystalline cellulose, although its genome contains a large cluster of genes involved in the cellulolysis process (15, 23). This cluster starts with the *cipA* gene encoding the scaffoldin CipA, followed by eight genes encoding mostly glycosylhydrolases derived from GH families 48, 9, and 5. *C. acetobutylicum* secretes very small quantities of a cellulosome of ~665 kDa devoid of activity on crystalline cellulose and possessing very low activity on carboxymethyl cellulose or phosphoric-acid-swollen cellulose (22, 23). Recently, the gene encoding a truncated CipA was overexpressed in the bacterium, leading to the formation of a minicellulosome in *C. acetobutylicum* (24). As with normal cellulosome, this recombinant minicellulosome was found to be inactive against crystalline cellulose. The reasons for these cellulosomes being so poorly produced are not yet clearly established. Since *C. acetobutylicum* grows very well on cellobiose, it may be possible to engineer the bacterium to grow on cellulose by introducing cellulases from another cellulolytic bacterium. The mesophilic *Clostridium cellulolyticum* was chosen as the donor for *cel* genes. This bacterium produces a cellulosome of ~700 kDa which efficiently degrades crystalline cellulose (1, 6). Almost all *cel* genes are clustered on a 26-kb fragment including *cipC*, coding for the scaffoldin CipC (18). Several components of the cellulosome have been extensively studied from the biochemical and structural points of view (1, 7, 12, 18, 19, 20, 21, 25, 26). The dockerin domains of the cellulases interact closely with the cohesin domains of the scaffoldin (16), and it has been demonstrated that the cohesins of CipC recognize all the dockerin-containing enzymes from *C.*

cellulolyticum (18). On the other hand, recognition between the cohesins and dockerins of the two clostridial species *C. cellulolyticum* and the thermophilic *Clostridium thermocellum* was shown to be species specific (17). Based on these observations, chimeric miniscaffoldins containing a cohesin from each species have been built by Fierobe et al. (2, 3). These chimeric proteins allowed the in vitro reconstitution of minicellulosomes containing two different cellulases, one possessing a *C. cellulolyticum* dockerin (cellulase_c) and the other harboring a *C. thermocellum* dockerin (cellulase_t). It was shown that the binding of various enzyme pairs on the hybrid scaffoldins induced a significant increase in activity toward crystalline cellulose, especially when the hybrid scaffoldin contained a cellular binding module (CBM). Our goal is thus to produce in *C. acetobutylicum* the most efficient minicellulosomes containing a chimeric scaffoldin and two selected enzymes.

In this study, we describe the production in *C. acetobutylicum* of two heterologous miniscaffoldins: miniCipC1 containing CBM3a, one X2 module, and the cohesin 1 module of CipC of *C. cellulolyticum*, and the chimeric Scaf3, in which the cohesin 3 module of the scaffoldin CipA of *C. thermocellum* was fused with the C-terminal region of miniCipC1.

Cloning of *cipC1* in *C. acetobutylicum*. The DNA fragment coding for miniCipC1, including the signal peptide, was amplified from *C. cellulolyticum* genomic DNA using the primers *cipC1D* and *cipC1R* (Table 1). The internal *Bam*HI site of the fragment was suppressed by PCR using the primers MutB-D and MutB-R. This fragment was introduced between the *Bam*HI and *Nar*I sites of the pSOS95 shuttle vector (kindly provided by P. Soucaille, INSA, Toulouse, France) under the control of the strong constitutive promoter of the *C. acetobutylicum* thiolase gene (*thl*). The transcriptional terminator used was that of the acetoacetate decarboxylase gene (*adc*). Unfortunately, no *Escherichia coli* transformant colony was obtained. It was hypothesized that the *thl* promoter was recognized by the *E. coli* transcription machinery and that the production of miniCipC1 would disrupt the secretion machinery, leading to cell death. This hypothesis was confirmed by the cloning of the gene encoding the mature form of miniCipC1 using the same

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TABLE 1. Oligonucleotide primers used in this study

Name	Sequence (5' → 3') ^a	Localization	Underlined sequence
SosA	TCCTGCAGGTGCGACTTTTAAACAA	<i>thl</i> promoter	<u>SaI</u> restriction site
SosB	ATTGTTATCCGGCTCA441TTCAACTTAATTATACCCACTATTATATTTT	<i>lac</i> operator 1	
SosD	ATTGTGAGCGG4744CA41TTTGAAGAAAACCGTATAAAATTAGGGATAAACTATGGA	<i>lac</i> operator 1	
SosE	TAAATTCCTGGATCCCTACGGGGTAAACAGA	<i>thl</i> promoter	<i>Bam</i> HI restriction site
LacD2	TCGATCTAGAA477GTGAGCGGAT44CA41TAAAGCTCCTGCAGG	<i>lac</i> operator 2	<i>SaI</i> extension
LacR2	TCGACCTGCAGGAGCTTT441TGTATCCGCTCAC441TTCTAGA	<i>lac</i> operator 2	<i>SaI</i> extension
CipC1D	GGGGATCCAGAA41TTAAAGGAGGGAT44AAATGCGTAAAAAGTCTTTAGCA	<i>cipC1</i>	<i>Bam</i> HI restriction site
CipC1R	TTCCGGGCGCTTATCTACTCTAATTAAGTTCCTTTG	<i>cipC1</i>	<i>Nar</i> I restriction site
MutB-D	CAGCTGGAGGTTCCATAGAGAT	<i>Bam</i> HI mutation in <i>cipC1</i>	
MutB-R	ATCTCTATGGAAACCTCCAGCTG	<i>Bam</i> HI mutation in <i>cipC1</i>	
rMC1SOS1	GGTGGGGGATCCTTCGAACTACTCGAGTTCCTTTGTAGGTTGAGTACC	Used for construction of <i>scaf3</i>	<i>Bam</i> HI, <i>Asu</i> II, <i>Xho</i> I restriction sites

^a *lac* operator sequences are italicized; stop codons are in boldface.

vector. Intracellular overproduction of the protein was observed in *E. coli* DH5 α (data not shown).

The promoter region of the pSOS95 vector was therefore modified by adding two *lac* operators (21 bp each) immediately upstream and downstream of the thiolase promoter region, which thus remained intact, in the pSOS952 plasmid (Fig. 1B). The primers used are listed in Table 1. The first *lac* operator was generated by three PCR steps, using pSOS95 as the matrix. The first step generated an 86-bp fragment, using the forward primer SosA and the reverse primer SosB. The second step generated a 111-bp PCR fragment, using the forward primer SosD and the reverse primer SosE. The entire fragment (178 bp) containing the *lac* operator was amplified by PCR from a mixture of both overlapping fragments by using the external primers SosA and SosE. After double digestion with *SaI*I and *Bam*HI, the *lac* operator 1 fragment was subsequently ligated into a similarly digested vector pSOS95, resulting in the vector pSOS95m. The second *lac* operator was inserted into the pSOS95m plasmid at the *SaI*I restriction site. The DNA fragment was obtained by simple hybridization of oligonucleotides LacD2 and LacR2.

E. coli SG-13009, containing the pREP4 repressor plasmid, was used as the recipient strain for the resulting pSOS952 plasmid and its recombinant forms. The DNA fragment coding for miniCipC1 with the signal peptide was cloned successfully using pSOS952. The resulting pSOS952-cipC1 plasmid was methylated in vivo in *E. coli* ER 2275 carrying the pAN1 methylating plasmid (10) and was used to transform *C. acetobutylicum* by electrotransformation (14).

One clone was selected to inoculate 2YT medium (containing, per liter, 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl) supplemented with cellobiose (5 g/liter) and erythromycin (40 μ g/ml). After growth overnight, the cells were harvested and the supernatant was adjusted to pH 6.5 with 1 M phosphate buffer and loaded onto an Avicel (Fluka PH 101) column equilibrated with 50 mM phosphate buffer, pH 6.5. After two washes with 50 and 20 mM phosphate buffer, pH 6.5, respectively, the protein was eluted from Avicel with 1% triethylamine solution, dialyzed, and concentrated in an Amicon apparatus using a polyether-sulfone (10-kDa cutoff) membrane. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), this fraction was found to contain mainly a protein, called miniCipC1_{cab}, with a molecular mass of 43,000 Da, which is in good agreement with the theoretical mass of miniCipC1 (43,500 Da). Furthermore, the electrophoretic profile was identical to that of the control protein miniCipC1_{eco} [recombinant miniCipC1 produced in *E. coli* BL21(DE3) from pETcipC1] (16) (Fig. 2A). These two proteins were specifically recognized by the polyclonal antibodies raised against CBM3a (Fig. 2B). N-terminus microsequencing of the protein produced in *C. acetobutylicum* (AGTGV) matched perfectly with the N terminus of *C. cellulolyticum* cellulosomal CipC, thus confirming that the purified protein is miniCipC1. Finally, it was estimated that ~15 mg of pure miniCipC1 can be obtained from 1 liter of overnight culture. These results showed that, as expected, the presence of the two *lac* operators did not prevent the expression of the gene coding for miniCipC1 in *C. acetobutylicum*.

Production of Scaf3 by *C. acetobutylicum*. Since miniCipC1 was easily synthesized by *C. acetobutylicum*, the production of

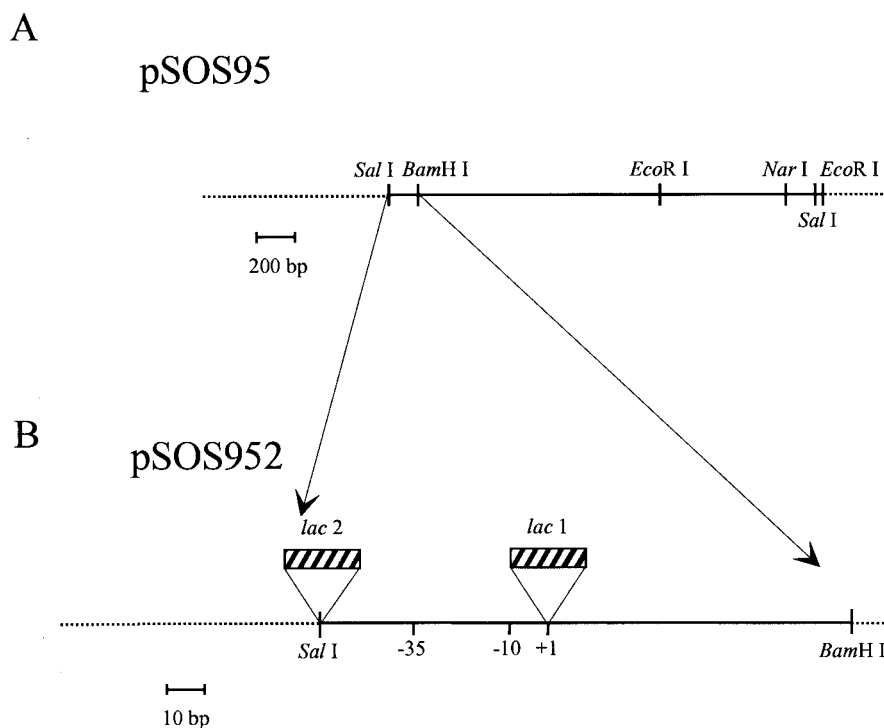


FIG. 1. Schematic representation of the cloning region of pSOS95 (A) and position of the two *lac* operators inserted upstream and downstream of the thiolase of the promoter region (B) to construct the pSOS952 derivatives.

the chimeric protein Scaf3 was carried out. Scaf3 consists of *C. cellulolyticum* miniCipC1 followed by cohesin 3 from *C. thermocellum* CipA. To obtain this construct, the 3' end of *cipC1* in pSOS952-cipC1 was modified by introducing an *XhoI* site upstream of the stop codon and *AsuII* and *BamHI* sites downstream, using the primers CipC1D (forward) and rMC1SOS1 (reverse). The amplified fragment, digested by *BamHI*, was ligated into *BamHI*-linearized pSOS952. Then, the cohesin 3 coding sequence, obtained from *XhoI* digestion of pETscaf3 (2), was ligated into the *XhoI* site of the recombinant pSOS952 plasmid previously obtained, leading to the plasmid pSOS952-scaf3. In the Scaf3 protein produced from this construction, the

two cohesin modules are separated by a 44-amino-acid linker. The same protocol as for miniCipC1 was used to produce Scaf3. The fraction eluted from cellulose mainly contained a protein with an apparent mass of 64 kDa on SDS-PAGE, which is in good agreement with the theoretical mass (61,6467 Da) calculated from the chimeric Scaf3 (2) (Fig. 3A). This protein was also recognized by the polyclonal antibodies raised against CBM3a (Fig. 3B) and was produced in its complete form, showing that the long linker located between the two cohesin domains was not cleaved. It was estimated that 10 mg of pure protein can be obtained from 1 liter of culture.

Interaction of miniCipC1 and Scaf3 with dockerin domains from *C. cellulolyticum* and *C. thermocellum*. To verify the functionality of the cohesins in miniCipC1 and Scaf3 produced by *C. acetobutylicum*, specific cohesin-dockerin interactions were performed. After SDS-PAGE, miniCipC1_{cab} and the control miniCipC1_{eco} were blotted onto nitrocellulose membranes and overlaid with either the *C. cellulolyticum* cellulase Cel48F_c or Cel9E_c. The blotting membranes were subsequently incubated with antiserum raised against Cel48F or Cel9E. In both cases, a band corresponding to miniCipC1 (control or miniCipC1_{cab}) was revealed, showing that Cel48F_c or Cel9E_c was able to interact with the cohesin domain of miniCipC1 produced by *C. acetobutylicum* (Fig. 4).

A second set of experiments was carried out to control the functionality of the two cohesins in Scaf3 produced in *C. acetobutylicum* (Scaf3_{cab}). MiniCipC1_{cab} and Scaf3_{cab}, as well as two control proteins, Scaf3_{eco} (2) and C2-CBMt_{eco} (3) (the latter is a fragment of CipA from *C. thermocellum* that contains

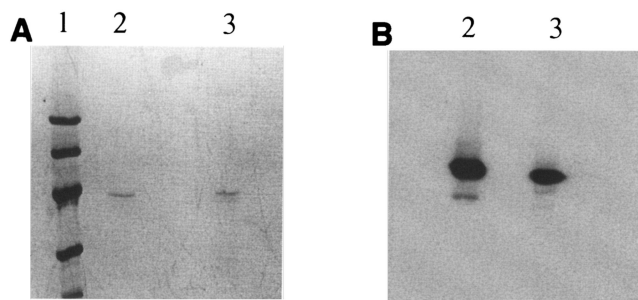


FIG. 2. Production of CipC1 by *C. acetobutylicum*. Lane 1, molecular mass markers (LMW; Amersham) (from top to bottom: 94, 67, 43, 30, 20, and 14 kDa); lanes 2, CipC1_{eco} (0.1 mg/ml) purified from *E. coli* BL21(DE3) recombinant clone; lanes 3, sample of CipC1_{cab} produced in *C. acetobutylicum*. (A) SDS-PAGE gel stained with Coomassie blue; (B) Western blot; detection with antiserum raised against CBM3a.

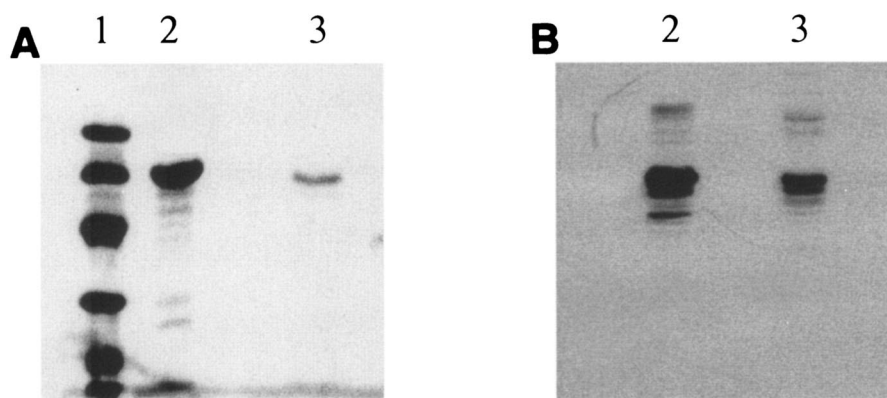


FIG. 3. Production of Scaf3 by *C. acetobutylicum*. Lane 1, molecular mass markers (LMW; Amersham) (from top to bottom: 94, 67, 43, 30, 20, and 14 kDa); lanes 2, control Scaf3_{eco} (1.1 mg/ml) produced in *E. coli* BL21(DE3); lanes 3, sample of Scaf3_{cab} produced in *C. acetobutylicum*. (A) SDS-PAGE gel stained with Coomassie blue; (B) Western blot; detection with antiserum raised against CBM3a.

cohesin 2 and CBM3a), were blotted onto nitrocellulose membranes and overlaid with either Cel9E_c or Cel9E_t (harboring a *C. thermocellum* dockerin). The membranes were subsequently incubated with antiserum raised against Cel9E. As can be seen in Fig. 5A, only miniCipC1_{cab}, Scaf3_{eco}, and Scaf3_{cab} were stained when the membrane was overlaid with Cel9E_c. On the other hand, only the two control proteins C2-CBMt and Scaf3_{eco}, as well as Scaf3_{cab}, were revealed when the blot was overlaid with Cel9E_t (Fig. 5B). All these data suggest that *C. acetobutylicum* is able to produce functional foreign cohesin domains originating from mesophilic or thermophilic clostridia.

These experiments showed that it is possible to produce a hybrid scaffoldin protein in *C. acetobutylicum*, which is the first step in the production in vivo of a well-defined minicellulosome. The challenge now is to produce, in an active form, the more suitable enzymes selected from a *C. cellulolyticum* cellulase library. Previous studies have shown that the most efficient minicellulosome was obtained with the complex including the endoprocessive cellulase Cel48F and the endocellulase Cel9G bound onto a single CBM-containing scaffoldin. Such a com-

plex is ~4-fold more active on Avicel than the mixture Cel48F plus Cel9G in the free state (3).

So far, only one heterologous expression of a cellulase gene (*engB* from *Clostridium cellulovorans*) has been reported in *C. acetobutylicum* ATCC 824 (8). The secretion in the extracellular medium was so poor that the recombinant cellulase could be detected only by Western blotting. Two glycoside hydrolases, Cel6A and Cel5D, from the eukaryotic organism *Neocallimastix patriciarum* have been produced in *Clostridium beijerinckii* (9). The two genes were functionally expressed, and the resulting enzymes were excreted into the extracellular medium. Nevertheless, it seems that the production of these enzymes was also very low, not enough to promote significant degradation of cellulose.

Attempts to produce Cel48F and Cel9G with suitable dockerins in *C. acetobutylicum* are underway. If successful, it will be possible to build a *C. acetobutylicum* strain able to secrete the most efficient minicellulosome, containing two cellulases and a hybrid scaffoldin. This would constitute a starting point for the development of an industrial process to convert cellulose directly into solvents.

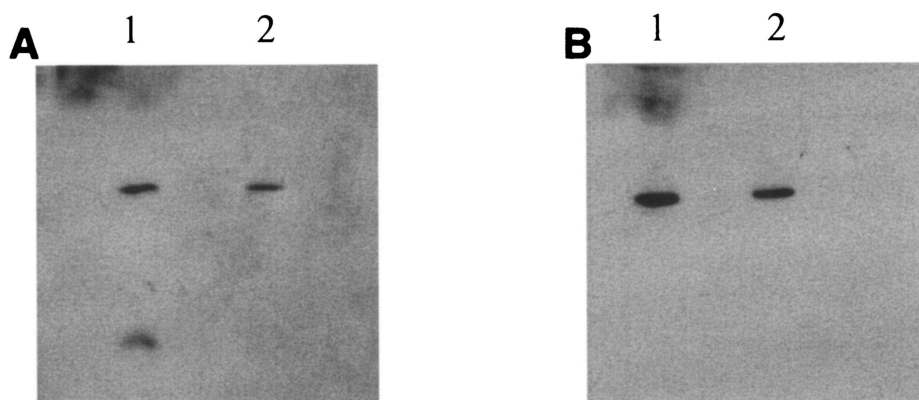


FIG. 4. Interaction of miniCipC1 with Cel48F_c and Cel9E_c. Lanes 1, control miniCipC1_{eco}; lanes 2, sample of miniCipC1_{cab} produced in *C. acetobutylicum*. (A) Blot overlaid with Cel9E_c and revealed with antiserum raised against Cel9E. (B) Blot overlaid with Cel48F_c and revealed with antiserum raised against Cel48F.

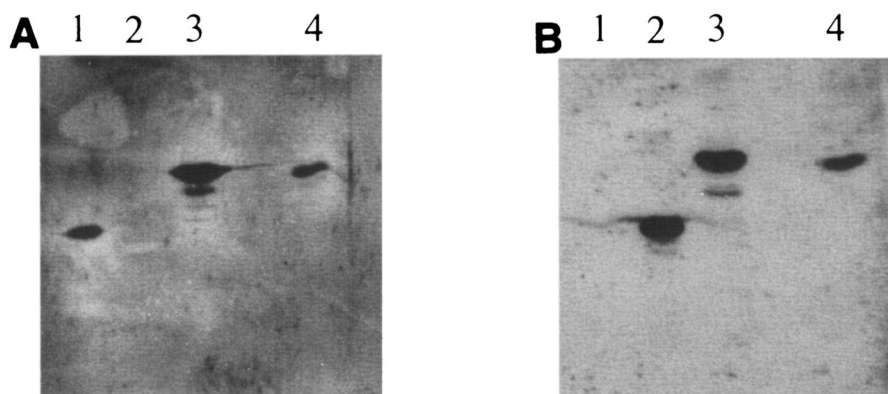


FIG. 5. Interaction of miniCipC1 and Scaf3 with Cel9E_C and Cel9E_E. Lanes 1, control miniCipC1_{eco}; lanes 2, control C2-CBMT_{eco}; lanes 3, control Scaf3_{eco}; lanes 4, Scaf3_{cab}. (A) Blot overlaid with Cel9E_C. (B) Blot overlaid with Cel9E_E. The two blots were revealed with antiserum raised against Cel9E.

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