Cell-surface binding domains from *Clostridium cellulovorans* can be used for surface display of cellulosomal scaffoldins in *Lactococcus lactis*

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Abstract
Engineering microbial strains combining efficient lignocellulose metabolism and high-value chemical production is a cutting-edge strategy towards cost-sustainable 2nd generation biorefining. Here, protein components of the *Clostridium cellulovorans* cellulosome were introduced in *Lactococcus lactis* IL1403, one of the most efficient lactic acid producers but unable to directly ferment cellulose. Cellulosomes are protein complexes with high cellulose depolymerization activity whose synergistic action is supported by scaffolding protein(s) (i.e., scaffoldins). Scaffoldins are involved in bringing enzymes close to each other and often anchor the cellulosome to the cell surface. In this study, three synthetic scaffoldins were engineered by using domains derived from the main scaffoldin CbpA and the Endoglucanase E (EngE) of the *C. cellulovorans* cellulosome. Special focus was on CbpA X2 and EngE S-layer homology (SLH) domains possibly involved in cell-surface anchoring. The recombinant scaffoldins were successfully introduced in and secreted by *L. lactis*. Among them, only that carrying the three EngE SLH modules was able to bind to the *L. lactis* surface although these domains lack the conserved TRAE motif thought to mediate binding with secondary cell wall polysaccharides. The synthetic scaffoldins engineered in this study could serve for assembly of secreted or surface-displayed designer cellulosomes in *L. lactis*.

KEYWORDS
biorefinery, cellulosic biomass, cellulosome, metabolic engineering, S-layer homology domain

1 | INTRODUCTION

Lignocellulose is the most abundant raw material on the Earth. Its low price makes it an ideal feedstock for 2nd generation biorefining aimed at replacing fossil-derived production of fuels and chemicals.¹ However, lignocellulose has been selected to be recalcitrant to microbial and enzyme activity; therefore, its conversion through biological process is both technically and economically challenging.¹ Nowadays, industrial fermentation of lignocellulose is complex and expensive since multiple bioreactors in series are generally required.²³ Development of single-step fermentation (i.e., consolidated bioprocessing, CBP) of biomass is considered as one of the most promising strategies to reduce the costs of 2nd generation biorefinery and make them competitive with those of oil refinery.³⁴ The most straightforward path to achieve this goal is by using microbial strains that can directly ferment plant biomass and produce high-value compounds with high efficiency. Since such microbes have not been found in nature, so far, metabolic engineering can hopefully be used to develop them through gene modification techniques.⁵⁶

Recombinant cellulolytic strategies (RCS) aim at endowing cellulolytic ability in microbial strains producing high-value compounds. RCS have been applied to several microbial models such as...
Saccharomyces cerevisiae, Kluyveromyces marxianus and Zymomonas mobilis (i.e., ethanol producers), Clostridium acetobutylicum (i.e., solvent producer), Corynebacterium glutamicum (i.e., glutamate producer) and lactic acid bacteria (LAB).\[^{15,16}\] LAB have significant industrial application in production of lactic acid (LA) and as probiotics.\[^{18}\] In addition, LAB have been considered as candidates for synthesizing other high-value compounds such as ethanol, polyhydroxalkanoates, polyols, and exopolysaccharides.\[^{9}\] However, LAB generally cannot directly ferment complex carbohydrates, namely few of them can ferment starch and no LAB isolated so far can depolymerize lignocellulosic feedstocks.\[^{7}\] Attempts to engineer minimal (hem)cellulase systems in LAB strains have therefore been reported (extensively reviewed in\[^{7}\]). RCS are based on mimicking natural biochemical systems for plant biomass depolymerization, consisting of multiple enzymes with different substrate specificities and catalytic mechanisms.\[^{5}\] These approaches generally refer to two main paradigms, that is, the non-complexed model of aerobic microorganisms, and the complexed (i.e., cellulose) model of anaerobic strains.\[^{10}\] In particular, cellulosomes are among the most efficient machineries for the degradation of lignocellulosic biomass, owing to close proximity between enzymes and microbial cells resulting in improved synergistic activity.\[^{11}\] For biotechnological purposes, designer cellulosomes based on artificial scaffoldins have been used to control the composition and architecture of cellulosomes and reduce the size of these complexes so as to diminish burden related to their heterologous expression.\[^{12,13}\]

Previously, we have expressed two non-complexed glycoside hydrolases, that is, a β-glucosidase (BglA) and an endoglucanase (EngD), from Clostridium cellulovorans in Lactococcus lactis enabling L. lactis to directly ferment celldextrins up to 8 glucose units to LA with high efficiency.\[^{14}\] Use of C. cellulovorans as a source of cellulolytic enzymes for expression in L. lactis was chosen owing to: i) similar GC content (31.2% in C. cellulovorans vs. 35.3% in L. lactis), which suggests similar codon usage;\[^{15,16}\] similar growth temperatures (37°C for C. cellulovorans vs. 30°C for L. lactis). Both these characteristics may facilitate expression and activity of C. cellulovorans proteins in L. lactis with respect to proteins derived from other established cellulolytic models such as Thermobifida fusca or Clostridium thermocellum which show higher GC content and/or higher growth temperature.\[^{12,17}\] Actually, both C. cellulovorans BglA and EngD were biosynthesized and efficiently secreted using their original signal peptides; thus, suggesting that genes from C. cellulovorans could be expressed in L. lactis without any prior modification/optimization.\[^{14}\]

The cellulolytic system of C. cellulovorans mainly consists of a cellulosome which synergistically collaborates with non-complexed enzymes.\[^{16,18}\] The present study was focused on the expression of components of the C. cellulovorans cellulosome in L. lactis and, in particular, on scaffolding proteins (also called scaffoldins) and proteins involved in anchoring the cellulosome to the cell surface. An additional advantage of using C. cellulovorans as a source of plant-biomass depolymerizing enzymes is that this bacterium can ferment all the main plant polysaccharides (namely cellulose, hemicelluloses and pectins)\[^{19,20}\] while other cellulolytic microorganisms have more restrained substrate range (e.g., C. thermocellum can metabolize cellulose only\[^{21}\] and Clostridium cellulolyticum cannot directly use pectin\[^{19}\]). Since cellulosome assembly through interaction between scaffoldins and enzyme subunits is generally species-specific,\[^{22}\] use of C. cellulovorans as cellulosome source can provide a larger cellulosomal enzyme panel, without the need of extensive protein engineering. Scaffoldins are pivotal elements of the cellulosome architecture owing to the multiple functions they provide.\[^{11}\] Primary scaffoldins are usually able to bind enzyme subunits through multiple cohesin (Coh) domains.\[^{123}\] Additional domains may enable scaffoldins to bind to the substrate (through carbohydrate binding modules, i.e., CBM) and/or anchoring the microbial surface through covalent or non-covalent linkages.\[^{11}\] Cellulosomes may contain from one to several scaffoldins contributing to these different functions. Although most cellulosomes described so far are tethered to the cell surface, cell-free scaffoldins and/or cellulosomes have been observed in C. thermocellum, Clostridium clariflavum and Acetivibrio cellulolyticus.\[^{11}\] The main scaffoldin of C. cellulovorans cellulosome is CbpA which consists of a family 3 CBM (that can bind crystalline cellulose and chitin), nine Coh domains and four hydrophilic domains (Figure 1A).\[^{24}\] The latter belong to the pfam PF03442 (pfam.xfam.org) of carbohydrate binding domains X2 (X2). Tandem associated CBM 3 and X2 domains have been found to promote the hydrolysis of insoluble polysaccharides.\[^{25}\] X2 modules have been described to bind to different polysaccharides, such as cellulose and chitin,\[^{18,19}\] and those found in bacterial cell walls,\[^{25}\] but also to possibly stabilize the structure of the neighboring Coh domains.\[^{26}\] More in detail, the binding affinity of CbpA X2 domains for C. cellulovorans cell wall fragments is lower than that for cellulose or chitin.\[^{24}\] Surface-display of C. cellulovorans cellulosomes is probably mediated by multiple proteins. Another main player is the endoglucanase E (EngE) (Figure 1A).\[^{27}\] N-terminal sequence of EngE comprises three tandem repeated S-layer homology (SLH) domains showing high homology with the S-layer protein RsaA from Caulobacter crescentus.\[^{27,28}\] Moreover, the small scaffoldins CbpB, CbpC and HbpA and the endoglucanase G may further help anchoring the cellulosome to the C. cellulovorans surface.\[^{29-31}\] However, the main role of CbpA and EngE in C. cellulovorans cellulosome structure and function is supported by the fact that they are among the most abundant components of this complex.\[^{32}\] As for other SLH domain-containing proteins, experimental evidence has been brought that EngE does not bind peptidoglycan but secondary cell wall polysaccharides (SCWPs).\[^{24,27,33}\] The mechanism of binding of CbpA X2 modules is different, but it is not known yet.\[^{24}\]

In the present study, different scaffoldins have been engineered by using domains issued from CbpA and EngE of C. cellulovorans, with special attention to X2 and SLH domains because of their possible involvement in cell-surface anchoring. The genes encoding these recombinant proteins were expressed in L. lactis IL1403 so as to test the ability of their protein products to be secreted and displayed at the cell surface. A common general structure of the cell wall is found in Gram-positive bacteria such as Clostridia and LAB (recently, also renamed as parietal monoderm bacteria).\[^{33,34}\] It consists of a thick layer of peptidoglycan decorated with other polysaccharides and glycopolymers (e.g., teichoic and lipoteichoic acids) and proteins. However, specific chemical modification and/or composition and/or decoration of
FIGURE 1  Schematic representation of the tertiary structure of: A) the cellulosomal main scaffoldin CbpA and endoglucanase E (EngE) from C. cellulovorans; B) the recombinant scaffoldins obtained in this study by assembling protein domains derived from C. cellulovorans CbpA and EngE. Carbohydrate binding modules (CBM) mediate linkage with cellulose or other polysaccharides. The function of X2 domains is still not clear and may include binding with different polysaccharides, stabilizing the structure of neighboring cohesin (Coh) domains and promoting hydrolysis of crystalline cellulose. S-layer homology (SLH) domains enable binding with bacterial cell wall polysaccharides. Coh domains mediate binding with enzymes or other protein components carrying a dockerin (Doc) domain. EngE is equipped with a catalytic activity supported by its family 5 glycosyl hydrolase (GH5) domain and a Doc domain.

cell-wall components characterizes each bacterial strain. Lactis IL1403 seems to have a simpler cell wall structure than other L. lactis strains since it includes only one type of SCWP that consists of a linear backbone of rhamnose irregularly substituted with a trisaccharide carrying glycerophosphate groups. Additionally, the structure of a poly (glycerolphosphate) teichoic acid of L. lactis IL1403 was recently determined. These characteristics significant affect the physiology of L. lactis, such as the electric charge distribution on cell surface which influences surface protein binding.

In this study, we were able to introduce three recombinant scaffoldins with different combinations of X2/SLH domains in L. lactis. Two of them were secreted in the extracellular medium, while the third one was displayed at L. lactis surface showing that cell-surface binding domains of C. cellulovorans are also able to recognize structural motifs on L. lactis cell wall.

2 | EXPERIMENTAL SECTION

2.1 | Bacterial strains and media

The bacterial strains used in this study are listed in Table S1. Growth media and culture conditions were previously described. Plasmids and primers used in this study are listed in Tables S1 and S2, respectively.

2.2 | Recombinant scaffoldin gene construction, cloning and transformation of L. lactis IL1403

Enzymes and protocols for DNA amplification, digestion, ligation, purification and transformation were previously described. Plasmids and primers used in this study are listed in Tables S1 and S2, respectively.

DNA fragments of interest were amplified from genomic DNA (gDNA) isolated from overnight cultures of C. cellulovorans as previously described. mini-cbpA (miniC), encoding the N-terminal fraction of CbpA (consisting of its CBM, X2, and Coh1, 2 domains, Figure 1B) was amplified by using the cbpA-D mini-cbpA-R primer pair (Table S2). A slightly modified version of mini-cbpA encoding a miniC with a 6 His tag at its C-terminus (miniCH) was amplified by means of the cbpA-D/mini-cbpA-His-R primer pair (Table S2). r-cbpA24 (C) and r-cbpA5547 bp

A) CBM X2 Coh1 Coh2 Coh3 Coh4 Coh5 Coh6 Coh7 Coh8 X2 X2 Coh9

B) Construction Short name Gene length (bp)

mini-cbpA(H) miniC(H) 1734

r-cbpA SLH4 C 2988

r-cbpA SLHE CE 3564

Signal peptide Carbohydrate binding module Carbohydrate binding module X2 S-layer homology domain Cohesion domain Catalytic domain (glycosyl hydrolase family 5) Dockerin domain

otherwise stated, recombinant Escherichia coli harboring pMG36eaΔ-based vectors were grown in LB medium supplemented with 100 μg mL⁻¹ ampicillin. Recombinant L. lactis strains harboring pMG36eaΔ-based vectors were grown at 30°C in GM17 medium plus 5 μg mL⁻¹ erythromycin without shaking.
fragment of cbpA (encoding CBM, X21, Coh1, Coh2 and X22 domains) was amplified with the cbpA-D/linkerX23-R primer pair (Table S2); thus, generating the portion encoding the N-terminal part of C/CE; ii) the fragment encoding the C-terminal fragment of C (consisting of X23, X24 and Coh9 domains of CbpA) was amplified by using the linkerX23-D/cbpA-R primer pair (Table S2); iii) the fragment encoding the C-terminal fraction of CE (comprising SLH1, SLH2 and SLH3 domains of EngE) was amplified by using the linker EngE-D/EngE-R primer pair (Table S2). Fragments encoding N- and C-terminal portions of C and CE, respectively, were assembled through fusion PCR. For assembling C, cbpA-D/cbpA-R primer pair was used while cbpA-D/EngE-R primer pair was used for CE (Table S2). miniC(H), C and CE were cloned in the pMG36ea linkerX23-D/cbpA-R primer pair (Table S2); iii) the fragment encoding the C-terminal fragment of CE (consisting of X23, X24 and Coh9 domains of CbpA) was amplified by using the linker X23-D/cbpA-R primer pair (Table S2). ii) the fragment encoding the C-terminal fragment of C (consisting of X23, X24 and Coh9 domains of CbpA) was amplified by using the linkerX23-D/cbpA-R primer pair (Table S2); iv) the fragment encoding the C-terminal fragment of CE (consisting of X23, X24 and Coh9 domains of CbpA) was amplified by using the linkerX23-D/cbpA-R primer pair (Table S2).

2.3 Production of anti-scaffoldin specific antibodies

MiniCH was purified from culture supernatants of L. lactis miniCH. Bacterial cells were grown overnight in 2 L of GM17 medium. Biomass and culture broth were separated by centrifugation (3005 × g, 25 min, 4°C). Proteins in acellular supernatant were precipitated by adding (NH4)2SO4 until 80% saturation and stirring overnight at 4°C. Precipitated proteins were recovered through ultracentrifugation (53,792 × g, 30 min, 4°C), re-suspended in 50 mM potassium phosphate buffer pH 6 and concentrated by Vivaspin 20 ultrafiltration devices (Sartotius Steffen, Goettingen, Germany) with 30 kDa cut off polyethersulfone (PES) membrane. MiniCH scaffoldin was purified through Immobilized Metal Affinity Chromatography by using Chelating Sepharose Fast Flow (GE Healthcare Life Science) with immobilized Ni, according to manufacturer’s instructions. Elution of the mini-scaffoldin was performed by using 300 mM imidazole in 50 mM sodium phosphate buffer pH 7. The purified protein (1.5 mg) was sent to Eurogentec (Seraing, Belgium) for production of polyclonal anti-miniCH specific antibodies in rabbit.

2.4 Protein quantification

Protein concentration was determined by the 2-D Quant Kit (GE Healthcare Life Science, Chicago, IL) and/or the DC Protein Assay Bradford protein assay (Biorad), using Bovin Serum Albumin (BSA) as the standard.

2.5 Cellulose binding assay

Cellulose Binding Assay was performed on extracellular fraction of L. lactis cultures as previously described[14] with slight modifications. Recombinant L. lactis cells were grown in 50 mL of GM17 until mid (OD600 nm = 2) exponential phase. Culture broths were separated from biomass by centrifugation (3005 × g, 20 min, 4°C), syringe filtered (0.45 μm cut off) and incubated with 100 mg of crystalline cellulose (Sigmacell, Sigma-Aldrich) for 1 h at 25°C. After centrifugation (3005 × g, 10 min, 4°C), pellets were washed twice with ice-cold 50 mM potassium phosphate buffer pH 6 and re-suspended in 100 μL of SDS-PAGE loading buffer.[14] Samples were then boiled and centrifuged to remove cellulose and supernatants were analyzed by SDS-PAGE and gels were stained with Coomassie Brilliant Blue as previously described.[14]

2.6 Detection of scaffoldins displayed on the L. lactis cell surface by immunofluorescence

Scaffoldin adhesion to cell surface was analyzed through immunofluorescence microscopy. Recombinant L. lactis cells were grown until the middle exponential phase (OD600 nm = 2) while C. cellulovorans was grown in CCM medium[14] plus 0.5% cellulose until OD600 nm = 0.7. Cells were harvested, washed twice with phosphate-buffer saline (PBS) (8 g L−1 NaCl, 0.2 g L−1 KCl, 1.44 g L−1 Na2HPO4, 0.24 g L−1 KH2PO4, pH 7.4) (3005 × g, 10 min, 4°C) and re-suspended in 800 μL PBS + 200 μL Fixing Buffer (12% formaldehyde, 150 mM Na3HPO4). Samples were incubated 15 min at room temperature with mild agitation and then 1 h in ice. After recovery by centrifugation (3005 × g, 5′ min, 4°C), cells were washed three times with cold PBS and re-suspended in a volume of GTE buffer (25 mM Tris-HCl pH 8, 10 mM EDTA, 50 mM glucose) so that the OD600nm of this suspension was around 1. 50 μL of this suspension was placed on polylysine-coated microscope slides and dried. Slides were washed with cold PBS and blocked by incubation with 2% BSA dissolved in PBS at 37°C for 15 min in pre-warmed moist chamber. After further washing with PBS, anti-miniCH antibody (1:3000 dilution) was added onto the slides to detect scaffoldins on the cell surface. Samples were incubated 1 h at 37°C in moist chamber. After washing with PBS, goat anti-rabbit IgG antibody conjugated to Atto 488 Dye (5 μg mL−1, Sigma-Aldrich) was added and incubated 1 h at 37°C in moist chamber. Slides were then washed again with PBS and DNA was stained with 3 μg mL−1 Propidium Iodide (15 min, 37°C, in moist chamber). After one last washing with PBS, samples were dried and covered with DABCO mix mounting medium (19.5 mM glycerol, 24 mL H2O, 9.6 g polyvinyl alcohol, 2.5 g 1,4-diazabicyclo[2.2.2]octane (DABCO), 48 mL Tris-HCl 0.2 M pH 8.5) and a coverslip. Fluorescence images were taken using an Olympus Fluoview 200 laser scanning confocal system (Olympus America Inc., Melville, NY, USA) mounted on an inverted IX70 Olympus microscope, equipped with 60x Uplan Fl (NA 1.25) oil-immersion objective. The antibody conjugated to Atto 488 Dye and Propidium Iodide dyes were excited with a
Ar/Kr laser at 488 and at 568 nm, respectively. Images acquired at 60x magnification were processed and analyzed with ImageJ software (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MA).

2.7 Detection of bacterial cell adhesion to cellulose membrane

In order to evaluate the binding of recombinant *L. lactis* strain to cellulose mediated by heterologous scaffoldins, cells were incubated with a cellulose membrane and detected with fluorescence microscope or counted in a Burker chamber. Additionally, the same analysis was performed on *E. coli* cells since they should not be able to bind to cellulose.

Recombinant *L. lactis* and *E. coli* TOP10 cultures were grown until the middle exponential phase OD$_{600nm}$ = 2. Cells were separated from culture broth through centrifugation (3005 × g, 10 min, 4°C) and washed twice with cold PBS. Pellet was re-suspended in a volume of 2% BSA in PBS so that the OD$_{600nm}$ = 20. In the meanwhile, a square slice (around 1.44 cm²) of cellulose dialysis membrane (cut off 30 KDa, Sigma-Aldrich) was leaned on glass slides, coated with 2% BSA in PBS and incubated 15 min at 30°C in pre-warmed moist chamber. After incubation, membrane was washed with cold PBS. Thirty microliter of the cell culture broth through centrifugation (3005 g) was dropped onto BSA-treated membrane and incubated 1 h at 30°C in moist chamber. In order to remove not attached bacteria, membrane was washed four times with PBS. Membrane-attached cells were then detected by fluorescence microscopy or cell counting.

For fluorescence microscopy observation, 0.2 µg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) was added onto the membrane and incubated 15 min at 30°C in moist chamber to stain DNA. After washing with PBS, membrane was covered with DABCO mix mounting medium and a coverslide. Cells were detected with an Olympus IX50 fluorescence microscope.

To quantify the amount of cells attached to cellulose membrane, membrane was stained for 1 min with Gram’s crystal violet solution (Sigma-Aldrich), further washed with PBS and placed into a Burker chamber. Cells in the 0.0025 mm² squares were counted through a Wild Leitz GMBH microscope (40x magnification). Three independent determinations (i.e., biological replicates) were performed for each *L. lactis* strain. For each biological replicate, seven squares were analyzed.

2.8 Statistical analyses

Data were analyzed by means of the Student’s t-test and considered as significantly different when $p < 0.05$.

2.9 Protein sequence analysis

Protein sequence alignments were performed with Geneious version 8.1 (Biomatters) (http://www.geneious.com). Search for SLH conserved motif was performed through the dedicated PROSITE tool (https://prosite.expasy.org). Blast search was performed at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Protein sequence logo was generated through WebLogo3 software (http://weblogo.threeplusone.com/).

3 RESULTS

3.1 Construction of recombinant scaffolding proteins

Three recombinant scaffoldins were constructed in this study by using protein domains of CbpA and EngE from *C. cellulovorans* as building blocks (Figure 1B). Each of these recombinant scaffoldins contains the 578 aa N-terminal portion of CbpA comprising its: original signal peptide, carbohydrate binding module (CBM), first X2 domain (X21) and first two cohesin domains (Coh1, 2). This corresponds to the whole structure of miniC scaffoldin (Figure 1B). A second version of miniC was also constructed which contains a 6 histidine-tag at its C-terminus (miniCH). C and CE scaffoldins were constructed by fusing miniC, respectively, with: X22,3,4 and Coh9 of CbpA (C); the X22 of CbpA and the three SLH domains of EngE (SLH1,2,3) (CE) (Figure 1B). Scaffoldin C, therefore, contains all the X2 domains of CbpA. Additionally, Coh9 was included in the design of this recombinant scaffoldin since previous studies had established its high binding affinity for dockerin domains of key cellulosomal enzymes of *C. cellulovorans*.[26] Both X2 domains of CbpA and SLH domains of EngE were reported to bind C. *cellulovorans* cell wall fragments.[24,27] MiniC, C and CE recombinant scaffolding proteins contain 1, 4 and 5 domains potentially anchoring bacterial cell wall through non-covalent binding, respectively (Figure 1B).

The genes encoding miniC(H), C and CE were constructed, cloned in the pMG36eαE. *coli*-L. *lactis* vector.[14] and finally transformed in *L. lactis* IL1403; thus, obtaining *L. lactis* miniC(H), C and CE, respectively.

3.2 Growth parameters of recombinant *L. lactis* strains

Final biomass (OD$_{600nm}$) and specific growth rate ($\mu$) of *L. lactis* miniC(H), C and CE were determined (Figure 2). Growth parameters of recombinant strains were the same as the parent *L. lactis* strain (pMG36eαA), except for *L. lactis* C which showed significant ($p < 0.05$) reduction of growth rate (32%) and final biomass (6%).

3.3 Recombinant scaffoldin secretion by *L. lactis*

*L. lactis* miniC, C and CE were grown in GM17 medium and harvested in the middle exponential phase. The presence of recombinant scaffoldins in the extracellular medium was analyzed by using the cellulose binding assay.[14] Actually, each scaffoldin engineered in this study contains a CBM at its N-terminus with high affinity for cellulose; thus, enabling their selective precipitation by incubation with crystalline cellulose. The extracellular fraction of *L. lactis* harboring the empty pMG36eαA
FIGURE 2 Specific growth rate (A) and final biomass (OD$_{600nm}$) (B) and of L. lactis strains expressing engineered scaffoldins obtained in this study. Three independent replicates were performed for each L. lactis strain. Symbol * indicates data that significantly ($p < 0.05$) differ from those measured in the parent (L. lactis pMG36eaΔ) strain.

FIGURE 3 Secretion of recombinant scaffoldins by L. lactis. The extracellular fraction of L. lactis harboring the empty pMG36eaΔ vector (pMG36eaΔ) and L. lactis miniC, C, and CE, harboring the plasmids encoding the recombinant scaffoldins, was analyzed by cellulose binding assay, followed by SDS-PAGE. A band corresponding to the molecular mass of scaffoldins miniC (61 kDa) and C (104 kDa) was present in the extracellular medium of L. lactis miniC and C cultures, respectively. A faint band corresponding to the M$_R$ of scaffoldin CE is likely present in the extracellular extract of L. lactis CE. Additional bands with lower M$_R$ are also present, which likely correspond to products of partial hydrolysis of the engineered scaffoldins.

3.4 Analysis of surface-displayed scaffoldins in L. lactis

The presence of miniscaffoldins on the surface of recombinant L. lactis was analyzed by two different approaches: i) immunofluorescence on whole recombinant L. lactis cells; ii) ability of recombinant L. lactis to adhere to cellulose membranes. The first methodology took advantage from the fact that specific anti-scaffoldin antibodies were obtained in this study. The second approach exploited the presence of a CBM in each recombinant scaffoldin engineered in this study. In parallel, the same analyses were performed on L. lactis harboring the empty pMG36eaΔ vector, as the negative control.

3.4.1 Immunofluorescence assays

Bacterial cells were fixed onto glass slides and incubated with primary (anti-scaffoldin) antibodies and fluorophore-labeled secondary antibodies. Propidium iodide was used to localize cells. This analysis was performed on L. lactis miniC(H), C and CE but also on L. lactis pM36eaΔ and on C. cellulovorans cells which served as negative and positive control, respectively (Figure 4). These analyses showed that scaffoldins are displayed on the surface of C. cellulovorans and L. lactis CE, while no
Figure 4 Detection of scaffoldins displayed at the surface of L. lactis and C. cellulovorans cells by immunofluorescence. Cells were stained with propidium iodide (PI) to detect DNA and localize cells and anti-scaffoldin (scaffoldin) antibodies. The merged images highlight the presence of scaffoldins on the surface of C. cellulovorans and L. lactis CE. Scale bars correspond to a length of 20 μm.

3.4.2 Ability of recombinant L. lactis to adhere to cellulose

Fluorescence was detected on the surface of L. lactis pM36eaΔ, miniC(H) or C (Figure 4).

The CBM of CbpA from C. cellulovorans is present in each mini-scaffoldin engineered in this study. L. lactis cells displaying these scaffoldins at their surface should therefore be able to adhere to cellulose. In order to test this ability, L. lactis pM36eaΔ, miniC(H), C and CE were incubated with a transparent cellulose dialysis membrane and cells adhering to the membrane were visualized by DAPI. An additional control, that is, E. coli TOP10, was tested as further negative control since this strain should not be able to bind to cellulose. No E. coli (data not shown) and very few L. lactis pM36eaΔ and C cells remained attached to the cellulose membrane, while this number was higher for L. lactis CE (Figure 5A–C). Cellulose membranes incubated with recombinant L. lactis cells were laid in a Burker chamber and the attached cells were counted. A higher number of L. lactis CE cells (8537 ± 2074 cells mm⁻²) was able to adhere to the cellulose membrane with respect to L. lactis pM36eaΔ (1807 ± 1103 cells mm⁻²) (p value = 4.61 x 10⁻¹⁴). According to these results, scaffoldin CE is displayed on the surface of L. lactis CE.
Cloning and expression of synthetic scaffoldins is an essential pre-requisite for introducing designer cellulosomes in heterologous microorganisms and has been performed in a number of microbial models\cite{37,38} including different LAB.\cite{12,17} Artificial scaffoldins allow precise control of designer cellulosome composition and architecture by mediating assembly of a minimal number of essential enzyme subunits; thus, reducing protein burden during heterologous expression.\cite{12,13} In the present study, three engineered scaffoldins, named miniC, C and CE, were constructed by using protein domains derived from two of the main components of the \textit{C. cellulovorans} cellulosome (i.e., the main scaffoldin CbpA and the endoglucanase EngE) and expressed in \textit{L. lactis}. More in detail, these scaffoldins consist of: one CBM; 2 (miniC and CE) or 3 (C) Coh domains for anchoring enzyme sub-units; 1 (miniC), 4 (C) or 5 (CE) domains possibly involved in anchoring the microbial cell surface. Our analyses clearly showed that all these proteins are biosynthesized and secreted by \textit{L. lactis} (Figure 3). In addition, CE scaffoldin was able to bind to the \textit{L. lactis} surface (Figures 4 and 5). Successful heterologous expression of miniC has previously been reported in \textit{Bacillus subtilis}.\cite{39} In the latter study, the original signal peptide was replaced with \textit{sacB} signal sequence for efficient protein secretion in \textit{B. subtilis} while in the present study the original signal peptide of CbpA from \textit{C. cellulovorans} was kept. This indicates that signal peptides from \textit{C. cellulovorans} can be recognized by \textit{L. lactis} secretion system; thus, confirming our previous results on the expression of \textit{C. cellulovorans} BglA and EngD in \textit{L. lactis}.\cite{14} More in general, the present results validate the initial assumption of this study, namely that \textit{C. cellulovorans} genes can be expressed in \textit{L. lactis} with few modifications, based on similar GC content and growth temperature of these bacteria. In the present study, scaffoldins including up to three Coh domains (i.e., that could potentially anchor up to three enzyme subunits) were transformed in \textit{L. lactis}, which are among the largest scaffoldins introduced in LAB, so far. Scaffoldins supporting assembly of more sophisticated cellulosomes were engineered in \textit{Lactobacillus plantarum} only.\cite{12} In particular, the latter study took advantage of sharing cell-surface binding and enzyme binding functions among different scaffoldin molecules; thus, reducing protein burden in host cells. It is worth noting that functionality of Coh domains included in the synthetic scaffoldins engineered in the present study was not tested here. However, the main focus of the present study was on protein domains related to cell-surface binding.

A number of surface-anchoring domains has previously been used to display heterologous proteins on the cell surface of LAB through covalent (i.e., sortase-mediated) or non-covalent (e.g., through LysM modules) binding.\cite{40–42} Examples of covalent binding of scaffoldins or cellulase system components to the LAB cell surface have also been reported.\cite{12,17} In these studies, heterologous proteins have generally been engineered with established surface-display motifs suitable for specific LAB host. In the present study, an original approach was used to develop anchoring scaffoldins for \textit{L. lactis} that used protein domains which are thought to mediate cell-surface anchoring of cellulosomes in \textit{C. cellulovorans}. More in detail, the four X2 domains of CbpA and the three SLH domains of EngE were included in the structure of the synthetic scaffoldins engineered in this study. The three scaffoldins engineered in this study differ for both the type and number of putative cell-surface anchoring domains. All the scaffoldins containing X2 domains derived from CbpA only, that is, miniC(H) and C, were not able to bind to the \textit{L. lactis} surface (Figures 4 and 5). The only scaffoldin able to anchor the \textit{L. lactis} surface was CE, that included the three SLH domains of EngE (Figures 4 and 5). According to the structure of CE scaffoldin, it could not be excluded that its surface-binding ability derives from the combination of CbpA X2 and EngE SLH domains. Nevertheless, these results indicate at least that EngE-derived SLH domains can bind to \textit{L. lactis} surface stronger than CbpA X2 domains, which is coherent with previous observations made on \textit{C. cellulovorans}.\cite{24} More in detail, recombinant scaffoldins including only CbpA X2\textsubscript{3-4} domains (Figure 1) showed 5.5-fold higher \textit{K}_d and 2-fold lower binding capacity than EngE for \textit{C. cellulovorans} cell wall.\cite{24} Consistently, scaffoldin CE (including CbpA X2\textsubscript{2-4} and EngE SLH domains) was displayed on the \textit{L. lactis} cells while scaffoldin C (including CbpA X2\textsubscript{2-4} domains) was not. These observations suggest that cell wall composition
of L. lactis and C. cellulovorans may have some similarity. Previous studies on C. cellulovorans also indicated that the higher the number of X2 domains in a protein the greater its binding affinity for the cell wall.[124] However, this did not have any major effect on L. lactis cell wall binding, since the degree of display of scaffoldins miniC(H) (one X2 domain) and C (four X2 domains) on L. lactis surface did not show any significant difference (Figures 4 and 5). Apart from binding affinity, it is known that the binding target(s) of CbpA X2 and EngE SLH domains on the cell wall of C. cellulovorans are different, since removal of SCWPs prevents EngE from binding to cell wall fragments while this does not influence CbpA X2 binding affinity.[24,27] Binding targets of CbpA X2 domains seem therefore absent or masked on the L. lactis surface.

A survey of the literature concerning S-layer proteins and SLH domains, indicates that there is still unclear and confusing understanding of their structure, their binding to target molecules in the bacterial cell wall and classification.[43] It has been speculated that SLH domains may have low sequence conservation between them because SLH binding sites in different bacterial species can significantly differ owing to the different composition of surface layer structure.[24,27,44] It has been shown that the C-terminal region of the S-layer protein S1pB (LcsB) of Lactobacillus crispatus K2-4-3 can also bind (although with different efficiency) to the cell wall of several other LAB (including different lacticobacilli, L. lactis and Streptococcus thermophilus) but not that of Lactobacillus casei.[45] High amino acid sequence conservation characterizes the SLH domains of EngE from C. cellulovorans (more than 63% identity)[28] but their structure is not currently available. Recently, the crystal structure of the SLH domain of S-layer proteins Sap from Bacillus anthracis[46] and SpaA from Paenibacillus alvei CCM 2015T[47] has been determined; thus, allowing to identify the amino acid residues which are essential for binding the cell surface. In particular, a conserved TRAE motif (SLH domain residues 42–45) is thought to mediate SLH domain binding with the negatively charged pyruvate ketal commonly found in SCWPs. An analysis of the EngE-derived domain sequence through the dedicated PROSITE tool (https://prosite.expasy.org) could not detect the presence of the SLH domain pattern (PDOC00823). The mechanisms enabling EngE SLH domains to bind to the bacterial cell wall remains therefore elusive. SLH domains from EngE were aligned and the consensus sequence was submitted to BLAST search for homologous sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). EngE SLH domains show high sequence identity with domains belonging to 11 clostridial glycosyl hydrolases and one from Herbinix luporum (Figure S1). These domains could possibly constitute a subgroup or another group of cell-associated protein domains, with a mechanism of binding that significantly differs from the most established SLH domains and which deserves further investigations.

In conclusion, this study led to construction of three synthetic scaffoldins with biotechnological relevance since they could potentially be used for future assembly of designer cellulosomes in L. lactis. MiniC and C could mediate assembly of soluble secreted mini-cellulosomes while CE could support display of designer cellulosomes on the surface of L. lactis. This a remarkable progress in the field of RCS applied to LAB, which is relatively underdeveloped as compared to the number of studies on other microbial models.[7] As far as we know, recombinant scaffoldins able to bind the surface of LAB have previously been developed only by the research group coordinated by Professor Martin in Canada [17] and that directed by Professors Mizrahi and Bayer in Israel.[41] With respect to these studies, the present investigation represents a further step towards industrial application of recombinant cellulolytic LAB, since constitutive expression of scaffoldins (i.e., without the need of expensive inducers) was demonstrated in L. lactis. However, application of scaffoldins obtained by the present study requires further analyses on their Coh domains to confirm their functionality. So far, all our attempts to introduce C. cellulovorans cellulosomal cellulases (i.e., the exoglucanase ExgS and the endoglucanases EngE, EngH and EngZ) in L. lactis (and confirm their ability to form complexes with the synthetic scaffoldins obtained in this study) were unsuccessful (data not shown). An ex vivo approach (i.e., by mixing scaffoldin-displaying L. lactis and C. cellulovorans cellulosomal subunits (i.e., the exoglucanase ExgS and the endoglucanases EngE, EngH and EngZ) in L. lactis (and confirm their ability to form complexes with the synthetic scaffoldins obtained in this study) were unsuccessful (data not shown).

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Loredana Tarraran and Chiara Gandini performed construction of genes for synthetic scaffoldins, Loredana Tarraran transformed these genes in Lactococcus lactis and performed most phenotypic characterization of engineered L. lactis strains. Anna Lugarini contributed to immunofluorescence detection of synthetic scaffoldins. Roberto
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