Appl Biochem Biotechnol (2011) 163:649–657
DOI 10.1007/s12010-010-9070-5

Functional Annotation of *Fibrobacter succinogenes* S85 Carbohydrate Active Enzymes

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Received: 25 April 2010 / Accepted: 16 August 2010 /
Published online: 28 August 2010

Abstract *Fibrobacter succinogenes* is a cellulolytic bacterium that degrades plant cell wall biomass in ruminant animals and is among the most rapidly fibrolytic of all mesophilic bacteria. The complete genome sequence of *Fisuc* was completed by the DOE Joint Genome Institute in late 2009. Using new expression tools developed at Lucigen and C5-6 Technologies and a multi-substrate screen, 5,760 random shotgun expression clones were screened for biomass-degrading enzymes, representing 2× genome expression coverage. From the screen, 169 positive hits were recorded and 33 were unambiguously identified by sequence analysis of the inserts as belonging to CAZy family genes. Eliminating duplicates, 24 unique CAZy genes were found by functional screening. Several previously uncharacterized enzymes were discovered using this approach and a number of potentially mis-annotated enzymes were functionally characterized. To complement this approach, a high-throughput system was developed to clone and express all the annotated glycosyl hydrolases and carbohydrate esterases in the genome. Using this method, six previously described and five novel CAZy enzymes were cloned, expressed, and purified in milligram quantities.

Keywords *Fibrobacter succinogenes* · Biomass · Genome screening · Cellulase · Hemicellulase · Rumen · Screening

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Background

Because of the uniqueness of its 16S RNA sequence, *Bacteroides succinogenes* was renamed *Fibrobacter succinogenes* [1] and has since been assigned as the sole genus in the phylum, *Fibrobacteres*. The organism was initially identified and characterized [2] as a gram-negative, strictly anaerobic, cellulolytic bacterium that degrades plant cell wall biomass in ruminant animals. The organism is capable of growth on glucose, cellobiose, lactose, and cellulose, but not xylose or xylan [3, 4]. Growing cultures digest cellulose at a rapid rate, but non-growing cells and cell extracts do not have detectable crystalline cellulase activity [5]. Digestion of cellulose by the organism does not appear to utilize freely secreted enzymes or cellulosome-type structures but does require attachment of cells to cellulose fibers. The mechanism by which cellulose is degraded is unclear; mutants have been isolated that are unable to grow on crystalline cellulose or both crystalline and amorphous cellulose [6], but the mutations were not in genes for crystalline cellulose-degrading enzymes.

Materials and Methods

Materials F. succinogenes S85 (ATCC 19169^T) is available from the American Type Culture Collection (Manassas, VA). Expresso™ T7 Cloning and Expression System, BL21 (DE3) chemically competent *Escherichia coli* cells, and pETite™ (a T7 promoter vector) were obtained from Lucigen, Middleton, WI. pET28a vector and Overnight Express medium were obtained from Merck Chemicals, San Diego, CA. Azurine cross-linked-labeled polysaccharides and low-viscosity barley β-glucan were obtained from Megazyme International (Wicklow, Ireland). 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbelliferyl-β-D-xylopyranoside, and 4-methylumbelliferyl-β-D-glucoyranoside were obtained from Research Products International Corp. (Mt. Prospect, IL). CelLytic IIB reagent and birchwood xylan were purchased from Sigma-Aldrich (St. Louis, MO). Ni-NTA agarose was obtained from Qiagen (Valencia, CA). magenta-glucoside was obtained from Inalco Pharmaceuticals (San Luis Obispo, CA). All other chemicals were of analytical grade.

Growth of Organisms Cultures of *F. succinogenes* S85 were grown in a modified Dehority medium [7] with 4 g cellulose/L for 48 h at 39 °C. YT plate media (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl and 16 g/l agar) was used in all *E. coli* molecular biology screening experiments. Terrific Broth (12 g/l tryptone, 24 g/l yeast extract, 9.4 g/l K_2HPO_4, 2.2 g/l KH_2PO_4, and 4.0 g/l glycerol added after autoclaving) was used for *E. coli* liquid cultures.

Enzyme Assays The *endo*-glucanase specificity of enzymes was determined in 0.50 ml of 50 mM acetate buffer, pH 5.8, containing 0.2% azurine cross-linked-labeled (AZCL) insoluble substrates and 50 μl of clarified lysate. Assays were performed at 40 °C, with shaking at 1,000 rpm, for 60 min in a Thermomixer R (Eppendorf, Hamburg, Germany). Tubes were clarified by centrifugation and absorbance values determined using a Bio-Tek EL_x800 plate reader. The exo-glucanase specificity of enzymes was determined by spotting 2.0 μl of clarified lysate directly on agar plates containing 10 mM 4-*methyl* umbelliferyl substrate. Plates were incubated in a 40 °C incubator for 60 min; after incubation, the plates were examined using a hand-held UV lamp and compared with negative and positive controls.

Enzyme-specific activity was measured using the modified Somogyi method for reducing sugars [8]. The reaction mixtures containing 200 μl of substrate (1% β-glucan

or birchwood xylan in 50 mM acetate buffer, pH 5.8) and 5 μl enzyme sample were incubated at 40 °C for 10 min. The samples were removed from the heating block and 200 μl of Reagent D (1.0 ml of Reagent B (150.0 g/l of $CuSO_4 \cdot 5H_2O$ containing 20 drops of concentrated H_2SO_4/l) combined with 25.0 ml of Reagent A (25.0 g/l Na_2CO_3, 25.0 g/l $KNaC_4H_4O_6 \cdot 4H_2O$, and 200 g/l g Na_2SO_4)) was added. The samples were vortexed briefly to mix and then incubated at 95 °C for 20 min. After incubation, the tubes were vortexed, incubated at room temperature for 5 min, and 600 μl of Reagent E (10.0 g/l of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.20 g/l $Na_2HAsO_4 \cdot 7H_2O$, and 8.4 ml/l of concentrated H_2SO_4) was added to each sample. The samples were vortexed and incubated at room temperature for 15 min, then centrifuged for 2 min at 13 K RPM to clarify. Aliquots, 200 μl of each, were transferred to a 96-well plate and the absorbance determined at 590 nm. Micromoles of sugars formed were determined using a glucose standard curve, and unit activity calculated as micromoles of reducing sugar per minute per milligram of protein.

Library Construction F. succinogenes S85 bacterial cell concentrate was lysed using a combination of SDS and proteinase K, and genomic DNA was purified using phenol/chloroform extraction [9]. The genomic DNA was precipitated, treated with RNase to remove residual contaminating RNA, and fragmented by hydrodynamic shearing (HydroShear apparatus, GeneMachines, San Carlos, CA) to generate fragments of 2–4 kb. The genomic DNA was sheared to 2–4 kb, end repaired, ligated to the pETite vector and transformed into BL21(DE3) *E. coli* competent cells. An aliquot of purified *F. succinogenes* genomic DNA was submitted to the Joint Genome Institute of the Department of Energy for whole genome sequencing.

Library Screening Transformants were grown overnight on YT+30 mg/l kan plates, and 5,620 random clones picked (corresponding to ~2× genome coverage). Clones were cultured overnight in 96 deep well plates using Overnight Express medium. Cells were pelleted by centrifugation, and the pellets were lysed using Cellytic B reagent. Aliquots of the lysate were transferred to the multiplex substrate assay reagent containing 0.2% AZCL-arabinoxylan, 0.2% AZCL-HE-cellulose, 0.02% methylumbelliferyl-β-D-xylopyranoside, and 0.02% magenta-glucoside in 50 mM acetate buffer, pH 5.8 and incubated overnight at 37 °C with shaking. Assay plates were centrifuged, and supernatant aliquots were transferred to a fresh 96 well plate for absorbance and fluorescence measurement.

Enzyme Cloning Additional genes of interest were selected from the completed genome for amplification and expression. These genes were amplified without leader sequences and cloned using the Lucigen Expresso™ T7 Cloning and Expression System following the manufacturer's instructions. Clones were cultured overnight in 96 deep well plates using Overnight Express medium. Cells were pelleted by centrifugation, and the pellets were lysed using Cellytic B reagent. Protein expression was checked by SDS-PAGE and enzyme activity by assay with the expected substrates. Active transformants were grown in 100 ml cultures overnight, lysed by sonication, and the proteins were purified using Ni-NTA Agarose resin according to the manufacturer's instructions.

Bioinformatics InterProScan Family analysis (http://www.ebi.ac.uk/Tools/InterProScan/), and Basic Local Alignment Search Tool [10, 11] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis tools were used to compare, identify and characterize predicted genes. Glycosyl hydrolase predictions were obtained from http://www.cazy.org/geno/acc_geno.html [12, 13]. The graph of CAZy genes was generated from the whole genome sequence using DNASTAR® GenVision software.

Results

Genome Sequencing Results

Whole genome sequencing of *F. succinogenes* S85 (http://genome.ornl.gov/microbial/fs85/) resulted in a completed assembly of 3.8 Mb in one contig. The GC content of the genome was 48% GC. Annotation identified 3,087 candidate protein-encoding gene models for *F. succinogenes*. Genes referenced in this work utilize the ORNL annotation numbers, which are also utilized by the CAZy and UniProt databases. These genes have reference numbers in the format Fisuc_xxxx. These are not identical to gene numbers previously and currently annotated by TIGR, identified by the format FSUxxx.

The CAZy database (http://www.cazy.org/Genomes.html) identified 104 glycosyl hydrolase genes in the *F. succinogenes* genome. These 104 CAZy genes represent 3.37% of its total genes, higher than the percentage present in the cellulose-degrading, thermophilic anaerobe, *Clostridium thermocellum*, with 2.2% of its total genes (Garret Suen, private communication). The genome also contains 12 predicted polysaccharide lyase genes, 17 carbohydrate esterase genes and 63 predicted CBMs. The 104 predicted glycosyl hydrolase genes are arranged randomly throughout the genome (Fig. 1). Structures resembling operons appear to be present. Enzyme functions are not segregated within these apparent operons, these operons appear to contain mixtures of enzymes able to degrade multiple polysaccharides.

Screening Results

We generated and screened 5,760 random shotgun expression clones, representing twofold genome expression coverage; 169 positive hits were recorded and 33 were unambiguously

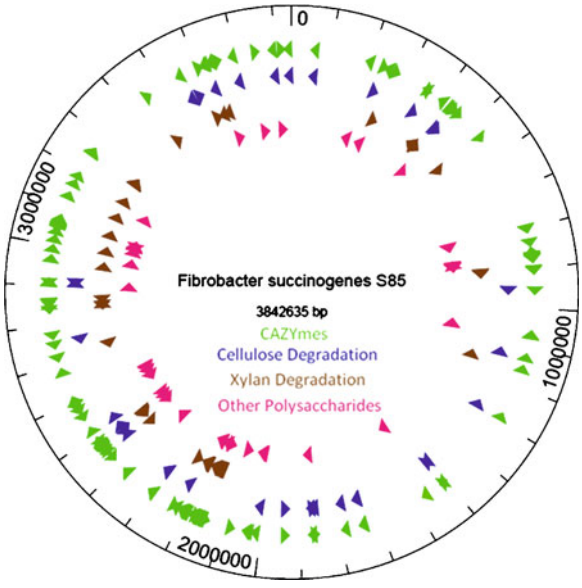

Fig. 1 CAZYme Annotation of *Fibrobacter succinogenes* glycosyl hydrolases. *Arrows from outmost circle to innermost circle*: *1*, all annotated CAZymes; *2*, cellulose-degrading enzymes; *3*, xylan degrading enzymes; *4*, other carbohydrases. Classification of glycosyl hydrolases based on InterproScan and BLAST analyses of CAZy-determined GH family members

Legend: Arrows, from outmost circle to innermost circle, 1- all annotated CAZymes; 2 - cellulose degrading enzymes; 3- xylan degrading enzymes; 4 - other carbohydrases.

identified by sequence analysis of the inserts. Eliminating duplicates, 24 unique CAZy genes were found by functional screening (Table 1). This is significantly higher percentage than with the same screen performed on a *C. thermocellum* DNA library (unpublished data). Of these 24, only four (XynB, XynC, XynE, and EndB) were previously described in the literature. Of the remaining 20, eight are annotated as cellulases, xylanases, or *endo*-glucanases and would be expected to be captured by the substrates used in the screening. The remaining 12 are annotated to genes not expected to be captured in the screen, indicating either broad substrate specificity for the enzymes or a failure of the electronic annotation to correctly match sequence to function.

Two of the genes captured in the screen, Fisuc_1425 and Fisuc_1426, (Fig. 2) are the first GH family 45 bacterial enzymes identified by screening, and two of only 13 bacterial GH family 45 enzymes identified by DNA sequence (four are present in *F. succinogenes*). The GH family 45 enzymes are predominantly eukaryotic proteins and are distantly related to plant expansins.

Gene Amplification Results

A total of 48 genes were selected for high-throughput amplification and expression of *Fibrobacter* CAZy genes. These genes included the 24 genes detected in the whole genome

Table 1 Enzymes obtained by random shotgun expression screening of *Fibrobacter succinogenes* DNA

Gene	Number of hits	Annotated product
Fisuc_0057	**1**	**Cellulase EndB**
Fisuc_0207	1	P37701 Endoglucanase 2
Fisuc_0362	**1**	**XynC**
Fisuc_0471	3	glycoside hydrolase family 8
Fisuc_0678	1	pectate lyase
Fisuc_0679	1	Pectin esterase
Fisuc_0730	2	Mannan endo-1,4-beta-mannosidase
Fisuc_0731	3	glycoside hydrolase family 57
Fisuc_1425	1	Cellulase—cel45A
Fisuc_1426	1	Cellulase
Fisuc_1764	1	Arabinoxylan arabinofuranohydrolase
Fisuc_1765	1	Glucuronoxylanase xynC
Fisuc_1773	1	Alpha-galactosidase
Fisuc_1774	1	Carbohydrate binding family 6
Fisuc_1793	**2**	**XynE Fibrobacter succinogenes S85**
Fisuc_1794	**2**	**XynB Fibrobacter succinogenes S85**
Fisuc_1932	1	Alpha amylase catalytic region
Fisuc_1991	1	Pectate lyase-like
Fisuc_2081	1	Coagulation factor 5/8 type domain protein
Fisuc_2201	1	Endo-1,4-beta-xylanase
Fisuc_2442	1	Endo-1,4-beta-xylanase
Fisuc_2579	1	Glycoside hydrolase family 8

Fibrobacter genes identified using the multiplex assay and the random shotgun library. Genes in bold are proteins that have been previously characterized in the literature

Fisuc_1425

MKYPLLRSLLTLGALALACSCSDDKSPNSGESSFVPCEDAWYLKDYSLLMYQDLKVTDLSGNEVGKLVPVQG
TLVAIVKDLSGNTIIPQVDLATTPVLTGDPTKCNANPKPQPSTAISSCIDAWYLAATKNYLLYADLTVTDEA
GTQVGTIVASSGSSLVNIVDLSGKPIINNIDLSKLPLITGDGVRYKILEPAFHLKDATGDYVIYQNTVVTKP
DGTPIGYADFATNSIKYIDQVTVLTTTSNILTLPILAPGGKCVDYAGVVASSSSTNPVYSSSSVYNPPNPNS
SSATPKSSSSKPKSSSSAPPPSSSSAPVTNQCPTIKTKGGGGSGWATRYWDCCKPHCSWPEHAGGNYSKQCT
NKGKTENTNWGDGSICSGGSQMTCTSQIPFTIDGCTEMAFAFAAVPAANGGQCGKCFQLTFTGTGKYSNDAN
IKRLKGKKLIIMATNVGGDVQQGQFDIMIPGGGVGIFNGCSSMGWGSQGAQYGGLLSDCETETKYAAGKYKS
CLTEKCNKSFANDEQARKGCLFLADWMGAAGNPEHNYVEVECPQVLKDKY

Fisuc_1426

MKNKLFKTLAIFGLSFIAWNCSDDPASAANNASDLTAVNAKPALEVDQSCWMITTGTQIF
LIVPNGTGTYLVTNEASVPVGTFDVATGTIVDANGAAVLTNVKLETLPVVNPDKTISYTD
GSKATIDGKTILLPGGIDPNAATTTPSTVVVASSSSAYIPSTTLPTVSSSSAKTQTPTPV
ASSSSQKTQQPVASSSSKQQTNSGTSDKQCNGQCYDSASGKCVAYYDQLTGSKGEKYAYD
NDCKVNCYYDPENKNCQNMTGSTPSQQPKSSSSVKSSSSQQQQQAKSSSSQQQQQPKSSS
SQPKSSSSQQQQQNNPNASAEEAKYLNAGAGGQQGFATRYWDCCMPHCSWPEHGGAAKTC
DAKGKTPISNTNGSICSGGQGTTCTSQIPIIVSDKLAYAFAATPGNDATCGKCFALTFTG
TGKYETKANHQALKGKTLVVMASNIGYDVQGGQFDIMIPGGGFGAFNGCSQMGWNIPQNT
TTYGGLLSDCEKEVGYNGNLLTLRKECLTKKCNSAFASDTQAKEGCLFLATWMEAAGNPN
HTYKEVECPAALKAKFH

Fig. 2 GH family 45 CAZYmes identified in random shotgun screen of *Fibrobacter succinogenes*

shotgun screen, a number of previously cloned and expressed genes and positive controls, and other potentially interesting genes. Amplification and cloning was done in a 96-well format, using the Lucigen Expresso™ kit. This can be done because the Expresso™ system eliminates the need for clean-up of PCR products or ligation of the PCR product to the vector. Of the 48 cloning reactions, 20 were evaluated for expression of active protein. Aliquots of the 20 transformations were plated and grown overnight, eight random colonies of each were picked and grown, and the cells were collected by centrifugation, lysed and assayed for activity. Of the 20 transformants selected, 17 were positive for the expected enzymatic activity. Of these 17, 11 were grown in 100 ml cultures, lysed and the enzymes were purified by Ni-NTA chromatography to >90% purity. A summary of the purification results is shown in Table 2.

Conclusions

Whole genome sequencing of *F. succinogenes S85* resulted in a completed assembly of 3.8 Mb in one contig. Annotation identified 3,087 candidate protein-encoding gene models for *F. succinogenes*, with 104 predicted glycosyl hydrolase genes, 12 predicted

Table 2 Enzymes obtained by PCR amplification of *Fibrobacter succinogenes* DNA

Gene	Annotation	Specific activity	Substrate
0057	**EndB (EGB)**	**1.5 u/mg**	**β-glucan**
0362	**XynC**	**56 u/mg**	**Xylan**
0471	Glycoside hydrolase family 8	0.2 u/mg	β-glucan
0678	Pectate lyase	1.6 u/mg	β-glucan
0897	**CelG**	**32.2 u/mg**	**β-glucan**
1794	**XynB (Xyn10B)**	**2.8 u/mg**	**Xylan**
1859	**CelE (End1)**	**26.4 u/mg**	**β-glucan**
2081	Coagulation factor 5/8 type domain protein	0.4 u/mg	β-glucan
2230	**Cel-3 (Eg3)**	**59 u/mg**	**β-glucan**
2442	Endo-1,4-beta-xylanase	3.3 u/mg	Xylan
2579	Glycoside hydrolase family 8	2.1 u/mg	β-glucan

Fibrobacter genes cloned from genomic DNA, expressed and purified as described in "Materials and Methods". Genes in bold are proteins that have been previously characterized in the literature. Enzyme activity measured as described in "Materials and Methods"

polysaccharide lyase genes, 17 carbohydrate esterase genes and 63 predicted CBMs in the *F. succinogenes* genome. The 104 predicted glycosyl hydrolase genes are arranged randomly throughout the genome, many in structures resembling operons. Phylogenetic analysis of the CAZy proteins using BLAST software shows little homology to other proteins outside the genus *Fibrobacter*.

Functional annotation of the genome was carried out using two methods. The first, random shotgun screening of the genome for *endo*-cellulase, *endo*-xylanase, *beta*-glucosidase, and *beta*-xylosidase positive clones, resulted in recovery of 169 positive clones, a very high hit rate of approximately 3%. Of these 169, 33 could be mapped to known CAZy genes in the genome and 24 unique CAZy genes were identified in the screen. The remaining 136 positive hits could not be linked to any known CAZy enzyme. A number of these may be false positives, and others may be mis-annotated or mis-assembled genes.

Of the 24 identified CAZy genes, only four had previously been reported in the literature, with the remaining 20 being novel enzymes. The reported electronic annotations of these 20 genes were misleading; of the 20 genes, only eight were annotated to their expected activities, with the remaining 12 being annotated to protein functions unrelated to the observed activities. This mis-annotation could be the result of broad substrate specificities in these proteins, or the existence of novel enzyme activities coupled to recognized structural components. With the substrates utilized, the random shotgun screen failed to detect any *beta*-glucosidase or *beta*-xylosidase genes. A total of 14 GH family 43 enzymes are present in the genome; many of these would be expected to have one of these activities. The failure to detect these family 43 enzymes may reflect an inability of the *Fibrobacte*r enzymes to recognize and hydrolyze the substrate analogs used in the assay. Work is continuing to identify the genes associated with the 136 activities that could not be mapped to known CAZy genes.

In a second approach to functional annotation, a high-throughput system was used to clone, express, and purify a total of 11 *Fibrobacter* enzymes, both previously reported and unreported enzymes. Specific activity results for the previously reported enzymes are in good agreement with the previously published values [14–16], showing the system reliably

generated authentic proteins. Many of the unreported enzymes show low specific activities; based on the results with the reported proteins it is unlikely that these low specific activities are an artifact of the high-throughput expression system. The low specific activities may reflect the choice of an incorrect substrate; a lack of some additional factor for maximum activity; or an inability to experimentally simulate the cell-localized environment in which the enzyme may typically operate. Cloning, expression, and purification of additional genes are continuing, with the goal of assembling a full set of biomass-degrading enzymes from the organism.

The CAZy analysis of the *F. succinogenes* genome offers few clues to the mechanism of crystalline cellulose degradation by the organism. The genome possesses no cellulases associated with either family 3 or family 2 CBMs, generally believed to be necessary for degradation of crystalline cellulose. This is in agreement with a published report that hydrolysis of crystalline cellulose is abolished by the deletion of two proteins, neither identified as a crystalline cellulose-degrading enzyme [6]. Published reports on *Fibrobacter* cellulases show poor activity on Avicel [17], showing that the annotated enzymes cannot explain the observed ability of the organism to rapidly degrade crystalline cellulose. This suggests the possibility that *F. succinogenes* may possess a novel mechanism for degradation of crystalline cellulose. A possible mechanism for crystalline cellulose degradation by *Fibrobacter* may involve a molecular motor similar to the actin-myosin system that utilizes hydrolysis energy to remove individual glucan chains from cellulose crystals and transport them through the outer membrane for cleavage. Such a system would be in agreement with electron micrograph views of *F. succinogenes* action on cellulose, which involves close physical contact and a lack of an extensive glycocalyx that would restrict movement of cells along the fiber axis (Fig. 3).

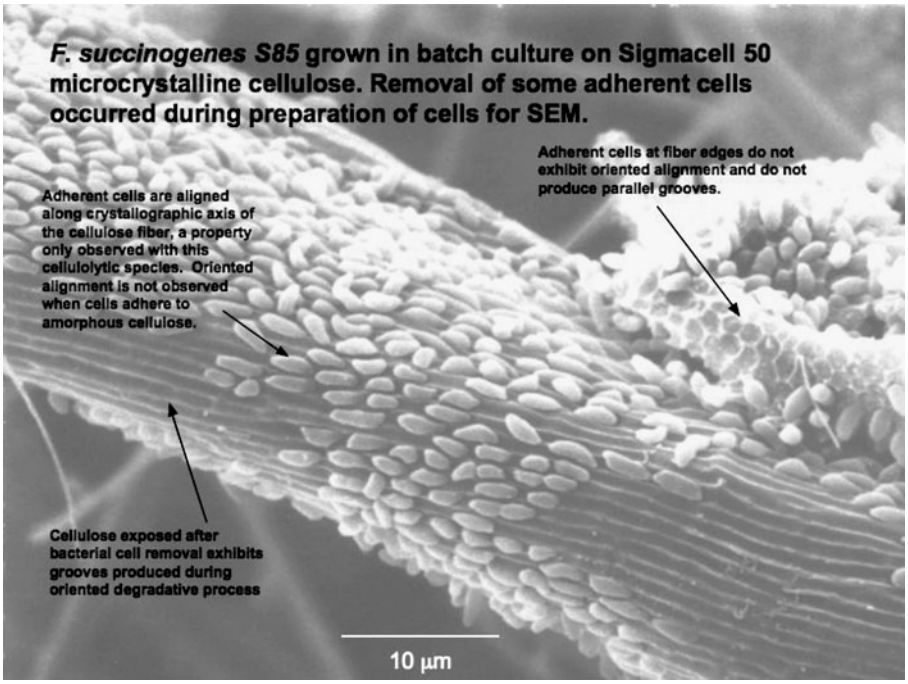

Fig. 3 Scanning electron micrograph of *Fibrobacter succinogenes* during degradation of crystalline cellulose

Acknowledgements This work was funded in part by DOE grant DOE DE-FG36-06GO16106, "Novel enzyme products for the conversion of defatted soybean meal to ethanol" and funded in part by the DOE Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494). The work conducted by the US Department of Energy Joint Genome Institute is supported by the Office of Science of the US Department of Energy under Contract No. DE-AC02-05CH11231.

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