AEM Accepts, published online ahead of print on 21 December 2007
Appl. Environ. Microbiol. doi:10.1128/AEM.02514-07

Genomic Differences Between *Fibrobacter succinogenes* S85 and *Fibrobacter intestinalis* DR7 Identified by Suppression Subtractive Hybridization

M. Qi[1], K.E. Nelson[2], S.C. Daugherty[2], W.C. Nelson[2], I.R. Hance[2], M. Morrison[3] and C.W. Forsberg[1*]
Department of Microbiology[1], University of Guelph, Guelph, ON, The J. Craig Venter Institute[2], Rockville, MD, Department of Animal Science[3], The Ohio State University, Columbus, OH

*Corresponding author

Department of Microbiology
University of Guelph
Guelph, Ontario
Canada, N1G 2W1
Email: cforsber@uoguelph.ca
Phone: (519)824-4120 Ext 53433
Fax: (519)837-1802

Abstract

Fibrobacter is a highly cellulolytic genus commonly found in the rumen of ruminant animals and cecum of mono-gastric animals. In this study, suppression subtractive hybridization was used to identify the genes present in *F. succinogenes* S85, but absent from *F. intestinalis* DR7. A total of 1082 subtractive clones were picked, plasmids purified, and inserts sequenced, and the clones lacking homology to *F. intestinalis* were confirmed by southern hybridization. By comparison of the sequences of the clones to one another and to the *F. succinogenes* genome, 802 sequences or 955 putative genes were identified that lack similarity with *F. intestinalis* chromosomal DNA and comprised approximately 409 kb of *F. succinogenes* genomic DNA. The functional groups of genes including those involved in cell envelope structure and function, energy metabolism, and transport and binding, had the largest number of genes specific to *F. succinogenes*. Low-stringency Southern hybridization showed that at least 37 glycoside hydrolases are shared by both species. A cluster of genes responsible for heme, porphyrin and cobalamin biosynthesis in *F. succinogenes* S85 was either missing or not functional in *F. intestinalis* DR7, which explains the requirement of vitamin B_{12} for the growth of the *intestinalis* species. Two gene clusters encoding NADH:ubiquinone oxidoreductase subunits probably shared by *Fibrobacter* genera appear to have an important role in energy metabolism.

KEYWORDS: Fibrobacter, Cellulase, Subtractive hybridization, Vitamin B_{12}

Introduction

Fibrobacteres is recognized as a main division (Phylum) within *Bacteria* that is closely related to the phyla of *Bacteroides* and *Chlorobi* (8, 19). *Fibrobacter* is the sole genus in this phylum. Bacteria of this genus are important anaerobic cellulose degraders and produce succinic acid as a major fermentation product (27). *Fibrobacter* strains were found in the rumen and ceca in cattle, sheep, horses, rats, pigs and other fiber consuming animals. Two species, *F. succinogenes* and *F. intestinalis,* were identified from phylogenetic analysis of 16S rRNA as well as phenotypic characterization. Considerable genetic diversity between the two species is apparent since they have 92% 16S rRNA similarity and less than 20% DNA-DNA similarity (4).

F. succinogenes S85 had been studied extensively because of its higher cellulolytic activity and important position in plant cell wall digestion in the rumen (9). More than one hundred carbohydrate active enzymes, including cellulases, xylanases, polysaccharide lyase, and esterases have been identified in the recently sequenced genome of *F. succinogenes* S85 (29). Recently, the gene coding for a major endoglucanase (*cel9B*) was identified, and three novel cellulases and two acetylxylan esterases were characterized (17, 32). Synergistic interactions were detected among the cellulases (32) and between a xylanase and the two acetylxylan esterases (17). In addition, genes coding for 13 cellulose binding proteins, which may be important for cellulose degradation, were identified in a proteomics study (16).

Previous studies of *F. intestinalis* identified two endoglucanases, one cellodextrinase and two xylanases, all with highest similarity to those in *F. succinogenes* S85 (7, 18). In addition, as in *F. succinogenes* S85 (11), a series of cellulose-binding proteins were also identified (26). Furthermore, *in vivo* ^{13}C NMR study of glucose and cellobiose metabolism in *F. intestinalis* and *F. succinogenes* has revealed marked homogeneity in their carbon metabolism (23). However, there are also some key differences between the species, notably, the sites of colonization within

the gastrointestinal tract that is favored by the two species, with *F. intestinalis* principally found or recovered from the ceca or hindgut of non-ruminant animals, including mice (3)

Suppression subtractive hybridization (SSH) (2) had been used to identify genes present in *F. intestinalis* DR7 that are absent from *F. succinogenes* S85 (33). Fifty five unique sequences were identified in *F. intestinalis* that do not exhibit detectable similarity to proteins either in *F. succinogenes* or GenBank. This study also showed that *F. intestinalis* encodes at least 30 plant cell wall degrading related proteins including 18 cellulases or xylanases, which have the highest similarity to those from *F. succinogenes*. Ninety of the sequences (including at least 30 transposases and 6 genes encoding restriction-modification systems) exhibit low or no homology to the *F. succinogenes* S85 genome. Furthermore, extensive genome reorganization was detected in *F. intestinalis* compared to *F. succinogenes*.

However, this study identified only genes that exist in *F. intestinalis* but were absent from *F. succinogenes*. To acquire a more in-depth appreciation of the genetic relatedness between the two species, and unique feature of the *Fibrobacter* genus, a further set of SSH experiments were conducted to identify genes in *F. succinogenes* that either do not exist or have low similarity to those in *F. intestinalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *F. succinogenes* S85 (ATCC 19169) and *F. intestinalis* DR7 (ATCC 43855) were grown under anaerobic conditions in a chemically defined medium (CDM) with 0.3% glucose as carbon source and carbon dioxide as gas phase at 37^{o}C as previously described (33).

To test the requirement for vitamin B_{12} by *F. intestinalis* DR7, 0.45 ml of CDM culture (OD675~1.0) was inoculated into 9 ml of CDM without vitamin B_{12} and was sub-cultured in the latter medium for *n* times (*n* ranged from 4~5 subcultures, see RESULTS section) until the cells stopped growing. Cells from the N-1 subculture were also inoculated into Vitamin B_{12}-substituted chemically defined media that lacked Vitamin B_{12}, but supplied with the same molar concentration of either of the precursors 5-aminolaevulinic acid (SIGMA) or porphobilinogen (SIGMA). The biotin requirement of *F. succinogenes* S85 was tested in CDM without biotin. In a separate trial both *F. intestinalis* and *F. succinogenes* were inoculated into the medium without either vitamin B_{12} or biotin and subcultured in the same medium for 10 times. Growth of the cells was monitored, in triplicate, by measuring the OD at 675 nm in a Lambda 2 spectrophotometer in a 1 cm cuvette.

DNA preparation. Genomic DNA was isolated from *F. succinogenes* S85 and *F. intestinalis* DR7 by the cetyltrimethylammonium bromide procedure as described by Ausubel *et al*. (5). Standard recombinant DNA techniques were performed as described by Sambrook and Russell (36).

Suppressive subtractive hybridization. SSH (2) was performed by using the Clontech PCR-Select Bacterial Genome Subtraction Kit as recommended by the manufacturer's instructions (Clontech, Palo Alto, CA). The resultant PCR amplicons from SSH that were enriched in *F. succinogenes* S85 specific sequences were purified by phenol/chloroform extraction and ethyl alcohol precipitation, followed by TA cloning into pGEM-T vector (Promega) and transformation into *Escherichia coli* JM109. The *E. coli* strains were then grown in LB medium supplemented with 100 µg/ml (w/v) ampicillin, and screened for clones using plates containing 1.6% agar 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 40 µg/ml 5-bromo-4-chloro-

3-indolyl-β-D-galactoside (X-gal). White colonies with inserts were restreaked for purity, and the plasmid inserts sequenced at The Institute of Genome Research (TIGR- now the JCVI) (Rockville, MD) as described by Qi *et al*. (33).

DNA dot blot analysis. DNA inserts from all the colonies selected were subjected to DNA dot blot analysis to identify false positives. Each of the clones was inoculated into 2 ml of LB medium containing ampicillin at 100 µg/ml and incubated at 37°C for 16 h at 150 rpm. Inserts from all of the SSH clones were amplified by PCR using nested primers 1 and 2R (Clontech Protocol) and Platinum Taq DNA Polymerase High Fidelity (Invitrogen) using 1 µl quantities of 16 h *E. coli* clones as the templates. The reactions were performed in 30 µl volumes for 25 cycles with an annealing temperature of 52°C. The PCR amplicons were purified by phenol/chloroform extraction as well as ethyl alcohol precipitation and the concentration determined using PicoGreen reagent (Molecular Probes) (1, 33). A 120 ng quantity of PCR amplicons from each clone was mixed with 400 µl 0.5 M NaOH and heated at 100°C and then spotted on duplicate Hybond N+ nylon membranes (Amersham) using a Bio-Dot SF Microfiltration Apparatus (BIO-RAD, Hercules, CA). Genomic DNA from *F. intestinalis* DR7 was digested with restriction enzyme Rsa I (Roche) and labeled with digoxigenin (DIG) using the Digoxigenin High-Prime labeling mixture (Roche). The hybridization and detection procedure that followed was carried out using 400 ng of the DIG-labeled genomic DNA. DNA dot-blots were pre-hybridized and hybridized at 60°C for high stringency or 37°C for low stringency as described by Sambrook and Russell (36). The DIG-detection was conducted following the manufacturer's instructions (Roche). PCR amplicons that hybridized with the *F. intestinalis* genomic DNA probes at high stringency indicated their high similarity to *F. intestinalis* genes. These sequences were treated as false positives, and were discarded. Amplicons that did not hybridize to *F. intestinalis*

genomic DNA at high stringency, but hybridized at low stringency conditions indicated that they had low sequence similarity to genes in *F. intestinalis*. Amplicons that did not hybridize to *F. intestinalis* genomic DNA at low stringency were treated as absent from *F. intestinalis.*

Sequence analysis. Insert sequences were used to query the *F. succinogenes* S85 genome by using the TIGR BLAST search program (http://tigrblast.tigr.org/ufmg/) and were subsequently mapped onto the genome. The translated amino acid sequences from the carbohydrate active enzyme genes in *F. succinogenes* were used to search against the GenBank non-redundant amino acid database (http://www.ncbi.nlm.nih.gov). The BLAST results were parsed and analyzed using Microsoft Excel 2000 and Visual Basic for Application, Version 6.3. Theoretical library size was estimated as described by Nesbo *et al.* (30).

RESULTS

Summary of the SSH library.

A total of 1152 clones were sequenced from both the 5' and 3' ends (Table 1). Sequences were assembled and redundant sequences were removed. By dot-blot analysis, 70 clones that hybridized with the *F. intestinalis* genomic DNA probe at high stringency (See Materials and Methods section) were treated as false positives and were removed. This resulted in 802 non-redundant sequences with an average length of 509.8 bp (409 kb in total). All of the sequences had 100% identity to those in the *F. succinogenes* genome. The G+C% of the unique clones was 47.3% (Table 1), which is slightly lower than the overall G+C% content of the *F. succinogenes* S85 (48.1%). Based on the incidence of duplicate clones, the theoretical library was estimated as 4100 clones with about 3100 unique clones. Therefore, assuming the average length of the clones was 510 bp, the DNA sequence present in *F. succinogenes* that was different from *F. intestinalis*

was 1581kb, or 41%, when taking the size of the genome (3843 kb) of *F. succinogenes* S85 into account.

Each of the SSH clones spanned up to three open reading frames (ORFs) in *F. succinogenes*, except for ten that resided in non-encoding regions. Altogether 955 ORFs were identified from the SSH clones (Appendix Table A1). Among the genes identified, 51.5% were not assigned any function. Of the remaining genes, those responsible for cell envelope metabolism, energy metabolism, as well as transport and binding were the most numerous (Table 2, Appendix Table A1).

The SSH sequences were mapped on the *F. succinogenes* genome (Figure 1) to determine whether there were any large contiguous regions of the genome that were absent from *F. intestinalis*. Most of the SSH sequences seemed to be randomly distributed along the chromosome, while some regions contained higher percentages of SSH sequences, indicating possible mutation or recombination hotspots.

Genes involved in energy metabolism.

Eighty-four genes in the functional category of energy metabolism showed low DNA similarity to the *F. intestinalis* genome. There were 69 SSH sequences that coded for 61 enzymes involved in plant cell wall degradation, including 19 cellulases and 16 hemicellulases, (Table 3 and Appendix Table A1). To determine whether the cellulases and hemicellulases identified by reverse SSH also exist in *F. intestinalis*, despite the observation that they diverged greatly in DNA sequence, a Southern dot blot analysis of the glycoside hydrolase genes at a low hybridization temperature was performed. Southern hybridization at low stringency showed that 32 of the 35 sequences identified by SSH hybridized to the *F. intestinalis* genomic DNA, indicating their existence in the latter genome. Only three glycoside hydrolase genes, which

overlapped in sequences RSGB_542 (FSU2294, GH10), RSGB_424 (FSU1894, GH45), and RSGB_704 (FSU2912, GH9) did not hybridize with the *F. intestinalis* genomic DNA at low stringency. Previously we had identified 18 cellulases or xylanases (33) in *F. intestinalis*. Thirteen of them were also identified in the present study. Assembling the results of the two SSH experiments together, a minimum of 37 glycoside hydrolase genes may be present in the *F. intestinalis* genome (Table 3). The non-redundant GenBank database was searched by the BLASTP program using the translated SSH cellulose gene sequences as the query. The names of organisms that contained glycoside hydrolases with the highest similarity to the respective *F. succinogenes* proteins are listed (Table 3).

Besides the glycoside hydrolase sequences, genes identified in the role of energy metabolism coded for proteins that are responsible for electron transport and fermentation. Among these, eight of 13 NADH dehydrogenase I subunits, which were in a cluster (FSU2661 to FSU2673, Cluster A), seemed to be either absent or diverged greatly in *F. intestinalis* (Appendix Table A1). Genes encoding the subunits of succinate dehydrogenase (FSU3061, FSU3062, FSU3070) were also identified.

Genes involved in transport and binding.

Fifty-five genes encoded proteins were involved in transport and binding. These included four proteins that were responsible for amino acid, peptide and amine transport, six involved with anion transport, two involved with carbohydrate transport, 20 responsible for cation and iron transport, and 21 other transporters of unknown substrate specificity.

Genes involved in DNA metabolism.

The genes involved in DNA metabolism included genes responsible for DNA replication, recombination and repair. Furthermore, there was a HU family DNA-binding protein that is known to be associated with the chromosome. However, no proteins responsible for DNA degradation were identified despite the previous characterization of an endonuclease in *F. succinogenes* S85 (20).

Genes involved in cell envelope metabolism.

The largest category of genes that differed from those in *F. intestinalis* encoded proteins for cell envelope metabolism. This included six genes involved in peptidoglycan biosynthesis, 35 involved in biosynthesis and degradation of surface polysaccharides and lipopolysaccharides (including at least eleven glycosyl transferases), and remarkably, there were 68 genes annotated as lipoproteins with unknown functions.

Genes involved in the biosynthesis of cofactors, prosthetic groups and carriers.

Twenty-three genes were identified that encoded proteins involved in the synthesis of cofactors such as biotin, folic acid, cobalamin (vitamin B_{12}), ubiquinone, pantothenate, pyridoxine and thiamine. Among these, a cluster of four genes that encoded enzymes responsible for cobalamin biosynthesis in *F. succinogenes* were identified (Figure 2). Three of them, *hemB* (FSU0299), *hemC* (FSU0303) and *hemL* (FSU0297), were shown to also exist in *F. intestinalis* DR7 as documented by low stringency Southern hybridization. However, the gene that encoded glutamyl-tRNA reductase (*hemA*) was not detected even at low stringency. Glutamyl-tRNA reductase catalyzes the conversion of glutamyl-tRNA to glutamate semi-aldehyde, which is known as the first step for cobalamin biosynthesis (34, 35) (Figure 2). In addition to the

cobalamin biosynthesis cluster, genes encoding biotin synthase (*bio*B, FSU1052) and para-aminobenzoate synthetase (*pab*B, FSU2014) were also identified.

Growth of *F. intestinalis* DR7 and *F. succinogenes* in the absence of vitamin B_{12} and/or biotin.

A supplementation experiment was carried out to determine if the absence of a functioning *hemA* gene is the basis for the inability of *F. intestinalis* DR7 to synthesize vitamin B_{12} (Figure 3). *F. intestinalis* DR7 was transferred from chemically defined medium to vitamin B_{12} free medium. After four subcultures in the latter medium (1:20 inoculum), *F. intestinalis* DR7 stopped growing. However, when the downstream product of glutamyl-tRNA reductase, 5-aminolaevulinic acid or porphobilinogen was added to the vitamin B_{12} free medium, growth of *F. intestinalis* DR7 was restored. A similar experiment was done with *F. succinogenes* S85, except using biotin free medium. After five subcultures, the growth ceased as well. However, when *F. intestinalis* DR7 and *F. succinogenes* S85 cells were mixed (1:1, determined by OD_{675}) and inoculated into the same medium without either vitamin B_{12} or biotin, the culture maintained growth even after 10 subcultures. The cells were harvested and subjected to PCR using both *F. succinogenes* and *F. intestinalis* DR7 specific primers and cells from the culture as the template. Both primer sets amplified DNA fragments with the corresponding sizes, indicating the two strains co-existed in the culture and provided for cross feeding of the missing intermediates.

DISCUSSION

The strains *F. intestinalis* DR7 and *F. succinogenes* S85 were shown to have 92% sequence similarity in 16S rRNA (27). The present study suggested approximately 41% of genes in *F. succinogenes* were either absent from or exhibited low similarity to those in *F. intestinalis*. Our

previous study indicated that 33% of genes were specific to *F. intestinalis* DR7 (33). These results indicated there are large differences between the genomes of the two species.

Despite the major differences between the DNA sequences of the two species, the cellulases and xylanases within *F. succinogenes* and *F. intestinalis* are well conserved. In our previous study (33), it was reported that most of the genes identified in *F. intestinalis* exhibited greater homology to those in *F. succinogenes* than to other organisms, which also indicates the close relationship between the two species. This is further supported by this study in that most of the *F. succinogenes* cellulases and xylanases identified in this study hybridized to *F. intestinalis* total genomic DNA at low stringency. Recently, our group characterized several glycoside hydrolases and these enzymes demonstrated synergistic interactions with cellulose degradation (32). Homologues of these *F. succinogenes* enzymes, including the two major endoglucanases Cel9B (endoglucanase 1) and Cel51A (endoglucanase 2), a family 5 endoglucanase Cel5H, a family 8 endoglucanase Cel8B and the chloride stimulated cellobiosidase (Cel10A) were all identified from the our SSH studies (Table 3 and Qi *et al*. 2005). Synergistic interaction may occur with these *F. intestinalis* cellulases as well. The conservation of the cellulases in the two species emphasizes the unique nature of the glycoside hydrolase system of the *Fibrobacter* genus.

Adhesion of *F. succinogenes* cells to cellulose appears to be a prerequisite for cellulose hydrolysis. Recently, thirteen cellulose binding proteins, which are thought to be important for cellulose adhesion, were identified from this organism (16). Seven of these CBPs were found to be present in *F. intestinalis* (data not shown). These findings indicate that the cellulose adhesion mechanism of the two species may be conserved as well.

Many of the glycoside hydrolases identified in *F. succinogenes* have highest similarity to the genes of *Cytophaga hutchinsonii, Clostridium thermocellum* and *Saccharophagus degradans* that belong to three distinct phyla, *Bacteroides, Firmicutes* and *Proteobacteria,* respectively.

Besides the 16S rRNA sequence and genomic content differences, there were several known phenotypic differences between the two species (27, 28). These differences include vitamin requirements of different strains. The SSH experiment identified four genes in a cobalamin biosynthesis gene cluster that are either missing or diverged greatly in *F. intestinalis* DR7. Figure 2 shows the cobalamin biosynthetic pathway that has been found in many bacteria. Cobalamin is involved as a cofactor in a variety of enzymatic reactions and is synthesized by some bacteria and Archaea (35). In the genome of *F. succinogenes*, the gene cluster responsible for uroporphyrinogen III biosynthesis and several genes in heme biosynthesis pathway were identified (Figure 2) which addressed the capability of *F. succinogenes* to synthesize vitamin B_{12} via this pathway. The genes encoding glutamyl tRNA reductase (*hemA*), glutamate-1-semialdehyde-2,1-aminomutase(*hemL*), porphobilinogen synthase (*hemB*) and porphobilinogen deaminase (*hemC*) were found to be missing or greatly diverged in *F. intestinalis* DR7. At low stringency, *hemA* did not hybridize with the *F. intestinalis* genomic DNA, which indicates that it might be absent from the genome. The missing genes would cause a block in synthesis of uroporphyrinogen III in *F. intestinalis*, which is the precursor of cobalamin (35). This finding was supported by restoration of growth of *F. intestinalis* by inclusion of either 5-aminolaevulinic acid or porphobilinogen in the medium. Interestingly, addition of porphobilinogen did not fully restore its growth, which may be due to the lack of a specific transporters/permease.

A large number of genes that differed from those in *F. intestinalis* encoded proteins for cell envelope metabolism. In addition many genes involved in biosynthesis and biodegradation of

cell surface polysaccharides and lipopolysaccharides were also identified in this and previous studies (33). These suggested substantial difference in the surface structure of the two species.

NADH:ubiquinone oxidoreductase complex I, is the first complex of the respiratory chain, which provides the proton motive force required for energy consuming processes like the synthesis of ATP (10). There are two sets of NADH dehydrogenase I subunits that form clusters (Cluster A: Fsu2661~Fsu2674; Cluster B: Fsu2886~Fsu2895) in the genome of *F. succinogenes* (29). The two clusters were on two different strands of the genome and separated by approximately 200kb. Interestingly, all eight genes that encode subunits B, C, D, E, G, H, I, M identified by SSH belonged to the same cluster (cluster A) while no genes in cluster B were identified. This may indicate that the genes in cluster B are highly conserved within the members of the *Fibrobacter* genus.

Our present and previous SSH experiments demonstrated that SSH is an effective approach to identify species-specific genes in the *Fibrobacter* genus. The strain-specific sequences identified may account for the different niches occupied by the two bacteria strains. Cellulases and hemicellulases identified in *F. intestinalis,* which have high similarity to those in *F. succinogenes*, indicate that bacteria in the *Fibrobacter* genus probably share similar mechanisms of plant cell-wall degradation. Finally, studies on the proteins with unknown functions, especially those conserved in the *Fibrobacter* genus but with no homology to other organisms, will also help us to understand additional features of this unique phylum.

ACKNOWLEDGMENTS

This research was supported by the Initiative for Future Agriculture and Food Systems Grant no. 2000-52100-9618 from USDA-CSREES, to the North American Consortium for Genomics of Fibrolytic Ruminal Bacteria, and by the Natural Science and Engineering Research

Council of Canada. The *Fibrobacter succinogenes* genome sequencing project was conducted at The Institute for Genomic Research (now the J. Craig Venter Institute), and also supported by the USDA-CSREES funds listed above.

SUPPLEMENTARY DATA

Table A1. DNA sequences in *Fibrobacter succinogenes* S85 that have low similarity to *F. intestinalis* DR7 as identified by suppressive subtractive hybridization.

Table A2. DNA sequences identified from this study.

Reference List

1. **Ahn, S. J., J. Costa, and J. R. Emanuel**. 1996. PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. Nucleic Acids Res. **24**:2623-2625.

2. **Akopyants, N. S., A. Fradkov, L. Diatchenko, J. E. Hill, P. D. Siebert, S. A. Lukyanov, E. D. Sverdlov, and D. E. Berg**. 1998. PCR-based subtractive hybridization and differences in gene content among strains of *Helicobacter pylori*. Proc.Natl.Acad.Sci.U.S.A **95**:13108-13113.

3. **Amann, R. I., L. Krumholz, and D. A. Stahl**. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J.Bacteriol. **172**:762-770.

4. **Amann, R. I., C. H. Lin, R. Key, L. Montgomery, and D. A. Stahl**. 1992. Diversity among *Fibrobacter* isolates - towards a phylogenetic classification. Syst.Appl.Microbiol. **15**:23-31.

5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Sidman, J. A. Smith, and K. Struhl**. 1994. Current Protocols in Molecular Biology, p. 2.4.1-2.4.5. *In* Greene Publishing Associates and John Wiley & Sons, Inc., NewYork.

6. **Bera-Maillet, C., V. Broussolle, P. Pristas, J. P. Girardeau, G. Gaudet, and E. Forano**. 2000. Characterisation of endoglucanases EGB and EGC from *Fibrobacter succinogenes*. Biochim.Biophys.Acta **1476**:191-202.

7. **Bera-Maillet, C., Y. Ribot, and E. Forano**. 2004. Fiber-degrading systems of different strains of the genus *Fibrobacter*. Appl.Environ.Microbiol. **70**:2172-2179.

8. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje**. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31**:442-443.

9. **Forsberg, C. W., E. Forano, and A. Chesson**. 2000. Microbial adherence to plant cell wall and enzymatic hydrolysis, p. 79-88. *In* P. B. Cronje (ed.), Ruminant physiology digestion, metabolism, growth and reproduction. CABI Publishing, Wallingford, Oxon.

10. **Friedrich, T. and D. Scheide**. 2000. The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. FEBS Lett. **479**:1-5.

11. **Gong, J. H., E. E. Egbosimba, and C. W. Forsberg**. 1996. Cellulose binding proteins of *Fibrobacter succinogenes* and the possible role of a 180-kDa cellulose-binding glycoprotein in adhesion to cellulose. Can.J.Microbiol. **42**:453-460.

12. **Huang, L., C. W. Forsberg, and D. Y. Thomas**. 1988. Purification and characterization of a chloride-stimulated cellobiosidase from *Bacteroides succinogenes* S85. J.Bacteriol. **170**:2923-2932.

13. **Iyo, A. H. and C. W. Forsberg**. 1994. Features of the cellodextrinase gene from *Fibrobacter succinogenes* S85. Can.J.Microbiol. **40**:592-596.

14. **Iyo, A. H. and C. W. Forsberg**. 1996. Endoglucanase G from *Fibrobacter succinogenes* S85 belongs to a class of enzymes characterized by a basic C-terminal domain. Can.J.Microbiol. **42**:934-943.

15. **Jun, H. S., J. K. Ha, L. M. Malburg, Jr., G. A. Verrinder, and C. W. Forsberg**. 2003. Characteristics of a cluster of xylanase genes in *Fibrobacter succinogenes* S85. Can.J.Microbiol. **49**:171-180.

16. **Jun, H. S., M. Qi, J. Gong, E. E. Egbosimba, and C. W. Forsberg**. 2007. Outer membrane proteins of *Fibrobacter succinogenes* with potential roles in adhesion to cellulose and in cellulose digestion. J.Bacteriol. **189**:6806-6815.

17. **Kam, D. K., H. S. Jun, J. K. Ha, G. D. Inglis, and C. W. Forsberg**. 2005. Characteristics of adjacent family 6 acetylxylan esterases from *Fibrobacter succinogenes* and the interaction with the Xyn10E xylanase in hydrolysis of acetylated xylan. Can.J.Microbiol. **51**:821-832.

18. **Lin, C. and D. A. Stahl**. 1995. Comparative analyses reveal a highly conserved endoglucanase in the cellulolytic genus *Fibrobacter*. J.Bacteriol. **177**:2543-2549.

19. **Ludwig, W. and K. H. Schleifer**. 2001. Overview: A phylogenetic backbone and taxonomic framework for prokaryotic systamatics, p. 49-65. *In* D. R. Boone and R. W. Castenholz (eds.), Bergey's Manual of Systematic Bacteriology. Springer-Varlag, Berlin.

20. **MacLellan, S. R. and C. W. Forsberg**. 2001. Properties of the major non-specific endonuclease from the strict anaerobe *Fibrobacter succinogenes* and evidence for disulfide bond formation *in vivo*. Microbiology **147**:315-323.

21. **Malburg, L. M., Jr., A. H. Iyo, and C. W. Forsberg**. 1996. A novel family 9 endoglucanase gene (celD), whose product cleaves substrates mainly to glucose, and its adjacent upstream homolog (celE) from *Fibrobacter succinogenes* S85. Appl.Environ.Microbiol. **62**:898-906.

22. **Malburg, S. R., L. M. Malburg, Jr., T. Liu, A. H. Iyo, and C. W. Forsberg**. 1997. Catalytic properties of the cellulose-binding endoglucanase F from *Fibrobacter succinogenes* S85. Appl.Environ.Microbiol. **63**:2449-2453.

23. **Matheron, C., A. M. Delort, G. Gaudet, and E. Forano**. 1998. *In vivo* ^{13}C NMR study of glucose and cellobiose metabolism by four cellulolytic strains of the genus *Fibrobacter*. Biodegradation **9**:451-461.

24. **McGavin, M. and C. W. Forsberg**. 1988. Isolation and characterization of endoglucanases 1 and 2 from *Bacteroides succinogenes* S85. J.Bacteriol. **170**:2914-2922.

25. **McGavin, M. J., C. W. Forsberg, B. Crosby, A. W. Bell, D. Dignard, and D. Y. Thomas**. 1989. Structure of the cel-3 gene from *Fibrobacter succinogenes* S85 and characteristics of the encoded gene product, endoglucanase 3. J.Bacteriol. **171**:5587-5595.

26. **Miron, J. and C. W. Forsberg**. 1999. Characterisation of cellulose-binding proteins that are involved in the adhesion mechanism of *Fibrobacter intestinalis* DR7. Appl.Microbiol.Biotechnol. **51**:491-497.

27. **Montgomery, L., B. Flesher, and D. Stahl**. 1988. Transfer of *Bacteroides succinogenes* (Hungate) to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes* comb. nov. and description of *Fibrobacter intestinalis* sp. nov. Int.J.Syst.Bacteriol. **38**:430-435.

28. **Montgomery, L. and J. M. Macy**. 1982. Characterization of rat cecum cellulolytic bacteria. Appl.Environ.Microbiol. **44**:1435-1443.

29. **Morrison, M., K. E. Nelson, I. K. O. Cann, C. W. Forsberg, R. I. Mackie, J. B. Russell, B. A. White, K. Amava, B. Cheng, M. Qi, H. Jun, S. Mulligan, K. Tran, H. A. Carty, H. Khouri, W. Nelson, S. Daugherty, and C. M. Fraser**. 2003. The *Fibrobacter succinogenes* strain S85 genome sequencing project. Abstract of 3rd ASM-TIGR Conference of Microbial Genomes. p.33.

30. **Nesbo, C. L., K. E. Nelson, and W. F. Doolittle**. 2002. Suppressive subtractive hybridization detects extensive genomic diversity in *Thermotoga maritima*. J.Bacteriol. **184**:4475-4488.

31. **Paradis, F. W., H. Zhu, P. J. Krell, J. P. Phillips, and C. W. Forsberg**. 1993. The *xynC* gene from *Fibrobacter succinogenes* S85 codes for a xylanase with two similar catalytic domains. J.Bacteriol. **175**:7666-7672.

32. **Qi, M., H. S. Jun, and C. W. Forsberg**. 2007. Characterization and synergistic interactions of *Fibrobacter succinogenes* glycoside hydrolases. Appl.Environ.Microbiol. **73**:6098-6105.

33. **Qi, M., K. E. Nelson, S. C. Daugherty, W. C. Nelson, I. R. Hance, M. Morrison, and C. W. Forsberg**. 2005. Novel molecular features of the fibrolytic intestinal bacterium *Fibrobacter intestinalis* not shared with *Fibrobacter succinogenes* as determined by suppressive subtractive hybridization. J Bacteriol. **187**:3739-3751.

34. **Raux, E., H. L. Schubert, and M. J. Warren**. 2000. Biosynthesis of cobalamin (vitamin B12): a bacterial conundrum. Cell Mol.Life Sci. **57**:1880-1893.

35. **Roth, J. R., J. G. Lawrence, and T. A. Bobik**. 1996. Cobalamin (coenzyme B12): synthesis and biological significance. Annu.Rev.Microbiol. **50**:137-181.

36. **Sambrook, J. and D. W. Russell**. 2001. Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

37. **Teather, R. M. and J. D. Erfle**. 1990. DNA sequence of a *Fibrobacter succinogenes* mixed-linkage beta-glucanase (1,3-1,4-beta-D-glucan 4-glucanohydrolase) gene. J.Bacteriol. **172**:3837-3841.

1 Table 1. Summary of *F. succinogenes* cloned DNA fragments enriched by SSH that did not
2 hybridize to *F. intestinalis* DNA under the conditions used.

Classification	Number
Number of Sequences:	1082
Unique sequences:	802
Average length (nts):	509.8
Total unique sequences (nts):	408823
Estimated library size (in clones):	4100
Unique sequences in estimated library:	3100
Total length of unique sequences in estimated library (nts):	1581 k
GC percentage:	47.3%

3

4

Table 2. Roles of the unique *F. succinogenes* genes identified by SSH that exhibit low similarity to those in *F. intestinalis.*

Functional Role(s)	**Number of genes** [a]	
Amino acid biosynthesis	17	(19.1%)
Biosynthesis of cofactors, prosthetic groups, and carriers	23	(26.7%)
Cell envelope	137	(33.8%)
Cellular processes	21	(33.3%)
Central intermediary metabolism	8	(29.6%)
DNA metabolism	22	(23.4%)
Energy metabolism	84	(40.2%)
Fatty acid and phospholipid metabolism	10	(30.3%)
Mobile and extrachromosomal element functions	5	(62.5%)
Protein fate	19	(25.0%)
Protein synthesis	26	(19.8%)
Purines, pyrimidines, nucleosides, and nucleotides	12	(24.0%)
Regulatory functions	22	(30.6%)
Signal transduction	16	(59.3%)
Transcription	13	(33.3%)
Transport and binding proteins	55	(32.5%)
Unknown function	497	(28.4%)
Total number of genes identified [b]	955	(29.4%)

[a] This table lists the functional roles of (each) genes identified, which shows the comprehensive random coverage of the genome by SSH. See Appendix Table A1 for genes in each category. Numbers in parentheses are the percentages of the genes identified versus total genes of each class in the genome.

[b] The total number of genes identified was less than the sum of the genes included in each function role (988) because some proteins had more than one role.

Table 3. Cellulases and xylanases genes in *F. succinogenes* (FS) and *F. intestinalis* (FI) identified by reverse and forward SSH using genomic DNA from FI and FS, respectively, as the drivers.

FSU#	Gene name [a]	Prior name [b]	FS Genes that share similarity to FI [c]	Genes unique to FS [d]	FI Genes that share similarity to FS [e]	Non-*Fibrobacter* Organisms of Top Blast match[f]
2622	Cel16A		+ (RSGB_633)			*Bacillus circulans*
1893	Cel45A		+ (RSGB_423)			*Cellvibrio japonicus*
1894	Cel45B			+ (RSGB_424)		*Cellvibrio japonicus*
1947	Cel45C				+	*Cellvibrio japonicus*
0382	Cel51A	CelF (22, 24)	+ (RSGB_074)		+	*Alicyclobacillus acidocaldarius*
1685	Cel5A		+ (RSGB_380)			*Cytophaga hutchinsonii*
2070	Cel5C	CedA (13)	+ (RSGB_476,477)			Unidentified bacterium
2290	Cel5E	Cel5K[g]				*Saccharophagus degradans*
2534	Cel5F		+ (RSGB_612)		+	*Orpinomyces joyonii*
2772	Cel5G	Cel-3 (25)	+ (RSGB_666,667)		+	*Cytophaga hutchinsonii*
2914	Cel5H				+	*Orpinomyces joyonii*
1346	Cel5K	CelG (14)	+ (RSGB_289)			*Saccharophagus degradans*
2866	Cel74A		+ (RSGB_694)		+	*Cytophaga hutchinsonii*
1680	Cel8A				+	*Cytophaga hutchinsonii*
2303	Cel8B		+ (RSGB_549)			*Cytophaga hutchinsonii*
3149	Cel8C		+ (RSGB_771)		+	*Cytophaga hutchinsonii*
2013	Cel9A		+ (RSGB_459)			*Xanthomonas axonopodis*
2361	Cel9B	CelE (21, 32)	+ (RSGB_564)		+	*Pseudomonas sp.*
2362	Cel9C	CelD [g](21)				*Cytophaga hutchinsonii*
2558	Cel9D		+ (RSGB_618)			*Vibrio parahaemolyticus*
2912	Cel9E			+ (RSGB_704,705)		*Cytophaga hutchinsonii*
0134	Cel9F		+ (RSGB_018)		+	*Bacillus pumilus*
0451	Cel9G	EGB [g] (6)				*Cytophaga hutchinsonii*
0809	Cel9H		+ (RSGB_149)		+	*Clostridium cellulovorans*
0810	Cel9I		+ (RSGB_150,151)		+	*Acetivibrio cellulolyticus*
0257	Cel10A	ClCBase (12)	+ (RSGB_042)			*Cytophaga hutchinsonii*
0226	Lic16C	LicA (37)			+	*Cytophaga hutchinsonii*
2181	Man26B		+ (RSGB_513)		+	*Clostridium thermocellum*

1165	Man26C		+ (RSGB_231)			*Piromyces sp.*
1166	Man5B		+ (RSGB_231)			*Saccharophagus degradans*
2292	Xyn10A	XynD (15)	+ (RSGB_541)			*Saccharophagus degradans*
2293	Xyn10B	XynE [g] (15)				*Cytophaga hutchinsonii*
2294	Xyn10C	XynB (15)		+ (RSGB_542)		*Saccharophagus degradans*
2851	Xyn10D		+ (RSGB_686,687)			*Cytophaga hutchinsonii*
1195	Xyn10F		+ (RSGB_237)		+	*Thermotoga neapolitana*
0777	Xyn11C	XynC [g] (31)				*Piromyces sp.*
2265	Xyn30A		+ (RSGB_532,533)			*Cytophaga hutchinsonii*
0206	Xyn30B		+ (RSGB_032)			*Clostridium thermocellum*
1373	Xyn39A		+ (RSGB_298)			*Clostridium acetobutylicum*
2263	Xyn43B		+ (RSGB_530)			*Clostridium thermocellum*
2264	Xyn43C		+ (RSGB_531)		+	*Clostridium thermocellum*
2520	Xyn43G		+ (RSGB_606)			*Bacteroides thetaiotaomicron*
2622	Xyn43J				+	*Clostridium thermocellum*
0651	Xyn8B		+ (RSGB_123)			*Cytophaga hutchinsonii*
0889	Xyn8C		+ (RSGB_172)			*Cytophaga hutchinsonii*

[a] The names of the genes as per the genome of *F. succinogenes* S85.

[b] Previous name of the gene and references were provided if applicable.

[c] Cellulase and xylanase genes identified in this study with *F. succinogenes* S85 as tester and *F. intestinalis* DR7 as driver that hybridized to DIG-labeled *F. intestinalis* genomic DNA in low stringency (Hybridized at 37°C) but not at high stringency (Hybridized at 60°C) in Dot-Southern Hybridization experiments. These genes are likely to be present in *F. intestinalis*, but have low DNA sequence similarity to their counterparts in *F. succinogenes*. The clone names of the genes identified were shown in parenthesis.

[d] Same as [c], except that these genes did not hybridized to *F. intestinalis* genomic DNA probe in either high or low stringency Southern-Blotting analysis, indicating they were absent from the genome of *F. intestinalis* DR7.

[e] Cellulase and xylanase genes identified from *F. intestinalis* in the Forward SSH experiment with *F. intestinalis* DR7 as tester and *F. succinogenes* S85 as driver (33). Although they had low similarity at the DNA sequence level, the amino acid sequences were quite similar to their counterpart in *F. succinogenes*.

[f] The amino acid sequences encoded were BLAST searched against the NCBI non-redundant amino acid database and the non-*Fibrobacter*-genus organisms containing a glycoside hydrolase with the highest score were listed.

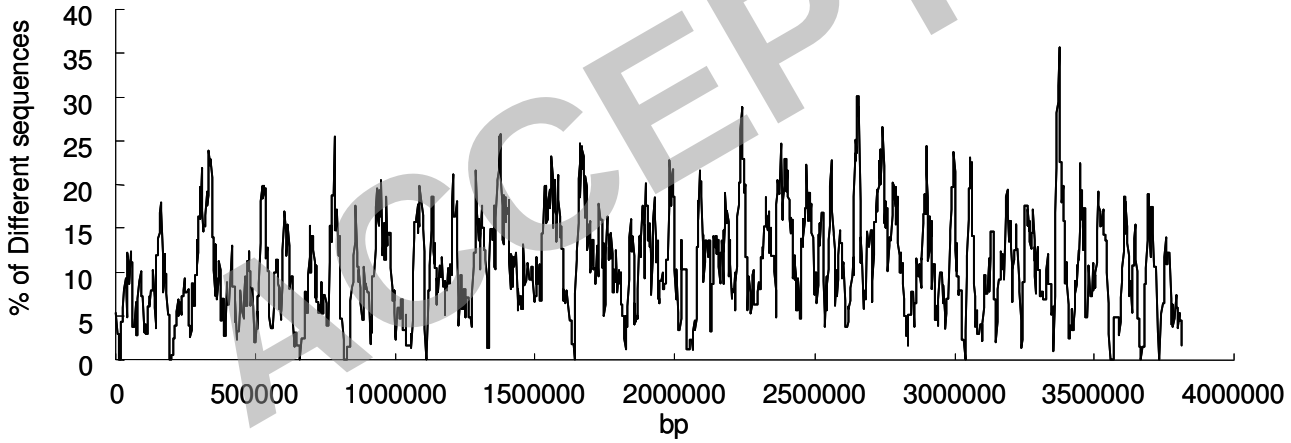
[g] These genes were not identified by either SSH experiments. However they were included to give a complete list of the cellulases and xylanases genes reported to date.

Figure legends

Figure 1. The distribution of the *F. succinogenes* specific genes on the chromosome of *F. succinogenes*. The x axis is the position on the chromosome. The y axis represents the percentage of the SSH sequences in each of a continuous 20 kb regions on the *F. succinogenes* chromosome.

Figure 2. A) Vitamin B_{12} biosynthetic pathway and genes involved in the first Five steps. B) Gene cluster for vitamin B_{12} biosynthesis in *F. succinogenes*. The genes highlighted by boxes (A) or cross-hatched arrows (B) are those with low homology to the respective counterparts in *F. intestinalis*. The *hemA* gene (filled arrow) was not detected under low stringency Southern Blotting.

Figure 3. Growth curve of *F. intestinalis* DR7 in Vitamin B_{12}-free media containing 5-aminolaevulinic acid (ALA, -■-) and porphobilinogen (PBG, -▲-); negative control of Vitamin B_{12}-free medium(-×-) and positive control of complete medium with Vitamin B_{12} (-O-) were also shown. Cells were subcultured in vitamin B_{12} free medium for 3 times before inoculated to the respective media. The growth rate for media with VB12, ALA and PBG were 0.171 h^{-1}, 0.137 h^{-1}, and 0.117 h^{-1} respectively. The mean ± standard deviation are illustrated by error bars.


% of Different sequences
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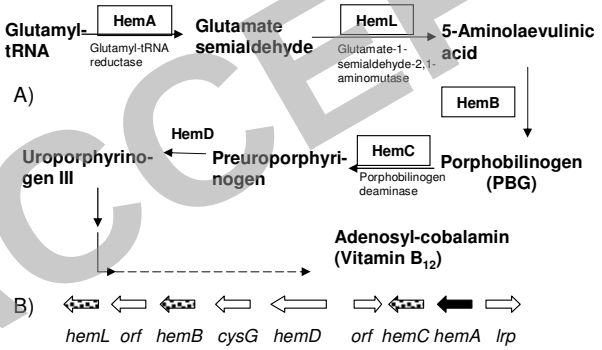
Glutamyl-tRNA
HemA
Glutamyl-tRNA reductase
Glutamate semialdehyde
HemL
Glutamate-1-semialdehyde-2,1-aminomutase
5-Aminolaevulinic acid
A)
HemB
HemD
Uroporphyrino-gen III
Preuroporphyri-nogen
HemC
Porphobilinogen deaminase
Porphobilinogen (PBG)
Adenosyl-cobalamin (Vitamin B12)
B)
hemL orf hemB cysG hemD orf hemC hemA lrp