Insights into the catalytic mechanism of cellulose hydrolysis by *Cytophaga hutchinsonii*

Cellulose is the most abundantly produced organic biopolymer in terrestrial ecosystems[1]. Each year, photosynthetic fixation of CO_2 yields more than 10 million tonnes of dry plant material worldwide and almost half of this material consists of cellulose[2]. The value of cellulose as a renewable source of energy and carbon has made cellulose hydrolysis the subject of scientific research and industrial interest for many years. Cellulosic wastes may be converted to glucose, soluble sugars, alcohol, single-cell protein and other industrially useful chemicals using enzymes such as cellulases. The conversion of cellulose to glucose involves at least three types of cellulases: (i) endoglucanases, which randomly cleave internal β-1,4 glucosidic linkages, (ii) cellobiohydrolases, which hydrolyse β-1,4 glucosidic linkages of cellulose from the ends liberating cellobiose, and (iii) β-glucosidases, which release two glucose residues from one cellobiose molecule[3].

Despite the information available on these enzyme systems and on the structure of plant cell walls, application of this knowledge to cellulose degradation has met with limited success. This may be attributed to at least two factors: (i) the inherent complexity and heterogeneity of native cellulose, and (ii) our limited understanding of the basic hydrolysis processes[3]. Therefore, an understanding of the molecular mechanisms underlying cellulose degradation in combination with new and superior enzymes may facilitate increased usage of this valuable renewable resource.

Cytophaga hutchinsonii is a Gram-negative soil bacterium isolated from sugarcane piles. It grows poorly in minimal medium with carbohydrate polymers such as cellulose, starch, cellobiose or xylan as the sole carbon source. But it is thought to be an important organism in the degradation of biomass[4]. This microorganism therefore probably produces an array of enzymes allowing it to digest cellulosic substrates. *C. hutchinsonii* thus represents a rich source of potentially effective cellulase enzymes that can be harnessed for conversion of biomass to simple sugars. These sugars can then be used as feedstock for ethanol production or other chemical syntheses.

Preliminary studies with *Cytophaga* grown on sugarcane bagasse revealed limited catalytic activity[5]. *Cytophaga* do not grow well in liquid or solid minimal media supplemented with carbon sources. The lack of a binding module (carbohydrate-binding module, CBM), which facilitates access to the cellulose substrates, might explain some of these limited activities. However, studies of Cel9A on different substrates showed the high efficiency of purified *Cytophaga* endoglucanases[6]. Cel9A without CBM seems to have an active catalytic domain capable of attacking polysaccharides. The mechanism by which *Cytophaga* operate in cellulose hydrolysis thereby remains unknown, as some of the common features required for catalysis are not present. In this study, we report the mutation of several residues within the catalytic domain of Cel9A, a β-1,4 endoglucanase and the subsequent catalytic activities. The present data should provide some insights into the molecular mechanism underlying the hydrolysis process by *Cytophaga* cellulases.

The amino acids that have been shown to be involved in catalysis in several E4 enzymes such as *Thermobifida fusca*, are also present in *Cytophaga* Cel9A (Figures 1 and 2). But the most intriguing feature about *Cytophaga* Cel9A is the absence of a CBM. This is not consistent with the current stand of today's literature on several industrial microorganisms. Most known cellulose degraders have a distinct structure showing a catalytic domain, an adjacent CBM, a Pro/Ser/Thr-rich linker and another CBM[7]. *Cytophaga* Cel9A differs from several Fam9 endoglucanases by structurally not having a linker region or any CBM domains. CBM is known to maintain a high concentration of the enzyme near the insoluble substrate. Other roles, such as disrupting crystalline cellulose to aid hydrolysis, have been suggested for the CBM[8]. The CBM has been considered as the limiting factor in hydrolysis. In the case of Cel9A, since there is no CBM present, would it be easier to achieve maximum increase in specific activity using *C. hutchinsonii* in an industrial setting?

A search for homologous proteins revealed a glycosyl hydrolase family 9 catalytic domain located from amino acid 30 to 590 and an immunoglobulin-like (Ig-like) domain from amino acid 600 to 890 (Figure 2). The Cel9A domain structure is shown in Figure 2. A CLUSTAL alignment of eight family 9 catalytic domains performed with the DNA STAR MEGALIGN Program, also

```
Cytophaga_hutchinsonii              AGSKISSPRGWYDAGDYNKYIVNSGISTYTLLAAYEH-------FSTYYD
Dyadobacter fermentans              ENTLISSTRGWYDAGDYNKYIVNSGITMGTLLSLYED-------FPLFFE
Saccharophagus degradans            ADTSFAAPKGWYDAGDYGKYIVNSGISTYTLMAAYEH-------FPSFYK
Pseudomonas sp.                     AESVISSPQGWYDAGDYGKYIVNSGISTYTLLAALEH-------YPELYA
Fibrobacter succinogenes S85        ---TIQSSKGWYDAGDYGRYIVNSGITTYTLLSLYEH-------FPEYFN
Clostridium cellulolyticum H10      ---TIQSSKGWYDAGDYGRYIVNSGITTYTLLSLYEH-------FPEYFN
Thermobifida fusca          CDYSLDVSGGWYDAGDHGKYVVNGGISVHQIMSIYERSQLADTAQPDKLA
Cellulomonas flavigena      CDYTLDVTGGWYDAGDHGKYVVNGGISVAQLLATYERTLHVEGASTEALA
                                    : . *******.:.*.**.**:  ::: *
```

Figure 1. A sequence alignment of *Cytophaga hutchinsonii* Cel9A showing the conserved residues (underlined) common to most family 9 cellulases.

Figure 2. Different domains characteristic of the structure of family 9 cellulase genes. Most known cellulases have the following structure: a signal sequence (Sig), a carbohydrate-binding module (CBM) attached to a catalytic domain by a short linker, and another CBM. *Cytophaga* do not reflect this commonly found structure.

Table 1. Total activity of mutants of Cel9A on different polysaccharides

	Activity μmol cellobiose/min/ μmol of enzyme*				Ratio of soluble/ insoluble reducing sugars
Enzymes	CMC	SW	BMCC	FP	Processivity
CEL9A-68	216	11.6	2.07	0.280	0.3
D358A	0.58	0.136	0.033	–	–
D358B	0.63	0.189	0.046	–	–
D361A	0.86	0.207	0.073	–	–
D361B	0.99	0.230	0.056	–	–

*Activity was calculated at 10% digestion for SW (phosphoric acid-swollen cellulose) and BMCC (bacterial microcrystalline cellulose from *Acetobacter xylinum*) and 5% digestion for CMC (carboxymethyl cellulose) and FP (filter paper). All assays were done for 16 h at 30°C. Cel9A-68 is the wild type, D358A/B are two different mutants from Asp at position 358. D361A/B are also two different mutants from the Asp at position 361.

revealed 30% similarity between them (Figure 1). There is an Ig-like domain module in Cel9A from amino acid 600 to 890 that is homologous to domains found in four other endoglucanases. This Ig-like domain is only weakly homologous to the three Ig-like repeats found in the family 9 enzyme *T. fusca*[8]. Thus far, the functions of these domains, which consist of repeating units of ~120 amino acids, remain unknown for microbial carbohydrates[9].

Family 9 catalytic domains are unusually homologous; however, the domain structure of these proteins varies markedly, as is shown in Figure 3, along with the specific activities of the different enzymes[9]. The published nomenclature has been used in comparison studies. The crystal structure of the Cel9 catalytic domain has been solved in *T. fusca*[9]. This structure has contributed greatly to understanding the family 9 cellulases. Cel9 has a cleft along one face of the protein. The cleavage site is located before the cleft. Therefore based on these findings, the crystal structure and the published literature, we suggest that Cel9A from *C. hutchinsonii* is a non-processive endocellulase. The Cel9A amino acid sequence was submitted to SWISS-MODEL and a theoretical model of Cel9A was generated based on the Cel9 structure. The overall model produced a good fit, the Cel9A residues, Y204, Y406, and D504, which line the cleft have Cel9 counterparts, as do the proposed catalytic residues.

The results of site-directed mutagenesis are listed in Table 1. Preliminary site-directed mutagenesis and computer modelling of Cel9A, suggested that within the conserved domain, Asp358 and Asp361 are important residues (Table 1). Mutation of these residues with Ala resulted in a 70% loss of activity. The idea of protein folding leading to a conformational change, which might have caused the loss in activity has been rejected by follow-up studies (unpublished data). This result is consistent with the literature and mutations of Cel9 of *T. fusca* and *C. fimi*[9]. *Cellulomonas hutchinsonii* fits the model; however, it differs structurally from other cellulose degraders. With a different gene structure, questions are being raised as to how *C. hutchinsonii* operates in cellulose hydrolysis. The following scenario has been proposed: *C. hutchinsonii* might randomly cleave the cellulose chain to release cellobiose and then take in cellobiose as an energy source for metabolism. However, this hypothesis remains to be tested.

Cytophaga cellulases are not well studied. Being a member of the phylum Bacteroidetes, these organisms are also not closely related to the standard model organism for cellulose utilization. As studies of starch utilization by *Bacteroidetes thetaiotamicron* have revealed many unusual features[10], it is therefore not surprising that these organisms are unique among the studied cellulose degraders. Here we provide in-depth studies of *Cytophaga* Cel9A, by reporting the mutation of several residues within its catalytic domain (CD). These studies produced some intriguing results. As previously reported, Cel9A was found to produce 80% insoluble sugars, when tested for the production of soluble and insoluble reducing ends, which makes Cel9A a non-processive endocellulase. In addition, although *Cytophaga* lack a binding domain, all the residues known to be involved in bacterial cellulose hydrolysis are present within CD of *Cytophaga* Cel9A, and their mutations respond accordingly to the catalytic activity. The SWISS-MODEL also produces a perfect fit. Several reports suggested that endoglucanases activity is usually hampered by substrates–sites accessibility[11]. Based on the findings in this study, *Cytophaga* do not seem to fit this category. This would suggest that *Cytophaga* use a different unknown mechanism for cellulose hydrolysis and that aspartate appears to play a key role in this mechanism. Further studies should shed more light on the mechanism of cellulose hydrolysis of *Cytophaga* by focusing, for example, on mutations beyond aspartates.

For both energy and chemical production, biomass is an important alternative that is renewable and environmentally sustainable. By enzymatically hydrolysing cellulose, the major component of biomass, to simple sugars and fermenting them to ethanol, one can produce a liquid fuel capable of substituting our dwindling fossil-fuel supply. Cellulase enzymes can hydrolyse cellulose to cellobiose, a double sugar molecule, which can then be converted to glucose. Research efforts worldwide for the past several decades have provided substantial insights into the world of cellulases. However, there are major gaps that need to be filled in terms of our understanding of how cellulases attack crystalline cellulose. In this study, we have provided some interesting insights into the mechanism of cellulose hydrolysis by the gliding bacteria *C. hutchinsonii*. These findings will significantly improve our understanding of cellulose hydrolysis, which may eventually lead to the design of novel cellulases enzymes.

1. Schlesinger, W. H., *Biogeochemistry: An Analysis of Global Change*, Academic, San Diego, 1991, p. 443.
2. Eriksson, K. E., Blanchette, R. A. and Ander, P., *Microbial and Enzymatic Degradation of Wood and Wood Components*, Springer-Verlag, New York, 1990.
3. Leschine, S. B., *Annu. Rev. Microbiol*., 1995, **49**, 399–426.
4. Walker, E. and Warren, F. L., *Biochem. J*., 1938, **32**(1), 31–43.
5. Louime, C., Abazinge, M., Johnson, E. and Latinwo, L., *Fla. Sci*., 2006, **69**, 44–48.
6. Louime, C., Abazinge, M., Johnson, E., Latinwo, L., Ikediobi. C. and Clark, A. M., *Appl. Biochem. Biotechnol*., 2007, **141**(1), 127–138.
7. Prosite, Expasy Proteomics Server, 2002; www.expasy.org/prosite
8. Rosano, C., Bisso, A., Izzo, G., Tonetti, M., Sturla, L., Flora, A. and Bolognesi, A., *J. Mol. Biol*., 2000, **303**, 77–91.
9. Zhou, W., Irwin, D., Kousen, J. E. and Wilson, D. B., *Eur. J. Biochem.*, 2004, **43**, 9655–9663.
10. Fields, M. W., Ryals, P. E. and Anderson, K. L., *Anaerobe*, 1997, **3**(1), 43–48.
11. Carrard, M. L., Forro, L. M. and Pekker, S., *Synth. Met*., 1996, **80**, 29–34.

ACKNOWLEDGEMENTS. We thank the United States Department of Agriculture for support through the capacity building Grant Program # USDA/CSREES 00/38820/9526.

Received 8 September 2010; revised accepted 14 March 2011

CLIFFORD LOUIME*
OGHENEKOME ONOKPISE
HEMANTH VASANTHAIAH

College of Engineering Sciences, Technology and Agriculture, FAMU BioEnergy Group, Florida A&M University, 217 Perry-Paige Bldg, Tallahassee, FL 32307, USA
**For correspondence.*
e-mail: Clifford.Louime@famu.edu

Deformed and dragged red bole horizon near Pune, Maharashtra

The Deccan Volcanic Province (DVP) is unique in the geology of India because of its prodigious volcanism and its pivotal role in studies of volcanology. The Deccan volcanics have erupted close to the Cretaceous–Tertiary (*K*/*T*) boundary at about 65 Ma. It has been estimated that today the Deccan Traps cover an area of 500,000 sq. km in western and Central India, consisting dominantly of subaerial flows of tholeiitic basalts with subordinate picrites, picritic basalts and alkaline basalts[1]. The emplacement of the Deccan Traps was a discontinuous process and the quiescence periods have been recorded by many intrabasaltic bole beds which are the products of weathering during major hiatus[2]. Thus bole beds are indeed paleosols formed over the lava flows they cap and they witness the processes of weathering occurring between emplacements of the successive lava flows. Hence, bole beds can be effectively used to construct the time intervals between successive lava flows in the Deccan Traps[3]. Intrabasaltic paleosols (fossil soils) are preserved in many of the flood basalt provinces of the world throughout the geological time and until recently, they were studied using primary qualitative methods. In recent years, palaeopedology has shifted from a largely qualitative field based on comparisons with modern analogues to an increasingly quantitatively endeavour[4] with the development of a variety of semi-quantitative and quantitative tools to examine past weathering and pedogenesis, which help reconstruct both palaeoenvironmental and palaeoclimatic conditions at the time that the palaeosols formed. According to Sayyed and Hundekari[5], and Ghosh *et al*.[6], the bole beds occur as prominent horizons composed of fine-grained earthy material having colours in shades of red to chocolate brown, green, purple or grey, having formed by pedogenesis of the underlying basalts on the basis of geochemistry of bole beds in

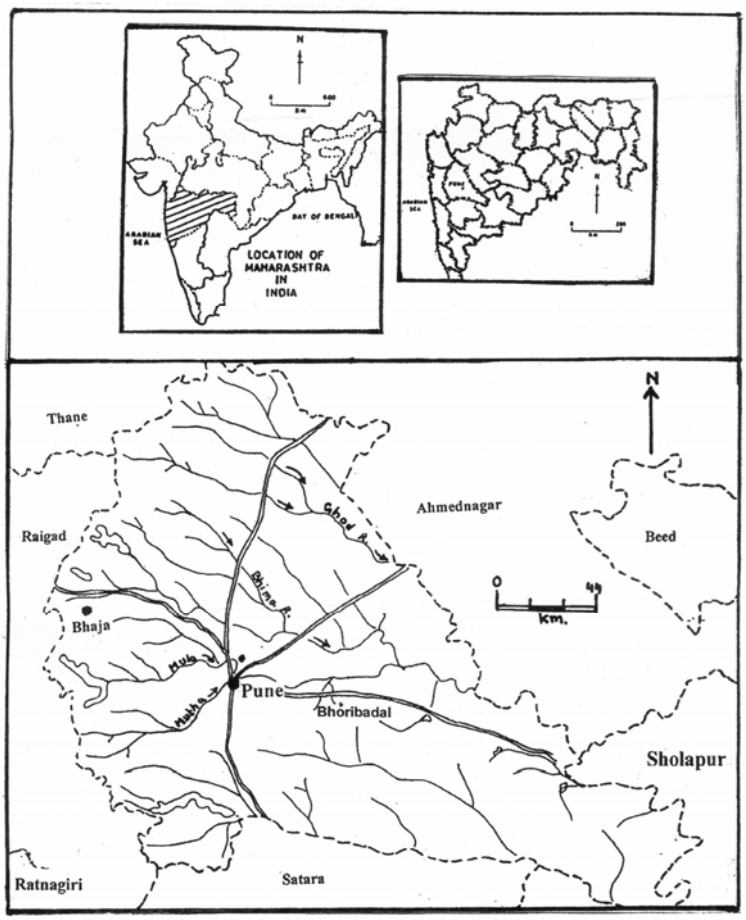

Figure 1. Location map of the study area.