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Clostridium cellulolyticum: model organism of mesophilic cellulolytic clostridia

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Abstract

Clostridium cellulolyticum ATCC 35319 is a non-ruminal mesophilic cellulolytic bacterium originally isolated from decayed grass. As with most truly cellulolytic clostridia, *C. cellulolyticum* possesses an extracellular multi-enzymatic complex, the cellulosome. The catalytic components of the cellulosome release soluble cello-oligosaccharides from cellulose providing the primary carbon substrates to support bacterial growth. As most cellulolytic bacteria, *C. cellulolyticum* was initially characterised by limited carbon consumption and subsequent limited growth in comparison to other saccharolytic clostridia. The first metabolic studies performed in batch cultures suggested nutrient(s) limitation and/or by-product(s) inhibition as the reasons for this limited growth. In most recent investigations using chemostat cultures, metabolic flux analysis suggests a self-intoxication of bacterial metabolism resulting from an inefficiently regulated carbon flow. The investigation of *C. cellulolyticum* physiology with cellobiose, as a model of soluble cellodextrin, and with pure cellulose, as a carbon source more closely related to lignocellulosic compounds, strengthen the idea of a bacterium particularly well adapted, and even restricted, to a cellulolytic lifestyle. The metabolic flux analysis from continuous cultures revealed that (i) in comparison to cellobiose, the cellulose hydrolysis by the cellulosome introduces an extra regulation of entering carbon flow resulting in globally lower metabolic fluxes on cellulose than on cellobiose, (ii) the glucose 1-phosphate/glucose 6-phosphate branch point controls the carbon flow directed towards glycolysis and dissipates carbon excess towards the formation of cellodextrins, glycogen and exopolysaccharides, (iii) the pyruvate/acetyl-CoA metabolic node is essential to the regulation of electronic and energetic fluxes. This in-depth analysis of *C. cellulolyticum* metabolism has permitted the first attempt to engineer metabolically a cellulolytic microorganism.

Keywords: Bacterial metabolism; Cellulose degradation; Cellulosome; Metabolic flux analysis; Cellulolytic clostridia; Metabolic engineering

Contents

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1. Introduction

Cellulose is a linear insoluble biopolymer composed of the repeated union of β-D-glucopyranose linked by β-1,4 glycosidic bonds (Fig. 1(a)). Consequently, and in contrast to other glucan polymers such as starch or callose, the repeating structural unit in cellulose is not glucose but the disaccharide cellobiose. With a degree of polymerisation (DP) ranging from 2 to 7, the β-1,4 glucose oligomers, also called cellodextrins or cello- oligosaccharides, are water soluble [1]. In cellulose, the glucan chain can reach a length of more than 25,000 glucose residues [2]. The association of cellulose macromolecules leads to the formation of a microfibril containing 15–45 chains in a regular crystalline arrangement (Fig. 1(b)). At the microscopic scale, the association of these microfibrils formed a cellulose fibril also called macrofibril or fibre at the macroscopic scale [3]. Native cellulose is paracrystalline since within the microfibril alternates amorphous and crystalline regions. Moreover, cellulose fibres contain various types of irregularities such as twists or voids, which increase their total surface area.

Despite its low density, cellulose is the most prominent, resistant and stable natural-organic compound known and, as a consequence, it tends to accumulate in the environment [4]. According to the most recent estimation, the net primary production of biomass in terrestrial ecosystems would be of 60 milliard tonnes of carbon per year and about half of this carbon would be fixed under the form of cellulose [5]. It is generally assumed that cellulose is synthesised by plants [6,7] but this polymer can also be produced by some animals, algae and bacteria [8,9]. In plant, cellulose is generally associated with other biopolymers, i.e. hemicelluloses, pectins, proteins and lignin, and it then designated lignocellulose [2,10]. Depending on the plant, tissue and stage of development considered, the structural organisation and proportion of the different polymers in the lignocellulose are highly variable; in grass for example, cellulose,

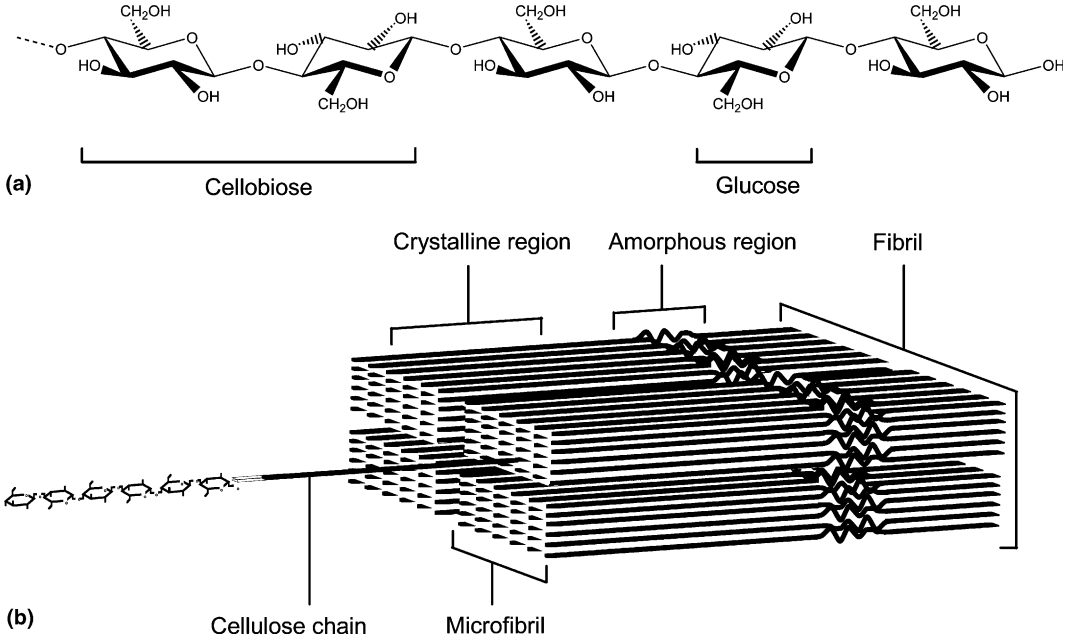

Fig. 1. (a) Scheme of the primary structure of cellulose. (b) Scheme of the structure of a cellulose fibril.

hemicelluloses, lignin and pectins represent 45–60%, 20–40%, 5–10% and 1–5% of the plant cell wall [11,12].

While it was long assumed that cellulose digestion was exclusively a microbiological process [13,14], the existence of cellulase of animal origin is now firmly established [15]. In the environment, cellulose is essentially degraded by microorganisms where the final products of the conversion are H_2O and CO_2 in aerobic conditions and also CH_4 in anaerobiosis [3,16]. Among cellulolytic microorganisms, bacteria of the class *Clostridia* occupy a place of choice. These bacteria (i) are ubiquitous in cellulosic anaerobic environments [17], (ii) digest cellulose very efficiently via an extracellular enzymatic complex called the cellulosome [8], (iii) can convert cellulose into a large variety of metabolites notably ethanol [18], and (iv) can therefore be used in direct microbial conversion process, i.e. in consolidated bioprocessing (CBP) [19]. However, the recursive interest in cellulose utilisation for bioenergetic prospects, notably for the production of H_2, CH_4 or biofuel, requires a better understanding of the physiology and metabolism of these bacteria [20–23].

Following a campaign of isolation and characterisation of mesophilic cellulolytic clostridia [24–27], *C. cellulolyticum* ATCC 35319, formerly identified as the strain H_{10} (Herbe 10 isolate), was isolated from decayed grass compost at the Université de Nancy, France, by Petitdemange et al. [28]. *C. cellulolyticum* is a bacillus, straight to slightly curved rod that is 3–6 μm long and 0.6–1 μm wide, with peritricheous flagella. Its name was coined from c*ellulosum* (Gr. n.): cellulose, and *lyticus* (Gr. adj.): dissolving. *C. cellulolyticum* has been extensively studied on both enzymatic and metabolic aspects of cellulose hydrolysis and is considered as the model of mesophilic cellulolytic clostridia. While the studies of cellulose digestion have been mainly focused on the genetic, structure, function and interaction of the cellulasic systems involved, few review articles have been dedicated primarily to cellulolysis as a microbial phenomenon [29]. After describing briefly the cellulasic system of *C. cellulolyticum*, this review will focus on the metabolisation of cellulose by this bacterium.

2. The cellulasic system of *C. cellulolyticum*

By secretion of cellulases as single enzymes, single polypeptides containing several cellulasic domains or extracellular multi-enzymatic complex, microorganisms have developed several strategies to digest cellulose [8]. The concept of cellulosome was first introduced in *C. thermocellum* [30]. Such a complex has been described in several anaerobic microorganisms including *C. cellulolyticum*.

2.1. Phylogenetic considerations

Within the 23 phyla of the domain Bacteria only seven (Thermotogae, Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes, Fibrobacteres and Bacteroidetes) contain truly cellulolytic organisms [8,31,32]. Surprisingly, no cellulolytic prokaryotes have been identified within the domain Archaea. About 80% of the isolated cellulolytic bacteria are Gram-positive and are found in only two phyla, i.e. Actinobacteria and Firmicutes. All the Gram-positive anaerobic cellulolytic bacteria are found in this latter phylum and more particularly in the class Clostridia, order Clostridiales and with a majority belonging to the family Clostridiaceae, genus *Clostridium*.

From 16S rDNA gene analyses, a new phylogenetic arrangement was proposed where this genus was divided into 19 different clusters [33,34]. Six of these clusters contain cellulolytic clostridia, i.e. the cluster I, III, IV, V, X and XIV [31]. While cluster I contains *Clostridium butyricum*, the reference species of the genus *Clostridium*, most cellulolytic species are found in cluster III. Interestingly, this cluster contains mesophilic and thermophilic bacteria. Together with *C. papyrosolvens*, *C. josui*, *C. cellobioparum*, *C. termitidis*, *C. aldrichii*, *C. thermocellum* and *C. stercorarium*, the prokaryote *C. cellulolyticum* belongs to the domain Bacteria, phylum Firmicutes, class Clostridia, order Clostridiales, family Clostridiaceae, genus *Clostridium*, cluster III [34]. In a new proposed hierarchical structure for clostridia, *C. cellulolyticum* was also placed into the family 4, genus 2 [34].

To date, cellulosomal structure has only been described in anaerobic microorganisms including some members of the fungal groups, within the domain Eukarya, and some bacteria, essentially in the order Clostridiales [35]. In this order, bacteria possessing a cellulosome are found in the families Lachnospiraceae and Clostridiaceae and in various clusters of the genus *Clostridium*. In the cluster III, together with *C. thermocellum* and *C. cellulolyticum*, experimental evidence of the presence of a cellulosome exists for the species *C. josui* and *C. papyrosolvens* [35,36]. For the other truly cellulolytic species *C. aldrichii*, *C. cellobioparum* and *C. termitidis*, consistent experimental evidence is still awaited. However, for *C. stercorarium*, neither a genetic or biochemical approach could reveal its presence [8,37].

Genetic analyses have shown that *C. cellulovorans* and *C. acetobutylicum*, which belong to cluster I of the genus *Clostridium*, possess a cellulosomal gene cluster with a synteny and sequence homology very similar to the *cel* cluster of *C. cellulolyticum* [38]. To offer a possible explanation for the non-cellulolytic activity of *C. acetobutylicum*, these genes were originally described as cryptic, i.e. by possessing frameshifts in the open reading frame (ORF) and/or disabled promoters [8].

However, more recent investigation revealed that a cellulosome was effectively expressed by *C. acetobutylicum* but was inactive [39]. Considering the phylogenetic distance between these three species, the presence of a common bacterial ancestor does not seem the most probable hypothesis for explaining the close taxonomic relatedness of cellulosomal genes. Recently, two novel insertion sequences (IS) suggested as transpositionally active, called ISCce1 and ISCce2, have been reported in *C. cellulolyticum* [40]. Southern blotting analysis with different *Clostridium* species of the ISCce2 region, revealed some DNA fragments homology with *C. cellobioparum*, *C. papyrosolvens*, *C. termitidis* and *C. cellulovorans* chromosomic DNA. No hybridisation was observed with *C. acetobutylicum* chromosomic DNA. While further investigations are required, the involvement of these or other IS elements in the spreading of the overall cellulosomal genes between quite distant clostridial species by horizontal transfer event in the course of evolution remains an attractive possibility [41].

2.2. *The cellulosome of* C. cellulolyticum

As all cellulosomes described so far, the *C. cellulolyticum* cellulosome is organised around a specialized integrating protein or scaffolding protein, called CipC, where different catalytic components bind to it. In essence, this structure is present on the bacterial cell surface and is dedicated to cellulose depolymerisation. For the bacterial cell, the biosynthesis of a cellulosome presents several advantages: (i) a direct and specific adhesion to the substrate of interest permitting efficient competition with other microorganisms present in the same ecological niche, and (ii) the proximity of the cell to cellulose insures an efficient cellular uptake of soluble cello-oligosaccharides by avoiding their diffusion in the extracellular milieu [42]. From an enzymatic point of view, the cellulosome (i) allows optimum concerted activity and synergism of the cellulases, (ii) avoids non-productive adsorption of the cellulases, (iii) avoids competition between cellulases for the sites of adsorption, and (iv) allows optimal processivity of the cellulase all along the cellulose fibre [8].

2.2.1. *Structure and ultrastructure of* C. cellulolyticum *cellulosome*

While the cellulosome of the thermophilic clostridium *C. thermocellum* is undoubtedly the most studied, the *C. cellulolyticum* cellulosome is the model for mesophilic clostridial species [43]. In *C. cellulolyticum*, most cellulosomal genes are clustered in an approximately 26 kb long DNA fragment, the *cel* cluster, in which 12 genes have been identified to date, i.e. *cipC–cel48F–cel8C–cel9G–cel9E–orfX–cel9H–cel9J–man5K–cel9M–rgl11Y–cel5N* (Fig. 2(a)) [44]. Such a genetic organization is not always the rule since in *C. thermocellum* the cellulosomal genes seem randomly distributed all over the genome [45].

The first gene of the *cel* cluster, *cipC*, encodes a protein with modular organisation consisting of eight cohesion domains of type I (Coh_I) numbered from 1 to 8 from the N- to the C-terminus, in addition to a cellulose-binding domain (CBD) domain and two X-modules, also called X2-module or hydrophilic domains (HLDs), separated by short linker sequences (Fig. 2(b)) [46]. CipC has no catalytic activity. The cohesins permit the tight binding of complementary dockerin domain of type I (Doc_I) borne by the cellulosomal cellulases. The CBD of CipC belongs to the family IIIa and allows the tight binding of the entire cellulosome to cellulosic substrate. The exact function of the X-module, which seems to be involved in the cellulose degradation, is still speculative. Crystallisation of CipC tends to be proved extremely difficult. However, each structural representatives of cellulosome constitutive modules being currently available, i.e. Coh_I [47], CBD IIIa [48] and X-module [49], a modelling of the global structure could become available in the near future.

The attachment of *C. cellulolyticum* cellulosome to the bacterial cell surface is an intriguing problem yet to be resolved. In *C. thermocellum*, CipA possesses a type II dockerin domain (Doc_{II}) interacting with a type II cohesin domain (Coh_{II}) presents in cell surface proteins anchored in the S-layer, i.e. SdbA, OlpB and Orf2p [50]. In the case of CipC, as well as for CbpA and CipA, the scaffolding proteins of *C. cellulovorans* and *C. josui* respectively, such a Coh_{II} is absent and these cellulosomes are therefore adsorbed or anchored to the cell surface by alternative mechanism(s). As first proposed for the *C. cellulovorans* cellulosome, it has been suggested that the hydrophilic X-module of CipC could be involved in (i) the solubility and structural stability of the overall scaffolding protein and/or (ii) the cellulosome adsorption on the bacterial cell surface [43]. In *C. cellulovorans* it was proposed that the cellulosomal cellulase EngE was involved in the cell-surface display of cellulosome. In fact, EngE would be anchored via its N-terminus into the cell wall layer while the C-terminus Doc_I of the protein interacts with one of the Coh_I of CbpA [51]. While a homologue to EngE has been reported in *C. acetobutylicum* [52], such a cellulosomal cellulase has not been identified yet in *C. cellulolyticum*. Another hypothesis would be the anchoring of *C. cellulolyticum* cellulosome to the bacterial cell wall by a transpeptidase sortase [53]. The protein substrates cleaved by this enzyme generally harbour a conserved LPXTG sortase-cleavage site followed by a transmembrane-spanning hydrophobic domain and by a hydrophilic charged domain at the C-terminus. In clostridia, sortases have been identified and variations of the LPXTG motif have also been reported [54]. Therefore, the implication of such a system in the anchoring of *C. cellulolyt-*

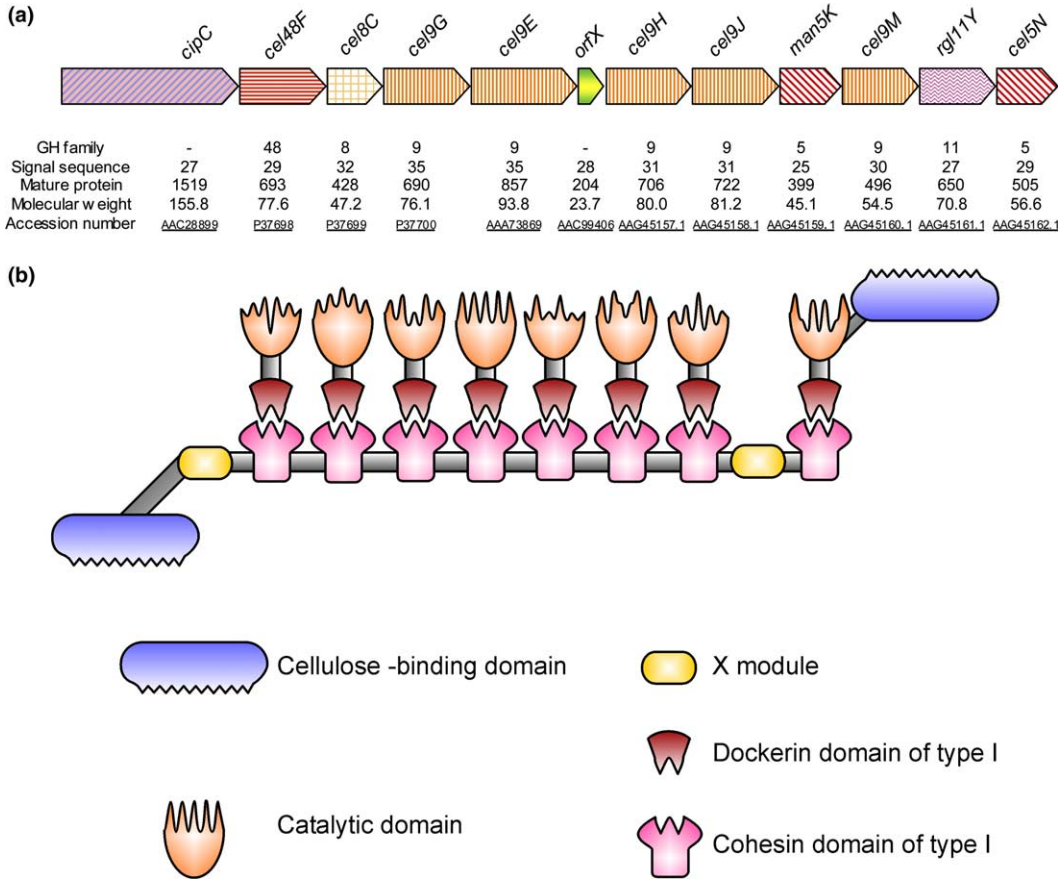

Fig. 2. (a) Scheme of the *cel* cluster of the cellulosomal genes in *C. cellulolyticum* and the corresponding encoded protein features. The signal sequence and mature size of the protein are given in number of amino acid residues. The molecular weight is expressed in kDa. (b) Scheme of the modular structure of the scaffolding protein CipC containing one cellulose-binding module, two X modules and eight cohesin modules.

icum cellulosome cannot be ruled out. At last, the attachment of cellulosome to the *C. cellulolyticum* cell surface could occur by a novel mechanism yet to be discovered. At the moment, none of these hypotheses have been experimentally confirmed for the *C. cellulolyticum* cellulosome. It is worth mentioning that in *C. cellulovorans*, several systems seem to permit the anchoring of cellulosome to the cell surface [51].

C. thermocellum possesses one of the most complex cellulosome with a size ranging from 2.0 to 6.5 MDa depending on the bacterial strains [55] and forming polycellulosomal protuberances of up to 100 MDa [42]. In *C. cellulolyticum*, cellulosome appears smaller and less complex with a stable protein structure of only 600 kDa [56] with cellulosomal aggregates of up to 16 MDa [57]. While the presence of cellulosomal protuberances on the cell surface similar to those first observed in *C. thermocellum* [58] or in *C. cellulovorans* [59] is generally assumed, no direct evidence is available; thus, the *C. cellulolyticum* cellulosome ultrastructure remains elusive. From the recent crystallisation of a cohesin–dockerin complex, it was proposed that Doc_I could be involved in the polycellulosome assembly [60].

In *C. thermocellum* cellobiose- and cellulose-grown cells exhibit polycellulosomal protuberances [50]. On the contrary, cellulosome biosynthesis appears tightly controlled in *C. cellulovorans* since few polycellulosomal structures are present on cells grown with a soluble-carbon substrate [61]. In *C. thermocellum*, it appears that the expression of cellulosomal component genes depends on the growth rate and the concentration of carbon source [62]. Under low growth conditions, the level of critical catalytic subunits and new cell surface complexes are increased in order to improve the ability of the bacterium to utilize cellulose. In *C. cellulolyticum*, information about the regulation of cellulosome under various growth conditions is not currently available. In addition to the cell-associated cellulosome, cell-free cellulosome production has also been reported in *C. cellulolyticum* [63]. In *C. thermocellum*, while the cellulosomes are intimately associated with the cell surface during the early- and mid-logarithmic growth phases, they begin to disengage from the cell during the late-exponential growth phase and are mostly detached in the stationary phase [64]. There is no experimental evidence that cell-free cellulosome makes up a major contribution of cellulolytic activity of actively growing bacteria on cellulose.

2.2.2. The catalytic components of C. cellulolyticum *cellulosome*

All the cellulases display similar catalytic mechanism; the β-1,4-glycosidic bonds of the cellulose molecule are hydrolysed by general acid catalysis [65]. According to their sequence in amino acids and fold, these various cellulases have been classified into different glycosyl hydrolase (GH) families and clans continuously updated on the carbohydrate-active enzymes web server (CAZy): http://afmb.cnrs-mrs.fr/CAZY [66]. To date, eight families (6, 8, 9, 45 and 48) and three clans, i.e. the clan GH-A (regrouping the families 1, 5, 10, 17, 26 and 39), GH-B (regrouping the families 7 and 16) and GH-C (regrouping the families 11 and 12), have been described for cellulases. In addition to their catalytic domain, some cellulases possess a CBD. CBD belongs to the greater class of carbohydrate-binding modules (CBM); as for the GH classification, the CBM classification emphasises sequence and structural homology rather than substrate specificity [67].

The cloning and overexpression approach of *C. cellulolyticum* cellulases was initiated in 1988 and mainly pursued over the years by Bélaïch's research group to characterise the cellulolytic system of this bacterium [38,68]. To date, eight cellulosomal enzymes have been biochemically characterised, including the cellulases Cc-Cel5A [69], Cc-Cel5D [70], Cc-Cel8C [71], Cc-Cel48F [72], Cc-Cel9G [73], Cc-Cel9E [74], Cc-Cel9M [75] and most recently of the rhamnogalacturonase Cc-Rgl11Y [44]. Furthermore, the three-dimensional structure of four of these enzymes was determined: Cc-Cel5A [76], Cc-Cel48F [77], Cc-Cel9G [78] and Cc-Cel9M [79]. Cc-Cel5I is the only non-cellulosomal cellulase identified so far in *C. cellulolyticum*. Cc-Cel5I is not found in the *cel* cluster but elsewhere on the chromosome of *C. cellulolyticum* [44,80]. Similarly Cc-Cel5A and Cc-Cel5D are not found in the *cel* cluster, their genes are expressed as monocistronic units, however, both are cellulosomal cellulases [69,81]. In addition to their catalytic domain Cc-Cel5D, Cc-Cel9E, Cc-Cel9H, Cc-Cel9J and Cc-Cel9G possess a CBD [38,73,74]. The remaining enzymes identified in the *cel* cluster, i.e. Cc-Cel9H, Cc-Cel9J, Cc-Man5K and Cc-Cel5N are still awaiting for an in-depth characterisation. Cc-Cel48F and Cc-Cel9E are the most abundant catalytic components of cellulosome [57].

Cc-Cel48F is regarded as a processive cellulase with an endo activity [72]. While the initial hydrolysis liberates soluble cellodextrins from cellobiose to cellohexaose with the cellotetraose as the major end product, longer incubation leads mainly to the formation of cellobiose [72]. Cc-Cel48F was also reported to exhibit an overall capacity to act in synergism with other cellulases.

Cc-Cel9E is an endocellulase retaining a certain capacity for random attack mode on cellulose [74]. Interestingly, Cc-Cel9E and Cc-Cel48F are both cellobiohydrolases which possess opposite processivity [82]. In addition to its catalytic domain, this cellulase also possesses a CBD belonging to the family IV. The deletion of this CBD induces a total loss of activity against soluble or insoluble substrates. While Cc-Cel9E alone shows a low level of activity, this enzymatic component displays surprisingly high synergistic properties when combined with other cellulosomal cellulases such as Cc-Cel5A, Cc-Cel8C, Cc-Cel48F, Cc-Cel9G or Cc-Cel9M [74,75]. Such an ability to induce synergism with other cellulosomal cellulases places Cc-Cel9E as a key enzyme in the crystalline cellulose degradation efficiency of cellulosome. It was suggested that such an effect could be induced by the generation of additional hydrolysable sites then accessible to the other cellulases [73].

Cc-Cel9G displays the highest activity against crystalline cellulose reported so far among cellusomal cellulases of *C. cellulolyticum* [75]. While mainly cellopentaose and cellotetraose are released during the initial phase of degradation, here again longer incubation time promotes the accumulation of cellobiose [73,75]. Like Cc-Cel9E, Cc-Cel9G possesses a CBD but it belongs to familly IIIc [73].

Cc-Cel5A [69], Cc-Cel5D [81] and Cc-Cel8C [71] are among the first cellulosomal cellulases of *C. cellulolyticum* that have been characterised at the genetic and biochemical levels. Initial characterisation of these enzymes indicated an endo mode of action where the main product of cellulose hydrolysis is cellobiose but with some substantial differences in the catalytic parameters. Interestingly, Cc-Cel5A can transglycosylate other soluble cellodextrins [71].

Cc-Cel9M is the most recently characterized cellulosomal cellulases in *C. cellulolyticum* [75]. Cc-Cel9M is a non-processive cellulase, which is consistent with the absence of a class III CBD. Even after long incubation time, the major product of cellulose hydrolysis is cellotetraose, which is in marked contrast with Cc-Cel9G, Cc-Cel9E or Cc-Cel48F [75,79].

Cc-Rgl11Y is the first cellulosomal pectinase characterised in *C. cellulolyticum* [44]. Pectins are mostly present in the primary plant cell wall but are absent from the secondary plant cell wall after lignification; the secondary cell wall of grasses is an exception since it possesses pectic polysaccharides [11,12].

This association of enzymes possessing various and complementary activities at a correct ratio together with the close proximity and appropriate spacing between the individual catalytic components are thought to be responsible for the highly efficient degradation of crystalline cellulose by the cellulosome [29]. As observed in *C. papyrosolvens*, a close relative of *C. cellulolyticum*, different cellulosomal particles with different enzymatic composition are produced by the bacterium [83]. Depending of the growth substrate, *C. cellulolyticum*

seems to be able to adapt the cellulosome composition in its catalytic components [63].

2.2.3. The extracellular assembly of C. cellulolyticum cellulosome

The extracellular assembly of individual cellulosomal components following their secretion on the bacterial cell surface is probably one the most intriguing mechanism related to the cellulosome complex which has not been addressed definitively in the literature [84]. To date, little is known about protein secretion in the bacteria of the genus *Clostridium*. Bioinformatic analyses of the signal peptides of cellulosomal components suggest that they are all secreted by the Sec (Secretion) apparatus.

It was proposed that ORFXp, the gene product of *orfX* present in the *C. cellulolyticum cel* cluster, could be anchored into the cytoplasmic membrane via an uncleaved signal sequence and could act as an intermediate in the docking of cellulase during the extracellular cellulosome assembly [46]. In fact, the cell surface protein OlpA of *C. thermocellum*, harbouring both a Coh_I and an S-layer module, was suggested to be involved in the binding of individual cellulosome components [50] and/or the temporary anchoring of cellulosomal cellulase before their final incorporation into the cellulosome [46]. However, this speculative model requires further investigations to validate it.

In the cellulosome, the attachment of cellulase subunits to the scaffolding protein results from the calcium-dependent specific recognition of a Doc_I and a Coh_I, respectively [85]. It was also found that the interaction between Doc_I and Coh_I of *C. thermocellum* and *C. cellulolyticum* is species specific [86]. As observed for *C. thermocellum* [87], the attachment of cellulosomal subunits to CipC does not seem to be specific of one particular Coh_I but would occur randomly along the scaffolding protein following a non-selective process. Nevertheless, preferred proximity relationships between specific catalytic domains cannot be excluded.

In *C. thermocellum*, the cellulosome contains 6–13% carbohydrate, most of them resulting from the *O*-glycosylation of threonine residues located in the linker regions of the scaffolding protein CipA [88]. In *C. cellulolyticum*, uncharacterised glycosylations have been reported for CipC and also for some catalytic components namely Cc-Cel9E, Cc-Cel48F and presumably Cc-Cel5D [57]. Such glycosylations could play a role in protection against proteases, structural stabilisation and in cohesin-dockerin recognition [89].

3. The physiology of *C. cellulolyticum* on cellulose

The first metabolic characterisation of *C. cellulolyticum* demonstrated that this mesophilic cellulolytic bacterium had an optimum growth temperature of 34 °C with a minimum and maximal growth temperature of 25 and 45 °C respectively [90]. In addition to cellulose, this bacterium can grow on xylan, soluble cellodextrins (from cellobiose to cellohexaose), glucose, xylose and weakly on some other sugars found in the hemicelluloses such as arabinose, fructose, galactose, mannose and ribose [63,90,91]. By determining the activity of enzymes at key points of the metabolism and by analogy to the catabolism of *C. thermocellum*, the cellobiose catabolic pathway of *C. cellulolyticum* was deduced as depicted in Fig. 3 [92,93].

Following insoluble substrate colonisation, cellulose catabolism begins with its depolymerisation by the catalytic components of cellulosome resulting in the release of soluble sugars, namely glucose and soluble cellodextrins, i.e. from cellobiose (DP = 2) to celloheptaose (DP = 7) [1]. Cellulosome permits the direct association of the cell and cellulose, resulting in a cellulose–enzyme–microbe (CEM) ternary complex [29]. In *C. cellulolyticum*, while the role of cellulosome in the bacterial cellulose adhesion is clear, glycocalyx biosynthesis has also been reported but its involvement, as well as other mechanisms such as fimbriae, and their respective contribution to such a process is unknown [94]. The released cello-oligosaccharides remain in close proximity to the cell in a stagnant region, between the cellulosome and the bacterial cell wall, in which contact corridors and/or glycocalyces may be present [29,35]. Considering that diffusion into the environment is restricted and that the uptake by an highly efficient ATP-Binding Cassette (ABC) transport system is rapid, it can be speculated that the time period spent by the soluble sugars between their release and their incorporation by the cell is very short, probably in a matter of seconds or less [95]. As organochemoheterotrophs, the clostridia generate ATP by the phosphorylation of the carbon substrate and the final electron acceptors are organic molecules [18,96]. As in any clostridia, carbon and electronic flows in *C. cellulolyticum* are intimately linked (Fig. 4). During glycolysis, some NADH is generated and a second oxidation occurs when the pyruvate is converted into acetyl-CoA by the pyruvate-ferredoxin oxidoreductase (PFO) with the formation of reduced ferredoxin (Fd). This thioclastic reaction has a key role in the metabolism since the acetyl-CoA can be directed towards various fermentation pathways (acid or solvent formation) or anabolic pathways as the biosynthesis of fatty acids [18]. From the reduced Fd, the electrons can be transfer to the hydrogenase, NADPH-Fd oxidoreductase or NADH-Fd oxidoreductase. NADH can be reoxidised by (i) dihydrogen production via the Fd, (ii) the ethanol pathway, and/or (iii) the lactate pathway.

The premiss that the main product of cellulolysis is cellobiose, has been used throughout the literature to circumvent bacterial cultivation on cellulose and justify the use of cellobiose as a carbon source to study the

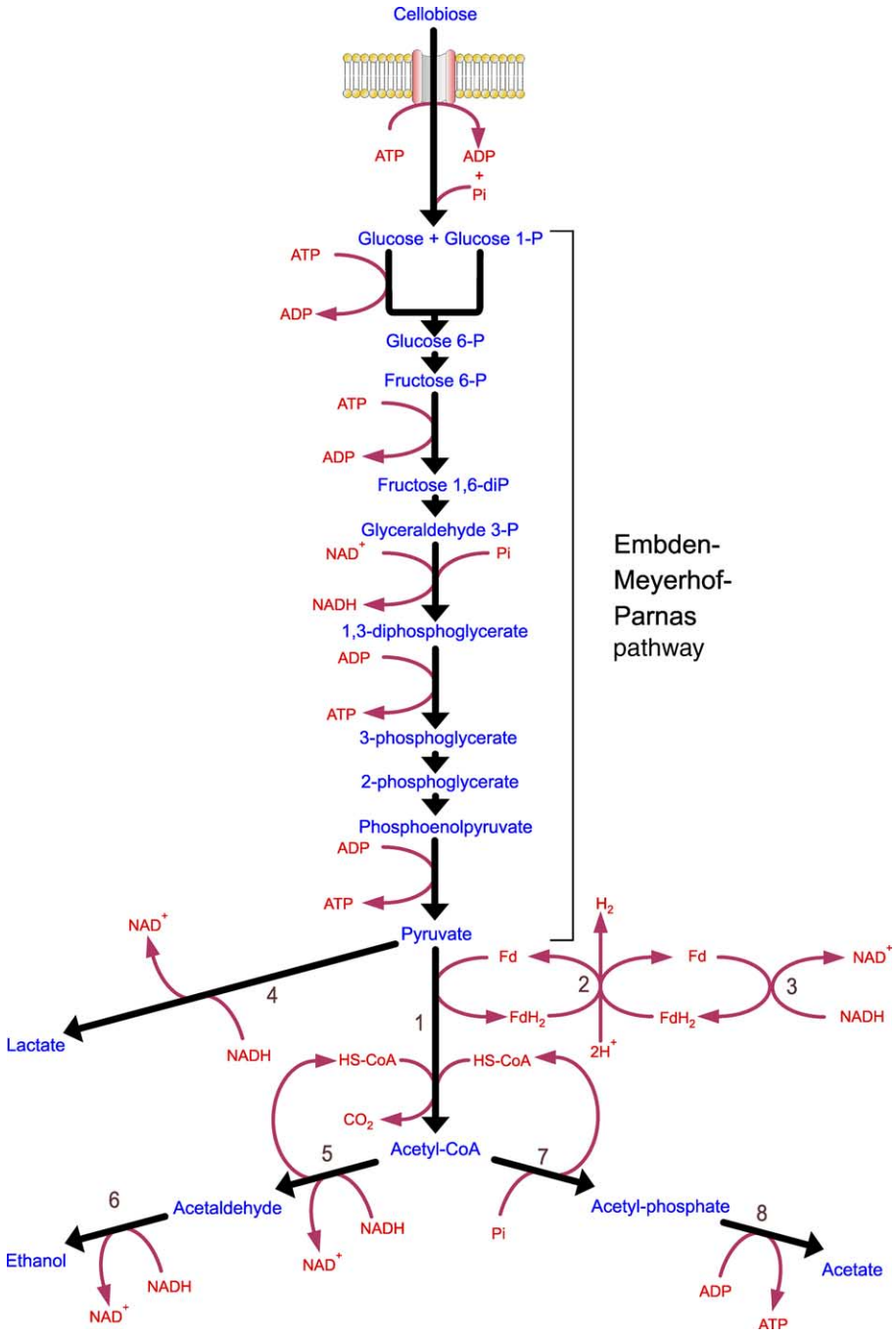

Fig. 3. The catabolic pathway of cellobiose in *C. cellulolyticum*. 1: pyruvate-ferredoxin oxidoreductase (EC 1.2.7.1); 2: hydrogenase (EC 1.18.99.1); 3: NADH-ferredoxin oxidoreductase (EC 1.18.1.3); 4: lactate dehydrogenase (EC 1.1.1.27); 5: acetaldehyde dehydrogenase (1.2.1.10); 6: alcohol dehydrogenase (EC 1.1.1.1); 7: phosphotransacetylase (EC 2.3.1.8); 8: acetate kinase (EC 2.7.2.1).

physiology of various cellulolytic microorganisms. It was assumed that (i) cellobiose was the major product of cellulose hydrolysis as suggested from cell growth on cellulose and enzymatic studies of individual cellulases [38,90], (ii) as a consequence, bacterial growth on cellobiose was representative of the bacterial growth on cellulose, (iii) the study of metabolic behaviour was more observable with a soluble substrate than a insoluble one [97]. Moreover, cellobiose is the most easily and commercially available soluble cellodextrin [1]. However, at least in *C. cellulolyticum*, cellobiose as the major end product of cellulose hydrolysis could be suspected to be a *petitio principii* considering that (i) the accumulation of cellobiose occurs only when cells are no more metabolically active, i.e. when the culture enters into the stationary phase as result of the medium acidification [98], and (ii) in vitro studies of cellulases show that while within the first minutes of initial hydrolysis, cello-

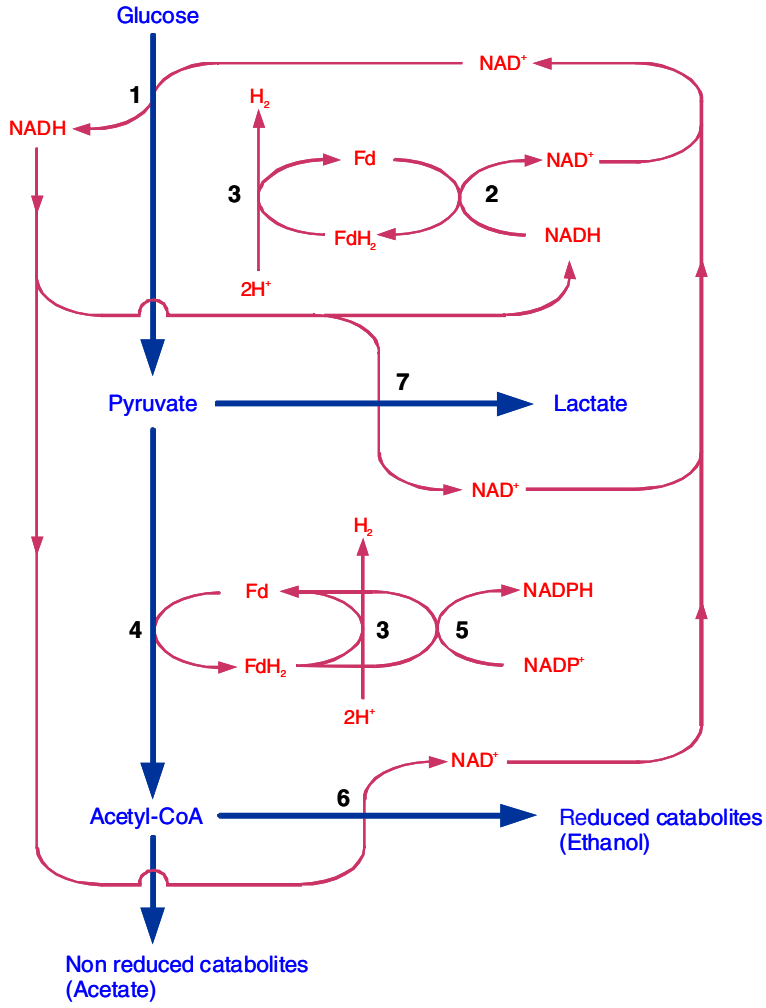

Fig. 4. Scheme of the interactions between the carbon flow (dark blue large arrows) and electron flow (red thin arrows) in *C. cellulolyticum*. 1: glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); 2: NADH-ferredoxin oxidoreductase (EC 1.18.1.3); 3: hydrogenase (EC1.18.99.1); 4: pyruvate-ferredoxin oxidoreductase (EC 1.2.7.1); 5: NADPH-ferredoxin oxidoreductase (EC 1.18.1.2); 6: acetaldehyde dehydrogenase (1.2.1.10) and alcohol dehydrogenase (EC 1.1.1.1); 7: Lactate dehydrogenase (EC 1.1.1.27).

dextrins with a high DP are released from cellulose, cellobiose as the major soluble cellodextrin appears only after several hours (see Section 2.2.2).

Early investigations of *C. cellulolyticum* physiology reported an early growth inhibition [90,97,99]. It is generally accepted that entry into the stationary phase, referring to a culture that shows no further increase in cell number, could be either the result of depletion of a particular nutrient from the culture medium or due to inhibition of cell growth [100]. It was suggested that rather than inhibition, this phenomenon was the result of nutritional limitation. Several unsuccessful attempts have been made to improve the growth of *C. cellulolyticum* namely by increasing the amount of all nutrients, and/or supplementing the medium with yeast extract, casamino acids or vitamins [97]. On the belief of non-determined special nutritional requirements, *C. cellulolyticum* was systematically cultivated in complex medium. Taking into account that the natural ecosystem of *C. cellulolyticum* is probably nutrient poor and even oligotrophic [101], Guedon et al. took the opposite view of the improvement of *C. cellulolyticum* growth with enriched culture medium by using a mineral-salt based medium in which the yeast extract was replaced by vitamins and oligo-elements [93]. In fact, with a maximum biomass of around 0.6 $g l^{-1}$, the use of a medium containing 5 $g l^{-1}$ of yeast extract could be considered as aberrant [92,97]. Such a chemically defined medium has allowed finer analysis of the cellular metabolism and bioenergetics, since the carbon substrate is the only source of both carbon and energy, and demonstrates to have a dramatic influence on bacterial metabolism [93].

The first metabolic investigations of *C. cellulolyticum* were performed in batch cultures without shaking, without pH regulation and in sealed penicillin flasks where the fermentation gases accumulated. An inherent feature of batch culture experiments is that the composition of the medium varies constantly during the course of

growth [102]. For this reason, *C. cellulolyticum* cultures in stable physiological conditions, i.e. chemostat cultures, were carried out. While the type of culture system used might appear trivial in the first instance, it is in fact crucial to rigorously study the metabolism and physiology of bacteria [103]. As pointed out early by J. Monod "Une culture en voie de croissance ne peut être considérée comme physiologiquement stable qu'au cours de la phase exponentielle de croissance, souvent trop courte pour les besoins de l'expérience. Encore la composition du milieu de culture se modifie-t-elle très rapidement au cours de cette phase", which can be translated by "A growing culture can be considered as physiologically stable only during the exponential growth phase, which is often too short for the experimental needs. Moreover, the composition of the medium is changing very quickly during this growth phase" [104]. In other words, batch culture is not the most appropriate culture system to study bacterial metabolism and chemostat culture is a much more powerful technique. It is worth remembering that a chemostat culture is a particular type of continuous culture; while chemostat is synonymous with continuous culture the opposite is not true. The chemostat is defined as a continuous culture where the feeding rate is an external factor and where the cell growth is limited by one nutrient [105]. This second condition is important since it means that the specific growth rate (μ), which is equal to the dilution rate (D) at the steady state of the system, is a function of only one limiting nutrient. The steady state of the system is reached only if both the cell density and the concentration of the limiting nutrient remain constant over time [104]. Thus, at the steady-state, a dynamic exists between the different components of the system, which is thermodynamically different from an equilibrium state [106].

Following investigations of *C. cellulolyticum* metabolism using batch cultures and continuous cultures under various growth conditions with cellobiose or cellulose as sole carbon and energy source, three key metabolic points were revealed (i) the cellulosome in the regulation of entering carbon flow, (ii) the phosphoglucomutase (PGM) in regulation of carbon flow towards the central metabolism, and (iii) the PFO metabolic node in regulation of energetic and electronic fluxes.

3.1. *The colonisation of cellulose by* C. cellulolyticum

With *C. thermocellum* and *C. cellulolyticum*, adhesion of the cell to cellulose appears to be required for rapid and efficient cellulose hydrolysis [107]. In the early stages of batch cultures, *C. cellulolyticum* is essentially found in close contact with cellulose and the release of bacterial cells at the end of growth is correlated with the exhaustion of accessible cellulose [108,109]. Gelhaye et al. showed that adhesion to cellulose was a two step process [110,111], where reversible and site specific cell–cellulose interaction occurs first, followed by cell–cell interaction due to aggregation of cells on the surface of the insoluble substrate. Cells in the upper layer of the biofilm are not in direct contact with the insoluble substrate. Study of the colonisation process indicated that part of the bacterial population is released from cellulose into the liquid phase before adhering and colonising another adhesion site on the cellulose fibre. Cellulose colonisation is proposed to occur through a cyclic process of adhesion–colonisation-release–readhesion [112]. Following release, cells probably encounter carbon starvation conditions. It was speculated that sporulation could ensure the perenniality of *C. cellulolyticum* in its ecological niche [113]. The spores of *C. cellulolyticum* are spherical with a 1.5 μm diameter, terminal and heat resistant (100 °C for 30 min) [90]. It was originally reported that these spores were rarely produced on media not containing cellulose. The adhesion of *C. cellulyticum* spores to cellulose is a non-specific process which would occur predominantly by hydrophobic interactions [114]. The current model of cellulose colonisation by *C. cellulolyticum* is depicted in Fig. 5 [114]. The vegetative bacterial cells first adhere to a specific site on the cellulose fibre and then colonise this site. Following the saturation or exhaustion of adhesion sites, the cells are re-

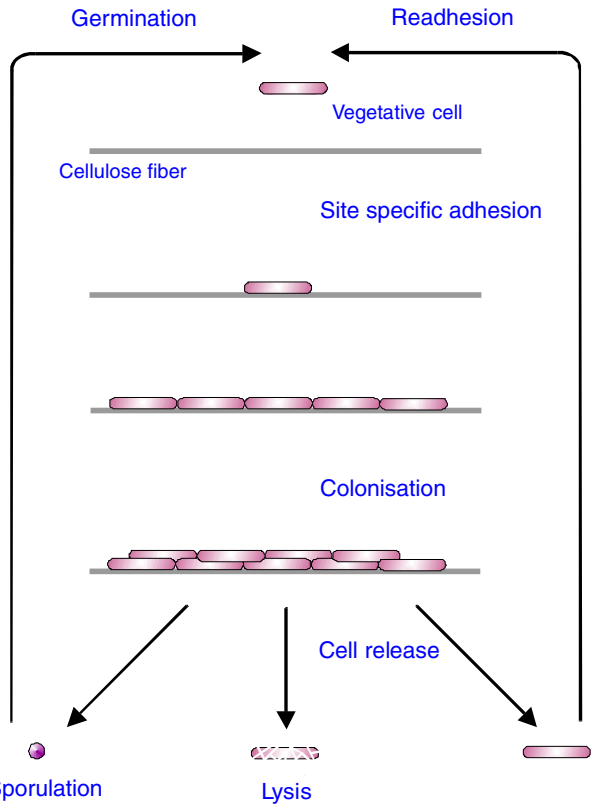

Fig. 5. Scheme of the model of the cellulose colonisation by *C. cellulolyticum*.

leased facing either lysis, readhesion to another site or sporulation until the germination thanks to the recovery of suitable growth conditions.

In contrast to the genus *Bacillus* where the regulation of sporulation has been investigated on a molecular basis [115,116], sporulation in clostridial species has not attracted much interest, especially in cellulolytic clostridia [117,118]. While it was long assumed that the sporulation process in *B. subtilis* could apply in most Gram-positive bacteria, the available genome sequences of several clostridia namely *C. acetobutylicum* [52], *C. perfringens* [119] and most recently *C. tetani* [120] revealed that the set of sporulation genes is quite different. One of the major differences is the absence of the genes involved in the phosphorelay system that functions in phase 0 of sporulation in *B. subtilis* and encoding such proteins as response regulator Spo0F, phosphotransferase Spo0B, histidine kinases KinA to KinE, and Rap family phosphatases. These facts suggest a unique sporulation process in clostridial species different from *Bacillus* species. Investigating the effect of carbon and nitrogen availability as well as the specific growth rate on *C. cellulolyticum* sporulation in chemostat cultures, it appeared that under cellobiose or ammonium limitation, sporulation mainly occurs at a low specific growth rate [121]. Using ''stop-flow chemostat'' experiments, where the feeding pump was stopped once the culture reached the steady-state, it was demonstrated that whatever the dilution rate used neither cellobiose nor ammonium exhaustion could increase the percentage of sporulation. While an excess of soluble carbon substrate repressed spore formation [121], increasing cellulose concentration promotes sporulation [118], suggesting that cell attachment and/or soluble sugar concentration could modulate and trigger a particular physiological regulation [122–124]. The remaining cellulose could serve as an exogenous energy source for sporulation by feeding bacterial cells continuously at a limited rate. This could also explain why no correlation between glycogen accumulation and endospore formation was detected in *C. cellulolyticum* [121]. Rather than the limitation or exhaustion of ammonium or carbon substrates as previously assumed, a low specific growth rate and mainly a low environmental pH in the presence of cellulose are the major factors inducing sporulation in *C. cellulolyticum* [118]. While clostridial-type bacteria are often considered to be sensitive to a low pH and restricted to less acidic ecological niches [125], anaerobic habitats are characterised by low pH conditions resulting from high concentrations of fermentation acids [3]. Thus, the sporulation of *C. cellulolyticum*, while attached to lignocellulosic compounds would allow the maintenance and integrity of the cell as well as successful survival and competition with others microorganisms in its microbiota [118]. It is worth mentioning that, the term ''nutrient limitation'' has been confusingly used to describe two completely different growth phenomena in the literature [126]. In batch cultures, nutrient limitation refers to the stoichiometric aspects of growth where the final cell density decreased with decreasing concentration of substrate. In continuous cultures of the type chemostat, nutrient limitation corresponds to a ''nutrient-limited growth'' and refers to conditions where the microbial specific growth rate (μ) is dictated by the substrate concentration when the bacterial growth is limited by one particular nutrient. Thus, the observation that cellobiose and/or ammonium limitation do not promote sporulation in *C. cellulolyticum* has to be replaced in the context of chemostat culture experiments, which correspond to nutrient-limited growth. This observation is far different from the observation that sporulation in *B. subtilis* is induced by carbon, nitrogen or phosphorus limitation when grown in batch cultures which then do not correspond to limiting growth substrates as sometimes assumed but refers only to the abundance of nutrients in the environment [115,116,127,128].

3.2. *The metabolisation of carbon in* C. cellulolyticum

From the first metabolic investigations of *C. cellulolyticum* in cellobiose batch cultures, it was observed that this bacterium undergoes an aceto-lactic fermentation [97]. During fermentation, the consumption of carbon substrate was very low since only 1–2 $g l^{-1}$ of cellobiose was fermented compared to the 30–100 $g l^{-1}$ of glucose usually consumed by other saccharolytic clostridia such as *C. acetobutylicum*, *C. pasterurianum*, or *C. butyricum* [97,129]. This low carbon consumption was associated with entry into the stationary phase at an early stage of growth. On cellulose, the production yields of lactate, acetate and ethanol were reported lower and the rate of cellulose degradation declined over time [99]. This decrease in cellulolysis rate was attributed to a change in the cellulose structure, such as an increase in the crystalline lattice due to the initial degradation by the cellulasic system. The controlling factor in bacterial growth was identified as the cellulolysis rate [130,131]. These investigations also suggested that the release of soluble sugars, mainly cellobiose, inhibits both cell growth and cellulase production [131]. At this stage, *C. cellulolyticum* was considered as a sluggish cellulolytic bacteria [90,99]. This was attributed mostly to its cellulasic system which needed to be genetically and biochemically studied to further improve the cellulose fermentation [130].

The cultivation of *C. cellulolyticum* in agitated bioreactor on a synthetic medium and with pH regulation clearly improved the growth and cellulolytic performance of the bacterium compared to the initial investigations [132]. Moreover, no modification of the cellulose crystallinity could be observed in the course of fermentation. Instead, the slowdown of cellulose hydrolysis is attributed to the change in distribution of the cellulasic activity between the cellulose fibres and

the supernatant [132]. With an initial cellulose concentration lower than 6.7 gl^{-1}, growth is limited by the substrate, and more exactly by the number of accessible adhesion sites on the cellulose fibres. The more cellulose is hydrolysed, the less the cellulasic system and the cells can find new adherence sites on the cellulose fibres. Thus, most of the total cellulasic activity is found in the culture supernatant at the end of fermentation and cannot participate in the residual cellulose degradation. Ethanol and acetate are the main fermentation products with an ethanol/acetate ratio increasing continuously towards 1 with increasing initial cellulose concentration. Kinetic analysis of the fermentation revealed that extracellular pyruvate is excreted in the course of fermentation but the cells rapidly reconsume it. With initial cellulose concentrations higher than 6.7 gl^{-1}, the percentage of cellulose degradation drops rapidly. Most of the cellulasic activity remains associated with cellulose and the final cell density does not increase. Lactate and acetate are the main final catabolites.

Whereas the effects of acidic conditions on the growth of cellulolytic rumen bacteria have been the subject of considerable research [133–137], few investigations have been devoted to cellulolytic clostridia. Contrary to the assumption that the buffering capacity of the culture medium is sufficient to compensate the acidification [97,99], reinvestigation of cellulose degradation by *C. cellulolyticum* demonstrated that the growth inhibition observed with batch cultures, performed in penicillin flasks, is essentially the result of a low pH due to acid production in the course of fermentation. Cellulose-limited chemostats confirmed the dramatic effect of environmental acidification which influences chiefly biomass formation rather than cellulose degradation and assimilation [98]. In fact, while cell density is lowered more than fourfold from pH 7.0 to 6.4 at constant D, the specific consumption rate of cellulose ($q_{cellulose}$) only slightly decreases. Growth yield ($Y_{X/S}$) continuously increases until pH 6.2 where a steady state of the continuous culture can not be established further since washout occurs [98]. Therefore, *C. cellulolyticum* does not show depressed yields and the transition to washout appears abrupt. This result would be consistent with a direct effect on a cellular constituent, such as the negative effect of acid or pH on enzyme(s) or transport protein(s) [134,135]. For *C. cellulolyticum*, the patterns of energetic growth yield (Y_{ATP}) and $Y_{X/S}$ as a function of pH are very similar to those obtained with *Fibrobacter succinogenes* [134]. In this bacterium, it was shown that while the extracellular pH dropped, the intracellular pH remained constant [136]. This kind of intracellular pH regulation would be common to most clostridia [136,138]. For *C. cellulolyticum*, the range of pH allowing maximum cell density is restricted and, therefore, strict control of pH is necessary to reach optimum cellulolytic performance.

As observed in any cellulose-limited continuous culture [139–141], while the cellulose fibres are colonised by *C. cellulolyticum* at all D, the fibres are completely covered mainly at low D (Fig. 6) [142,143]. In carbon-limited conditions using either cellobiose or cellulose, ethanol and acetate are the main end products of catabolism with an acetate/ethanol ratio always higher than 1 but decreasing with increasing D. However, with cellulose, there is no shift from an acetate–ethanol fermentation to a lactate–ethanol fermentation as observed on cellobiose when D increased [93,142]. The growth maintenance coefficient (m), maintenance energy (m_{ATP}) and true energetic yield of biomass (Y_{ATP}^{max}) are very similar either with cellobiose or cellulose [93,142]. This indicated the energetic needs of the cell are similar towards these two substrates. However, the true growth yield

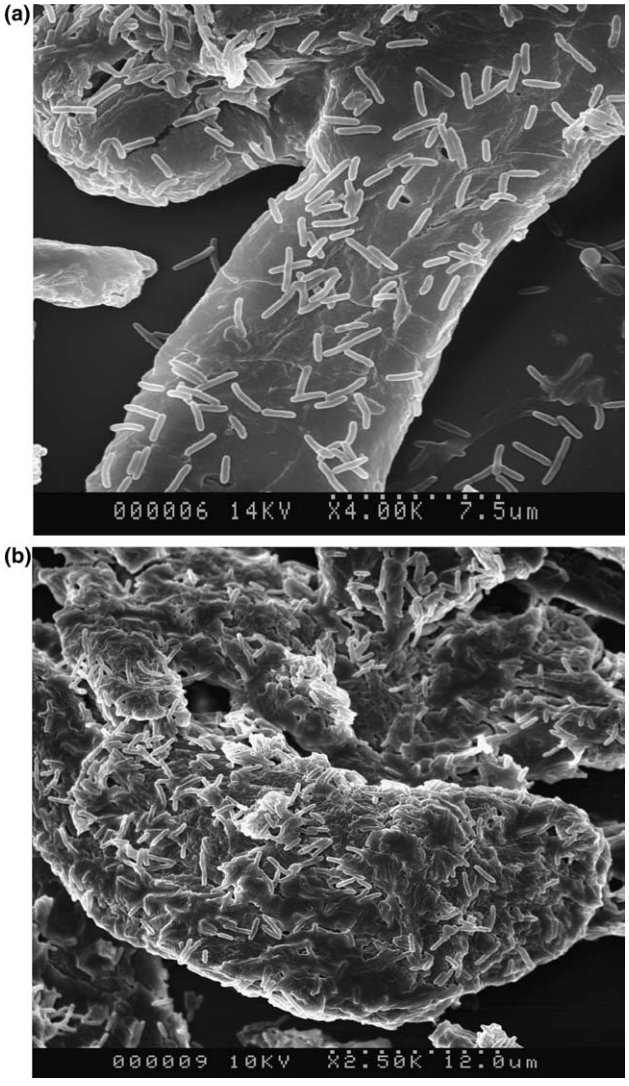

Fig. 6. (a) Scanning electron micrograph of *C. cellulolyticum* growing in cellulose-limited chemostat at $D = 0.083\ h^{-1}$. (b) Scanning electron micrograph of *C. cellulolyticum* growing in cellulose-limited chemostat at $D = 0.014\ h^{-1}$.

($Y^{max}_{X/S}$) determined on cellobiose is significantly lower than the one estimated on cellulose. This finding has a profound implication, since it means that bacterial growth on cellulose could reach a higher biomass for the same quantity of carbon consumed compared to cellobiose; thus, growth on cellulose is more beneficial to the cell than growth on cellobiose. Such a result could be explained by a transport and metabolisation mechanism of soluble cellodextrins similar to *C. thermocellum* [29,95]. In cellulose-sufficient continuous cultures, the $q_{cellulose}$ is higher than in cellulose-limited chemostats and results from a higher percentage of adhering cells to the cellulose fibres, which thus participates directly in cellulose digestion. As in cellulose-sufficient continuous cultures, in ammonium-limited conditions some catabolised hexose and hence ATP are no longer associated with biomass production as indicated by lower Y^{max}_{ATP} and $Y^{max}_{X/S}$ compared to cellulose-limited conditions [144]. Such an energetic uncoupling of anabolism and catabolism, is typical of chemostat cultures in nitrogen limitation; the biosynthesis pathways are limited by the nitrogen source while the carbon source in excess leads to an excess of energy. In such conditions, ATP production is higher than the cellular energetic needs and generally involves pathways dissipating the energy excess [145,146]. Some continuous cultures performed under limitation of nutrients, other than the carbon source, have higher rates of carbon substrate utilisation when the carbon is in excess than when the carbon is limited. These cultures have a greatly increased m_{ATP} requirement and use the remaining energy more efficiently than in carbon-limited chemostats [146–148]. However, in *C. cellulolyticum* since m_{ATP}, which corresponds to the expenditure of energy towards functions that are not directly related to the bacterial growth, does not increase in ammonium-limited conditions, wasting of energy associated with maintenance function does not occur [144]. Surprisingly, even in ammonium limitation, free amino acids are present in the culture supernatant, which suggests that the uptake of nutrients and the generation of biosynthetic precursor occur faster than their utilisation for biomass production.

Metabolic differences, observed during growth of *C. cellulolyticum* on cellobiose or cellulose, are associated with carbon consumption rates, which are always higher on cellobiose than on cellulose. The hydrolysis of cellulose fibres by the cellulosome, prior to the incorporation of soluble sugars by the cell, introduces an extra mean for the regulation of the entering carbon flow. While the use of cellobiose allows highlighting of metabolic limitation and regulation of *C. cellulolyticum*, some of these events should rather be interpreted as distortions of the metabolism or laboratory artefacts due to culture conditions far removed from those in which this bacterium has evolved in nature. In *C. cellulolyticum*, the first step in the carbon catabolism is the depolymerisation of insoluble cellulose into soluble cellodextrins via the cellulosome. In the course of evolution, the catabolic and anabolic pathways have been optimised as a function of the carbon flowing from cellulasic activities. The use of soluble sugars, such as cellobiose, completely shunts the cellulosome. However, the use of cellobiose permits to demonstrate (i) the strong influence of the cellulosome on the entering carbon flow, and (ii) that *C. cellulolyticum* is not adapted to catabolic rates as high as in other clostridia. From these investigations, *C. cellulolyticum* appeared to be adapted to low carbon flow, which is in good agreement with the degradation of lignocellulosic compounds. In nature, cellulose hydrolysis is generally a slow and incomplete process; degradation takes place over of period of months or years and is subject to seasonal variation [8].

3.3. The glucose 1-phosphate/glucose 6-phosphate metabolic node

In a remarkable study on *C. thermocellum*, Strobel et al. demonstrated that glucose and soluble cellodextrins are actively taken up by an ATP-dependent transport system and that following the uptake, the sugars are processed by cytosolic cellobiose phosphorylase (EC 2.4.1.20) and cellodextrin phosphorylase activities (EC 2.4.1.49) [95]. To date, this is the only investigation demonstrating cellodextrin transport by a cellulolytic *Clostridium*. The transmembrane transport and the involvement of cellodextrin phosphorylases in the incorporation and metabolisation of soluble cellodextrins with a DP higher than 5, i.e. cellohexaose and celloheptaose has never been reported since their synthesis and purification, especially the celloheptaose, is particularly difficult to achieve [1]. Although no stoichiometry between the transported soluble sugar and the ATP hydrolysed has been established in cellulolytic clostridia, the structure of ABC transporters suggests the hydrolysis of two ATP per substrate transported [149,150]. Similarly to *C. thermocellum* [151], with *C. cellulolyticum* the molar growth yield ($Y_{X/S}$) is higher on cellobiose than on glucose [97]. Together with the fact that a cellobiose phosphorylase activity is present in *C. cellulolyticum* [150], these data strongly suggest that the incorporation mechanism of glucose and soluble cellodextrins in *C. cellulolyticum* is similar to *C. thermocellum* [95].

Once the soluble cellodextrins are inside the cell compartment, they are converted first into glucose 1-phosphate (G1P) and then glucose 6-phosphate (G6P) (Fig. 3). Chemostat investigations in mineral salts medium in nitrogen-limited conditions using cellobiose as substrate first revealed the importance of the metabolic branch points of the G1P–G6P for the distribution of the carbon flow inside and outside the cell [152]. The G1P and G6P pools are connected by the PGM (EC

5.4.2.2). As the entering carbon flow increased, PGM becomes a limiting step in catabolism and consequently an inversion of the G1P/G6P ratio is observed. The accumulation of G1P leads to the rerouting of metabolism through, first, the production of glycogen and, second, the exopolysaccharide biosynthesis. In *C. cellulolyticum*, the presence of uncharacterised exopolysaccharides was suggested early [97]. The synthesis of glycogen was also reported but contrary to ruminal cellulolytic bacteria where it can represent between 30% and 60% of the dry weight [153], in *C. cellulolyticum* its accumulation is limited to between 3% and 5% [150]. The increase of glycogen turnover associated with a size of pool quite modest, suggests the glycogen is synthesised and degraded permanently. This argument is reinforced by the presence of biosynthetic activities, i.e. ADP-glucose pyrophosphorylase (EC 2.7.7.27) and glycogen synthetase (EC 2.4.1.21), and degradative activity, i.e. glycogen phosphorylase (EC 2.4.1.1), at all *D*. Such a glycogen cycling was reported in other cellulolytic bacteria [154,155]. In contrast to many bacteria, the synthesis of glycogen in *C. cellulolyticum* (i) is not subject to stimulation by limiting growth nutrient or (ii) does not occur during a limited growth period at the outset of the stationary phase [156–158]. The kinetics of glycogen accumulation from batch and continuous cultures suggests that the rapid synthesis and degradation of this intracellular polymer is associated with rapid growth and hence with high carbon flow [152].

With cellulose as a carbon source, the G6P–G1P branch point plays its entire role since the higher is the DP of incorporated soluble cellodextrins, the more G1P is generated (Fig. 3). Metabolic flux analysis (MFA) demonstrated that PGM stabilises the proportion of carbon flow directed towards biosynthesis and catabolites formation pathways. At the G6P–G1P branch point, the ratio *R*, representing the specific enzymatic activity to the metabolic flux through the considered metabolic pathway, is very close to 1 and reflects the tight control exerted by PGM on the partition of carbon flow at this junction [159]. At this metabolic node, the carbon surplus is dissipated by exopolysaccharide, soluble cellodextrins and intracellular glycogen synthesis [142–144]. The carbon surplus is balanced mainly by exopolysaccharide and glycogen biosynthesis at high *D* values, while cellodextrin excretion occurs mainly at lower ones. No cellodextrin with a DP higher than 3 could be detected; the presence of cellobiose and cellotriose in the supernatant is related to reversible phosphorylase activities [143]. The formation of these products buffers the increasing carbon flow from G1P, which could not be otherwise metabolised by PGM. In fact, the proportion of carbon directed towards G6P remains quite constant with increasing *D*. On cellulose, the proportions of carbon directed towards cellodextrins, glycogen and exopolysaccharide pathways are not as high as with cellubiose and result from lower specific consumption rates [144,152]. Under cellobiose-sufficient conditions, only cellotriose is detected in the supernatant [150,152]; however, the detection of cellobiose in addition to cellotriose in cellulose-sufficient conditions suggests that cellobiose could also be synthesised de novo during cell growth in cellobiose excess.

Considering *m*, which is estimated to 0.9 mmol of hexose equivalent (g of cells)$^{-1}$ h^{-1} [142] and the maximum glycogen concentration reported in ammonium-limited conditions which is of 164.0 mg (g of cells)$^{-1}$ [144], then the maximum survival time of *C. cellulolyticum*, relying only on glycogen to supply its functions of maintenance, would be 67.5 min [146]. This time will be even shorter if new biosyntheses are triggered by the cell; in other words, glycogen is unlikely to play the role of an energy source permitting the long-term survival of this bacterium, as sometimes suggested in literature. Rather than prolonging cell viability, glycogen biosynthesis in *C. cellulolyticum* would be involved in carbon flow regulation. By mobilising a small fraction of the carbon flow and by its redistribution within the central metabolism, the glycogen cycle would constitute a triggering reaction of glycolysis. Additionally, the provisional storage of intermediate metabolites in the form of glycogen avoids their accumulation by the cell at toxic levels. As a substrate cycle, another probable function of the glycogen cycle is the dissipation of energy [146,160]. In the same way, the exopolysaccharide pathway consumes excess energy but also evacuates the excess carbon. In fact, in *C. cellulolyticum* the glycogen synthesis as an unballasting pathway for the resumption of the carbon flow remains limited and the bacterium uses a second evacuating pathway directed towards exopolysaccharide biosynthesis. Interestingly, recent genomic analyses suggested that the lack of glycogen metabolism would be a trait associated with parasitic behaviour [161].

3.4. The pyruvate/acetyl-CoA metabolic node

From the first chemostat cultures of *C. cellulolyticum* where a complex medium containing cellobiose as the limiting nutrient was used [92], it appeared that despite higher energetic yield of biomass (Y_{ATP}), the cell density at the steady state of the system fell when μ was higher than 0.075 h^{-1}. In fact, the ethanol pathway, normally permitting the reoxidation of the reduced coenzymes, could not compensate for the acetate production pathway which implied the reorientation of the electron flux towards the NADH-Fd oxidoreductase and the hydrogenase [162]. An NADH/NAD$^+$ ratio as high 57 indicated this latter pathway was a limiting step. A direct consequence of the high concentration in reduced coenzymes was the inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [92,163]. Thus, limited growth of *C. cellulolyticum* appeared to be the result of a low rate of NADH reoxidation.

Comparing cellobiose-limited chemostat experiments using a complex medium and a salt-based medium, it appears (i) the specific cellobiose consumption ($q_{cellobiose}$) increases threefold, (ii) the specific growth rate reaches 0.138 h^{-1}, and (iii) the NADH/NAD^+ ratio ranges from 0.29 to 2.08 [93]. These results clearly indicates a better control of the carbon and electron flows on a mineral salts medium than on a complex medium. As the μ increases, a shift from an acetate–ethanol fermentation to a lactate–ethanol fermentation is observed. In fact, the specific production rate of lactate ($q_{lactate}$) parallels the increase of catabolic rate leading to a pyruvate overflow. To characterise in more detail the influence of carbon flow on the limited growth observed for *C. cellulolyticum*, continuous cultures were performed using a salt-based medium in which the concentration of carbon substrate in the feeding reservoir was progressively increased in a stepwise fashion [164,165]. Under these conditions, the cell density at the steady state was significantly increased since at a $D = 0.083$ h^{-1}, it reached 863 $mg l^{-1}$ with 6 $g l^{-1}$ of cellobiose in carbon-limited condition, against 487 $mg l^{-1}$ with 5 $g l^{-1}$ in carbon saturation. This indicates that *C. cellulolyticum* is not able to optimise its growth and carbon flow in response to a sudden increase in the concentration of cellobiose. MFA in bioreactor batch cultures revealed the excretion of pyruvate coincides with lactate production and abrupt growth arrest [132,165]. This pyruvate leak, indicating an intracellular accumulation of this metabolic intermediate, suggests the PFO could no longer support the carbon flow arising from glycolysis. However, in cellulose-limited chemostat, whatever the specific rate of cellulose consumption ($q_{cellulose}$), the lactate and extracellular pyruvate remain very low indicating a better control of the carbon flux with cellulose than with cellobiose [93,142]. This also means PFO and LDH do not compete for the carbon flowing from glycolysis and, as a consequence, the pyruvate leak is very limited on cellulose [93,142]. On synthetic medium, cellobiose is catabolised to produce ATP permitting its conversion into biomass; on complex medium, it serves mainly as an energy source and leads to an accumulation of intracellular metabolites when present in excess. Results obtained from experiments with the mineral salt-based medium definitively exclude a default in the redox balance as the main cause of limited growth. The unbalanced metabolism, observed on complex medium, could be the consequence of experimental conditions far different to the natural ecosystem of the bacterium, which is unable to cope with a surfeit of nutrients. Therefore, early growth arrest would be the consequence of a high and unregulated entering carbon flow correlated to the accumulation of intracellular toxic compound(s) as suggested by the presence of extracellular pyruvate.

In carbon-limitation, the electronic flux is mainly balanced by the NADH-Fd reductase and hydrogenase activities at low D and by the ethanol pathway as D increased [93,142]. This regulation is inverted in carbon-saturation since the reoxidation of NADH to NAD^+ involves, essentially, the ethanol pathway at low D and the NADH-Fd reductase and hydrogenase at higher D [143,150]. This metabolic behaviour is in marked contrast with other Gram-positive anaerobic bacteria where the formation of lactate increases concomitantly with the specific growth rate [166,167]. In fact, most lactate dehydrogenases (LDH) are allosterically regulated by the fructose-1,6-biphosphate (FBP) [168]. This does not seem to be the decisive factor for *C. cellulolyticum* since the intracellular concentration of FBP remains quite constant with increasing D. Interestingly, the absence of lactate production would result from rerouting of the carbon flow towards exopolysaccharide biosynthesis. In carbon-sufficient conditions, while the H_2/CO_2 ratio increases, the specific production rate of ethanol stabilises with increasing D [150]. However, the NADH/NAD^+ ratio is always lower than 1 with cellulose as a carbon source, whereas a ratio of as high as 1.51 was obtained with cellobiose excess [143,150]. In carbon-sufficient conditions, the simultaneous decline of the true energetic yield of biomass (Y_{ATP}^{max}) and true growth yield ($Y_{X/S}^{max}$) clearly indicates that an uncoupling growth phenomenon occurs and it is associated with lactate production accompanied by a pyruvate leak [142,143]. Intracellular inhibition could then explain the establishment of a steady state under conditions of excess of all nutrients [105].

While PFO is clearly a sensitive metabolic node involved in the regulation of both energetic and electronic fluxes, pyruvate as a direct cause of growth inhibition in bacteria has not been proved experimentally; such growth inhibition could be explained by the accumulation of its anionic form at toxic concentrations for the cell [169–171]. In *Selenomonas ruminantium*, intracellular pyruvate accumulation is responsible for LDH activation [166,172]. In *Eubacterium limosum*, the presence of extracellular pyruvate is associated with growth inhibition and reflects a limitation of the metabolic pathway generating acetyl-CoA [171]. It cannot be excluded that *C. cellulolyticum* growth inhibition is a consequence of the accumulation of other metabolic intermediate(s) whose formation is correlated to pyruvate excretion; few examples are available to date. In *Bacillus subtilis*, the accumulation of glucose 1-phosphate has been associated with the cell lysis [173], however, in *C. cellulolyticum* lysis is only observed when the carbon source is completely exhausted [121]. Some triose phosphates, such as glyceraldehyde 3-phosphate or dihydroxyacetone phosphate, have sometimes been incriminated [174,175]. However, in *C. cellulolyticum* the intracellular concentrations of these compounds vary only slightly

[93,150,152]. Another intracellular toxic compound reported as a growth inhibitor is acetaldehyde where its aldehyde group reacts with the amine function of proteins [176]. In *C. cellulolyticum*, the activity of ADH is always higher than the specific production rate of ethanol indicating that the enzyme is not limiting, and therefore the accumulation of acetaldehyde is improbable [93,150,152]. Another possibility would be the production of methylglyoxal which is a highly cytotoxic compound [177,178]. Methylglyoxal is a metabolic intermediate of the D-lactate pathway which is essentially produced as a result of an uncoupling between anabolism and catabolism [146,153]. Dihydroxyacetone phosphate is converted into methylglyoxal by a methylglyoxal synthetase (EC 4.2.99.11), and then into D-lactate by a methylglyoxalase (EC 4.4.1.5). In *C. cellulolyticum*, no D-lactate has been detected but an incomplete pathway cannot be excluded [93,150,152,179].

3.5. *Metabolic engineering of* C. cellulolyticum

Cocultures of *C. acetobutylicum* and *C. cellulolyticum* were among the first attempts to improve cellulose fermentation [180,181]. As with the vast majority of cellulose hydrolysis investigations, however, the research focus on the cellulase enzyme systems of *C. cellulolyticum* with some attempts in the improvement of cellulase production relying on random mutagenesis [130]. Following the enzymatic paradigm [29], the investigations later focused on the cellulosome for the specific incorporation of enzymatic and non-enzymatic components. This approach allows the design of defined chimeric cellulosome complexes using the cohesin–dockerin interaction as a selective type of molecular adapter for incorporating desired proteins into multicomponent complexes [182–184]. In the light of the investigations on *C. cellulolyticum* metabolism, it appears that with cellobiose and even with pure cellulose, carbon flow could be high enough to lead to pyruvate overflow, indicating that carbon flux through glycolysis is higher than the rate of processing by PFO and LDH (see Sections 3.2 and 3.3). In order to use *C. cellulolyticum* in CBP, i.e. where cellulase production, cellulose hydrolysis and cellulose fermentation are accomplished in a single step [29], the metabolism of this bacterium has been improved through metabolic engineering by Guedon et al. [185]. Such an approach was rendered possible following the development of molecular biology tools for *C. cellulolyticum* [186,187].

This metabolic engineering strategy consisted of the incorporation of a new heterologous catabolic pathway branched on the pyruvate metabolic node, constituted of pyruvate decarboxylase (PDC) (EC 4.1.1.1) and ADH [185]. The genes *pdc* and *adhII* from *Zymomonas mobilis* were expressed under the control of the ferredoxin promoter from *C. pasteurianum* and organised as an artificial operon inserted into the shuttle vector pMTL500F [188], i.e. pMG8. When the *C. cellulolyticum* recombinant strain Cc-pMG8 was grown in batch cultures on cellulose, the final cell density, cellulose degradation and CO_2, H_2, acetate and ethanol concentrations were all increased [185]. Surprisingly enough, the major fermentation end product was not ethanol but CO_2, H_2 and acetate. Therefore, this genetic construction could not completely reroute the carbon flow towards the formation of ethanol. At least two explanations could be given for this result:

- Firstly, although the genes *pdc* and *adhII* were expressed in *C. cellulolyticum*, the specific activity of PDC appeared lower than in other bacterial species where the heterologous expression of the genes had been tested. In *pdc*, the presence of rare codons for *C. cellulolyticum* has been reported (http://www.kazuka.or.jp/codon). Thus, if the expression of this gene is not optimal, the carbon flow cannot be rerouted correctly towards this new catabolic pathway. Ultimately, the heterologous expression of this gene could be improved by site-directed mutagenesis.
- Secondly, in all culture conditions studied so far, acetate is always the main catabolite produced (see Section 3.3), suggesting the importance of this pathway in the metabolism of *C. cellulolyticum* to maintain a correct energetic balance. As indicated by m_{ATP} and Y_{ATP}, the complete rerouting of the carbon flow away from this pathway is certainly physiologically impossible. Alternatively, a channelling phenomenon cannot completely be excluded [189].

In this context, an alternative approach could consist of improving the carbon flow by the incorporation of a PFO from another saccharolytic clostridium such as *C. acetobutylicum* or *C. pasteurianum* [31]. This strategy could circumvent the problem of rare codons since these bacterial species are more closely related to *C. cellulolyticum*. Moreover, from the acetyl-CoA produced (i) the carbon flow can still be directed towards the formation of acetate and ethanol to maintain a correct energetic and redox balance, (ii) other catabolites could be produced by incorporating others metabolic pathways such as butanol, acetone or isopropanol.

4. Concluding remarks and perspectives

Investigations of *C. cellulolyticum* metabolism using successively cellobiose and cellulose as sole carbon and energy sources allow solving the nature of carbon flow regulation. These in-depth analyses of *C. cellulolyticum* metabolism, using batch cultures and particularly chemostat technique, points out (i) the key role of cellulo-

some in the regulation of entering carbon flow, (ii) the major role of PGM in carbon flow regulation since its controls the entry of G1P towards the central metabolism, and (iii) the importance and sensitivity of PFO metabolic node in regulation of energetic and electronic fluxes. Results from MFA herald the metabolic engineering of this microorganism. Targeting PFO, which appears the most sensitive metabolic node of *C. cellulolyticum* catabolism, prolongs cell growth, and as consequences, higher cell density, cellulolysis and final concentrations of catabolites were reached [185]. Contrary to some assumptions of the enzymatic paradigm [190], these investigations clearly indicate that cellulose depolymerisation is not the limiting step of microbial cellulose digestion by *C. celluloyticum* and improvement of cellulolysis by *C. cellulolyticum* must primarily focus on bacterial metabolism rather than catalytic activity of cellulosome.

4.1. *The metabolism of cellulose in* C. cellulolyticum

The study of *C. cellulolyticum* metabolism could be embodied in a succession of investigations using growth conditions unrelated to the physiology of this bacterium respective to its natural ecosystem. In fact, *C. cellulolyticum* was first cultivated on a complex medium highly enriched mainly with yeast extract while its natural ecological niche is most likely oligotrophic. As with all anaerobic cellulolytic bacteria investigated so far, *C. cellulolyticum* is particularly sensitive to environmental acidification, but the influence of pH was ruled out from the first metabolic investigations. For a long time, the metabolism of this bacterium was studied with cellobiose, which physical nature is far different from natural lignocellulosic compounds. Contrary to what was long assumed in the literature, comparison of *C. cellulolyticum* growth with cellobiose and cellulose revealed that they do not result in the same metabolism. Therefore, the use of cellobiose for metabolic studies of cellulolytic microorganisms must be avoided, or at least carefully taken into consideration for the interpretation of results. Moreover, the status of cellobiose as the major cellulolysis end product is highly debatable in the context of microbial cellulose utilisation by microorganisms possessing a cellulosome. Cellulolytic bacteria inhabit natural environments where cellulose is very rarely found in a pure form but rather embedded in a dense matrix of hemicelluloses, pectins and lignin. The proportion and organisation of these biopolymers in lignocellulose certainly greatly influences the degradative capacities of *C. cellulolyticum*. Much remains to be learned about the N_2-fixing ability of cellulolytic clostridia. Thus, investigations of *C. cellulolyticum* metabolism with carbon and nitrogen substrates more closely related to natural ecosystem of the bacterium such as lignocellulosic compounds and atmospheric N_2, are necessary. It should be underlined that while *C. cellulolyticum* can grow on xylose and xylan, very little is known about their metabolisation in cellulolytic clostridia.

In the course of evolution, *C. cellulolyticum* has developed its ability to degrade natural cellulose very efficiently via the cellulosome. The catabolic pathways have been optimised as a function of the carbon flowing from cellulase activities, which degrades lignocellulosic compounds into soluble substrates incorporated by cells. Therefore, *C. cellulolyticum* is not adapted to high catabolic rates as in other saccharolytic clostridia such as *C. acetobutylicum*, *C. butyricum* or *C. pasteurianum* [18]. Instead, *C. cellulolyticum* appears restricted to a cellulolytic lifestyle. The use of a soluble carbon source, such as cellobiose, highlighted the metabolic limitation of *C. cellulolyticum*. It also pointed out the strong influence of both the physical nature of carbon source and the type of culture system used on bacterial metabolism. While the field of bacterial metabolism and physiology is arguably the oldest in microbiology [191], proper investigations of *C. cellulolyticum* metabolism, that must be addressed using chemostat technique, turned up lately. Ultimately, the results from these investigations should be completed by proteomic and transcriptomic analysis for an in-depth analysis of the molecular mechanisms involved; such approach will be facilitated by the sequencing genome of this microorganism. Further investigations are also needed to confirm whether or not these metabolic regulations are general features of cellulolytic clostridia and/or other cellulolytic bacteria. In fact, it should be stressed that very few studies have been dedicated to bacterial metabolism on cellulose; combination of batch culture, chemostat technique and MFA to investigate metabolic regulations when cellulolytic bacteria grow on cellulose is so far limited to *C. cellulolyticum*. Comparison of cellulose utilisation by cellulolytic bacteria essentially in terms of kinetics of cellulose degradation has been recently reviewed [29]. It must be pointed out, however, that observed differences could result from the use of different cellulosic substrates; crystallinity, allomorphs, porosity, capillarity and/or gross surface area of the cellulose used could result in different kinetics of cellulose degradation, which in turn influence bacterial metabolism [29,142]. Thus, attempts to compare metabolism of cellulolytic bacteria are rendered extremely difficult and should not be overinterpreted, a caveat which points out the necessity for standardisation of cellulose used in such studies for further direct comparison [142].

In *C. cellulolyticum*, glucose and cellodextrins transport systems have not been characterised yet. Better knowledge of this system is undoubtedly necessary since the uptake mechanism of these true growth substrates is central to the understanding of bacterial metabolism when the cell is growing on cellulose. It will permit

assessment of current hypotheses such as (i) the model of cellodextrin transport in *C. cellulolyticum* based on *C. thermocellum* model, (ii) the benefit for cells to grow on cellulose versus cellobiose, (iii) the bioenergetic advantage of incorporation of large cellodextrins by the cell, (iv) the incorporation of soluble cellodextrins with DP as high as 7, or (v) the extracellular hydrolysis of soluble cellodextrin prior to their incorporation. As pointed out by Lynd et al. [29], further data are needed to support the hypothesis that long cellodextrins are important for cells growing on cellulose. From this point of view, it would be important to reconcile our understanding of cellulolysis based on enzymatic studies to bacterial physiology [192]. In this respect, microbial catabolism of glucose and cellobiose has received a decade of attention and the studies predate the investigation of cellulose hydrolysis itself. On the other hand, the utilisation of soluble cellodextrins has received little attention, mainly for a reason of cost and feasibility. The understanding of cellulose degradation in the environment requires that data from monospecies laboratory cultures must be extrapolated to microbiota where, as observed with the rumen microflora, complex interactions between cellulolytic and non-cellulolytic microorganisms and environmental conditions take place [29,193–196]. In this context, the feeding of non-cellulolytic satellite bacteria either with cellodextrins synthesised de novo from cellulolytic bacteria hydrolysing cellulose via primarily cell-associated enzymes, or with cellodextrins lost by diffusion from the cellulolytic site, or both is an intriguing issue [29].

In *C. cellulolyticum*, the early growth arrest is related to high carbon flow resulting in self-intoxication of the cell where pyruvate excretion is the more visible consequence. Yet, pyruvate as directly responsible compound for intracellular growth inhibition in *C. cellulolyticum* has not been proven experimentally. One particularly attractive possibility would be the involvement of methylglyoxal, which needs to be investigated further. Such self-intoxication of the bacterial cell could be a phenomenon far more common than expected and should be considered by more systematic assays of intermediary metabolites [197]. These results must be paralleled to certain forms of viable-but-non-culturable (VBNC) phenomenon, which referred to bacteria having some metabolic activities but incapable of cell division [198–200]. The reason for a VBNC state is not clearly elucidated; the hypotheses of DNA damage, presence of autocrine factors, and/or generation of free radicals have been suggested [198,201]. The recovery of bacteria from various ecosystems generally implies the use of enrichment media to prevent any nutrient deficiency [202], but the transfer of bacterial cells to nutrient-rich medium and/or batch culture conditions could initiate imbalance in their metabolism. The use of a naturally derived nutrient source at ambient nutrient concentration, such as atmospheric N_2 as sole nitrogen source or complex organic compounds as sole carbon source are not always easy to identify with respect to the isolated microorganisms. This is then rarely applied for resuscitation experiments and/or in combination with an open continuous-culture system, which are more closely related to the natural ecosystem of a bacterium compared to a closed batch culture where metabolic fluxes cannot be controlled [126]. Thus, it is conceivable that the growth of bacteria more sensitive than *C. cellulolyticum* could be even more rapidly inhibited when fed at high metabolic rates with easily available and/or plentiful nutrient sources leading to the toxic intracellular accumulation of metabolite(s) due to bottleneck(s) at some metabolic node(s). This could be another alternative explanation to certain forms of VBNC.

4.2. Biotechnology and microbial cellulose degradation by C. cellulolyticum

As revealed in a recent review on cellulosomes from mesophilic bacteria [43], the extensive study of genetics, structure, function and interaction of cellulasic components in *C. cellulolyticum* makes this cellulase enzyme system the model of mesophilic clostridial cellulosomes. Until recently, in-depth investigation of the molecular biology of *C. cellulolyticum* was limited by the number of molecular tools available for this microorganism [40,186,187]. One of the most intriguing mechanisms, which still needs to be properly investigated, is related to the extracellular anchoring and assembly of the cellulosome on the bacterial cell surface. While the involvement of ORFXp in the cellulosome assembly has been suggested, deletion/complementation experiments are still awaited to validate this hypothesis. Besides its fundamental implications, the understanding of such process is essential for the optimum heterologous expression and display of designer cellulosomes by a variety of hosts [43]. Previous attempts to improve microbial cellulolysis relied on recombinant cellulolytic strategies by heterologous cellulase overexpression. This strategy, primarily investigated in bacteria (essentially *Z. mobilis* and some enteric bacteria) and in yeast (essentially *Saccharomyces cerevisiae*) had limited success [29]. With this approach, only overexpression of single cellulases secreted in the supernatant has been reported and heterologous expression of cellulosomes has not been achieved yet.

The common feature of cellulolytic bacteria isolated so far is their limited growth and catabolic capabilities towards cellulose, which jeopardize their potential utilisation in CBP [29]. In order to improve cellulose degradation and conversion into products of interests, metabolic engineering in cellulolytic bacteria has been extensively discussed [29]. Improvement of cellulolytic and catabolic properties of *C. cellulolyticum* constitutes the first and, to date, unique example of such an attempt

in any cellulolytic organism [185]. Importantly, this work demonstrates that the improvement of cellulolysis by *C. cellulolyticum* should primarily focus on the improvement of its metabolism, which is not adapted to high catabolic rates, rather than the catalytic activity of the cellulosome. While very high ethanol production could not be achieved, improvement of the catabolic flow at the pyruvate node has been also considered via site-directed mutagenesis of *pdc* or insertion of PFO from *C. acetobutylicum* [31]. However, it should be emphasized that inherent to the catabolism of *C. cellulolyticum*, PGM plays a major role in controlling and limiting the carbon flow directed towards glycolysis. Therefore, this approach should also be completed by the directed evolution of the metabolism in vivo in chemostat in order to improve *C. cellulolyticum* metabolism towards cellulose, i.e. both cellulose digestion and cell growth [203].

An alternative approach, overcoming both the difficulty of heterologous expression of a cellulosome and the limited catabolic activity of cellulolytic bacteria, arises from the study of *C. acetobutylicum*. This non-cellulolytic bacterium possesses a highly effective catabolism [18] and the sequencing of its genome revealed the presence of cellulosomal genes [52]. This cluster of genes, originally considered as cryptic, has high homology with the *cel* cluster in *C. cellulolyticum*. Recent studies have demonstrated this cellulosome is expressed but not effective against crystalline cellulose [39]. While it could be speculated that this lack of efficient activity against crystalline cellulose could be the result of absent or inactive major catalytic components of this cellulosome, such as Cel48F and Cel9E homologues, further investigations are required. *C. acetobutylicum* offers the advantage of being the clostridial species for which the metabolism and genetics are the best known and for which the most extensive range of genetic tools has been developed [18,52,204]. Ultimately, it can be expected that the recovery of an optimum cellulasic activity by this cellulosome would permit highly efficient conversion of lignocellulosic compounds into solvents by *C. acetobutylicum*. Considering the close taxonomic relatedness of the cellulosomal genes of *C. acetobutylicum* with *C. cellulolyticum*, the knowledge about *C. cellulolyticum* cellulosome is undoubtedly of great value in any attempt to recover the activity of *C. acetobutylicum* cellulosome.

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