A Thesis

For the Degree of Master of Science

Characterization of a novel mucolytic bacterium, *Prevotella mucinisolvens* sp. nov., isolated from rumen epithelium of Korean cattle

한우 반추위벽으로부터 분리한 신종 뮤신 분해 박테리아 *Prevotella mucinisolvens* sp. nov.의 특성 규명

February 2020

By

Soo Jong Hong

College of Agriculture and Life Sciences

Seoul National University

Characterization of a novel mucolytic bacterium, *Prevotella mucinisolvens* sp. nov., isolated from rumen epithelium of Korean cattle

한우 반추위벽으로부터 분리한 신종 뮤신 분해 박테리아 *Prevotella mucinisolvens* sp. nov.의 특성 규명

지도교수 **백 명 기**

이 논문을 농학석사 학위논문으로 제출함

2020 년 1 월

서울대학교 대학원 농생명공학부

홍 수 종

홍수종의 농학석사 학위논문을 인준함

2019 년 12 월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

Mucin, a heavily glycosylated protein produced by epithelial cells, plays an integral role in lubrication to chemical barriers and protection against pathogens. Although commensal mucolytic bacteria have been reported that they improve host health, there is a lack of research on mucolytic bacteria. In this study, mucolytic bacteria were isolated from the rumen epithelial tissue of the dorsal sac of Korean cattle steer using a targeted cultivation on a mucin defined medium as a sole carbon source in anaerobic conditions.

A novel bacterium, designated strain Rep29^T, was isolated and characterized in terms of phenotypic, biochemical, and chemotaxonomic characteristics and phylogenetic position based on 16S rRNA sequence analysis. The 16S rRNA sequence analysis showed that strain Rep29^T belonged to a member of the genus *Prevotella* and was most closely related to *Prevotella brevis* GA33^T with 89.8% similarity. Strain Rep29^T was Gram-negative, obligately anaerobic, and non-motile coccus. Growth of strain Rep29^T was observed at 35-45°C (optimum, 39°C), at pH 6.0-7.5 (optimum, 7.0), and in the presence of 0.0–1.5% (w/v) NaCl (optimum, 0.0–0.5%). The major cellular fatty acids of strain Rep29^T were $C_{16:0}$, $C_{18:1}$ ω9c, and iso-$C_{15:0}$. The G+C content of genomic DNA was 47.7 mol%. Analysis of enzyme profiles using API Rapid ID 32A and API ZYM identification kit revealed that this strain gave positive reactions for *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, and *N*-acetyl-*β*-glucosaminidase related to mucin degradation. In addition, functional annotation of the whole genome sequences of strain Rep29^T showed that this bacterium had a putative mucolytic pathway. Strain Rep29^T is expected to utilize mucins by Sus-like systems and had the metabolism of galactose, GlcNAc, sialic acid (Neu5Ac), and mannose. Strain Rep29^T was identified the presence of putative extracellular

polysaccharide biosynthesis mechanisms via Wzx/Wzy-dependent pathway and synthase-dependent pathway and virulence factors for bacterial adhesion.

On the basis of the above-mentioned findings, strain Rep29^T represents a novel species of the genus *Prevotella*, the name *Prevotella mucinisolevens* sp. nov. is proposed; the type strain is Rep29^T (= KCTC 15980^T).

Keywords : Rumen microbiota, epimural bacteria, mucin, *Prevotella*, novel species
Student Number : 2018-22941

Table of Contents

List of tables

Supplementary table S1. List of predicted starch utilization system (Sus) genes in the complete genome of strain Rep29^{T}

List of figures

List of abbreviations

ABB : Anaerobe basal broth

ADS : Anaerobic dilution solution

BHI : Brain heart infusion

CBM : carbohydrate-binding module

CDS : Coding sequences

DNA : Deoxy ribonucleic acid

EDTA : Ethylenediaminetetraacetic acid

Fuc : Fucose

Gal : Galactose

GalNAc : *N*-acetylgalactosamine

GH : Glycoside hydrolase

GI : Gastrointestinal

GlcNAc : *N*-acetylglucosamine

LB : Luria-Bertani broth

ML : Maximum-likelihood method

MP : Maximum-parsimony method

Neu5Ac : *N*-acetylneuraminic acid

Neu5Gc : *N*- glycolylneuraminic acid

NGS : Next-generation sequencing

NJ : Neighbor-joining method

OD : Optical density

PCR : Polymerase chain reaction

PCR-DGGE : Polymerase chain reaction-denaturing gradient gel electrophoresis

PYG : Peptone-yeast extract-glucose

rRNA : ribosomal ribonucleic acid

SEM : scanning electron micrograph

Ser : Serine

Sus : Starch utilization system

TEM : Transmission electron micrograph

Thr : Threonine

tmRNA : transfer-messenger RNA

tRNA : transfer RNA

TSB : Trypticase soy broth

VNTR : Variable number of tandem repeats

Units and marks

°C : Degree Celsius

% : Percent

mol% : Mole percent

g : Gram

mg : Milligram

ml : Milliliter

N : Normality

h : Hour

nm : Nanometer

Ⅰ. Introduction

Rumen in the ruminant gastrointestinal (GI) tract harbors a diverse and abundant microbiota. Rumen microbes are essential for the proper physiological development of the rumen and the host's digestive ability. Most rumen bacteria float freely in the rumen fluid or attach to feed particles (Puniya et al., 2015). Only less than 1% of total rumen bacteria adhere to rumen epithelium, which is called "epimural" bacteria (adherent epithelial bacteria) (Cheng et al., 1979; Sadet et al., 2007; Mead and Jones, 1981). Regardless of a relatively minor composition, they play important roles in several metabolic process of the host, not in the fermentation of feeds (McCowan et al., 1980; Sadet et al., 2007). Epimural bacteria perform hydrolysis of urea, oxygen scavenging, and tissue recycling due to distinctive characteristics of rumen epithelial environment (Cheng et al., 1979).

A mucus layer covering the GI tract serves as a protective barrier against pathogens, as well as a selective barrier that allows specific things to pass through and a lubricative barrier to help transports smoothly (Rose and Voynow, 2006). Mucus is a viscous and hydrated gel layer that coated the epithelial surface of organs composed of heavily glycosylated proteins (glycans), called mucins. Mucins are composed of diverse combinations of oligosaccharides that are attached to the proline, threonine, and serine-rich region of protein backbone via *O*- or *N*-glycosidic bonds (Brockhausen, 2010). Mucins are critical to the function of mucus as a barrier and can be a place for bacterial adhesion and substrates of nutrients for bacterial growth (Derrien et al., 2010). Although mucolytic bacteria had been considered as pathogens because of loss of the protective mucus layer at first, it is the normal system for epithelial regeneration (Levy and Aminoff, 1980; Norin et al., 1985). They protect the epithelium from

the attachment of pathogens as commensal bacteria and also provide nutrients such as monosaccharides or amino acids for other bacteria by degrading mucin glycans (Derrien et al, 2010).

Epimural bacteria are the bridge where host cells meet the rumen internal environment for the first time. Especially in the rumen microbiology, only rumen bacteria related to rumen fermentation were focused. Despite the commensal effects, the association between microbiota and mucus is poorly understood. Therefore, further studies on mucolytic bacteria from rumen epithelium are needed.

In this study, a novel mucolytic bacterium, strain Rep29^{T}, was successfully isolated by culturing on a medium containing mucin as a sole energy carbon source to enrich mucolytic bacteria from rumen epithelium of Korean cattle. Moreover, physiological and genetic characterizations of strain Rep29^{T} were investigated.

Ⅱ. Literature review

1. Rumen microbial community

1.1. Rumen and bacteria

Rumen is a highly evolved foregut in the ruminants. It has a complex bionetwork that provides proper conditions for microbial growth. The symbiotic microbial community in the rumen consists of a variety of archaea, bacteria, protozoa, and fungi (Puniya et al., 2015). Above all, rumen bacteria are physiologically and nutritionally important to the host. They can be classified into three groups depending on their location in the rumen: (i) planktonic population composed of bacteria free in the rumen fluid; (ii) population attached to feed particles (firmly/loose); (iii) population attached to the epithelium (Sadet et al., 2007; Puniya et al., 2015).

1.2. Diversity of bacteria in the rumen

The phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Lentisphaerae, Tenericutes, Spirochaetae, Chloroflexi, Cyanobacteria, Elusimicrobia, Synergistetes, Fibrobacteres,* and *Fusobacteria* were present in the rumen and the phyla *Bacteroidetes, Firmicutes,* and *Proteobacteria* account about 90% of the rumen bacterial ecology (Liu et al., 2016; De Mulder et al., 2017). The predominant taxa at the genus level include the genera *Prevotella, Clostridium, Turicibacter, Butyrivibrio, Succiniclasticum, Ruminococcus, Mogibacterium, Campylobacter, Desulfobulbus, Syntrophococcus, Acetitomaculum,* and *Treponema* (Liu et al., 2016). These various bacteria have different compositions depending on regional effects in the rumen and play a specific role to benefit the host's physiology.

The phylum *Bacteroidetes* (Gram-negative bacteria) are common and

abundant within the GI tract of animals. The majority of *Bacteroidetes* spp. belongs to the bacterial families *Bacteroidaceae, Prevotellaceae, Rikenellaceae,* and *Porphyromonadaceae* and the bacterial genera *Alistipes, Bacteroides, Parabacteroides, Prevotella, Paraprevotella, Alloprevotella, Barnesiella, Tannerella, Odoribacter,* and *Porphyromonas*. They produce succinic acid, acetic acid, and propionic acid as the metabolic end products in common (Mirjana and Willem, 2014). Some species of the genera *Bacteroides* and P*revotella* can digest complex plant and milk oligosaccharides such as starch, cellulose, xylans, pectins, and milk glycan (Wu et al., 1992; Morotomi et al., 2009; Marcobal et al., 2011; Sakamoto and Ohkuma, 2012). Since *Bacteroidetes* species have proteases at the cell wall, they have proteolytic activity, as well as ureolytic activity in some cases (Macfarlane et al., 1986, 1988; Yatsunenko et al., 2012). In addition, some species can grow on the mucus and use the mucins as energy sources (Salyers et al., 1977; Leitch et al., 2007; Huang et al., 2011).

The phylum *Firmicutes* (Gram-positive and negative bacteria) are the most affluent group of the GI microbiota. The major GI microorganisms are members of the class *Clostridia*, including the families *Ruminococcaceae* and *Lachnospiraceae* and the class *Bacilli*, including the families *Lactobacillus*, *Enterococcus*, and *Streptococcus*. According to enormous variety, *Firmicutes* in the GI tract represent several contradictory functions that are extended from health promotion with the probiotic effect of *Lactobacillus spp*. to the pathogenic *Clostridium difficile* (Mirjana and Willem et al., 2014). Some species are dominant producers of butyrate and also convert lactate to butyrate or propionate to help stabilize the colonic microbiota by inhibiting lactate accumulation and excess acidity (Belenguer et al., 2007; Louis et al., 2010; Flint et al., 2015). Furthermore, *Firmicutes* are generally found to contain highly specialized degraders of non-digestible polysaccharides that have fewer genes involved in polysaccharide

degradation than *Bacteroides spp*. (Ze et al., 2012; Kaoutari et al., 2013; Flint et al., 2015).

The phylum *Proteobacteria* (Gram-positive and negative bacteria) are commonly observed in various sites, including the oral cavity, skin, vaginal tract and GI tract (Shin et al., 2015). Since *Proteobacteria* take in a variety of pathogens, such as the genera *Escherichia*, *Salmonella*, *Vibrio*, and *Helicobacter*, an increased abundance of *Proteobacteria* may represent a microbial signature of metabolic disorders and diseases (Shin et al., 2015; Rizzatti et al., 2017).

1.3. Epimural bacteria

1.3.1. Rumen epithelium and epimural bacteria

Most rumen bacteria float freely in the rumen fluid or attach to feed particles. However, less than 1% of the total rumen bacteria adhere to rumen epithelium. (Cheng et al., 1979; Sadet et al., 2007; Li et al., 2012). The latter is also known as "epimural" bacteria (adherent epithelial bacteria) (Mead and Jones, 1981) (Supplementary figure S1).

The stratified squamous epithelium in the rumen maintains the static population of adherent bacteria by attaching to the glycocalyx of horny cells using their fibrous carbohydrate coats (Lavker et al., 1969; McCowan et al., 1978). Epimural community is also distributed in cavities within the epithelial cells (Dinsdale et al., 1980).

1.3.2. Distribution of epimural bacteria

Early studies about epimural community were described using electron microscopy and culture-dependent techniques in 1970s and 1980s (Li et al., 2012). Isolations of adherent bacteria from rumen epithelium using culture-

dependent methods were identified as several species involving in the genera *Acidaminococcus*, *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Clostridium*, *Corynebacterium*, *Eubacterium*, *Fusobacterium*, *Lachnospira*, *Lactobacillus*, *Micrococcus*, *Propionibacterium*, *Ruminococcus*, *Staphylococcus*, *Selenomonas*, *Streptococcus*, *Succinivibrio*, and *Treponema* (Cheng et al., 1979; Dehority and Grubb, 1981; Mead and Jones, 1981; Muller et al., 1984). These phenotype-based methods were difficult to assess the diversity of epimural bacterial community because uncultured bacteria and classification of genetically close species were not considered (Sadet et al., 2007; Li et al., 2012).

However, culture-independent methods with development of genetic techniques, such as 16S rRNA sequencing, real-time polymerase chain reaction (PCR), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and next-generation sequencing (NGS), have been used to further investigate the biodiversity different from rumen contents and functions of these bacteria (Cho et al., 2006; Sadet et al., 2007; Pei et al., 2010; Li et al., 2012; De Mulder et al., 2017). The variation of microbial composition between rumen contents and drumen epithelium was present at both the phylum and genus levels. At the phylum level, epimural bacterial community has higher relative abundances of the phyla *Proteobacteria, Firmicutes, Spirochaetae, Synergistetes, Tenericutes, Fibrobacteres, Fusobacteria, Elusimicrobia,* and *Actinobacteria* compared with the bacterial distribution of rumen contents. At the genus level, it presented lower percentage of the genera *Prevotella, Saccharofermentans, Succiniclasticum,* and *Ruminococcus*, while greater abundance of the genera *Butyrivibrio, Mogibacterium, Treponema, Syntrophococcus, Howardella, Campylobacter, Desulfovibrio,* and *Desulfobulbus* compared with bacterial distribution of rumen contents (Liu et al., 2016; De Mulder et al., 2017). In addition, most epimural bacteria are made up of a large number of Gram-positive bacteria, whereas Gram-negative bacteria

are predominant in the rumen contents (McCowan et al., 1978).

1.3.3. Function of the epimural bacteria

Epimural community has a relatively minor contribution to ferment feeds rather than other bacteria in the rumen contents. The bacteria attached to the host tissue are not so much closely associated with luminal bacteria as the host metabolism (McCowan et al., 1980; Sadet et al., 2007). They are exposed to urea and oxygen from the blood vessels across the rumen epithelium (Lavker et al., 1969; Cheng and Wallace, 1979). Because of the distinctive characteristics of rumen epithelial environment, they perform the following functions: (i) hydrolysis of urea; (ii) oxygen scavenging; (iii) tissue recycling (Cheng et al., 1979).

Hydrolysis of urea. In ruminants, urea supplies nitrogen for microbial protein biosynthesis. The remaining ammonia used by rumen bacteria is transferred to blood through the rumen mucosa, enters the liver, and finally it is transformed into the urea via the ornithine cycle to be discharged through the urine or directly into the rumen epithelium through saliva or rumen epithelium (Kennedy and Milligan, 1980). Epimural bacteria are mainly responsible for urea metabolism in the rumen epithelial cells and initiate the breakdown of sloughed epithelial cells. Furthermore, it is not only a crucial factor in the recycling of endogenous nitrogen but also controls the rate of urea influx (Wallace et al., 1979).

Oxygen scavenging. Oxygen is essential for the epithelial cells of the GI tract for aerobic respiration and consequently, contact of the epithelium with oxygen is inevitable. It is beneficial for epimural bacteria to scavenging oxygen diffusing from the blood vessel, thereby leading suitable conditions for the microbial ecosystem of oxygen-sensitive anaerobes. Hence, most bacteria adherent to the epithelium are facultative or not sensitive to oxygen toxicity in contrast to other obligate bacteria associated with the rumen contents (Cheng et al., 1979; Mead and Jones, 1981; Muller et al., 1984; Liu et al., 2015; Steele et al., 2016).

Tissue recycling. For normal functions of the epithelium as barriers separating the lumen from its surface, epithelial cells must be established, maintained, and modified the polarity of various membrane compositions, including enzymes, receptors, and channels that are responsible for the transepithelial transports of metabolites (Brown, 1989). These mechanisms are regulated by the process of tissue recycling. Epithelial bacteria degrade keratinized cells which cannot be digested by the host itself or sloughed cell. It continually makes barriers strong and prevents the passage of pathogens, as well as promotes the absorption of nutrients for the host health (Dinsdale et al., 1980; McCowan et al., 1978).

2. Mucin and mucolytic bacteria

2.1. Mucus and mucin

The mucus is a viscous and hydrated gel layer that coats the epithelial surface of respiratory, GI, and reproductive tracts and composed of water, salts, immunoglobulin, secreted proteins, and mucin (Bansil et al., 1995). It has some properties: (i) a protective barrier against pathogens and toxins that provides the innate defensive system; (ii) a selective barrier which allows specific things to pass through, like molecules, ions, or other small particles; (iii) a lubricative barrier to help transports smoothly (Rose and Voynow, 2006). In addition, the mucins which are critical to functions of the mucus serve as substrates for growth, adhesion, and protection of microbial cells (Derrien et al., 2010).

The mucins, the most abundant macromolecules in mucus, are heavily *O*-glycosylated proteins (*O*-linked glycans) that consist of approximately 50-90% carbohydrate by weight which are attached to the protein backbone (Figure 1A). They are characterized by variable number of tandem repeats (VNTR) called as 'PTS region' (proline, threonine (Thr), and serine (Ser) rich region) of the protein backbone and length and sequences of VNTR and numbers of repeats depend on the different types of mucins (Brockhausen., 2010). Furthermore, a diverse combination of galactose (Gal), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), and other residues like sialic acid (*N*-acetylneuraminic acid; Neu5Ac, *N*-glycolylneuraminic acid; Neu5Gc), sulfate, or fucose (Fuc) constitutes *O*-linked glycoprotein (Hoorens et al., 2011). These mucins are established in distinct size, charge, and branching, depending on mucosal sites. The compound of mucin oligosaccharides is synthesized starting from GalNAc which links the glycan to serine and threonine residues of the

mucin core (Carrington et al., 2012). This linkage cause eight-core structures of mucin *O*-linked glycan with the most general core 1 to 4: core 1 (Galβ1-3GalNAcα-Ser/Thr); core 2 (GlcNAcβ1-6(Galβ1-3)GalNAcα-Ser/Thr); core 3 (GlcNAcβ1-3GalNAcα-Ser/Thr); core 4 (GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-Ser/Thr) (Figure 1B). Based on the cores, it is extended to various glycan chains through the sequential performance of glycosyltransferases and sulfotransferases and frequently finishes with fucose or sialic acid as a terminal capping structure (Carrington et al., 2012; Tailford et al., 2015).

There are two kinds of mucins according to their structure: (i) secretory mucins or (ii) membrane-bound mucins (Figure 1C). Secretory mucins are secreted by particular cells of the epithelium, such as goblet cells, and have the ability to form a viscous mucus gel through linkage of protein monomers via disulfide bonds. Membrane-bound mucins, however, cannot construct oligomeric products for gel-forming but can anchor the specific domain of membrane. They play a role as cell surface receptor, the response of cell signaling, and regulation of cell adhesion for epithelial bacteria (Gendler and Spier, 1995; Brockhausen., 2010; Derrien et al., 2010). In the rumen epithelium, there is only membrane-bound mucin, such as MUC1, MUC16, and MUC20, without secretory mucins unlike other epithelial tissues of GI tracts, including abomasum, small and large intestines (Hoorens et al., 2011).

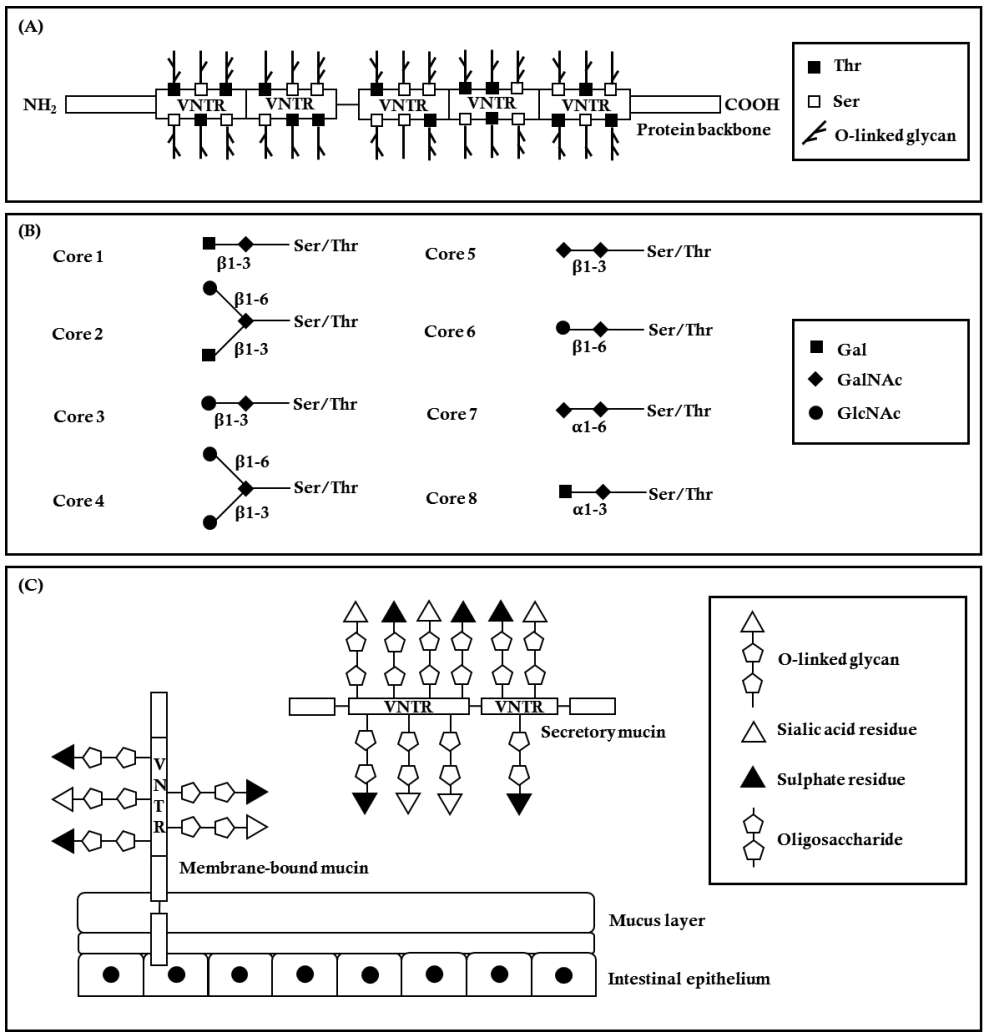


Figure 1. Structure of mucins: (A) mucin monomer; (B) eight core structures of mucins (core 1-8); (C) Two types of mucins: secretory mucin and membrane-bound mucin. Gal = galactose, GalNAc = *N*-acetylgalactosamine, GlcNAc = *N*-acetylglucosamine, Ser = serine, Thr = threonine, VNTR = variable number of tandem repeats (Darrien et al., 2010; Carrington et al., 2012; Tailford et al., 2015).

2.2. Mucolytic bacteria

Mucins provide a place for bacterial adhesion and sources of nutrients for bacterial growth (Florey, 1955; Darrien et al., 2004; Macfarlane et al., 2005). For these reasons, mucolytic bacteria are colonized within the mucosal layer. The distribution of mucolytic bacteria in the human is known to ~1% of total fecal microbiota (Hoskins and Boulding, 1981). Until now, bacterial species from the *Bacteroides, Firmicutes, Actinobacteria*, and *Verrucomicrobia* phyla and *Akkermansia*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Helicobacter*, *Prevotella*, *Ruminococcus*, *Streptococcus*, *Streptomyces*, and *Vibrio* genera have been investigated for their mucolytic ability (Derrien et al., 2010; Tailford et al., 2015).

The mucolytic bacteria were considered to be pathogenic in the past, as they remove mucins from the epithelial cells (Levy and Aminoff, 1980; Prizont, 1982; Slomiany et al., 1992). However, Norin et al. (1985) observed that mucin degradation is a normal process of epithelial regeneration after birth. Furthermore, consuming mucins by bacteria may have beneficial impacts on the host. The mucolytic bacteria stimulate hyperplasia and hypertrophy of goblet cells secreting mucins and obstruct abnormal accumulation of mucus in the GI tract by the degradation of secreted mucins (Kandori et al., 1996). In addition, they provide such as monosaccharides or amino acids released from mucin glycans to other bacteria and prevent the attachment of mucin to pathogens (Derrien et al, 2010). *Akkermansia muciniphila*, isolated from a human fecal sample, is one of the well-known commensal mucolytic bacteria (Darrien et al., 2004). Reduction in the abundance of *Akkermansia muciniphila* is related to various metabolic disorders, including inflammatory bowel disease, type 2 diabetes, cardiovascular diseases, autism, atopy or obesity (Derrien et al., 2004, 2017). Moreover, it can break down mucins, as well as stimulate mucin synthesis, indicating an automated catalytic process. Also, it enhances host immunity by

producing antimicrobial peptides and the proliferation of anti-inflammatory regulatory T cell (Shin et al., 2014; Derrien et al., 2017).

Despite the commensal effects, studies on the interaction between mucus and microbiota are still insufficient. Especially in the rumen microbiology, studies on mucolytic bacteria from rumen epithelium were rarely done during mid-1900s and further research is needed (Fina et al., 1961; Mishra et al., 1967, 1968).

2.3. Bacterial mucin degradation

Turnover of mucin removes surface particles like adherent microbes, toxins, and other environmental contaminants and maintains normal mucosal barrier functions (Carrington et al., 2012). As complex and diverse structures of mucin glycan, the coordination of various enzymes, including proteases, sulfatases, and glycoside hydrolases (GHs: galactosidases; fucosidase; neuraminidases/sialidases; *N*-acetylgalactosaminidases; *N*-acetylglucotosaminidases), is necessary to deconstruct their arrangements (www.cazy.org/) (Lombard et al., 2014; Tailford et al, 2015) (Table 1). GHs have carbohydrate-binding modules (CBMs) for attachment to certain carbohydrates. Ficko-Blean and Boraston (2012a) reported CBM families 32, 40, 47, and 51 which are specificity for terminal mucin glycans.

Table 1. Types of glycoside hydrolases related to degradation of mucins

Enzymes	GHs
β-Galactosidases	GH2, GH20, GH42
Fucosidases	GH29, GH95
Neuraminidases/sialidases	GH33
-*N*-acetylgalactosaminidases	GH101, GH129
α-*N*-acetylglucosaminidases	GH89
Exo- and endo-*β*-*N*-acetylglucosaminidases	GH84, GH85

Sequential degradation of mucins starts from cleaving sialic acid through GH33 sialidases since terminal sites of sialic acid residues in the mucin oligosaccharide branches prevent hydrolysis from the action of other GHs (Tailford et al, 2015). GH29 and GH95 fucosidases play a role in breaking fucosyl residues linked to galactose by α1,2 linkages or to GlcNAc by α1,3 linkages at the extremity of *O*-linked chains (Katayama et al., 2005; Tailford et al., 2015). Mucin glycan core structures are released from the Ser and Thr regions, the mucin protein backbone, by GH101 and GH129 endo-α-*N*-acetylgalactosaminidases (Fujita et al., 2005; Kiyohara et al., 2012). Finally, the mucins are degraded to monosaccharides by the rest of the GHs related to mucin glycan catabolism (Figure 2).

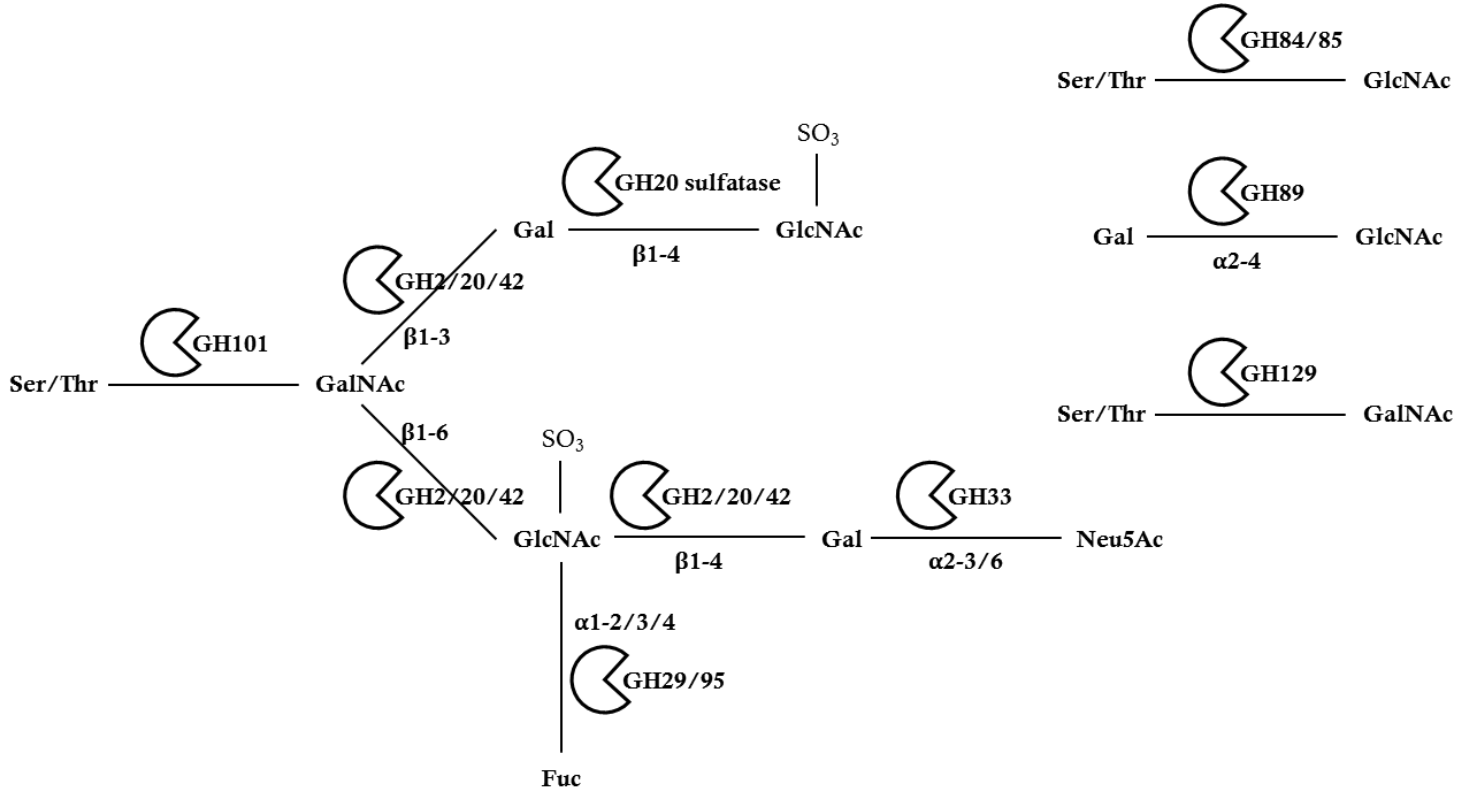


Figure 2. *O*-glycan structure showing activation sites of GHs related to mucin degradation. Fuc = fucose, Gal = galactose, GalNAc = *N*-acetylgalactosamine, GH = glycoside hydrolases, GlcNAc = *N*-acetylglucosamine, Neu5Ac = *N*-acetylneuraminic acid, Ser = serine, Thr = threonine (Tailford et al., 2015).

Ⅲ. Materials and Methods

1. Isolation and identification of mucolytic bacteria from the rumen epithelium

1.1. Media composition

Mucin defined medium and mucin medium were prepared with some modifications of medium 10 (Caldwell and Bryant, 1966). Mucin defined medium contained the following components: 2 g peptone, 0.5 g yeast extract, 2.5 g hog gastric mucin (Type Ⅲ; Sigma), 50 ml mineral solution 1 (6 g K_2HPO_4 per liter), 50 ml mineral solution 2 (12 g NaCl; 6 g KH_2PO_4; 1.2 g $CaCl_2.2H_2O$; 1.2 g $MgSO_4.7H_2O$; 12 g $(NH_4)_2SO_4$ per liter), 10 ml Pfenning's solution (0.5 g EDTA; 0.1 g $ZnSO_4.7H_2O$; 0.03 g $MnCl_2.4H_2O$; 0.03 g H_3BO_3; 0.2 g $CoCl_2.6H_2O$; 0.01 g $CuCl_2.2H_2O$; 1.5 g $FeCl_2.4H_2O$; 0.02 g $NiCl_2.6H_2O$; 0.03 g $Na_2MoO_4.2H_2O$; 0.01 g Na_2SeO_3 per liter), 1 ml hemin solution (0.5 g hemin; 10 ml 1 N NaOH per liter), 1 ml resazurin (0.1%; w/v), 20 ml cysteine sulfide solution (6.25 g NaOH; 25 g cysteine-HCl; 25 g $Na_2S.9H_2O$ per liter), 4 g $NaHCO_3$ per liter. Mucin medium consisted of mucin defined medium with 100 ml clarified rumen fluid per liter (Table 2).

Table 2. Composition of mucin medium (per 1,000 ml)

Component	Mucin defined medium	Mucin medium
Clarified rumen fluid	-	100 ml
Peptone	2 g	2 g
Yeast extract	0.5 g	0.5 g
Mineral solution 1	50 ml	50 ml
Mineral solution 2	50 ml	50 ml
Pfenning's solution	10 ml	10 ml
Cysteine sulfide solution	20 ml	20 ml
$NaHCO_3$	4 g	4 g
Hemin solution 0.1% (w/v)	1 ml	1 ml
Mucin	2.5 g	2.5 g
Resazurin 0.1% (w/v)	1 ml	1 ml

1.2. Sample collection and enrichment of mucolytic bacteria

The rumen epithelium tissue samples were collected from the dorsal sac of rumen of Korean cattle, directly after slaughtering at the abattoir in Bucheon (37°31'48.4"N 126°45'46.3"E) of South Korea. The collected tissue samples are transferred to the laboratory in a sterile container with rumen contents to minimize contact with oxygen. On arriving, samples were rinsed several times with an anaerobic dilution solution (ADS; 3 g K_2HPO_4, 6 g NaCl, 3 g KH_2PO_4, 0.6 g $CaCl_2.2H_2O$, 0.6 g $MgSO_4.7H_2O$, 6 g $(NH_4)_2SO_4$, 0.5 g cysteine-HCl, 0.5 g $Na_2S.9H_2O$, 0.625 g NaOH, 1 mg resazurin per liter) in an anaerobic chamber to remove rumen contents and non-adherent bacteria (Mead and Jones, 1981; Muller et al., 1984). Afterward the washed epithelium tissues were stripped from the muscle layer and five grams of epithelial surfaces were homogenized in 25 ml of ADS and serially diluted (10-fold). Every 0.3 ml of dilution was inoculated into 30 ml of mucin medium in a butyl rubber stoppered serum bottle and

incubated at 39°C in the anaerobic atmosphere (96% CO_2, 4% H_2) for 24 h for enrichment.

1.3. Isolation of mucolytic bacteria

Enrichments were serially diluted with ADS, spread onto mucin agar medium, and incubated at 39°C for 5 days under anaerobic conditions. Every single colony was picked, inoculated into 5 ml of mucin defined medium in a Hungate tube, and incubated at 39°C for 24–48 h. Cycles of liquid culture and single colony picking were repeated until an isolate was pure. The isolates were stored with 15% (v/v) glycerol stock solution at –80°C (Figure 3).

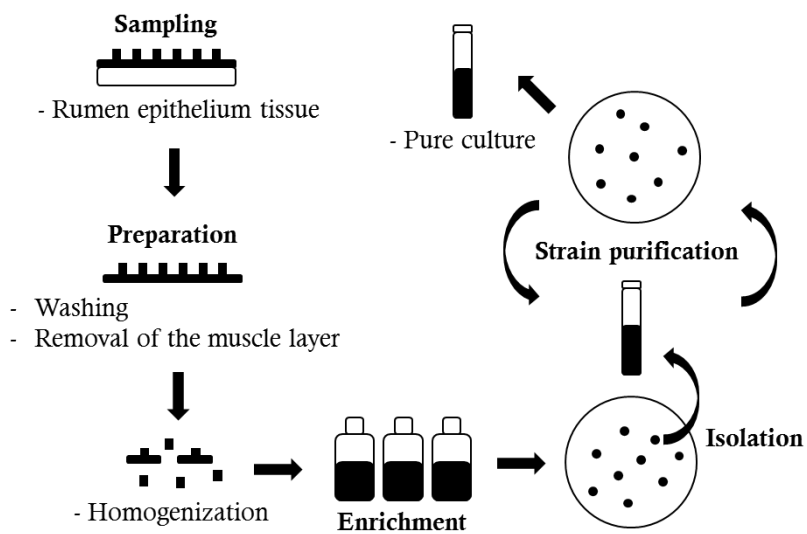


Figure 3. Overview of isolation of mucolytic bacterium from rumen epithelium

1.4. Identification of mucolytic bacteria

1.4.1. DNA extraction and 16S rRNA gene amplicon PCR

Genomic DNAs of the isolates were extracted using DNeasy PowerSoil Kit (Qiagen, Germany), as recommended by the manufacturer's protocol. The extracted DNAs were used as a template for PCR to amplify 16S rRNA genes using AccuPower® PCR PreMix (Bioneer, South Korea). The PCR primers were universal primers 10F (5′-AGT TTG ATC ATG GCT CAG ATT G-3′) and 1507R (5′-ACC TTG TTA CGA CTT CAC CCC AG-3′) (Lane, 1991). The PCR reactions were cycled as follows: initial denaturing (at 94°C for 3 min), 27 cycles of denaturing (at 94°C for 1 min), annealing (at 55°C for 1 min), and elongation (at 72°C for 1 min), and final extension (at 72°C for 7min). The amplified PCR products were purified using MEGAquick-Spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea), following the manufacturer's protocol.

1.4.2. Sequencing of 16S rRNA gene and data analysis

The resulting 16S rRNA sequences were analyzed at NICEM (Seoul National University, Seoul, South Korea). Three overlapping 16S rRNA gene fragments were generated in separate reactions by using the universal primers 340F (5′-CCT ACG GGA GGC AGC AG-3′), 518R (5′-ATT ACC GCG GCT GCT GG-3′), and 805F (5′-GAT TAG ATA CCC TGG TAG TC-3′). After checking the sequencing quality, sequences were assembled and edited using the program Geneious Prime (version 2019.2.1). The 16S rRNA consensus sequences were compared with those of all validated type strains using the

Nucleotide Similarity Search program in the EzBioCloud server (http://www.ezbiocloud.net/identify/) (Yoon et al., 2017).

2. Characterization of strain Rep29^T, a novel mucolytic bacterium adherent to the rumen epithelium

Strain Rep29^T was selected for phenotypic, biochemical, and chemotaxonomic characteristics and phylogenetic analysis to identify characteristics of a novel strain, considering the degree of growth on mucin medium among the uncultured isolates with less than 90% 16S rRNA gene sequence similarity with the closely related bacterium.

2.1. Construction of phylogenetic tree based on 16S rRNA gene sequence

The 16S rRNA sequence alignment for Strain Rep29^T as previously described and closely related taxa were used to construct an evolutionary phylogenetic tree using the program MEGA-X (version 10.0.05) with neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) algorithms (Kumar et al., 2016).

2.2. Cell morphology and Gram staining

A Gram staining kit (Sigma) was used for Gram staining, following the manufacturer's protocol. After Gram staining test through phase-contrast microscopy, cell morphology of strain Rep29^T was examined by transmission electron microscopy (LIBRA 120; Carl Zeiss) at 120 kV. Negative staining with phosphotungstic acid was applied to 200 copper mesh Formvar/carbon-support film grids (Ted Pella Inc.) using cells of strain Rep29^T incubated in mucin agar medium at 39°C for 3 days.

2.3. Determination of optimum growth conditions

To determine the optimum growth conditions, the growth pattern of Strain Rep29^T was observed in the optical density (OD) at a wavelength of 600 nm by culturing the cell under various terms of medium, temperature, pH and salt concentration. The medium was determined in mucin medium, tryptic soy broth (TSB; BD), brain heart infusion broth (BHI; BD), anaerobe basal broth (ABB; Oxoid), and Luria-Bertani broth (LB; Sigma) (Table 3) at 39°C for 48 h. The optimum temperature, pH and salt concentration tolerance ranges were evaluated via mucin medium growth tests subjected to different temperatures (5–45°C at 5°C intervals), pH (5.0–9.0 at 0.5 pH unit intervals, adjusting the pH with Na_2HPO_4-NaH_2PO_4 buffer at pH 5.0–7.0; Tris-HCl buffer at pH 7.5–9.0 before and after autoclaving), and NaCl concentrations (0.0–2.0% at 0.5% intervals). (Gomori, 1955).

2.4. Determinations of oxygen tolerance

Tolerance to oxygen was assessed by measuring growth in the OD at 600nm in mucin medium supplemented with aerobic or anaerobic gas in the headspace and the presence or absence of a reducing agent (cysteine sulfide solution).

Table 3. Medium compositions for optimum medium test

Component	Mucin medium	TSB	BHI	ABB	LB
Growth factors or undefined components	Clarified rumen fluid	–	Calf brains, infusion from 200 g	–	–
	–	–	Beef heart, infusion from 250 g	–	–
Nitrogen sources	Peptone	Pancreatic digest of casein	Proteose Peptone	Peptone	Pancreatic digest of casein
	Yeast extract	Peptic digest of soybean	–	Yeast extract	Yeast extract
	–	–	–	Arginine	–
Carbon sources	Mucin	Dextrose	Dextrose	Dextrose	–
	–	–	–	Starch	–
	–	–	–	Sodium pyruvate	–
	–	–	–	Sodium succinate	–
Reducing agents	Cysteine sulfide solution	–	–	L-cysteine-HCl	–
	–	–	–	Dithiothreitol	–
	–	–	–	Sodium thioglycolate	–
Mineral / Buffer / Vitamin sources	Mineral solution 1	NaCl	NaCl	NaCl	NaCl
	Mineral solution 2	K_2HPO_4	Na_2HPO_4	Ferric pyrophosphate	–
	Pfenning's solution	–	–	Hemin	–
	Hemin solution	–	–	$NaHCO_3$	–
	$NaHCO_3$	–	–	Vitamin K	–

2.5. Enzyme profile test and analysis of cellular fatty acid composition

Reference strains, *Prevotella melaninogenica* ATCC 25845^T which is the type species of the genus *Prevotella* and strain E39^T which is an unpublished mucolytic bacterium isolated from rumen epithelium in our laboratory were used to compare enzyme profiles and cellular fatty acid composition with strain Rep29^T. The freeze-dried cells of *Prevotella melaninogenica* ATCC 25845^T and strain E39^T were regenerated in peptone yeast extract-glucose (PYG) medium (KCTC medium 282) and mucin medium at pH 6.8-7.0 for 24-48 h at 37°C under anaerobic conditions, respectively.

For biochemical characterization, the enzyme profiles were determined using the API Rapid ID 32A and API ZYM identification kit (bioMérieux), followed by the manufacturer's instructions.

For chemotaxonomic characterization, analysis of cellular fatty acids was conducted according to a standard MIDI protocol. All of the strains were cultivated in PYG medium, except for strain E39^T cultivated in mucin medium. Fatty acid methyl esters were obtained from cells harvested at the late exponential phase by saponification, methylation, extraction, and base wash. Extracted fatty acid methyl esters were determined with gas chromatography (Hewlett Packard 6890) and identified using the RTSBA6 database of the Microbial Identification System (Sherlock ver. 6.0B) (Sasser, 1990).

2.6 Whole genome sequencing

Whole genome de novo sequencing was performed using both the Illumina HiSeq™ X and PacBio RSII platforms at Macrogen (Seoul, Korea; http://www.macrogen.com). Libraries were prepared using TruSeq nano DNA library prep kit and PacBio DNA Template prep kit 1.0 for Illumina and PacBio, respectively. After sequencing, trimmed reads were used for de novo assembly based on the HGAP3 using SMRT portal (v2.3) (Chin et al., 2013). To obtain a high-quality sequence, error correction of the assembled contig was performed using Illumina short reads through Pilon (v1.21). (Walker et al., 2014).

The complete genome sequence was annotated using the Prokka Galaxy tool (version 1.13) (https://usegalaxy.org/) (Cuccuru et al., 2014; Seemann, 2014). Functional annotation of predicted proteins was evaluated using the BlastKOALA tool of Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/blastkoala/) (Kanehisa et al., 2016). To analyze carbon-metabolism related enzymes, the genome sequence of strain Rep29^{T} was annotated according to the Carbohydrate-Active enZYmes (CAZy) database using a meta server for automated carbohydrate-active enzyme annotation (dbCAN2) (http://cys.bios.niu.edu/dbCAN2/) (Zhang et al., 2018). Prediction of virulence factors and antibiotic-resistant genes was performed using VRprofile (http://bioinfo-mml.sjtu.edu.cn/VRprofile/) (Li et al., 2018).

Ⅳ. Results

1. Isolation and identification of mucolytic bacteria from the rumen epithelium

Single colony picking of total 40 isolates was conducted from mucin agar medium. Only 12 isolates were grown on mucin defined liquid medium and identified by 16S rRNA gene analysis. The six isolates were most closely related to 2 cultured bacteria: *Paraclostridium benzoelyticum JC272*^{T} *and Citrobacter murliniae CDC 2970-59*^{T} with 99.8-99.9% and 99.5% 16S rRNA gene sequence similarities, respectively. And the others were most closely related to 3 uncultured bacteria: Uncultured rumen bacterium clone L206RT, Uncultured bacterium clone C3-2, and Uncultured rumen bacterium clone U28-G08 with 98.0%, 93.8-94.0%, and 88.0% 16S rRNA gene sequence similarities, respectively (Table 4).

Table 4. List of isolates identified by 16S rRNA gene sequence similarity

Order	Isolates (the number)	Most closely related bacteria	Similarity	Note
1	Rep4, Rep14, Rep21, Rep25, Rep30 (5)	*Paraclostridium benzoelyticum* JC272^T	98.8–99.9%	Cultured bacteria (6)
2	Rep12 (1)	*Citrobacter murliniae* CDC 2970-59^T	99.5%	
3	Rep18, Rep29 (2)	*Prevotella brevis* GA33^T	89.9, 89.7%	Uncultured bacteria (6)
4	Rep20, Rep27, Rep36 (3)	*Campylobacter fetus* subsp. *testudinum* 03-427^T	96.5-97.0%	
5	Rep26 (1)	*Prevotella bryantii* B14^T	86.6%	
Total	12 isolates			

2. Characterization of strain Rep29^T, a novel mucolytic bacterium adherent to the rumen epithelium

2.1. 16S rRNA gene sequence and phylogenetic tree of strain Rep29^T

The nucleotide sequence 16S rRNA gene of the strain Rep29^T (1486 nucleotides) was submitted to NCBI GenBank and provided with the accession number of MN540723. The results of 16S rRNA gene sequence alignment of the strain Rep29^T belonged to the genus *Prevotella* on the EzBioCloud server. *Prevotella brevis GA33^T* and *Prevotella ruminicola Bryant 23^T* were most closely related to strain Rep29^T with 89.8% and 89.4% 16S rRNA gene sequence similarities among cultured bacteria, respectively. The NJ (Figure 4), ML (Figure 5), and MP (Figure 6) phylogenetic trees were constructed using species involved in the genera *Prevotella, Alloprevotella, Pararevotella,* and *Bacteroides*.

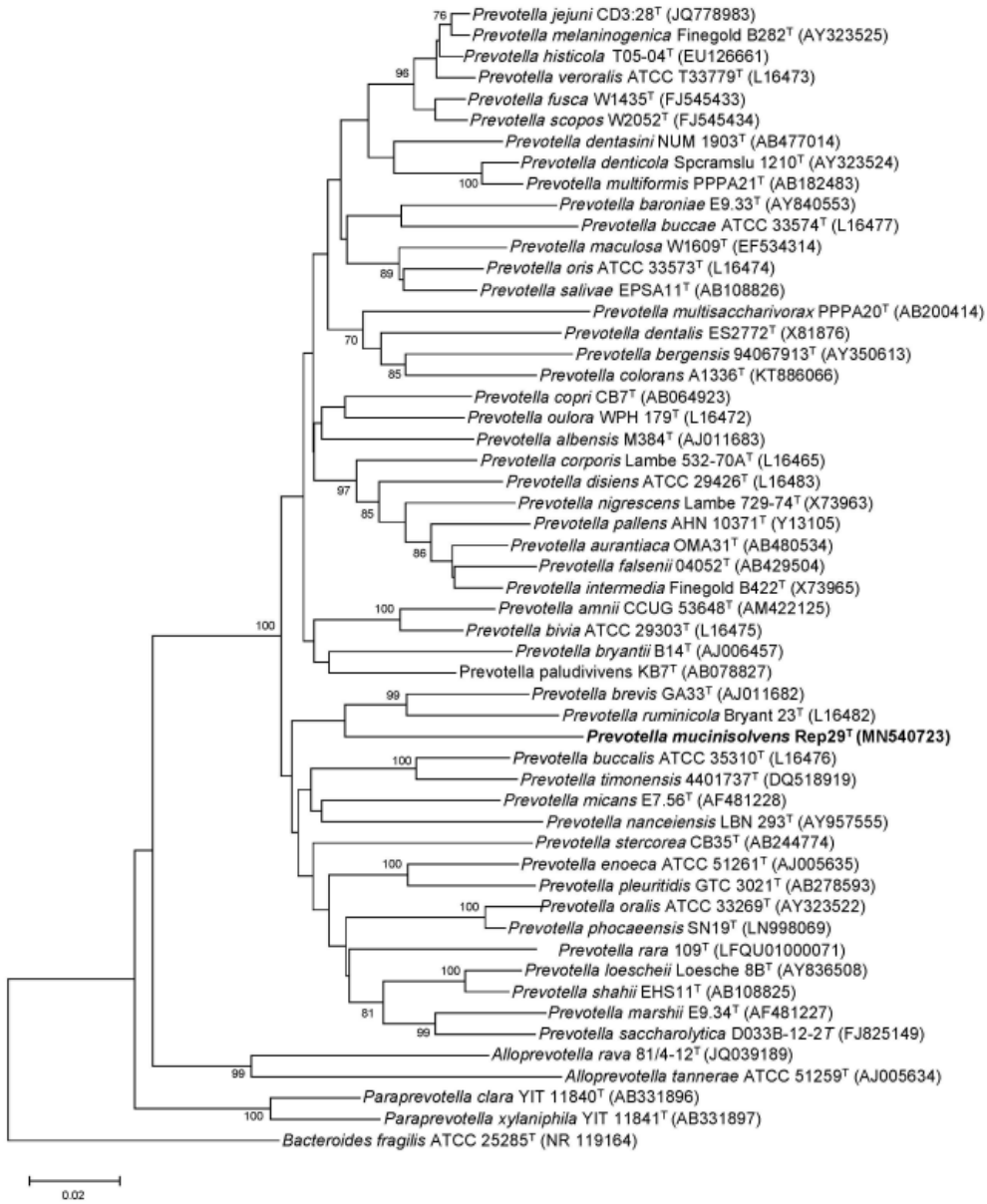


Figure 4. Neighbor-joining phylogenetic tree showing strain Rep29^T in relation to species belonging to the family *Prevotellaceae* based on 16S rRNA gene sequences. Bootstrap values are represented on node labels based on the percentage of 1,000 replicates. Only values higher than 70% are labeled. The tree was rooted with *Bacteroides fragilis* ATCC 25285^T (NR119164). Bar, 0.02 sequence divergence.

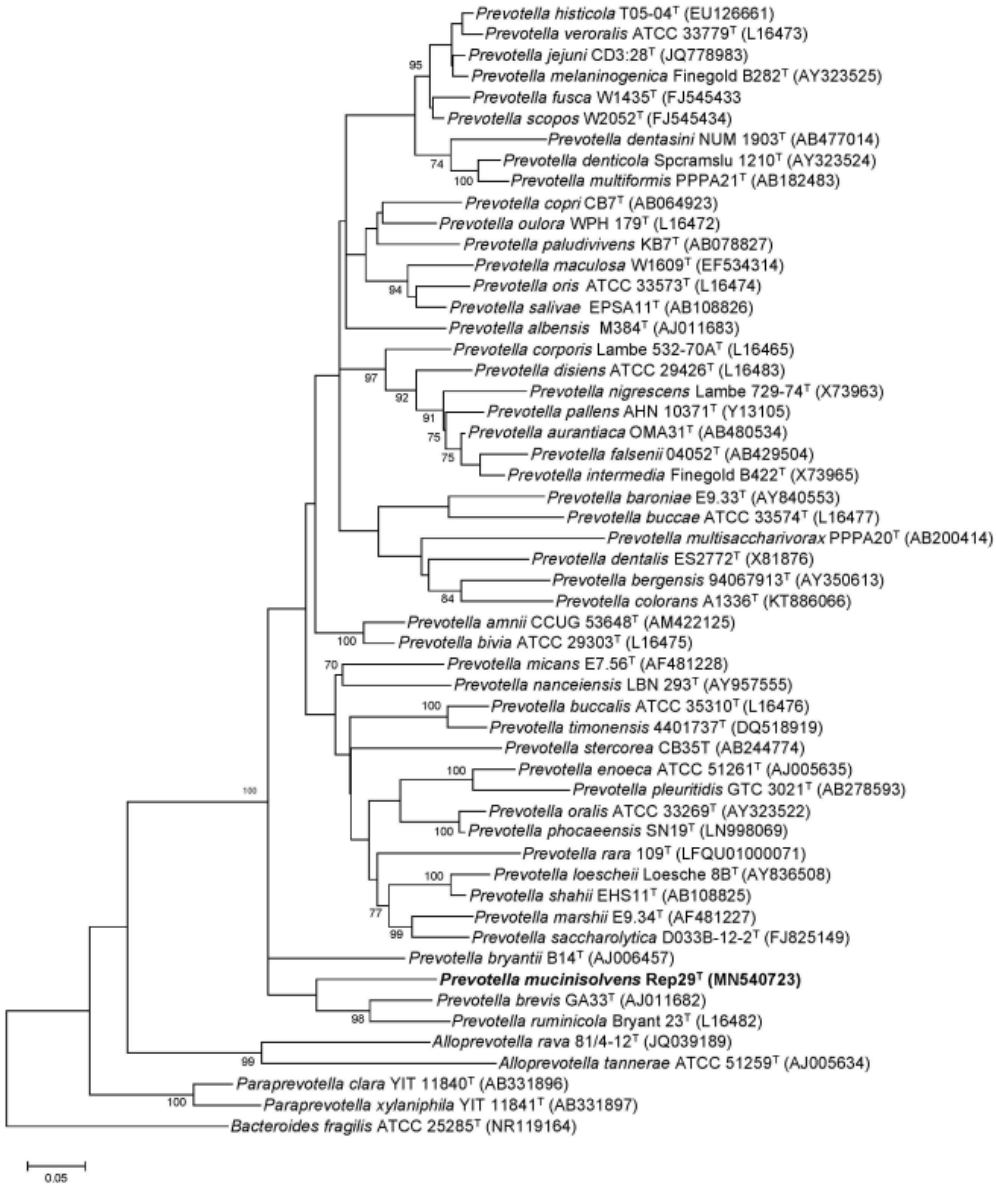


Figure 5. Maximum-likelihood phylogenetic tree showing strain Rep29^T in relation to species belonging to the family *Prevotellaceae* based on 16S rRNA gene sequences. Bootstrap values are represented on node labels based on the percentage of 1,000 replicates. Only values higher than 70% are labeled. The tree was rooted with *Bacteroides fragilis* ATCC 25285^T (NR119164). Bar, 0.05 sequence divergence.

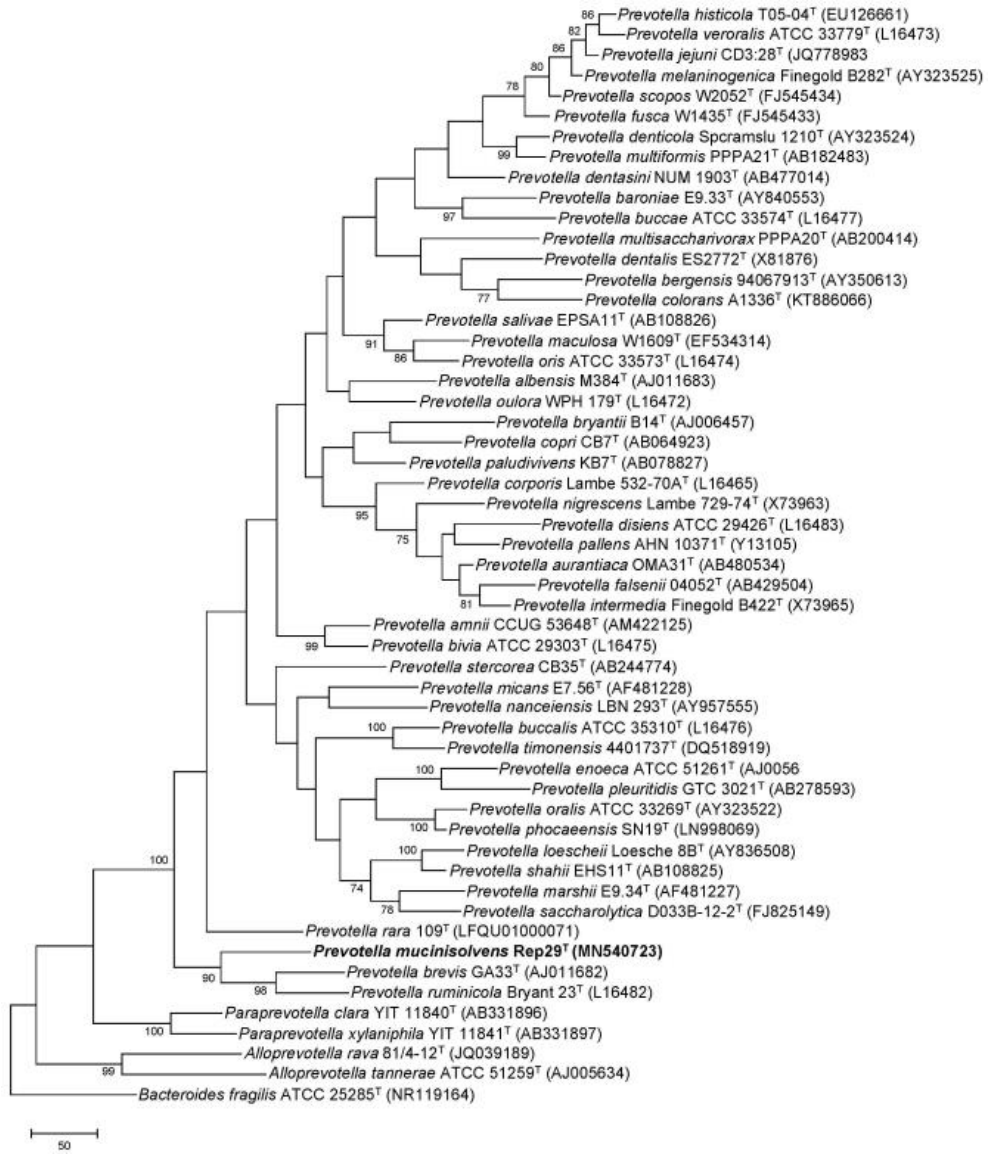


Figure 6. Maximum-parsimony phylogenetic tree showing strain Rep29^T in relation to species belonging to the family *Prevotellaceae* based on 16S rRNA gene sequences. Bootstrap values are represented on node labels based on the percentage of 1,000 replicates. Only values higher than 70% are labeled. The tree was rooted with *Bacteroides fragilis* ATCC 25285^T (NR119164). Bar, 50 sequence divergence.

2.2. Phenotypic and growth characteristics of strain Rep29^T

The cells of strain Rep29^T were Gram-negative, non-motile coccus without flagella (680–820 nm in diameter) and had filamentous structures from the cell surface (Figure 7).

Growth of strain Rep29^T occurred in mucin medium, but not in TSB, BHI, ABB, and LB media (Figure 8). Cells grew at 35-45℃, pH 6.0-7.5, and 0.0-1.5% NaCl with optimum at 39℃, pH 7.0, and 0.0-0.5% NaCl, respectively (Figure 9; Figure 10; Figure 11). Cells did not grow under aerobic conditions with or without presence of a reducing agent when the headspace was filled with oxygen. They showed growth regardless of a reducing agent in the headspace replaced with anaerobic gas. However, they were grown more vigorously in the presence of a reducing agent (Figure 12).

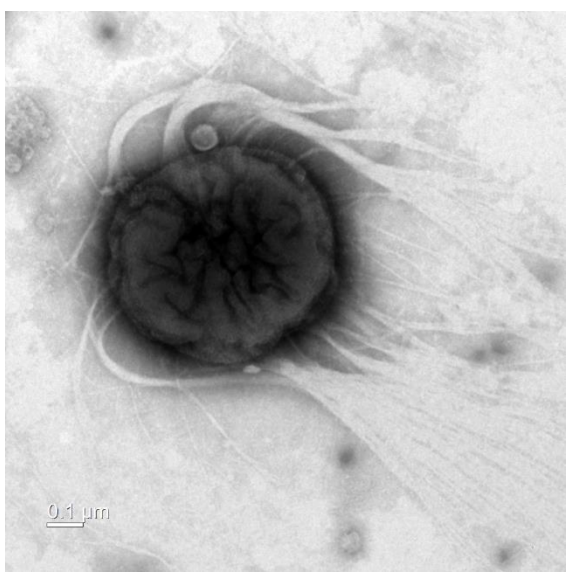


Figure 7. A transmission electron micrograph (TEM) of a negatively stained cell of strain Rep29^T incubated in mucin agar medium at 39°C for 3 days. Bar, 0.1μm.

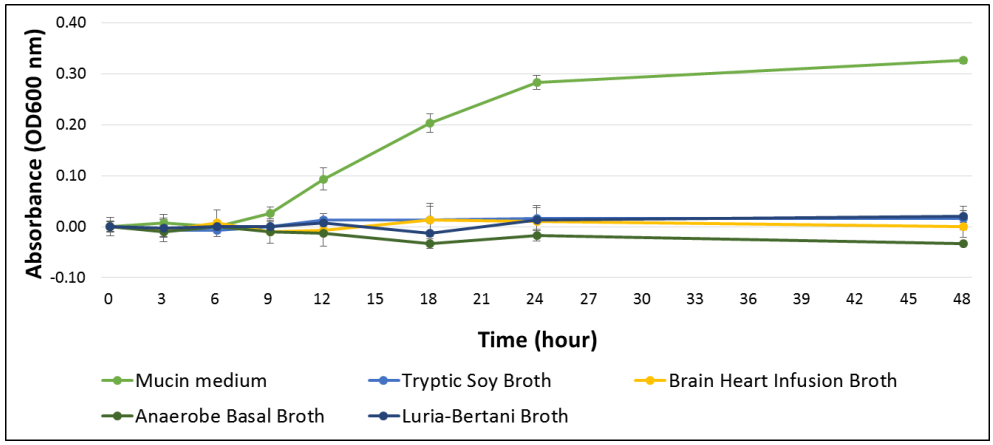


Figure 8. Growth performance of strain Rep29^{T} on different media.

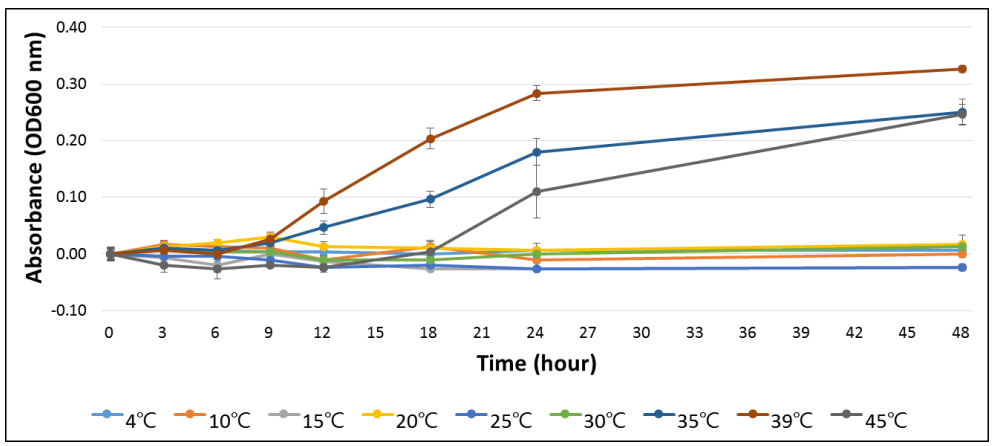


Figure 9. Growth performance of strain Rep29^{T} at different temperatures.

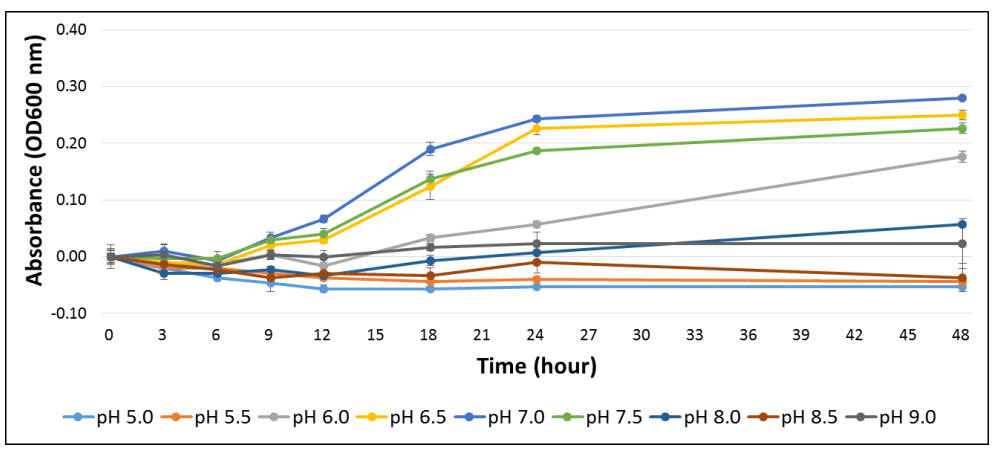


Figure 10. Growth performance of strain Rep29^{T} at different pH units.

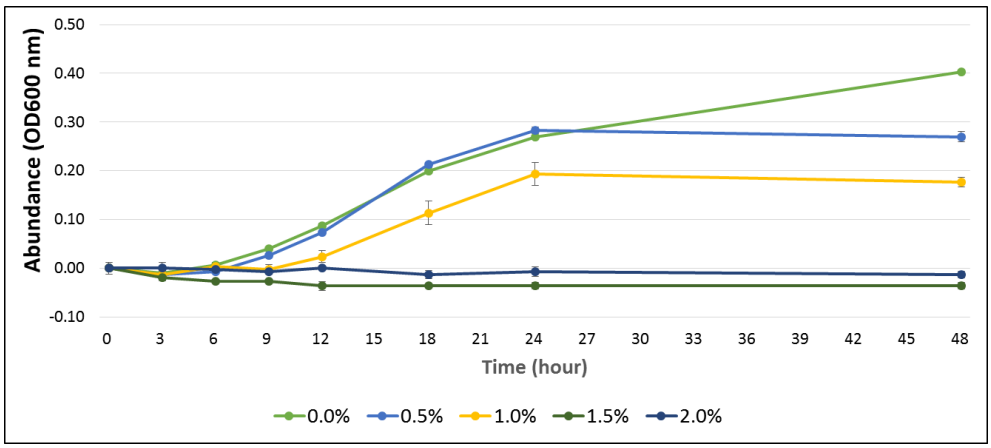


Figure 11. Growth performance of strain Rep29^{T} at different NaCl concentrations.

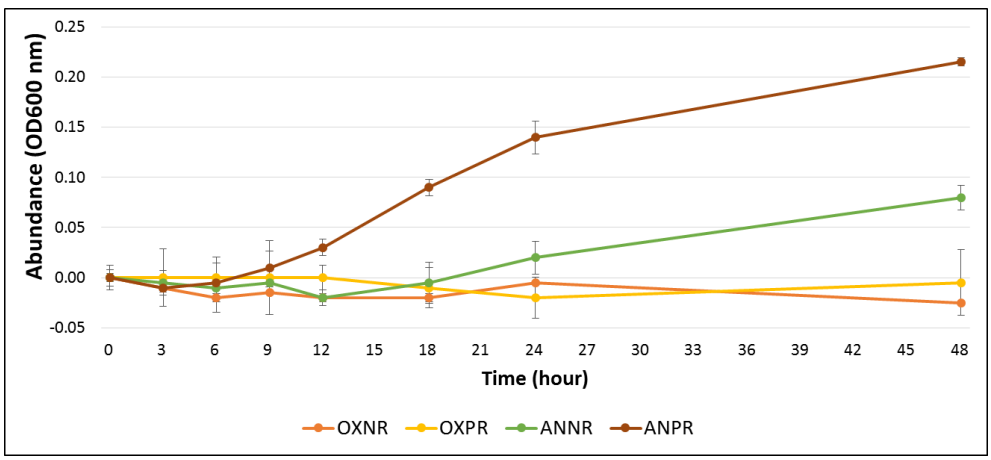


Figure 12. Growth performance of strain Rep29^{T} at different oxygen concentrations. OXNR: aerobic headspace and no reducing agent; OXPR: aerobic headspace and presence of reducing agent; ANNR: anaerobic headspace and no reducing agent; ANPR: anaerobic condition and presence of reducing agent.

2.3. Enzyme profile of strain Rep29^T

Positive reactions were obtained using API Rapid ID 32A panel for *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, *N*-acetyl-*β*-glucosaminidase, indole production, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, glycine arylamidase, and glutamyl glutamic acid arylamidase: negative reactions were obtained for urease, arginine dihydrolase, *β*-galactosidase 6 phosphate, *β*-glucosidase, *α*-arabinosidase, *β*-glucuronidase, mannose fermentation, raffinose fermentation, glutamic acid decarboxylase, *α*-fucosidase, reduction of nitrates, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, histidine arylamidase, and serine arylamidase.

Positive reactions were obtained using API ZYM for alkaline phosphate, acid phosphate, naphthol-AS-BI-phosphohydrolase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, and *N*-acetyl-*β*-glucosaminidase and reactions for esterase (C4), esterase lipase (C8), valine arylamidase, trypsin were weak of the kit used; negative reactions were obtained for lipase (C14), leucine arylamidase, cysteine arylamidase, *α*-chymotrypsin, *β*-glucuronidase, *β*-glucosidase, *α*-mannosidase, *α*-fucosidase (Table 5).

Table 5. Differential characteristics of strain Rep29^T and related species of the family *Prevotellaceae*. Strain: 1, strain Rep29^T (this study); 2, *Prevotella loescheii* JCM 8530^T; 3, *Prevotella oralis* JCM 12251^T; 4, *Prevotella salivae* JCM 12084^T; 5, *Prevotella copri* (data for five strains, including the type strain); 6, *Prevotella stercorea* JCM 13469^T; 7, *Prevotella veroralis* JCM 6290^T; 8, *Prevotella melaninogenica* ATCC 25845^T; 9, *Prevotella multisaccharivorax* PPPA20^T (Sakamoto et al., 2005); 10, *Prevotella shahii* JCM 12083^T; 11, *Prevotella marshii* JCM 13450^T; 12, strain E39^T. Data for reference were obtained from Hayashi et al. (2007) unless indicated otherwise. All strains are positive for acid phosphate, naphthol-AS-BI-phosphohydrolase. All strains are negative for lipase (C14), leucine arylmidase, cysteine arylmidase, *β*-glucosidase.

Characteristic	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8***	**9**	**10**	**11**	**12***
Enzyme activities of												
Alkaline phosphate	+	+	+	+	+	+	+	+	+	w	+	+
α-Galactosidase	+	+	+	+	+	w	w	-	-	w	-	+
β-Galactosidase	+	+	+	+	+	+	+	+	+	w	-	-
α-Glucosidase	+	+	+	+	+	+	+	+	+	w	+	-
N-Acetyl-*β*-glucosaminidase	+	+	+	+	-	+	+	+	+	+	-	+
Esterase (C4)	w	-	-	-	-	-	-	+	-	-	-	w
Esterase lipase (C8)	w	-	-	-	w	-	-	+	-	-	-	w
Valine arylmidase	w	-	-	-	-	-	-	-	-	-	-	-
Trypsin	w	-	-	-	-	-	-	-	-	-	-	w
α-Chymotrypsin	-	-	w	-	w	-	-	-	-	-	w	-
β-Glucosidase	-	-	w	+	+	-	-	-	+	-	-	-
α-Mannosidase	-	-	w	-	-	w	-	-	-	-	-	-
α-Fucosidase	-	w	w	w	-	+	w	-	-	w	-	+
DNA G+C content (mol%)	47.7†	46.6†	44.5†	41.7†	44.9†	48.9†	41.8†	40.9†	48.3†	44.3†	47.4†	47.8†

+, positive; –, negative; w, weak.
*Data from this study.
†The DNA G+C content was calculated based on its genome sequence.

2.4 Cellular fatty acid composition of strain Rep29^T

The major cellular fatty acids (>5% of the total fatty acids) of strain Rep29^T were $C_{16:0}$, $C_{18:1}$ ω9c, iso-$C_{15:0}$ and summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c). Among strain Rep9^T along with the related taxa of genus *prevotella*, $C_{16:0}$ and iso-$C_{15:0}$ were commonly detected as the major cellular fatty acids (Table 6).

Table 6. Cellular fatty acid compositions (%) of strain Rep29^T and related strain. Strain: 1, strain Rep29^T (this study); 2, *Prevotella brevis* ATCC 19188^T; 3, *Prevotella ruminicola* ATCC 19189^T; 4, *Prevotella melaninogenica* ATCC 25845^T; 5, strain E39^T. Data for reference were obtained from Minato et al. (1988) unless indicated otherwise. Data are expressed as percentages of the total fatty acids and fatty acids quantity less than 0.5% in all strains are not shown. Major components (>5.0%) are highlighted in bold. tr, trace amount (<0.5%); –, not detected.

Fatty acid	1*	2	3	4*	5*
Saturated:					
$C_{12:0}$	0.5	tr	tr	tr	0.7
$C_{13:0}$	–	1.6	2.3	–	tr
$C_{14:0}$	4.9	2.2	4.3	2.0	3.3
$C_{15:0}$	–	**23.0**	**23.2**	–	**17.5**
$C_{16:0}$	**16.3**	**5.9**	**10.8**	**8.0**	0.5
$C_{17:0}$	0.5	1.2	**5.0**	tr	**10.9**
$C_{18:0}$	1.4	tr	1.4	0.7	4.1
Unsaturated:					
$C_{13:1}$ at 12-13	–	–	–	**5.6**	0.6
$C_{14:1}$ ω5c	1.7	–	–	0.8	0.5
$C_{15:1}$ ω5c	0.7	–	–	–	–
$C_{17:1}$ ω8c	1.8	–	–	tr	–
$C_{18:1}$ ω9c	**36.1**	–	–	**14.0**	**13.3**
$C_{18:3}$ ω6c (6,9,12)	1.4	–	–	0.7	2.0
iso-$C_{17:1}$ ω5c	0.9	–	–	tr	–
anteiso-$C_{17:1}$ ω9c	1.3	–	–	–	–
Hydroxy:					
$C_{11:0}$ 2-OH	0.5	–	–	tr	–
$C_{15:0}$ 2-OH	0.7	–	–	tr	tr
$C_{16:0}$ 2-OH	–	–	–	**6.9**	2.2
$C_{17:0}$ 2-OH	tr	–	–	0.8	–
iso-$C_{17:0}$ 3-OH	2.3	–	–	**7.0**	2.1
Branched:					
iso-$C_{15:0}$	**9.5**	**12.6**	**5.3**	**7.4**	2.5
iso-$C_{16:0}$	tr	**8.7**	1.6	0.8	0.7
iso-$C_{17:0}$	tr	3.2	2.3	1.2	0.5
iso-$C_{19:0}$	2.3	–	–	**6.5**	2.7
anteiso-$C_{13:0}$	0.5	1.8	1.1	tr	tr
anteiso-$C_{14:0}$	0.7	–	–	–	tr
anteiso-$C_{15:0}$	3.6	**26.2**	**35.5**	**22.7**	1.7
anteiso-$C_{17:0}$	1.1	3.6	2.8	0.7	0.5
Summed feature†:					
3	**5.5**	–	–	2.5	2.5

7	1.0	–	–	–	–
8	3.9	–	–	2.9	2.1

*Data from this study.

†Summed features represent groups of two fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3, $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; summed feature 7, $C_{19:1}$ ω6c and/or $C_{19:1}$ ω7c and/or $C_{16:1}$ 19cy; summed feature 8, $C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c.

2.5. Whole genome sequencing of strain Rep29^T

To obtain a more accurate complete genome, both the Illumina and PacBio platform were conducted. After the first de novo assembly using PacBio, mapping the Illumina reads to first assembled genome sequence were applied for sequence compensation to construct contigs more correctly. Moreover, self-mapping was performed to get a whole genome sequence with higher quality (Table 7).

The complete genome of strain Rep29^T was composed of a 2,730,135 base pairs circular chromosome with 47.67 mol% genomic DNA G+C content. It was determined that the genome contains 2,445 genes, 2,374 coding sequences (CDSs), 61 transfer RNA (tRNA), 1 transfer-messenger RNA (tmRNA), and 9 rRNA. Functional annotation on 962 CDSs of total 2,374 CDSs (47.1%) were identified. An analysis of carbohydrate-active enzymes revealed that the strain Rep29^T contains 106 enzymes, including 51 GHs, 2 carbohydrate-binding modules, 45 glycosyltransferases, 1 polysaccharide lyase, 6 carbohydrate esterases, and 1 auxiliary activity. The overall genome features of strain Rep29^T are summarized in Table 8. In addition, it were classified into various metabolisms in 6 KEGG categories, including metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, and human diseases (Table 9). The genome of strain Rep29^T was submitted to the NCBI Genome under the accession number of CP047159.

Table 7. De novo assembly results of strain Rep29^T

(A) Stats of PacBio raw data

Contigs	Total contig bases	N50[a]	Max length	Min length	Mean length
1	2,730,116	2,730,116	2,730,116	2,730,116	2,730,116

(B) Stats of Illumina raw data

	Total reads bases	Total reads	GC (%)	Q20 (%)[b]	Q30 (%)[c]
Raw dataset	1,468,595,800	9,725,800	47.95	96.7	92.08
Filtered dataset	964,666,822	6,388,522	47.541	99.560	98.227

(C) Stats after assembly correction

Contigs	Total contig bases	N50 [a]	Max length	Min length	Mean length
1	2,730,135	2,730,135	2,730,135	2,730,135	2,730,135

(D) Overall mapping stats

Library name	Total reads	Mapped reads	Coverage (%)[d]	Depth[e]	Ins.size (Std.)[f]
Rep29	6,388,522	6,386,155	100.00	348.11	454 (461.12)

(E) Results of assembly: 1 contig was formed

Contig name	Length	GC (%)	Depth[g]	Circular[h]	Alias[i]
contig1	2,730,135	47.69	179	YES	Contig1
Total	2,730,135	47.69	179		

[a]N50: 50% of all bases come from contigs longer than this value
[b]Q20 (%): Ratio of bases that have phred quality score over 20
[c]Q30 (%): Ratio of bases that have phred quality score over 30
[d]Coverage (%): The percentage of mapped sited (>= 1x)
[e]Depth: Average mapping depth
[f]Ins.size (Std.): The length between adapters and standard deviation of predicted length
[g]Depth: The number of reads that aligned to each contig
[h]Circular: 5' end and 3' end are connected
[i]Alias: The alias is named based on the BLASTN (v2.7.1+) result
The following two conditions are used to create an alias:
a. Query cover 80% or more
b. Similarity between genome size
If both conditions are met, it is named Chromosome or Plasmid.
If not, named it Contig.

Table 8. Genomic features of strain Rep29^{T}

Number of Contigs	1		
Genome Size (bp)	2,730,135		
G+C content (mol%)	47.69		
Number of Genes	2,445	Protein encoding genes	2,374
		transfer RNA	61
		transfer-messenger RNA	1
		ribosomal RNA	9
Proteins with Predicted Functions	1,118		
Hypothetical or Uncharacterized Proteins	1,327		
Proteins with KEGG Annotations	962		
Carbohydrate-Active Enzymes	106	Glycoside Hydrolase	51
		Carbohydrate-Binding Module	2
		Glycosyltransferases	45
		Polysaccharide Lyase	1
		Carbohydrate Esterase	6
		Auxiliary Activities	1
Virulence Factors	12		
Antibiotic-Resistant Genes	4		

Table 9. Genome annotation results of strain Rep29^{T}

No.	Categories	Sub-catergories	Pathways	Genes
1	metabolism	Carbohydrate metabolism	15	149
		Energy metabolism	7	80
		Lipid metabolism	6	21
		Nucleotide metabolism	2	55
		Amino acid metabolism	12	95
		Metabolism of other amino acids	8	21
		Glycan biosynthesis and metabolism	8	41
		Metabolism of cofactors and vitamins	11	78
		Metabolism of terpenoids and polyketides	6	17
		Biosynthesis of other secondary metabolites	8	19
		Xenobiotics biodegradation and metabolism	2	11
2	Genetic information processing	Transcription	1	3
		Translation	3	78
		Folding, sorting and degradation	4	24
		Replication and repair	5	61
3	Environmental information processing	Membrane transport	2	24
		Signal transduction	5	24
4	Cellular processes	Transport and catabolism	2	6
		Cell growth and death	4	16
		Cellular community-prokaryotes	3	17
		Cell motility	2	2
5	Organismal systems	Immune system	4	5
		Endocrine system	8	8
		Digestive system	1	1
		Excretory system	1	1
		Nervous system	2	4
		Aging	2	4
		Environmental adaptation	2	3
6	Human diseases	Cancers: Overview	4	5
		Cancers: Specific types	1	1
		Immune diseases	1	1
		Neurodegenerative diseases	1	1
		Cardiovascular disease	1	3
		Endocrine and metabolic diseases	2	2
		Infectious diseases: Bacterial	4	8
		Drug resistance: Antimicrobial	3	15
		Drug resistance: Antineoplastic	1	4
Total	6 categories	37 sub-categories	154 pathways	908 genes

2.5.1. Mucin degradation and utilization

Among 51 GHs, strain Rep29^T had 14 GHs related to putative mucolytic enzymes, including *β*-galactosidases (GH2, GH20), *α*-*N*-acetylgalactosaminidases (GH101), *α*-*N*-acetylglucosaminidases (GH89), sialidases (GH33), fucosidases (GH29, GH95) (Table 10). Strain Rep29^T was predicted to use a starch utilization system (Sus)-like system for the utilization of mucin glycan. It was identified that strain Rep29^T had putative SusCD through a BlastP analysis against the genome of strain Rep29^T (Supplementary table S1).

The putative mucin-degrading pathway of strain Rep29^T was constructed based on the KEGG pathway and Blastp analysis. Strain Rep29^T had genes involved in the metabolism of carbon sources such as galactose, GlcNAc, sialic acid (Neu5Ac), and mannose according to the results of KEGG pathways (Figure 13). However, it is also incomplete because of the absence of galactose 1-phosphate uridyltransferase (EC 2.7.7.12) and *N*-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25) associated with galactose and GlcNAc metabolisms, respectively.

Table 10. List of predicted mucolytic enzymes in the complete genome of strain Rep29^T

Mucolytic enzymes	Glycoside hydrolases (GHs)	Locus ID[a]	KEGG ID	Prokka annotation
β-galactosidases	GH2	DJPCDFCF_01074	K01190 (*β*-galactosidase)	*β*-galactosidase
		DJPCDFCF_02139	K01190 (*β*-galactosidase)	*β*-galactosidase
	GH20	DJPCDFCF_00755	K12373 (*β*-hexosaminidase)	Hypothetical protein
		DJPCDFCF_01740	–	Hypothetical protein
		DJPCDFCF_01799	K12373 (*β*-hexosaminidase)	*β*-hexosaminidase
		DJPCDFCF_02310	K12373 (*β*-hexosaminidase)	Hypothetical protein
	GH42	–	–	–
Endo-*β*1,4-galactosidases	GH98	–	–	–
α-*N*-acetylgalactosaminidases	GH101	DJPCDFCF_01301	K01604 (methylmalonyl-CoA decarboxylase subunit alpha)	Propionyl-CoA carboxylase beta chain
	GH129	–	–	–
Exo- and endo-*β*-*N*-acetylglucosaminidases	GH84	–	–	–
	GH85	–	–	–
α-*N*-acetylglucosaminidases	GH89	DJPCDFCF_00315	K01205 (*α*-*N*-acetylglucosaminidases)	Hypothetical protein
Sialidases	GH33	DJPCDFCF_00633	–	Hypothetical protein
		DJPCDFCF_00914	K01186 (sialidase-1)	Sialidase
		DJPCDFCF_01114	–	Hercynine oxygenase
Fucosidases	GH29	DJPCDFCF_01076	K01206 (*α*-L-fucosidase)	Hypothetical protein
		DJPCDFCF_02312	K01206 (*α*-L-fucosidase)	Hypothetical protein
	GH95	DJPCDFCF_02222	K15923 (*α*-L-fucosidase 2)	Hypothetical protein

[a]Locus IDs are the results of CAZyme annotation using dbCAN2 tool.

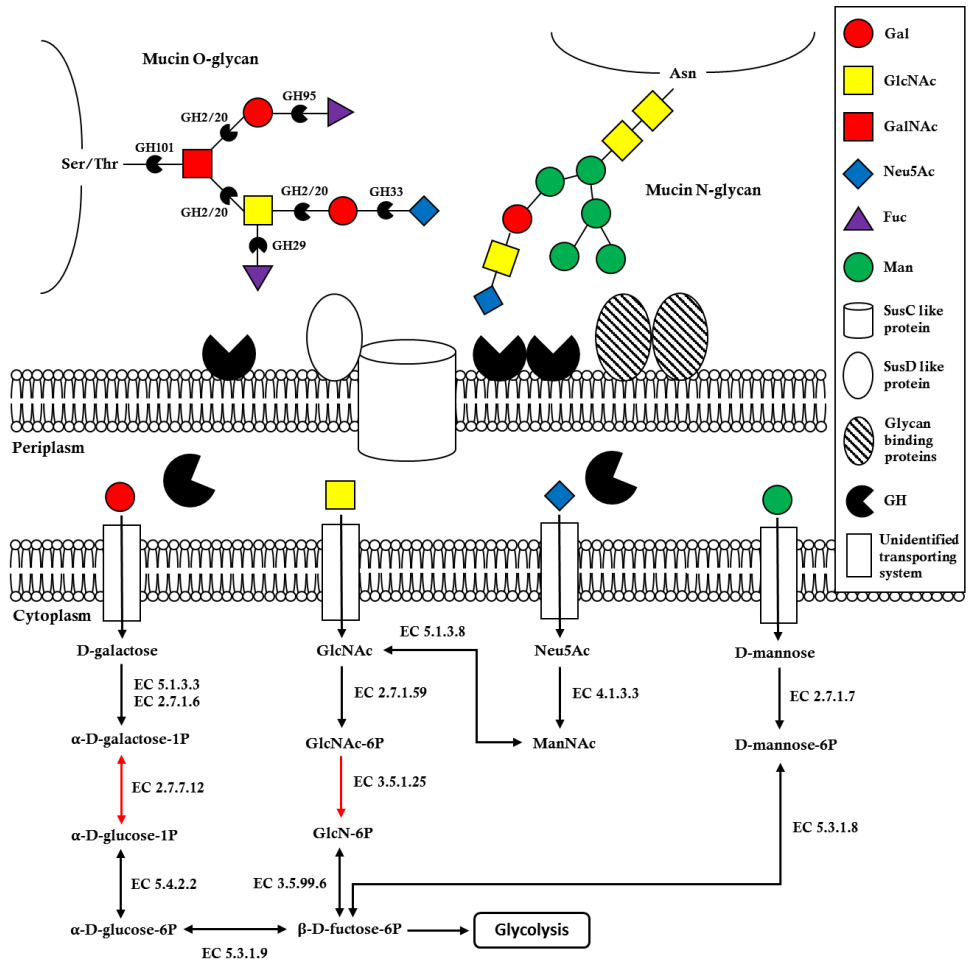


Figure 13. Putative mucin-degrading pathway of strain Rep29^{T}. Metabolic pathways that are present in the strain Rep29^{T} are depicted in black, and metabolic pathways that are absent in the strain Rep29^{T} are depicted in red. Asn = asparagine, Fuc = fucose, Gal = galactose, GalNAc = *N*-acetylgalactosamine, GH = glycoside hydrolases, GlcNAc = *N*-acetylglucosamine, Man = mannose, Neu5Ac = *N*-acetylneuraminic acid, Ser = serine, Sus = starch utilization system, Thr = threonine.

2.5.2. Extracellular polymeric substances biosynthesis

Characteristic phenotype trait of the Strain Rep29^T included the formation of branched-shaped extracellular structures (Figure 7). Through Blastp against the genome of strain Rep29^T, strain Rep29^T were confirmed the presence of putative extracellular polysaccharide biosynthesis mechanisms, including Wzx/Wzy-dependent pathway and synthase-dependent pathway. Strain Rep29^T had 4 putative glycosyltransferases (DJPCDFCF_00841, DJPCDFCF_00842, DJPCDFCF_00844, and DJPCDFCF_00852), 3 acetyltransferases (DJPCDFCF_00827, DJPCDFCF_00835, and DJPCDFCF_00845), 1 flippase (DJPCDFCF_00787), 1 polymerase (DJPCDFCF_00850), 1 polysaccharide co-polymerase (DJPCDFCF_00782), and 1 outer membrane transport protein (DJPCDFCF_01169) for the Wzx/Wzy-dependent pathway. Moreover, strain Rep29^T had 1 putative synthase (HasA; DJPCDFCF_00894), 3 precursors of synthesis (HasB; DJPCDFCF_00799 and DJPCDFCF_00834 and HasC; DJPCDFCF_00851) for synthase-dependent pathway.

2.5.3. Virulence factors

Strain Rep29^T had 12 putative virulence factors. They contain factors associated with adherence (hasB, KpsF, and htpB), stress reactions (clpP and clpC), antiphagocytosis (hasB and bcs1), O-antigen (gmd, fcl, and galE), and metabolic adaption (panD) (Table 11).

Table 11. List of predicted virulence factors in the complete genome of strain Rep29^T

No.	Locus ID	Length(aa)	VRprofile[a] ID	Ha-value	Product	VFDB[b] category
1	DJPCDFCF_00298	221	VFG0077	0.45	clpP (ATP-dependent Clp protease proteolytic subunit)	Stress protein
2	DJPCDFCF_00549	862	VFG0079	0.46	clpC (Chaperone protein ClpB)	Stress protein
3	DJPCDFCF_00799	434	VFG0963	0.55	hasB (Nucleotide sugar dehydrogenase)	Antiphagocytosis; Adherence; Tissue invasion
4	DJPCDFCF_00887	318	VFG1971	0.48	kpsF (KpsF/GutQ family protein)	Adherence; Phase variation
5	DJPCDFCF_00939	542	VFG1855	0.58	htpB (60 kDa chaperonin)	Adherence
6	DJPCDFCF_01015	845	VFG0079	0.45	clpC (ATP-dependent Clp protease ATP-binding subunit ClpC)	Stress protein
7	DJPCDFCF_01134	361	VFG2365	0.64	gmd (GDP-mannose 4,6-dehydratase)	O-antigen
8	DJPCDFCF_01135	390	VFG2364	0.44	fcl (GDP-L-fucose synthase)	O-antigen
9	DJPCDFCF_01304	115	VFG1416	0.51	panD (Aspartate 1-decarboxylase)	metabolic adaptation
10	DJPCDFCF_01438	345	VFG2361	0.46	galE (UDP-galactose 4-epimerase)	O-antigen
11	DJPCDFCF_02188	255	VFG0700	0.44	bcs1 (bifunctional; ribulose 5-phosphate reductase; CDP-ribitol pyrophosphorylase)	Antiphagocytosis
12	DJPCDFCF_02392	420	VFG1823	<0.64	mbtC (3-oxoacyl-)	Iron uptake; Siderophore

[a]VRprofile: a web-based tool for in silico profiling of virulence and antibiotic resistance traits encoded within genome sequences of bacteria.
[b]VFDB: a reference database for bacterial virulence factors.

Ⅴ. Discussion

Microbial community in the rumen plays a central role in the fermentation and productivity of ruminants. Among them, epimural bacteria (rumen epithelial adherent bacteria) are responsible for the association with luminal bacteria in relation to the metabolic process of the host (McCowan et al., 1980; Sadet et al., 2007). Studies on epimural bacteria were mainly done using electron microscopy with cultural technique only in the 1970s and 1980s (Bauchop et al.,1975; McCowan et al., 1978; Wallace et al., 1979; Mead et al., 1981; Mueller et al., 1984). As genetic technology advanced, it revealed that a large variety of microbial community resides in the rumen epithelium (Li et al., 2012). In general, mucolytic bacteria colonize on the mucosal layer beneath the epithelial tissue attached to mucins as a nutrient source (Darren et al., 2010). Recently, a targeted cultivation approach through basal medium supplemented with mucin as a single carbon source allowed to isolate a new intestinal bacterium, *Akkermansia muciniphila,* from human fecal samples (Derrien et al., 2004). This commensal mucolytic bacteria, *Akkermansia muciniphila*, could maintain the microbial colonization of the mucosal layer associated with other beneficial bacteria and improve various metabolic disorders such as inflammatory bowel disease, type 2 diabetes, cardiovascular diseases, autism, atopy or obesity (Derrien et al., 2004, 2017).

In this study, epithelial tissue samples obtained from the root of the dorsal rumen. Because of low abrasive action with digest in the dorsal sac, the root of the dorsal rumen was most densely colonized by epimural community (McCowan et al., 1980). Treatment for detachment of the bacteria involved either homogenization or washing tissue to remove feed particle and non-

adherent bacteria was conducted. And then, mucolytic bacteria adherent to the rumen epithelium were isolated using mucin defined medium as a sole carbon source. The 12 isolates were identified as 2 cultured bacteria and 3 uncultured bacteria by 16S rRNA gene analysis. In two kinds of cultured bacteria, five isolates were most closely related to *Paraclostridium benzoelyticum* JC272^T with 98.8-99.9% similarity and one isolate was most closely related to *Citrobacter murliniae* CDC 2970-59^T with 99.5% similarity. In three kinds of uncultured bacteria, two isolates were most closely related to *Prevotella brevis* GA33^T with 89.9% and 89.7% each similarity, three isolated were most closely related to *Campylobacter fetus* subsp. *testudinum* 03-427^T with 96.5-97.0% similarity, and one isolate was most closely related to *Prevotella bryantii* B14^T with 86.6% similarity (Table 4).

The isolate Rep29 was selected for characterization because it grew well in mucin medium among the uncultured isolates with less than 90% rRNA gene sequence similarity with the closely related bacterium.

The 16S rRNA gene sequence of strain Rep29^T was phylogenetically analyzed with those of species in the family *Prevotellaceae* using NJ, ML, and MP algorithm. The phylogenetic analysis showed that strain Rep29^T was affiliated with the family *Prevotellaceae* and closed to the genus *Prevotella* (Figure 4, 5, and 6). In the phylogenetic tree reconstructed from aligned sequences, *Prevotella brevis* and *Prevotella ruminicola* were the most closely related to strain Rep29^T. Also, the 16S gene sequence of strain Rep29^T had the highest similarity in order of *Prevotella brevis* (89.8%) and *Prevotella ruminicola* (89.4%), respectively. The range of similarity percentages of Rep29^T with species of the genus *Prevotella* was 85.9-89.8%. In general, less than 90% rRNA gene sequence similarity is one of the evidences of novel genus. However, until now, the species named genus *Prevotella* have also been classified as genus *Prevotella* by

~90% similarity (Bhul et al., 2016; Efimov et al., 2018). For comparative phylogenetic and 16S gene sequence similarity analysis, it clearly indicated that the strain Rep29^T represents a member of the genus *Prevotella*.

Like the coccoid cells of *Prevotella brevis* and *Prevotella ruminicola*, cells of the strain Rep29^T were Gram-negative, non-motile coccus. According to TEM inspection, strain Rep29^T had filamentous structures similar to mucolytic bacteria such as *Akkermansia muciniphila* isolated from human feces and strain E39^T previously isolated from rumen epithelium in our laboratory (Figure 7) (Derrien et al., 2004).

Cells exhibited growth at a temperature of 35-45℃ and optimum temperature at 39℃. Given that the temperature of rumen is typically controlled between 38℃ and 41℃, strain Rep29^T can be seen that growth is optimized in the rumen (Figure 9). Although pH of the rumen maintained between pH 5.5 and 6.9, the cells were grown at a relatively high pH of 6.0 to 7.5 and showed optimal growth at pH 7.0 (Figure 10). Alkaline secretion from epithelial cells into the mucus layer provides the first defense against the acid discharged from the stomach (Williams and Turnberg, 1981; Sababi et al., 1995). Thus, the cells appeared to grow at higher pH than internal rumen by attaching to the rumen epithelium. The cells did not grow under aerobic conditions in spite of the presence of reducing agents (Figure 12). This is common in that rumen remains anaerobic. However, epithelial cells in the GI tract are necessary for contact with oxygen for aerobic respiration. The cells represented growth under anaerobic conditions in the absence of reducing agents. It is suggested that the cells had some resistance to oxygen and have properties of epimural bacteria that consume oxygen and make a suitable environment for oxygen-sensitive anaerobes in the rumen (Cheng et al., 1979; Mead and Jones, 1981; Muller et al., 1984).

The enzyme profiles test showed that there was a negative activity on *α*-fucosidase related to mucin degradation. But strain Rep29^T had positive activities on mucolytic enzymes, including *α*-galactosidase, *β*-galactosidase, *α*-glucosidase and *N*-acetyl-*β*-glucosaminidase (Derrien et al., 2010). In addition, a negative reaction on urease activity suggested strain Rep29^T may not hydrolyze urea, which is one of the important roles of epimural bacteria.

The analysis of CAZyme annotation using the dbCAN2 tool showed that strain Rep29^T had a total 51 GHs, of which 14 GHs were identified as enzymes involved in mucin degradation (Table 10). Compared with 66 GHs of *Bacteroides thetaiotaomicron* and 28 GHs of strain E39^T which are Gram-negative mucolytic bacteria, the strain Rep29^T had fewer but similar kinds of mucolytic GHs. Strain Rep29^T is predicted to utilize mucin glycan by Sus-like systems, such as *Bacteroides thetaiotaomicron*. According to Blastp analysis, the putative Sus genes, SusCD, were identified in the genome of strain Rep29^T (Supplementary table S1). Initially, mucin glycans are bounded by outer membrane proteins, such as glycan-binding SusD proteins. Surface-bounded glycans are partially degraded by various GHs to produce smaller oligosaccharides. Afterward, SusC proteins transport oligosaccharide from an extracellular place to the periplasm. After transportation, oligosaccharides are degraded into their component mono- or disaccharides by GHs. Finally, mucin-derived saccharides are imported to the cytoplasm through inner membrane transporters (Martens et al., 2009). Through the results of KEGG pathways, strain Rep29^T had the metabolic process of galactose, GlcNAc, sialic acid (Neu5Ac), and mannose (Figure 13). Similar to the results of the identification kit test, it was shown that the lack of enzymes for fucose metabolism made it impossible to use fucose as a substance for energy source. However, although strain Rep29^T was shown a negative reaction in the mannose fermentation, the

mannose pathway is clearly identified. To understand the mucin-degrading metabolic process of Rep29^T, further studies on the system of inner transporters are needed. Strain Rep29^T revealed the growth pattern at the low OD compared with strain E39^T, mucolytic bacteria, previously isolated from rumen bacteria in our laboratory. This is suggested by the fact that fewer mucolytic enzymes and absence of fucose metabolism.

Extracellular polysaccharides produced by microbes can be important for microbial biofilm formation. These play a crucial role in a variety of adhesion to the bacterial cells or surfaces, protection from environmental stress and disinfectants, and structure of colonization (Czaczyk and Myszka, 2007). Strain Rep29^T was confirmed the formation of branched-shaped extracellular structures by the result of TEM visualization (Figure 7). These structures were assumed to act as adhesion to rumen epithelium as the bacterial glycocalyx, polysaccharide-containing structures of bacterial origin (Costerton et al., 1981). Strain Rep29^T was identified the presence of putative extracellular polysaccharide biosynthesis mechanisms, Wzx/Wzy-dependent pathway and synthase-dependent pathway, by Blastp against the genome of strain Rep29^T. In the Wzx/Wzy-dependent pathway, glycosyltransferases assemble repeating units and a flippase (Wzx protein) translocate the units across the cytoplasmic membrane. The repeating units are extended by a polymerase (Wzy protein) and transported across the outer membrane through a polysaccharide export protein (Schmid et al., 2015). Strain Rep29^T had 4 putative glycosyltransferases, 3 acetyltransferases, 1 flippase, 1 polysaccharide co-polymerase, and 1 outer membrane transport protein for the Wzx/Wzy-dependent pathway. In the synthase-dependent pathway, a single synthase protein secretes complete polymer strands across the membrane and the cell wall without a flippase for translocation and required only one type of sugar precursor. Hyaluronic acid

synthesis reaction mechanism is the combined activity of polymerization and translocation (Schmid et al., 2015). Also, strain Rep29^T had 1 putative synthase (*hasA*), 3 precursors of synthesis (*hasB*, *hasC*) for hyaluronic acid synthesis of synthase-dependent pathway.

Bacterial virulence factors enable bacteria to survive in the host (Bakour et al., 2016). They include bacterial toxins that contribute to the pathogenicity of the bacterium, surface-exposed proteins that mediate adhesion, colonization, and invasions, and capsular polysaccharides that protect a bacterium (Wu et al., 2008). Strain Rep29^T had 12 putative virulence factors related to adherence (hasB, KpsF, htpB), stress reactions (clpP, clpC), antiphagocytosis (hasB, bcs1), O-antigen (gmd, fcl, galE), and metabolic adaption (panD) (Table 11). Because of the absence of genes coding bacterial toxins, strain Rep29^T was characterized as showing low-pathogenicity for the host. The virulence factors encoded in the genome may allow to attach and colonize on the rumen epithelium by evading the host's immune systems. Further studies on pathogenic effects at molecular approaches or *in vivo* were needed to determine the pathogenicity of the strain Rep29^T.

In conclusion, the phylogenetic, physiological and chemotaxonomic features represent a novel species of the genus *Prevotella*, for which the name *Prevotella mucinisolevens* sp. nov. is proposed. This study contributes to the discovery of a novel mucolytic bacterium from the rumen epithelium and provides valuable functional data on host-rumen microbial interactions. Besides, genetic analysis of putative mucin-degrading ability and pathogenicity can be used as a basis to elucidate commensal bacteria and health indicators. Future studies should be investigated potential functions of strain Rep29^T on the host's physiology.

Ⅵ. Literature cited

Avgustin G, Wallace RJ, Flint HJ. Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. *Int J Syst Bacteriol* 1977;47:284–288.

Bakour S, Sankar SA, Rathored J, Biagini P, Raoult D, Fournier PE. Identification of virulence factors and antibiotic resistance markers using bacterial genomics. *Future Microbiol* 2016;11:455-466.

Bansil R, Stanley E, LaMont JT. Mucin biophysics. *Annu Rev Physiol* 1995;57:635–657.

Belenguer A, Duncan SH, Holtrop G, Anderson SE, Lobley GE, Flint HJ. Impact of pH on lactate formation and utilization by human fecal microbial communities. *Appl Environ Microbiol 2007*;73:6526–6533.

Buhl M, Willmann M, Liese J, Autenrieth IB, Marschal M. *Prevotella colorans* sp. nov., isolated from a human wound. *Int J Syst Evol Microbiol* 2016;66:3005–3009.

Brockhausen I. Biosynthesis of Complex Mucin-Type *O*-Glycans. In: Mander L, Lui H-W, Wang PG (eds). Comprehensive Natural Products II Chemistry and Biology, Volume 6: Carbohydrates, Nucleosides and Nucleic Acids.

Elsevier. pp 315–350; 2010.

Brown D. Membrane recycling and epithelial cell function. *Am J Physiol* 1989;256:F1–F12.

Bryant MP, Small N, Bouma C, Chu H. *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica*; the new genus and species; species of succinic acid-producing anaerobic bacteria of the bovine rumen. *J Bacteriol* 1958;76:15–23.

Bauchop T, Clarke RT, Newhook JC. Scanning electron microscope study of bacteria associated with the rumen epithelium of sheep. *Appl Microbiol* 1975;30:668–675.

Caldwell DR, Bryant MP. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Appl Microbiol* 1966;14:794–801.

Carrington SD, Irwin JA, Liu L, Rudd PM, Matthews E, Corfield AP. Analysing mucin degradation. *Methods Mol Biol* 2012;842:191–215.

Cheng KJ, McCowan RP, Costerton JW. Adherent epithelial bacteria in ruminants and their roles in digestive tract function. *Am J Clin Nutr* 1979;32:139–148.

Cheng KJ, Wallace RJ. The mechanism of passage of endogenous urea through

the rumen wall and the role of ureolytic epithelial bacteria in the urea flux. *Br J Nutr* 1979;42:553–557.

Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, Clum A, Copeland A, Huddleston J, Eichler EE, Turner SW, Korlach J. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nat Methods* 2013;10:563–569.

Cho SJ, Cho KM, Shin EC, Lim WJ, Hong SY, Choi BR, Kang JM, Lee SM, Kim YH, Kim H, Yun HD. 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. *J Microbiol Biotechnol* 2006;16:92–101.

Czaczyk K, Myszka K. Biosynthesis of Extracellular Polymeric Substances (EPS) and Its Role in Microbial Biofilm Formation. *Pol J Environ Stud* 2007;16:799–806.

De Mulder T, Goossens K, Peiren N, Vandaele L, Haegeman A, De Tender C, Ruttink T, De Wiele TV, De Campeneere S. Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiol Ecol* 2017;93.

Dehority BA, Grubb JA. Bacterial population adherent to the epithelium on the root of the dorsal rumen of sheep. *Appl Environ Microbiol* 1981;41:1424–1427.

Derrien M, Vaughan EE, Plugge CM, de Vos WM. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol* 2004;54:1469–1476.

Derrien M, van Passel MWJ, van de Bovenkamp JHB, Schipper R, de VOS W, Dekker J. Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes* 2010;1:254–268.

Derrien M, Belzer C, de Vos WM. *Akkermansia muciniphila* and its role in regulating host functions. *Microb Pathog* 2017;106:171–181.

Dinsdale D, Cheng KJ, Wallace RJ, Goodlad RA. Digestion of epithelial tissue of the rumen wall by adherent bacteria in infused and conventionally fed sheep. *Appl Environ Microbiol* 1980;39:1059–1066.

Downes J, Sutcliffe I, Tanner AC, Wade WG. *Prevotella marshii* sp. nov. and *Prevotella baroniae* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* 2005;55:1551–1555.

Efimov BA, Chaplin AV, Shcherbakova VA, Suzina NE, Podoprigora IV, Shkoporov AN. *Prevotella rara* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2018;68:3818–3825.

Ficko-Blean E, Boraston AB. Insights into the recognition of the human glycome by microbial carbohydrate-binding modules. *Curr Opin Struct Biol* 2012;22:570–577.

Fina LR, Hay CA, Bartley EE, Mishra B. Bloat in cattle. V. The role of rumen mucinolytic bacteria. *J Anim Sci* 1961;20:654–658.

Flint HJ, Duncan SH, Scott KP, Louis P. Links between diet, gut microbiota composition and gut metabolism. *Proc Nutr Soc* 2015;74:13–22.

Florey, H. Mucin and the protection of the body. *Proc R Soc Lond B Biol Sci* 1955;143:147–157.

Fujita K, Oura F, Nagamine N, Katayama T, Hiratake J, Sakata K, Kumagai H, Yamamoto K. Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type *O*-glycan-specific endo-alpha-*N*-acetylgalactosaminidase from Bifidobacterium longum. *J Biol Chem* 2005;280:37415–37422.

Gendler SJ, Spicer AP. Epithelial mucin genes. *Annu Rev Physiol* 1955;57:607–634.

Gianmauro C, Orsini M, Pinna A, Sbardellati A, Soranzo, N, Travaglione A, Uva P, Zanetti G, Fotia G. Orione, a web-based framework for NGS

analysis in microbiology. *Bioinformatics* 2014;30:1928–1929.

Gomori G. Preparation of buffers for use in enzyme studies. *Methods Enzymol* 1955;1:138–146.

Hayashi H, Shibata K, Sakamoto M, Tomita S, Benno Y. *Prevotella copri* sp. nov. and *Prevotella stercorea* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2007;57:941–946.

Hoorens PR, Rinaldi M, Li RW, Goddeeris B, Claerebout E, Vercruysse J, Geldhof P. Genome wide analysis of the bovine mucin genes and their gastrointestinal transcription profile. *BMC Genomics* 2011;12:140–151.

Hoskins LC, Boulding ET. Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. *J. Clin. Invest* 1981;67:163–172.

Huang JY, Lee SM, Mazmanian SK. The human commensal *Bacteroides fragilis* binds intestinal mucin. *Anaerobe* 2011;17:137–141.

Kandori H, Hirayama K, Takeda M, Doi K. Histochemical, lectin-histochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice. *Exp Anim* 1996;45:155–160.

Kanehisa M, Sato Y, Morishima K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J Mol Biol* 2016;428:726–731.

Kaoutari AE, Armougom F, Gordon JI, Raoult D, Henrissat B., Sato Y, Morishima K. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol* 2013;11:497-504.

Katayama T, Fujita K, Yamamoto K. Novel bifidobacterial glycosidases acting on sugar chains of mucin glycoproteins. *J Biosci Bioeng* 2005;99:457-465.

Kennedy PM, Milligan LP. The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants: a Review. *Canadian Journal of Animal Science* 1980;60:205-221.

Kiyohara M, Nakatomi T, Kurihara S, Fushinobu S, Suzuki H, Tanaka T, Shoda S, Kitaoka M, Katayama T, Yamamoto K, Ashida H. α-*N*-acetylgalactosaminidase from infant-associated bifidobacteria belonging to novel glycoside hydrolase family 129 is implicated in alternative mucin degradation pathway. *J Biol Chem* 2012;287:693–700.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1874.

Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds). Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons. pp 115–175; 1991.

Lavker R, Chalupa W, Dickey JF. An electron microscopic investigation of rumen mucosa. *J Ultrastruct Res* 1969;28:1–15.

Leitch EC, Walker AW, Duncan SH, Holtrop G, Flint HJ. Selective colonization of insoluble substrates by human faecal bacteria. *Environ Microbiol* 2007;9:667–679.

Levy GN and Aminoff D. Purification and properties of a-*N*-acetylgalactosaminidase from *Clostridium perfringens*. *J Biol Chem* 1980;255:11737–11742.

Li J, Tai C, Deng Z, Zhong W, He Y, Ou HY. VRprofile: gene-cluster-detection-based profiling of virulence and antibiotic resistance traits encoded within genome sequences of pathogenic bacteria. *Brief Bioinform* 2018;19:566–574.

Li M, Zhou M, Adamowicz E, Basarab JA, Guan LL. Characterization of bovine ruminal epithelial bacterial communities using 16S rRNA sequencing, PCR-DGGE, and qRT-PCR analysis. *Vet Microbiol* 2012;155:72–80.

Liu J, Zhang M, Zhang R, Zhu W, Mao S. Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. *Microb Biotechnol* 2016;9:257–268.

Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res* 2014;42:D490–D495.

Louis P, Young P, Holtrop G, Flint HJ.. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ Microbiol* 2010;12:304–314.

Macfarlane GT, Cummings JH, Allison C. Protein degradation by human intestinal bacteria. J *Gen Microbiol* 1986;132:1647–1656.

Macfarlane GT, Allison C, Gibson SA, Cummings JH. Contribution of the microflora to proteolysis in the human large intestine. *J Appl Bacteriol* 1988;64:37–46.

Macfarlane S, Woodmansey EJ, Macfarlane GT. Colonization of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. *Appl Environ Microbiol* 2005;7:7483-7492.

Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, Lebrilla CB, Weimer BC, Mills DA, German JB, Sonnenburg JL. *Bacteroides* in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host Microbe* 2011;10:507–514.

Martens EC, Koropatkin NM, Smith TJ, Gordon JI. Complex glycan catabolism by the human gut microbiota: the *Bacteroidetes* Sus-like paradigm. *J Biol Chem* 2009;284:24673–24677.

McCowan RP, Cheng KJ, Bailey CBM, Costerton JW. Adhesion of bacteria to epithelial cell surfaces within the reticulo-rumen of cattle. *Appl Environ Microbiol* 1978;35:149–155.

McCowan RP, Cheng KJ, Costerton JW. Adherent Bacterial Populations on the Bovine Rumen Wall: Distribution Patterns of Adherent Bacteria. *Appl Environ Microbiol* 1980;39:233-241.

Mead LJ, Jones GA. Isolation and presumptive identification of adherent epithelial bacteria ("Epimural" bacteria) from the ovine rumen wall. *Appl Environ Microbiol* 1981;41:1020–1028.

Minato H, Ishibashi S, Hamaoka T. Cellular fatty acid and sugar composition of representative strains of rumen bacteria. *J Gen Appl Microbiol* 1988;34:303–319.

Mishra BD, Fina LR, Bartley EE, Claydon TJ. Bloat in cattle. XI. The role of rumen aerobic (facultative) mucinolytic bacteria. *J Anim Sci* 1967;26:606–612.

Mishra BD, Bartley EE, Fina LR, Bryant MP. Bloat in cattle. XIV. Mucinolytic activity of several anaerobic rumen bacteria. *J Anim Sci* 1968;27:1651–1656.

Morotomi M, Nagai F, Sakon H, Tanaka R. *Paraprevotella clara* gen. nov., sp. nov. and *Paraprevotella xylaniphila* sp. nov., members of the family '*Prevotellaceae*' isolated from human faeces. *Int J Syst Evol Microbiol* 2009;59:1895–1900.

Muller RE, Iannotti EL, Asplund JM. Isolation and identification of adherent epimural bacteria during succession in young lambs. *Appl Environ Microbiol* 1984;47:724–730.

Norin K, Gustafsson BE, Lindblad B, Midtvedt T. The establishment of some microflora associated biochemical characteristics in feces from children during the first years of life. *Acta Paediatr Scand* 1985;74:207–212.

Pei CX, Mao SY, Cheng YF, Zhu WY. Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. *Animal.* 2010;4:20–29.

Prizont R. Degradation of intestinal glycoproteins by pathogenic *Shigella flexneri*. *Infect Immun* 1982;36:615–620.

Puniya AK, Singh R, Kamra DN. Rumen microbiology: An overview. In: Choudhury PK, Salem AZM, Jena R, Kumar S, Singh R, Puniya AK (eds). Rumen Microbiology: From Evolution to Revolution. Springer. pp 3–47; 2015.

Rajilic-Stojanovic1 M, De Vos W.M. The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiol Rev* 2014;38:996-1047.

Rizzatti G, Lopetuso LR, Gibiino G, Binda C, Gasbarrini A. Proteobacteria: A Common Factor in Human Diseases. *Biomed Res Int* 2017;9351507.

Rose MC and Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;86:245–279.

Sababi M, Nilsson E, Holm L. Mucus and alkali secretion in the rat duodenum: effects of indomethacin, N^{ω}–omega-nitro-*L*-arginine, and luminal acid. *Gastroenterology* 1955;109:1526-1534.

Sadet S, Martin C, Meunier B, Morgavi DP. PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium.

Animal 2007;1:939–944.

Sakamoto M, Umeda M, Ishikawa I, Benno Y. *Prevotella multisaccharivorax* sp. nov., isolated from human subgingival plaque. *Int J Syst Evol Microbiol* 2005;55:1839–1843.

Sasser M. Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. Newark, DE: MIDI Inc; 1990.

Salyers AA, Vercellotti JR, West SE, Wilkins TD. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. *Appl Environ Microbiol* 1977;33:319-322.

Schmid J, Sieber V, Rehm B. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Front Microbiol* 2015;6:496.

Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.

Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, Bae JW. An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* 2014;63:727–735.

Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol* 2015;33:496–503.

Slomiany BL, Murty VL, Piotrowski J, Grabska M, Slomiany A. Glycosulfatase activity of H. pylori toward human gastric mucin: effect of sucralfate. *Am J Gastroenterol* 1992;87:1132–1137.

Steele MA, Penner GB, Chaucheyras-Durand F, Guan LL. Development and physiology of the rumen and the lower gut: Targets for improving gut health. *J Dairy Sci* 2016;99:4955–4966.

Tailford LE, Crost EH, Kavanaugh D, Juge N. Mucin glycan foraging in the human gut microbiome. *Front Genet* 2015;6:81.

Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK, Earl AM. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 2014;9:e112963.

Wallace RJ, Cheng KJ, Dinsdale D, Ørskov ER. An independent microbial flora of the epithelium and its role in the ecomicrobiology of the rumen. *Nature* 1979;279:424–426.

Watabe J, Benno Y, Mitsuoka T. Taxonomic study of *Bacteroides oralis* and

related organisms and proposal of *Bacteroides veroralis* sp. nov. *Int J Syst Bacteriol* 1983;33:57–64.

Williams SE, Turnberg LA. Demonstration of a pH gradient across mucus adherent to rabbit gastric mucosa: evidence for a 'mucus-bicarbonate' barrier. *Gut* 1981;22:94–96.

Wu C-C, Johnson JL, Moore WEC, Moore LVH. Emended descriptions of *Prevotella denticola, Prevotella loescheii, Prevotella veroralis,* and *Prevotella melaninogenica*. *Int J Syst Bacteriol* 1992;42:536–541.

Wu HJ, Wang AH, Jennings MP. Discovery of virulence factors of pathogenic bacteria. *Curr Opin Chem Biol* 2008;12:93–101.

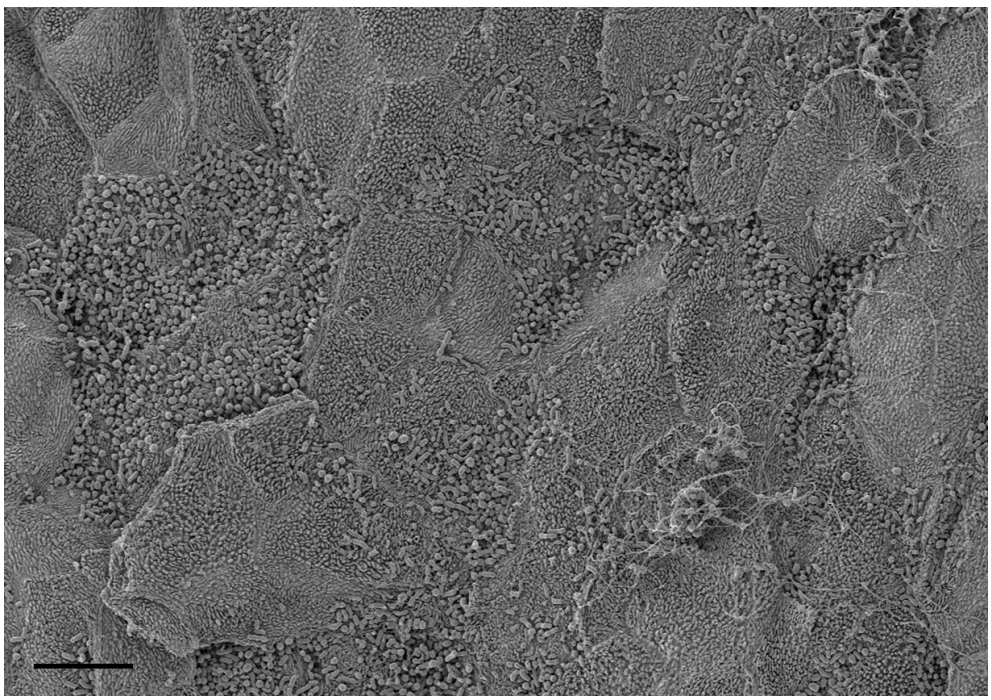
Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI. Human gut microbiome viewed across age and geography. *Nature* 2012;486:222–227.

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J. Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.

Ze X, Duncan SH, Louis P, Flint HJ. *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. *ISME J* 2012;6:1535–1543.

Zhang H, Yohe T, Huang L, Entwistle S, Wu P, Yang Z, Busk PK, Xu Y, Yin Y. dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res* 2018;468:W95–W101.

Ⅶ. Supplements



Supplementary figure S1. A scanning electron micrograph (SEM) showing rumen bacteria adherent to the rumen epithelium from dorsal sac; epimural bacteria. Bar, 10 μm.

Supplementary table S1. List of predicted starch utilization system (Sus) genes in the complete genome of strain Rep29^{T}

Gene	Locus ID	KEGG ID	Prokka annotation
SusC	DJPCDFCF_00002	–	Hypothetical protein
	DJPCDFCF_00066	–	Hypothetical protein
	DJPCDFCF_00068	–	Hypothetical protein
	DJPCDFCF_00072	–	TonB-dependent receptor SusC
	DJPCDFCF_00180	–	Hypothetical protein
	DJPCDFCF_00363	–	TonB-dependent receptor SusC
	DJPCDFCF_00481	–	TonB-dependent receptor SusC
	DJPCDFCF_00483	–	Hypothetical protein
	DJPCDFCF_01522	–	Hypothetical protein
	DJPCDFCF_01753	K01951 (GMP synthase)	TonB-dependent receptor SusC
	DJPCDFCF_01951	–	Hypothetical protein
	DJPCDFCF_01956	–	Hypothetical protein
SusD	DJPCDFCF_00065	K21572 (starch-binding outer membrane protein)	Hypothetical protein
	DJPCDFCF_00362	K21572 (starch-binding outer membrane protein)	SusD-like protein
	DJPCDFCF_00484	K21572 (starch-binding outer membrane protein)	Hypothetical protein

Ⅷ. Summary in Korean

본 연구는 한우의 반추위벽으로부터 당단백질인 뮤신을 분해하는 박테리아를 분리하여 특성을 규명하기 위해 수행하였다. 혐기적 조건에서 유일한 탄소원으로 뮤신을 첨가한 배지를 통한 표적 배양으로 도축된 한우 거세우의 등 쪽 반추위벽으로부터 뮤신 분해 박테리아를 분리하였다. 총 40개의 단일 콜로니 중에서 12개가 뮤신만 첨가된 배지에서 성장하였다. 분리된 균주의 16S rRNA 유전자 분석을 통해 2종의 이미 밝혀진 박테리아 (*Paraclostridium benzoelyticum*와 *Citrobacter murliniae*)와 3종의 신종 박테리아로 확인되었다. 그리고 분리된 신종 균주 중에서 strain Rep29의 표현형, 생화학적, 화학 분류상 및 계통학적 분석을 하였다. strain Rep29는 그람 음성, 운동성이 없는 구형 혐기성 균으로, 35-45°C (최적 39°C), pH 6.0-7.5 (최적 7.0) 및 0.0–1.5 % NaCl (최적 0.0-0.5%)에서 성장을 확인하였다. 주요 지방산은 $C_{16:0}$, $C_{18:1}$ ω9c와 iso-$C_{15:0}$으로 확인하였다. API Rapid ID 32A와 API ZYM identification kit (bioMe´rieux)를 이용한 효소 분석을 통해 strain Rep29는 뮤신 분해 효소인 α-galactosidase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase 활성을 보였다. 또한 전체 유전자 서열 분석을 통해 Sus-like 시스템을 이용하여 뮤신을 에너지원으로 이용할 것으로 예상되며, galactose, GlcNAc, Neu5Ac 및 mannose 대사를 확인하였다. 그리고 반추위벽에 부착하기 위한 세포 외 다당류 생합성 경로 (Wzx/Wzy-dependent pathway와 synthase-dependent pathway)와 독성인자를 확인하였다. 16S rRNA 유전자의 서열을 기반으로 시행한 계통 분석을 통해 strain Rep29는 *Prevotella brevis* GA33^{T}와 가장 가까운 유사성을 보이며, genus *Prevoetlla*에 속하는 새로운 species임을 확인하였다. 균주의 이름

은 *Prevotella mucinisolevens*라 명명하였으며 type strain은 Rep29^{T}이다 (= KCTC 15980^{T}).