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Cellulase Gene Expression in *Fibrobacter succinogenes* S85: Insights from Rams and Bucks

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Running title:

***Fibrobacter succinogenes* S85 cellulase gene in the Bucks vs. Rams**

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Abstract: In this study, two experiments were conducted. The first one was performed to isolate pure colonies of *Fibrobacter succinogenes* S85 from rumen liquor of adult rams and bucks. Samples were collected by stomach tube from 3-3.5 years old rams and bucks at the Agriculture Experimental Station, Faculty of Agriculture, Cairo University, under sterilized conditions. In the second experiment, cellulase gene alignment, PCR primer design and phylogeny tree for cellulolytic ruminal bacteria was performed. PCR and electrophoresis separations were done to assure the target strain to quantify the cellulase gene expression in *Fibrobacter succinogenes* S85 using Real Time PCR. At the end of the experiment, it was concluded that, the high digestion efficiency in bucks than rams are due to the cellulase gene expression in *Fibrobacter succinogenes* S85 derived from adult buck's rumen liquor being 3.24-fold higher than the same from adult ram's rumen liquor. Also, the phylogenetic tree clarifies the relationship between cellulolytic bacteria on basis of cellulase gene sequence alignment.

Key words: *Fibrobacter succinogenes* S85, cellulase gene expression, rams, bucks and real time PCR.

Introduction

Increasing global energy demand and the continuing depletion of fossil fuels has resulted in an urgent need to establish energy security through the exploration of fuel alternatives such as biofuels. Industrial scale biotechnological production of biofuels such as ethanol and butanol is a reality, but it is not sustainable, as the production process currently utilises food-based feedstocks. Non-food based lignocellulose biomass – comprising cellulose, hemicellulose and lignin – is an emerging sustainable feedstock alternative. The recalcitrant nature of lignocellulose necessitates a two-step process for biofuel production: (i) saccharification for the generation of fermentable sugars (pre-treatment) and (ii) fermentation to biofuels. The primary bottleneck in the production of economically viable lignocellulosic bio-based commodity chemicals is saccharification. Current industrial scale lignocellulosic biofuel generation is expensive, as the production process is heavily dependent upon energy-intensive physical and chemical saccharification steps. With more intensive research efforts, biological saccharification using lignocellulose-degrading microorganisms could be a viable alternative. Consolidated bioprocessing (CBP), i.e. use of native or recombinant microorganisms for both saccharification and fermentation, will be a major breakthrough for the realization of cost-effective and sustainable lignocellulosic biofuels [1].

Ruminants are hoofed mammals that have a unique digestive system that allows them to better use energy from fibrous plant material than other herbivores. Unlike monogastrics such as swine and poultry, ruminants have a digestive system designed to ferment feedstuffs and provide precursors for energy for the animal to use. Much of the plant biomass consumed by herbivores is degraded by symbiotic microorganisms in the host digestive tract. The rumen bacterial flora contains over 300 species that vary in their primary role and range of substrate utilized. Among the cellulolytic rumen bacteria, the most intensively studied species were *R. flavefaciens*, *R. albus* and *F. succinogenes* [2,3].

F. succinogenes S85 is an efficient lignocellulose degrader isolated from the rumen of herbivores. Intensive investigations over the past three decades indicates that *F. succinogenes* S85 uses an orthogonal lignocellulose degradation system compared to model lignocellulose-degrading microorganisms, as it does not possess either a cellulosome as seen in *Clostridium thermocellum* [4] or a free cellulolytic enzyme secretion system as seen in *Trichoderma reesei* [5]. A hallmark of F. succinogenes is its ability to efficiently hydrolyze many plant polysaccharides it encounters in the rumen, which include cellulose, pectin, starch, glucomannan, arabinogalactan, and various

forms of xylan, only cellulose was found to be both hydrolyzed and metabolized. A large number of other polysaccharides were found to be hydrolyzed without being metabolized [1].

Cellulose, a β-(1,4) homopolymer of glucose, is the most prevalent biopolymer, it is composed of very long, linear glucan chains, or microfibrils, with each individual chain containing between 3000 and 15,000 glucose units. Cellulose microfibrils, along with lignin and hemicellulose, comprise the plant cell wall. As a result of the uniform structure of the individual chains, cellulose is highly crystalline; this crystallinity gives structural rigidity to the plant cell wall. The effective and low-cost enzymatic conversion of cellulose to glucose is regarded as essential for the production of biofuels from biomass. Therefore, *Fibrobacter succinogenes* S85 is considered the most effective among the rumen bacteria in the utilization of cellulose from plant tissues [6].

There are four factors regulating ruminant fiber digestion which are (1) plant structure and composition, which regulate bacterial access to nutrients; (2) nature of population densities of predominant fiber-digesting microorganisms; (3) microbial factors that control adhesion and hydrolysis by complexes of hydrolytic enzymes of the adherent microbial populations, and (4) animal factors that determine the availability of nutrients through mastication, salivation and digesta kinetics [7,8].

The objectives of this study were to determine the effectiveness of ruminal bacteria in rams and bucks to clarify the main reasons for increased fiber digestibility in bucks compared to rams. Also to isolate one of the common cellulolytic bacteria, *Fibrobacter succinogenes* S85, from rams and bucks and to compare by molecular genetics the efficiency of cellulase gene in each of them.

Materials and methods

Two experiments were conducted in this study. The first one to isolate Fibrobacter succinogenes S85 from Egyptian rams and buck's rumen liquor, and the second for characterizing its cellulase gene expression and also phylogeny tree for cellulolytic ruminal bacteria was performed.

Experiment I: Isolation of Fibrobacter succinogenes S85 from rumen liquor

Rumen samples of about 0.5 kg were withdrawn after 4 hrs after feeding through the stomach tube from live mature rams and bucks, then kept in flasks that were filled to the top and plugged with rubber stoppers to minimize the air contact with the samples. Those samples were used for

isolation of cellulolytic anaerobes after enrichment of serum bottle 250 ml capacity filled with, the modified, medium 10 supplemented with cellulose powder as the sole carbohydrate source.

The medium and the anaerobic dilution solution were prepared under oxygen-free gassing, according to Hungate technique [9]. All ingredients were dissolved in distilled water and boiled at least for 10 min under a stream of oxygen-free CO_2, and cooled under the same phase. The final pH was adjusted at 6.8 – 7.0 and autoclaved at 121°C for 20 min. The ingredients of the medium were prepared according to Holdman *et al.*, 1977 [10].

To avoid precipitation, $CaCl_2$ and $MgSO_4$ were mixed first in 300 ml distilled water until it was completely dissolved. Then 500 ml distilled water were added and the remaining salts were slowly added while swirling. Then, the volume was adjusted to one liter (sterilized distilled water). To prepare the dilution blank solution, 50 ml of the salts stock solution, plus 50 ml water containing 0.2 g gelatin and 0.4 ml of resazurin 0.025% solution were mixed together. The solution was usually still slightly pink even after adding the 0.05%, of the reducing agent cysteine. It was added after cooling.

The two required gases for anaerobes are nitrogen and carbon dioxide. These gases must be purified to be oxygen free, in the sense that oxygen content should be below one ppm, for this purpose, Copper furnace system was used

All procedures and tools were accomplished under oxygen free gassing and sterilization. The fresh rumen contents were used in the isolation trails. The large particles of the contents were avoided after gravity sedimentation. A series of dilution tubes from 10^1 to 10^5 were prepared using 9 ml anaerobic dilution blank solution per tube. One milliliter of clear rumen fluid was used to inoculate 9 ml of the first dilution and thoroughly mixed. Each dilution was used to inoculate the agar roll tubes, which contained 3 ml of the medium no 10 with 2% agar and 5% cellulose powder as the sole carbon source. The agar tubes were kept in a water bath at 47-49°C. Five agar tubes were inoculated from each dilution with 1 ml per tube. The inoculated tubes were plugged with butyl rubber stoppers under oxygen free carbon dioxide and crimped with an aluminum cap as recommended by **Hungate, 1966 [11].** Isolation of rumen bacteria was performed using the roll tube method of **Hungate (1950) [12]** as illustrated by **Shockey and Dehority (1989) [13].**

Experiment II: cellulase gene alignment, PCR primer design and phylogeny tree for cellulolytic ruminal bacteria

This experiment was carried out in the Molecular biology (Rumen Biotechnology) lab belonging to the project of "Production of Transgenic Aerobic Cellulolytic Microflora to Utilize Farm Waste" Animal Physiology lab, Animal Production Department, Faculty of Agriculture, Cairo University and the Center of Excellence of Biotechnology Research, King Saud University, Saudi Arabia.

Cellulase gene alignment and PCR primer design.

Multiple sequence alignment was performed by CLUSTAL W Multiple Sequence Alignment Program [14] on three species of cellulolytic bacteria, using default parameters. The PCR primer was tested by sequence manipulation suite software [15], to indicate each primer location on the cellulase gene. Also PCR designed primer was verified by *in silico* PCR software included in Fast PCR V6.0 package and PCR primer stats in the package of sequence manipulation suite Java script programs.

Cellulase gene PCR product was calculated utilizing NCBI primer BLAST server http://www.ncbi.nlm.nih.gov/tools/primer-blast on the genome of *Fibrobacter succinogenes* S85. Furthermore using this solution for screening any further matches with the designed primers on the most of culturable microorganisms.

Nucleotide sequence alignment phylogeny analysis was performed by the same program and drew by phylodendron phylogenetic tree drawing software version 0.8d [16].

Primer design for *Fibrobacter succinogenes* S85

Designed primer calculation resulted as NCBI primer BLAST server output at molecular weight of 557 bp single and *Fibrobacter.succinogenes* S85 specific product in the putative sequence of cellulase gene (Bioneer corporation, Korea).

primer structure was designed as follow: Forward primer CGCAGGCGCCAACGAACC

Reverse primer CGCGGCCCTGCAAATGGAG. in addition, the reference gene was GAPDH housekeeping gene with forward primer: CGACCACTTTGTCAAGCTCA and the reverse: GAGGGTCTCTCTCTTCCTCT.

Total RNA isolation and purification for *F. succinogenes* S85

The total RNA was extracted from 5 ml of filtrated fresh rumen liquid from 10 different samples (5 from rams and 5 from bucks) paralleled with standard strain of *Fibrobacter succinogenes* subsp. *succinogenes* S85 (GenBank: CP002158.1). The samples were centrifuged at speed of 14,000 rpm for 2 min. Pellet was re-suspended in 100 µl of Tris - EDTA buffer solution, at pH 8.0. This contained 0.4 mg/ml lysozyme, where lysozyme mixture was incubated at room temperature for 5 minutes prior to adding 75 µl of RNA lysis buffer (referring to protocol of SV total RNA isolation system, Cat#Z3101 Promega Corporation, USA). Then 350 µl of RNA dilution buffer, after mixing. Ethanol, 200 µl, 95% were added to the lysis mixture to increase the binding of RNA to silica spin column filter. The mixture was transferred to the assembled spin column for retaining the nucleic acid to the filter. It was then centrifuged at 14,000 rpm for 1 min before discarding the liquid in the collection tube of the spin column assembly.

The RNA washing steps was performed identically as the procedure of SV total RNA isolation system technical manual for removing organic contaminants plus bacterial genomic DNA and other organic contaminants.

cDNA synthesis for *F. succinogenes* S85

Purified mRNA was used as a template for producing a complementary strand of DNA (cDNA) which was used as a template for amplification in the real time PCR system detailed below. This process was done via reverse transcription reaction by utilizing reverse transcriptase enzyme which acts as polymerase such adding the deoxy-ribotriphosphate unit in the extended strand of DNA in complementary to the RNA template [17].

The reaction prepared in final volume of 25 µl contained 1 µg of purified mRNA, 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Cat.# M170A, Promega corporation, USA), 200 pmole dNTP mix (10 mM each), 1X M-MLV RT reaction buffer, 25 units Recombinant RNasin Ribonuclease Inhibitor (Cat.# N2511, Promega corporation, USA), and 100 pmole each specific primer. With noting that each RNA and primer mix were preheated at 70^{o}C for 5 min and checked on ice before adding to the reaction mix to prevent secondary structure from reforming of the single strands, the reaction was incubated at 42^{o}C for 1 hr.

Conventional PCR setup

Conventional PCR was used as a trail experiment prior to real time PCR experiment for testing the optimal thermal conditions for reducing the non-specific amplification which causes false interlaced results in the real time PCR experiment. Annealing temperature of the amplification cycles were adjusted at 56^{o}C in 35 cycles of amplification began with 95^{o}C of amplification for 30 seconds, 56^{o}C of annealing for 20 seconds, and 72^{o}C of extension for 30 seconds. Cycles were encircled between step of predenaturation at 95^{o}C for 3 min and step of finalizing elongation at 72^{o}C for 7 min [17].

Reaction was prepared in a final volume of 25 µl contained 1X of GoTaq® Colorless Master Mix (Cat# M7132, Promega corporation, USA), 10 pmol primer, and 1 µl from the previous reverse transcription reaction (cDNA) product.

Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) is the easiest and most common way of separating and analyzing DNA. The agarose gel, when completely polymerized, serves as a molecular sieve which, in the presence of electric current, separates DNA according to size or molecular weight. AGE is not only commonly used to visualize DNA but also for quantification or to isolate a particular band. The DNA is visualized in the gel by staining with of ethidium bromide (EtBr). EtBr binds strongly to DNA by intercalating between the bases and it is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light.

However, EtBr is reported to be highly toxic and carcinogenic, so it should be handled with extreme awareness and care. In an attempt to find safer and non-toxic substitutes for staining DNA, SYBR safe was developed as an alternative which is reported to be more sensitive and completely safe, [18].

Conventional PCR products were loaded in a gel medium contained 1 g of agarose in a 100 ml of 1X TAE buffer pre-melted at 100^{o}C before pouring it in the horizontal gel casting unit. The gel is polymerized by cooling at room temperature prior to soaking in 1X TAE buffer in the chamber of a horizontal electrophoresis unit. After PCR products loaded behind the DNA ladder well, electrophoresis voltage was adjusted as 2.5 volts per centimeter.

Gel was stained, post migration, by soaking in a pool contained 10 mg/l ethidium bromide dye powder in water. By staining, nucleic acids in the gel medium can be visualized under exposure of

ultraviolet transilluminator unit included in the UVB GelDoc-It® TS Imaging System (CAT#TS 310, UVP, LLC, USA).

Real Time PCR set up

Synthesized cDNA used as a template to analyze the level of gene expression representative for the quantity of expressed mRNA of the desired gene (Cellulase). Reaction was prepared in volume of 25 µl contained 1X of GoTaq® Colorless Master Mix (Cat# M7132, Promega corporation, USA), 10 pmol primer, and 1 µl from the previous reverse transcription reaction (cDNA) product. Also 1X Syber green reporter dye was included in the reaction to quantify the progression of amplification.

Amplification cycles program is cited up in Applied Biosystems 7500 Instrument guide. First step of pre-denaturation was adjusted at 95^{o}C for 5 minutes followed by 40 cycles of amplification began at 95^{o}C for 30 seconds, 56^{o}C of extension of annealing for 20 seconds and 72^{o}C of extension for 30 seconds.

The finalization step, adjusted at 72^{o}C for 10 minutes, end point analysis cycles began with denaturation step at 95^{o}C for 30 seconds and annealing temperature at 70^{o}C for 30 seconds. The temperature increased by 0.5^{o}C per second of ramping for continuous scanning for the decreasing of the reporter dye until it reached 95^{o}C of complete denaturation.

The previous steps which are related to PCR were done at the Molecular Biology (Rumen Biotechnology) lab. and Rumen Microbiology lab., Department of Animal Production, Faculty of Agriculture, Cairo University, Egypt.

The step of Real Time PCR was done at the Center of Excellence of Biotechnology Research, King Saud University, Saudi Arabia.

RESULTS AND DISCUSSION

Experiment I: Isolation of Fibrobacter succinogenes S85 from rumen liquor

Isolation trials were carried out from the rumen contents withdrawn from living mature rams and bucks via stomach tube, four hours after feeding roughages in MBL.

The inoculated tubes were checked for colonies formation after one week of incubation at 39°C and up to one month. Colonies formation, due to the digested cellulose was used as an indicator for the pure strain of cellulolytic anaerobes Fibrobacter succinogenes S85 according to Varel and Yen (1997) [19]. After colonies formation; these colonies were Fibrobacter succinogenes S85.

Some tubes showed the presence of VFA production between vasper layer and the medium surface indicates a positive growth of Fibrobacter succinogenes S85 and been sured that was our target strain by staining with Gram stain and microscopic examination. The other tubes showed that there was no space between the media and the vasper indicates a negative growth. The negative growth tube by Gram stain and microscopic examination showed a mixture of ruminal bacteria.

Trials were made for further purification of the isolated anaerobic cellulolytic bacteria. Colonies were picked up anaerobically from agar roll tubes and Petri dishes, transferred into medium no. 10 with 5% cellulose powder as the sole carbon source and incubated at 39°C. Tubes were observed daily, revealing no clear zones were formed after incubation periods up to three weeks.

In fact, the cellulolytic bacteria are closely associated with non-cellulolytic bacteria as reported by many investigators [7,20,21]. Pochon (1941) [22] found that in fermenting cellulose, non-cellulolytic associated organisms are needed. The types of the mutual interactions between cellulolytic anaerobes and their associates were observed by Latham and Wolin (1979) [23]. They found that the growth efficiency of R. flavefaciens increased in the presence of Methanobacterium rumination.

Experiment II

Cellulase gene alignment and phylogeny tree for cellulolytic ruminal bacteria

Multiple sequences alignment was shown in Fig.1. and Table (1) indicated a high variable distance among cellulolytic bacteria on the bases of gene sequence. It is also confirmed by the phylogeny tree shown in Fig. 2. and digitally in Table 1. This variability in cellulase gene sequence was utilized to design the specific primer for *Fibrobacter succinogenes* S85 cellulase gene able to specific amplification to quantify mRNA of cellulase gene derived from *Fibrobacter succinogenes* S85 cells.

The phylogenetic tree relationship between *Acetovibrio cellulolyticus*, *Clostridium phytofermentans*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Ruminococcus cellulase* and *Ruminococcus albus.* Based on cellulase gene nucleotide sequence alignment is presented in Fig. 2 and Table 2. This clarifies the relationship between cellulolytic bacteria on basis of cellulase gene sequence alignment.

cDNA synthesis for *Fibrobacter succinogenes* S85

Complementary DNA strands produced by specific reverse transcription reaction to amplify Cellulase gene mRNA were obtained as a 556 bp molecular weight of the PCR product band (Fig. 3).

Real time PCR analysis for *F. succinogenes* S85

The Cycle threshold (CT) value-based comparative analysis of a cellulase gene as represented by *Fibrobacter succinogenes* S85 from a cellulase producing bacteria in the rumen is calculated in favor of buck cellulolytic ruminal bacteria. It showed an average CT value in average of 27.91. Ram cellulolytic ruminal bacteria showed an average of CT value 29.61. This indicates that buck rumen bacteria have higher expression of cellulase gene than ram rumen bacteria.

Amplification plot indicated highly expressed cellulase gene sample in the buck 4 sample (CT value = 22.86). On the other hand, the melt curve end point analysis represents single PCR products except some individuals with noisy background. In addition, four samples shown the same TM value at 87.1649^{o}C and the others around this TM is proved the reality of presenting single PCR product representing the cellulase gene.

Real time PCR analysis for *F. succinogenes* S85

The CT value-based comparative analysis of cellulase gene represented by *Fibrobacter succinogenes* S85 cells as cellulolytic bacteria in the rumen favored buck rumen bacteria. Results showed that buck rumen derived samples CT value had an average of 27.91. Ram rumen derived samples shown this average in value of 29.61. The lower or earlier CT value means a larger amount of starting cDNA copies. These confirm that buck rumen bacteria have higher expression of the cellulase gene than ram rumen bacteria. A mathematical model was used to compare the level of cellulase gene expression using the equation of Livak *et al.,* (2001) [24] as follows:

$$\textbf{Ratio}_{(\text{Sample1:Sample2})} = \mathbf{2}^{(\Delta \text{ Ct of Sample1} - \Delta \text{ Ct of Sample2})}$$

$$= \mathbf{2}^{29.6054 - 27.9099} = \mathbf{2}^{1.6955} = \mathbf{3.24}$$

Where CT is real time PCR cycle number at which detectable signal of reporter dye is achieved.

The previous equation result indicated that cellulase gene expression in *Fibrobacter succinogenes* S85 derived from buck rumen is **3.24-fold** higher than that derived from ram rumen. Amplification

plot Indicated that the highly expressed cellulase gene in the buck 4 sample (CT value = 22.8564). On the other hand, the melt curve end point analysis represents single PCR products except individuals background noises in both G3 and SH1 samples and not significantly affect the single peak shape of the melt curve. However, samples shown the same Tm value at 87.1649^{o}C and the others around this Tm which proved the reality of presenting of single PCR product represent the cellulase gene.

- **Ethical Approval:**

The study protocol has been reviewed and approved by the Faculty of African Postgraduate Studies – Cairo University ethics committee, ensuring that the research adheres to ethical guidelines and safeguards the rights and welfare of participants.

- **Consent to Participate**

I confirm that the authors have obtained consent from participants to participate in the study described in the manuscript. This ensures that participants have willingly agreed to be part of the research, understanding its purpose, procedures, and potential risks.

- **Consent to Publish**

I confirm that the authors have obtained consent to publish any potentially identifiable information or images included in the manuscript.

-Authors Contributions

Each author's role in the research, including conceptualization, data collection, analysis, and manuscript preparation, is thoroughly documented. This transparency ensures appropriate credit and accountability for the work presented.

- **Funding**

Not applicable

- **Competing** Interests

I confirm that the authors have adequately disclosed any competing interests in the manuscript. There are no conflicts of interest that could have influenced the research outcomes or interpretations presented in the study.

- **Availability of data and materials**

I confirm that the availability of data and materials is thoroughly addressed in the manuscript. The authors have provided clear information on data access, ensuring transparency and reproducibility of the study. All necessary materials are appropriately documented and readily available for further research.

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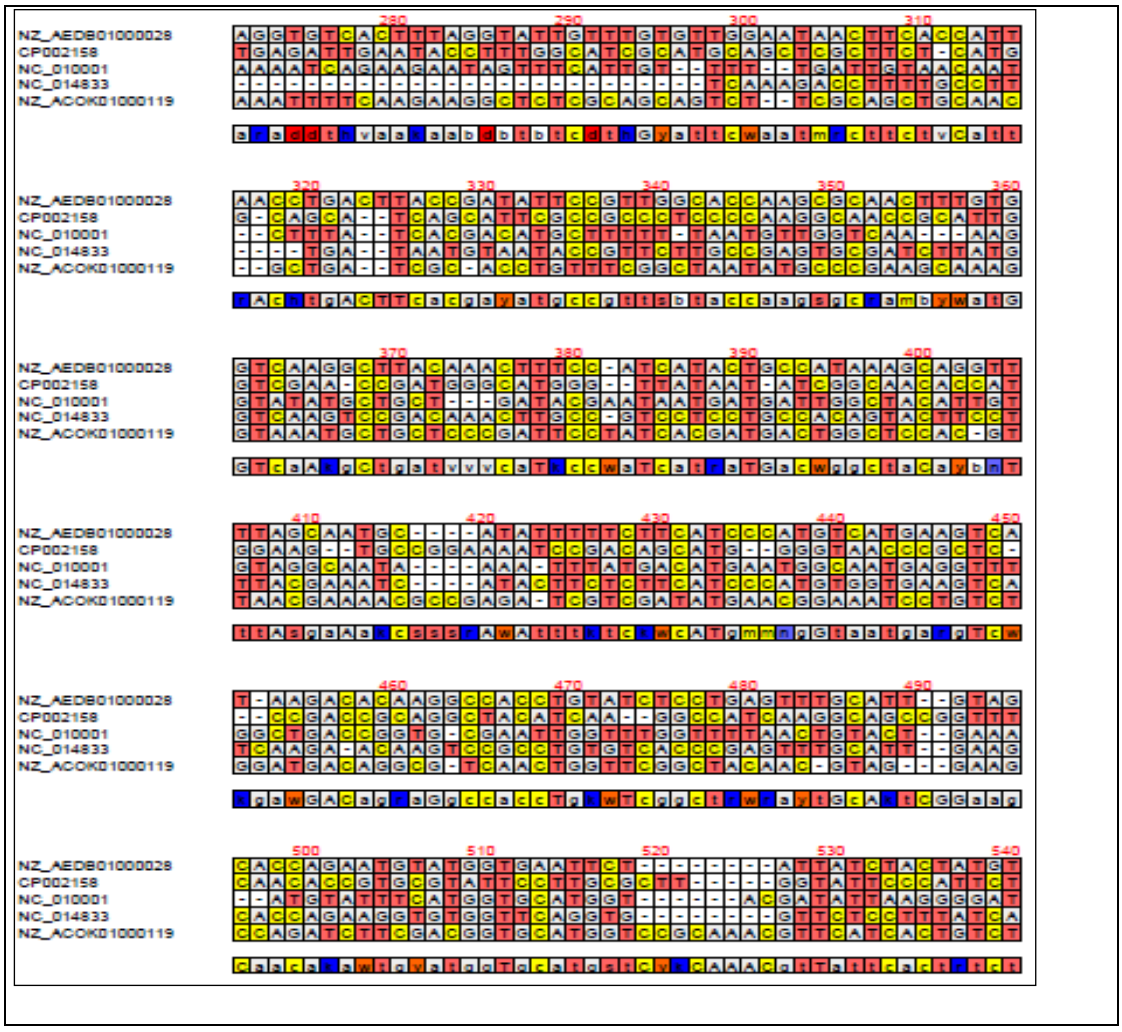
Table 1: Cellulase gene molecular weight of microorganisms used in multiple sequence alignment and its NCBI accession number

Accession No.	Organism	Molecular weight
NZ_AEDB01000028	Acetivibrio cellulolyticus	1653
CP002158	F. succinogenes S85	2181
NC_010001	Cl. phytofermentans	1407
NC_014833	Ruminococcus albus 7	1941
NZ_ACOK01000119	R. flavefaciens	2127

Table 2: Distance matrix of the phylogeny tree

Distance Matrix	NZ_AEDB010	CP002158	NC_010001	NC_014833
CP002158	1.0822			
NC_010001	1.0985	1.0405		
NC_014833	0.4976	1.2875	1.1793	
NZ_ACOKO01000119	1.0741	1.0166	0.541	1.2199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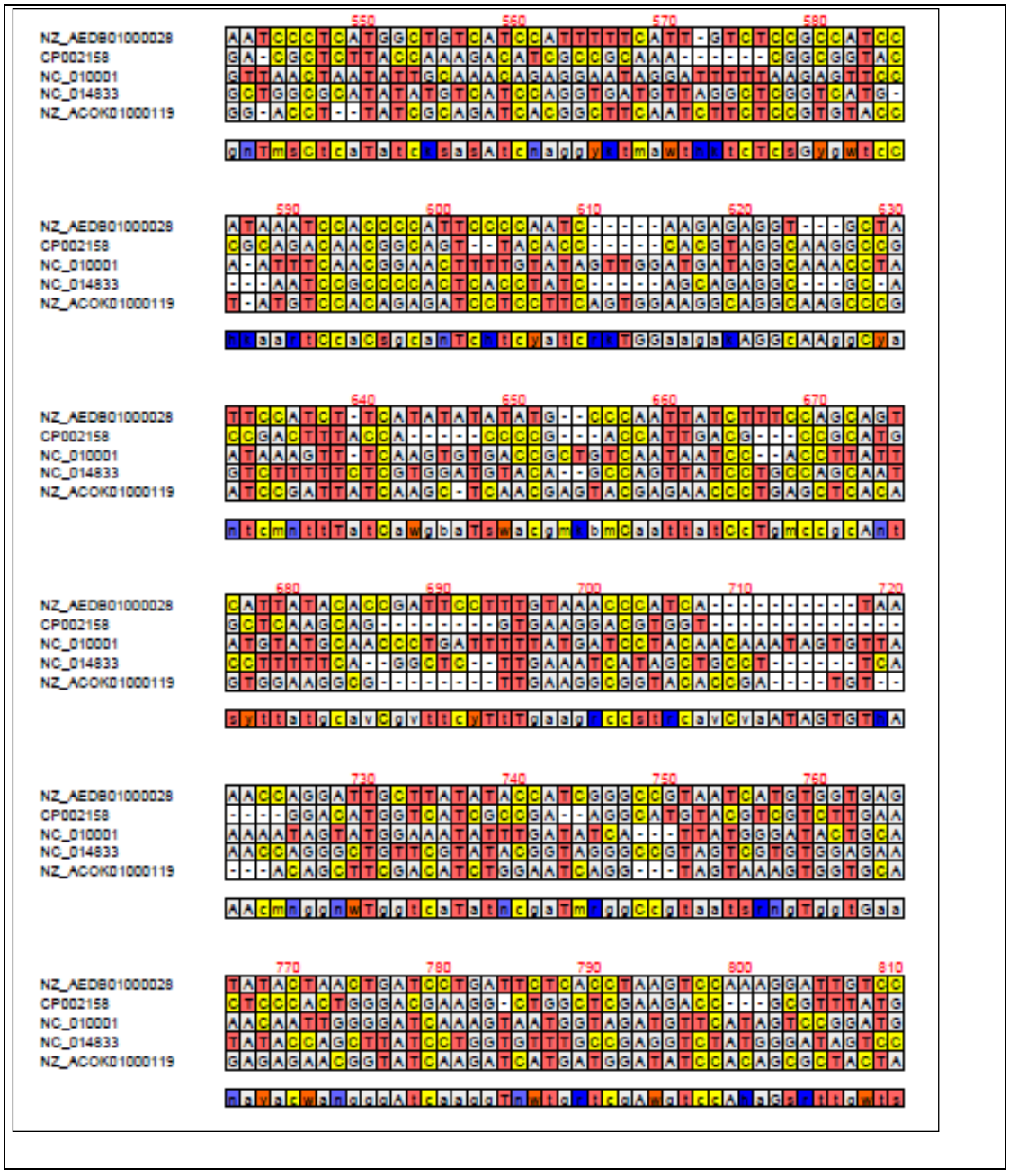
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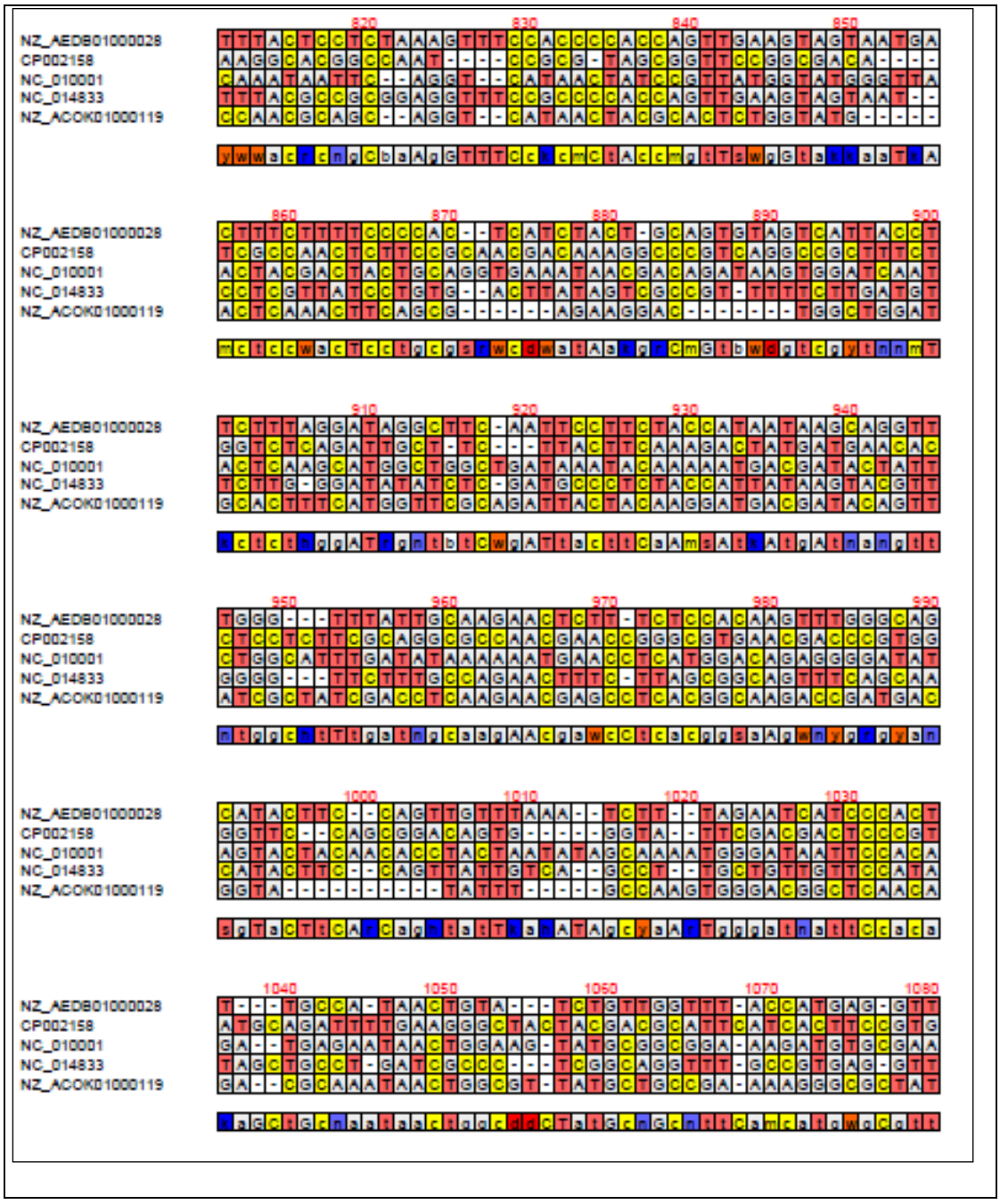
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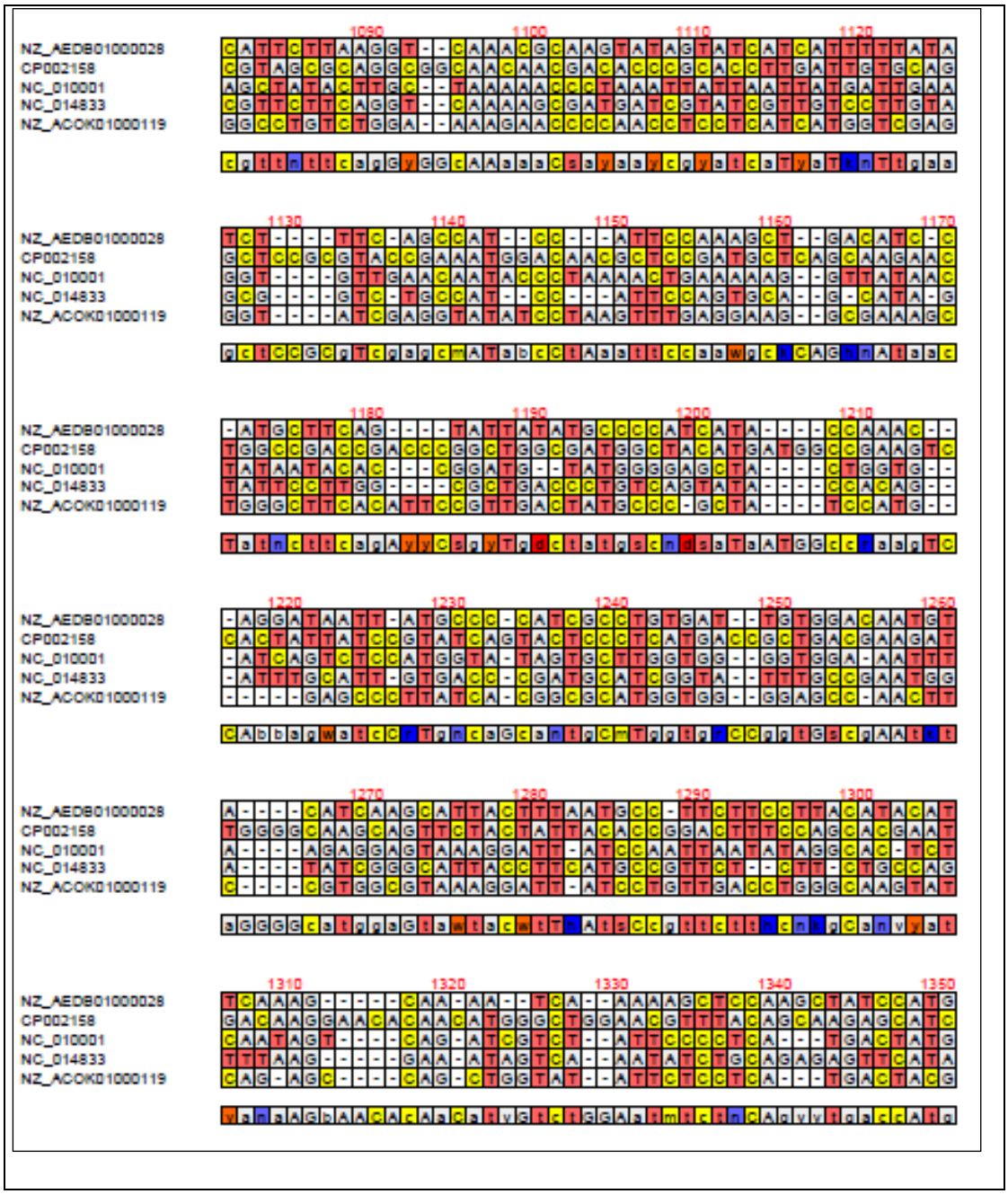
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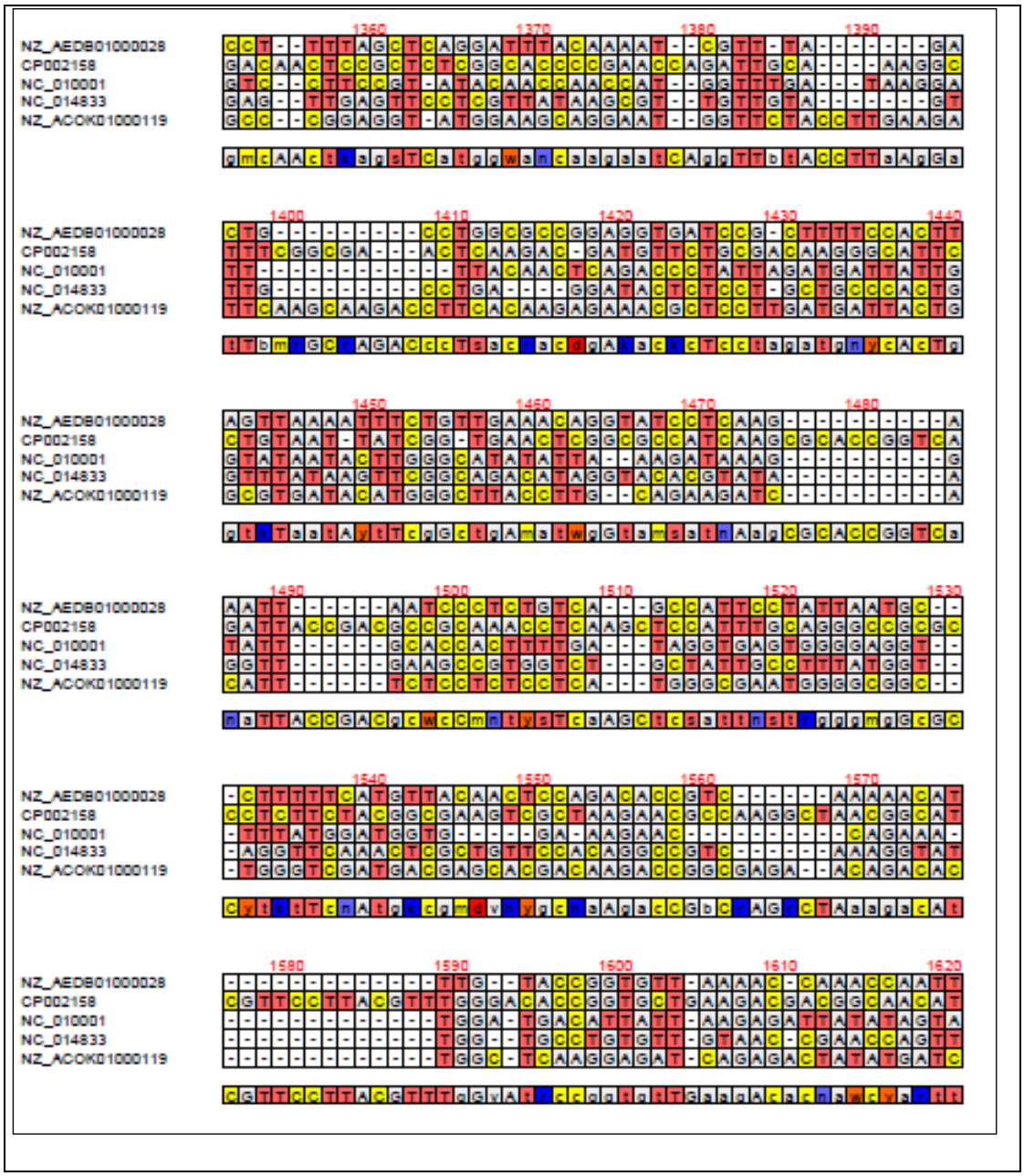
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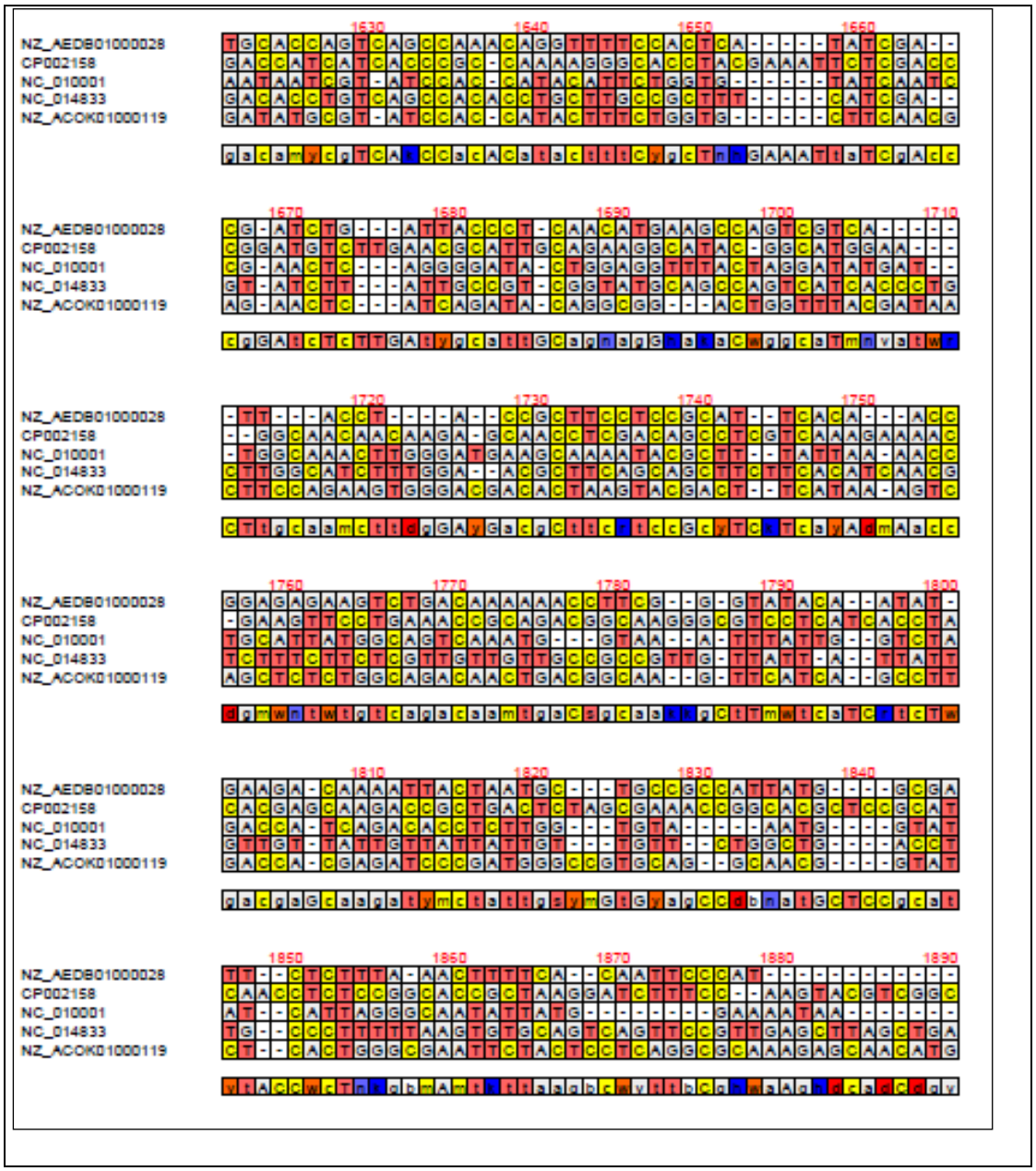
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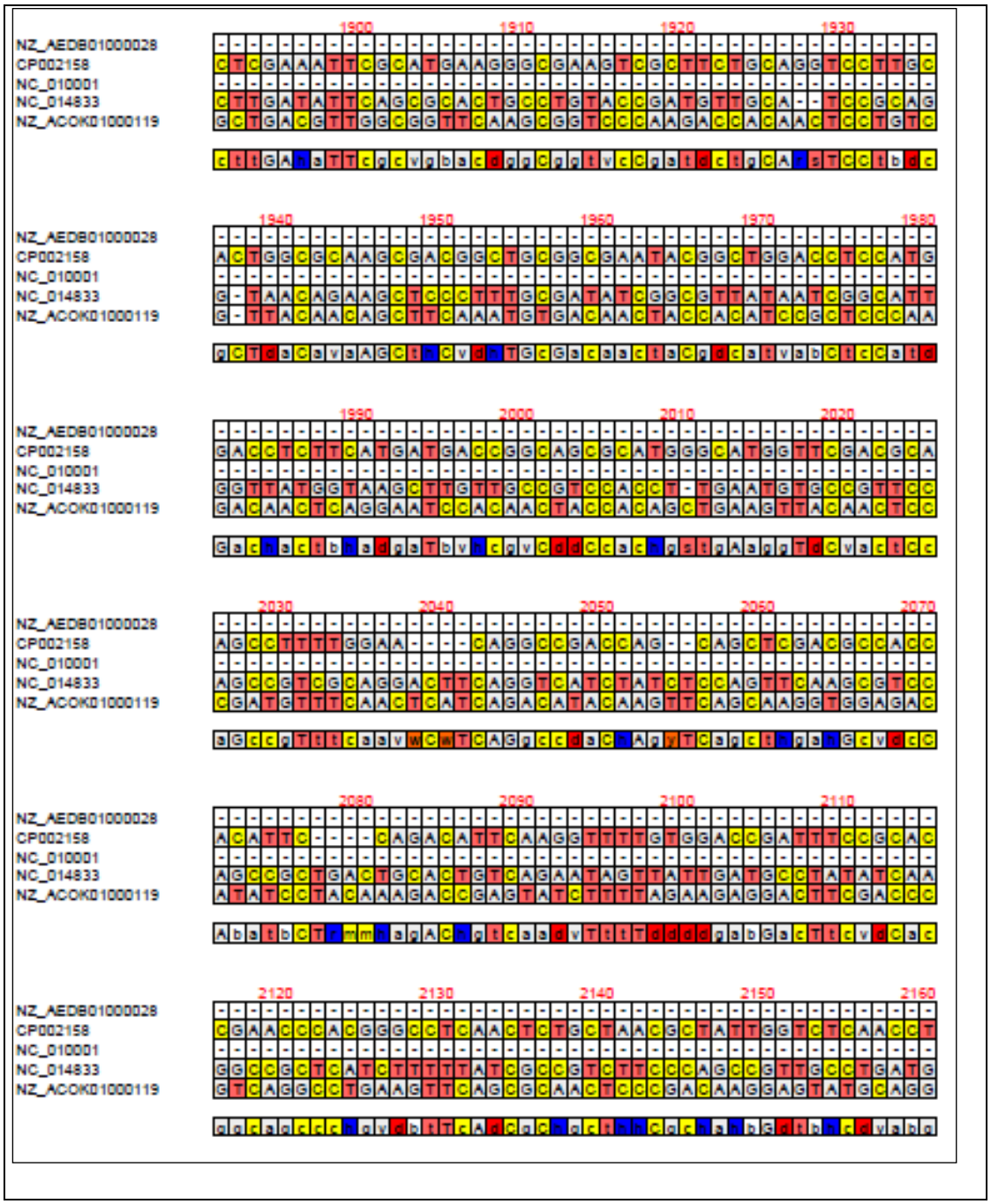
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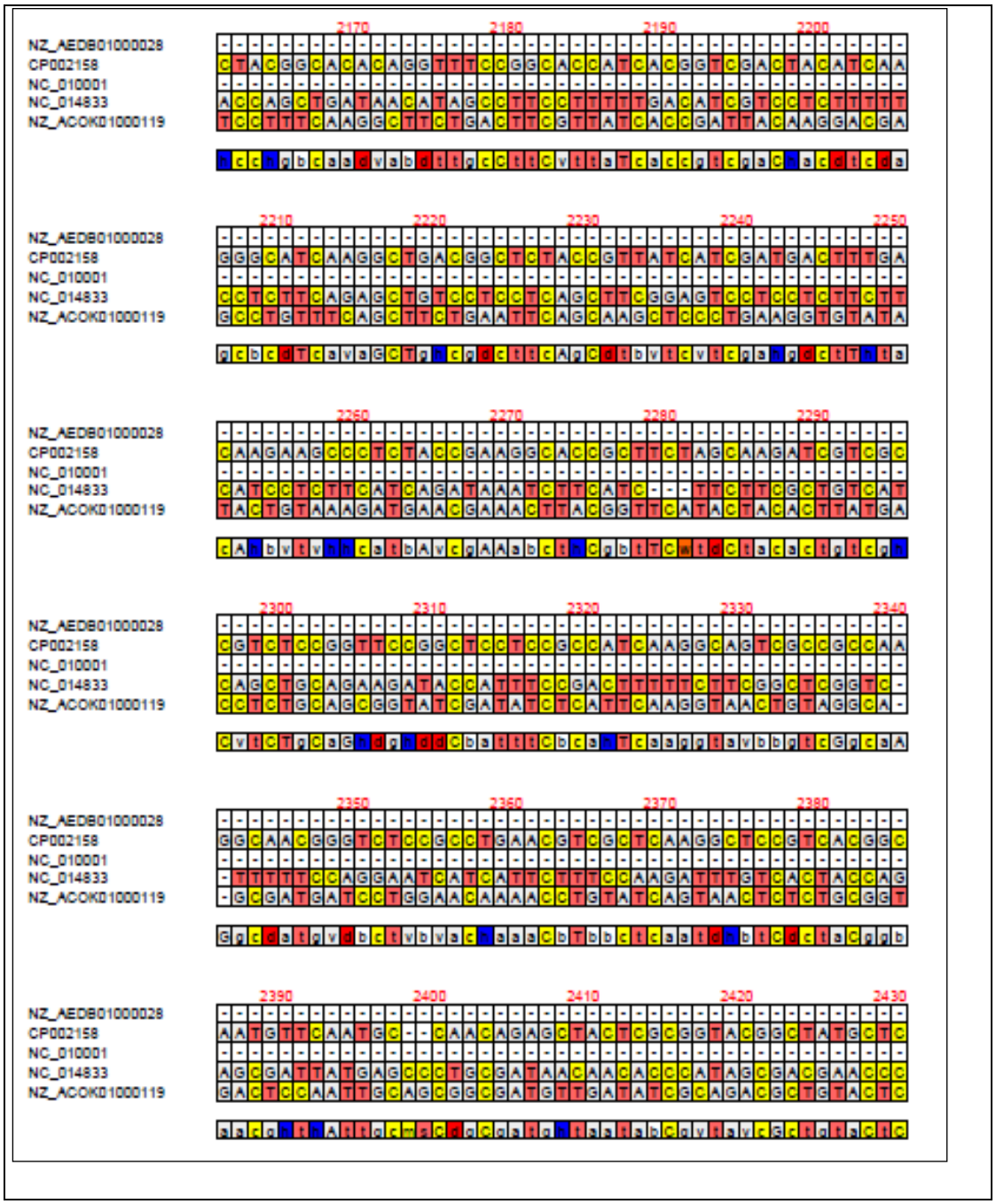
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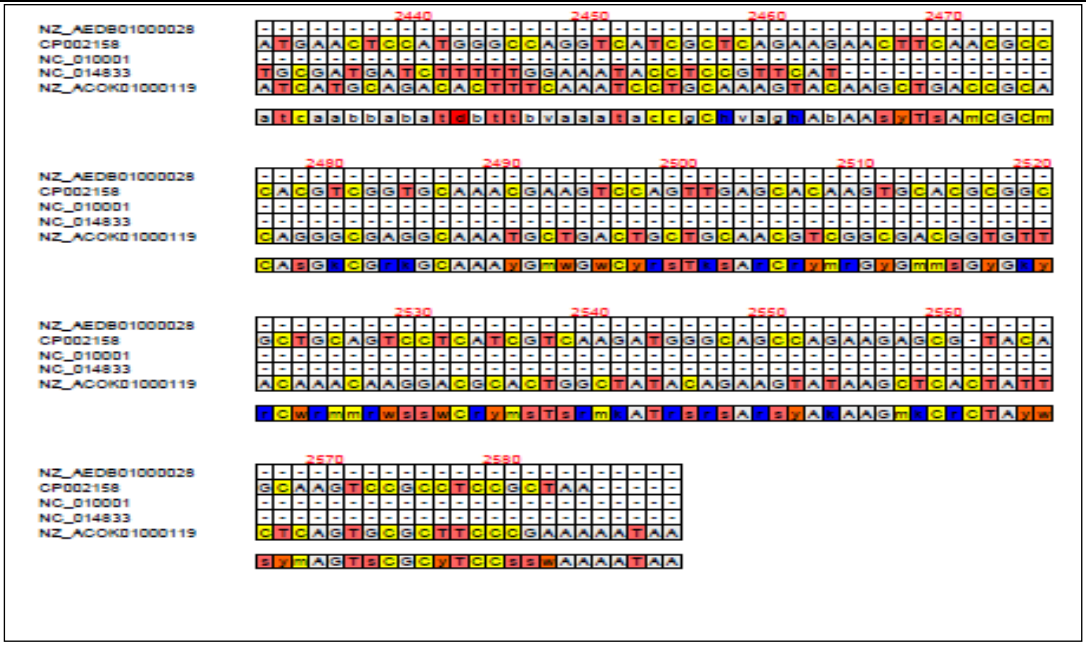

Figure 1: Clustal W Multiple sequence alignment of cellulase gene of *F. succinogenes* S85 isolated from rams and bucks' ruminal fluid.

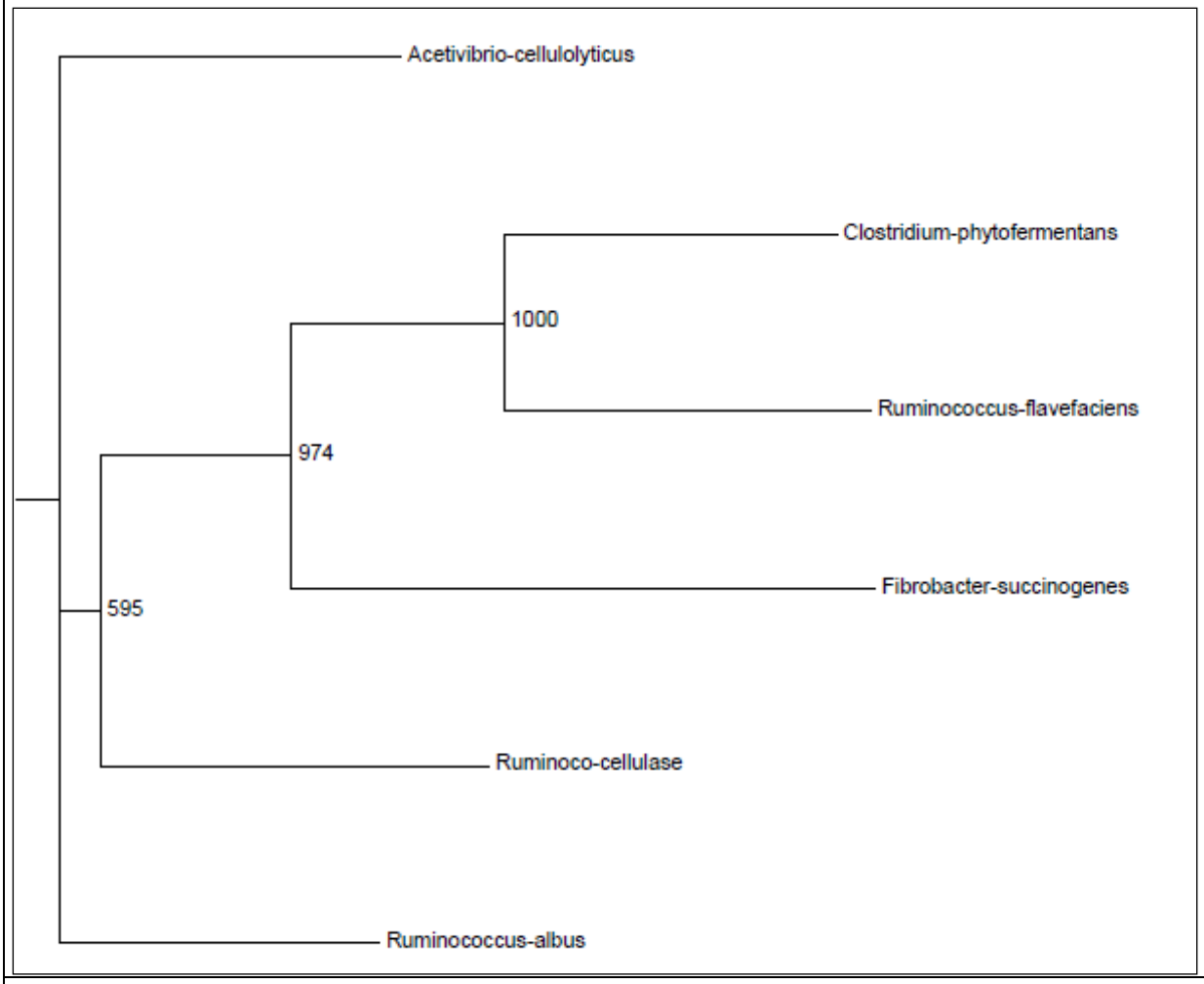

Fig. 2. Dendogram of the cellulolytic microorganisms used in the multiple sequence alignment.

Fig. 3. Primer optimization of the *F. succinogenes* S85 of ram and buck ruminal samples.

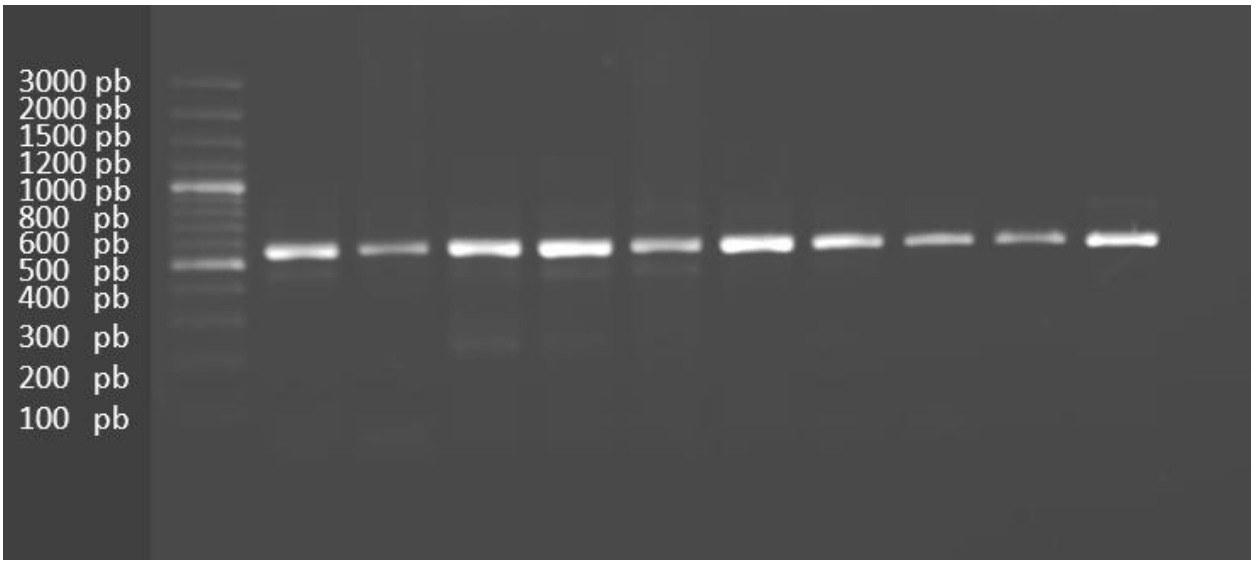

Fig. 4. PCR for ram and buck ruminal samples.