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Genomic Analysis of *Actinomyces* sp. Strain CtC72, a Novel Fibrolytic Anaerobic Bacterium Isolated from Cattle Rumen

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A xylanolytic and cellulolytic anaerobic bacterium strain CtC72 was isolated from cattle rumen liquor. Phylogenetic analysis of 16S rRNA gene sequences revealed that strain CtC72 shared only 97.78% homology with its nearest phylogenetic affiliate *Actinomyces ruminicola*, showing its novelty. The strain could grow on medium containing xylan, carboxymethyl cellulose and avicel producing CO_2, acetate, and ethanol as major fermentation products. The whole genome analysis of the strain CtC72 exhibited a broad range of carbohydrate-active enzymes required for the breakdown and utilization of lignocellulosic biomass. Genes related to the production of ethanol and stress tolerance were also detected. Further there were several unique genes in CtC72 for chitin degradation, pectin utilization, sugar utilization, and stress response in comparison with *Actinomyces ruminicola*. The results show that the strain CtC72, a putative novel bacterium can be used for lignocellulosic biomass based biotechnological applications.

Keywords: **Anaerobe, avicel, bioethanol, biomass, cellulose, lignocellulose**

Introduction

Lignocellulosic biomass is the most abundant renewable energy source present in the world [1]. The cellulose and hemicellulose components of lignocellulose contain around 70% of fermentable sugars, which can be converted in to methane or ethanol and used as renewable and sustainable fuels [2]. Development of second generation biofuel technologies based on lignocellulosic biomass requires lignocellulolytic microbes. In this regard, anaerobic bacteria are of immense importance due to their ability to produce highly efficient extracellular multi-enzyme complexes, called cellulosomes [3]. Rumen, which houses highly diverse group of anaerobic microbes is an ideal habitat for finding efficient strains of lignocellulolytic anaerobic bacteria [4].

Several anaerobic bacteria of rumen origin *viz. Fibrobacter succinogenes, Ruminococcus flavefaciens, Ruminococcus albus, Prevotella ruminicola, Butyrivibrio fibrisolvens*, etc. are considered as efficient lignocellulose degraders [3]. Also, the recent advances in genome sequencing technologies have enabled researchers to decipher the genes and pathways involved in biomass degradation [5]. Here, we report genome characteristics of a xylanolytic and cellulolytic anaerobic bacterium strain CtC72 isolated from rumen liquor of cattle.

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Materials and Methods

Isolation and identification

The strain CtC72 was isolated from cattle rumen liquor following enrichment and isolation in bacterial culture medium [6] using rice straw (1 mm size) for enrichment and microcrystalline cellulose (avicel) for isolation [7]. The genomic DNA of strain CtC72 was extracted using GenElute Bacterial Genomic DNA Kit (Sigma) following the manufacturer's instruction. The 16S rRNA gene was amplified (using 27f and 1492r primers) and outsourced for sequencing (1st BASE, Singapore) [8]. The 16S rRNA sequences were used to ascertain the identity of strain CtC72 using BLASTN search at NCBI.

Metabolite analysis

The strain CtC72 was grown for 24 h in bacterial culture medium [6] containing xylan, carboxymethyl cellulose (CMC) or avicel to evaluate its ability to utilize different components of lignocellulosic biomass. The culture was used to estimate fermentation metabolites like gas, volatile fatty acids (acetate, propionate, and butyrate) and ethanol using gas chromatography [9, 10].

Genome sequencing, assembly, and annotation

Whole genome sequencing of the strain CtC72 was performed using Ion Torrent PGM sequencer with 200-bp library chemistry applying the 316^{TM} sequencing chip, according to the manufacturer's instructions (Life Technologies, USA). De novo assembly was performed using version 3.9.1. of SPAdes assembler. The genome was annotated using Rapid Annotation Subsystem Technology (RAST) server [11] and BASys, a web-based bacterial annotation system [12]. Accessory data was uploaded to KEGG database [13] for studying the metabolic pathways. Carbohydrate-Active Enzyme (CAZymes) annotation analysis was performed using web server dbCAN [14]. The phylogenetic tree was constructed using MEGA 6.0 by the neighbor-joining method with 1,000 replications in the bootstrap test [15].

The whole genome sequence of strain CtC72 has been deposited in GenBank under the accession number NZ_MTPX02000000.

Digital DNA-DNA hybridization and Average Nucleotide Identity

To investigate the taxonomic novelty of the strain CtC72, digital DNA-DNA hybridization (dDDH) comparison was performed using Genome-to-Genome Distance Calculator (GGDC) web browser (DSMZ, Braunschweig, Germany) [16] between strain CtC72 (query) and the nearest phylogenetic affiliate. Average Nucleotide Identity (ANI) is defined as a pairwise calculation of overall similarity between two genome sequences of study. ANI has been widely used for defining species boundary between prokaryotes by calculating their ANI values between the genome sequences [17]. Here our genome CtC72 was compared with the type strain *Actinomyces ruminicola* DSM 27982.

Genome comparison using circular genome map

Blast Ring Image Generator (BRIG) software was used to create circular genome image comparisons. The genome sequences of query and reference strains were submitted in the '.fna' format to create the figure. BLAST analysis was carried out for the closely related genomes against the reference strain to create the circular genome comparison map [18].

Prediction of metabolites and antibiotic resistance genes

The prediction of antibiotic resistance and secondary metabolite biosynthetic gene clusters from the genome of the strain CtC72 was performed using antiSMASH web server. The antiSMASH provides the prediction by integrating a large number of in silico secondary metabolite analysis tools [19]. The bacteriocin prediction was carried out by BAGEL 3 web server. The genetic information analysis is based on the input data evaluated against a curated dataset of bacteriocins [20].

Antibiotic resistance genes in the genome of strain CtC72 was predicted using RAST annotation as well as by the web-based server Comprehensive Antibiotic Resistance Database (CARD). The CARD server analyses the genome sequences using BLAST and Resistance Gene Identifier (RGI) software for gene prediction [21].

Results and Discussion

Taxonomic novelty

The 16S rRNA gene sequence similarity search

revealed that the strain CtC72 shared only 97.78% sequence similarity with *Actinomyces ruminicola* DSM 27982 (valid type strain: B71^T, accession number: DQ072005/NR043523), indicating the novelty of strain CtC72 [16, 22]. The genus *Actinomyces* is classified under family *Actinomycetaceae*, order *Actinomycetales*, class *Actinobacteria* of phylum *Actinobacteria* within domain Bacteria [23]. The other genera of this group include *Actinobaculum*, *Arcanobacterium*, *Mobiluncus*, *Trueperella*, and *Varibaculum*. Of these, the genus *Actinomyces* has been reported to be quite diverse with 47 recognized species isolated from diverse habitats. Almost all species are Gram positive, rich in G+C content and shows anaerobic, facultative anaerobic or aerotolerant growth patterns [24]. A phylogenetic tree based on the 16S rRNA gene sequences was constructed to show the relationship between the strain CtC72 and closely related *Actinomyces* species (Fig. S1). The tree displayed a clear branching of strain CtC72 from its nearest phylogenetic affiliate with 100% bootstrap support.

General genome architecture

The genome of the strain CtC72 consisted of 3,413,161 bp with 68% G+C content, comparable to *A. ruminicola*7 DSM 27982 and *A. radicidentis* CCUG 36733. The final assembly contained 99 contigs with an N50 value of 74,975 bp with the largest contig assembled measured. A total of 3828 coding sequences, 49 tRNA and three rRNA and 322 subsystems were identified through RAST annotation. Most of the annotated genes determined carbohydrate metabolism (540), amino acids and derivatives synthesis (259), protein metabolism (209), cofactors, vitamins, prosthetic groups and pigment formations (145), and RNA metabolism (74). Genome statistics of strain CtC72 are given in Table 2. Sub-system distribution of the strain CtC72 is shown in Fig. 1 based on BASys annotation server analysis.

Genome comparison of strain CtC72 with reference genomes

The digital DNA-DNA hybridization using the genome of the strain CtC72 with its closest phylogenetic neighbor *A. ruminicola* DSM 27982 showed only 33.30% similarity, with a difference of 1.70% in G+C content, a threshold value of 70% DNA-DNA similarity is recommended for the definition of bacterial species to desig-

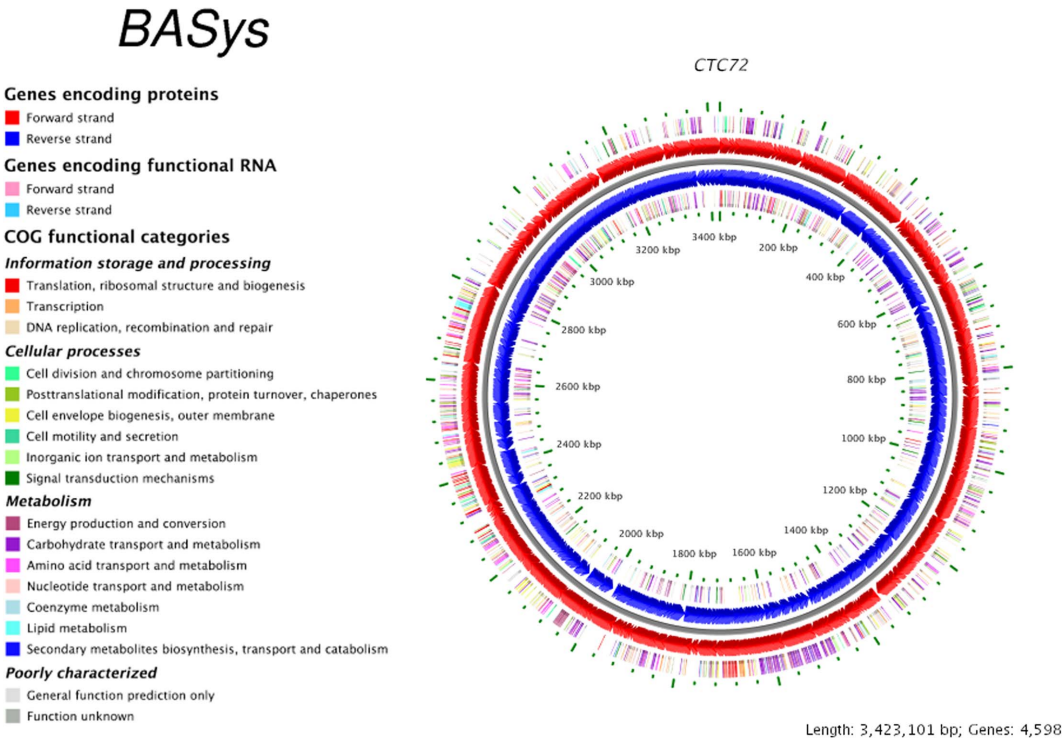

Fig. 1. Distribution and counts of genes in COG categories for genome of strain CtC72 from BASys annotation.

Table 1. Comparison of major metabolites of strain CtC72 with *A. ruminicola* DSM 27982 on different substrates.

Bacterial strain	CtC72	27982	CtC72	27982	CtC72	27982
Substrate / Metabolite	Avicel		CMC		Xylan	
Total VFA (ppm)	2121 ± 8	1632 ± 12	1687 ±28	1975 ± 18	2730 ± 17	2193 ± 5
Acetate (ppm)	2037 ± 12	1477 ± 4	1647 ± 24	1854 ± 18	2625 ± 21	2083 ± 5
Ethanol (ppm)	1647 ± 24	1854 ± 18	25 ± 7	38 ± 7	634 ± 9	129 ± 4

nate as a novel species [24]. Additionally ANI values between these two genomes were only 87.34% further confirming the novelty of strain CtC72, as ANI values less that 95-96% is proposed species cut-off value [17]. Genome comparisons by visualization especially as a circular image have become invaluable in determining genotypic differences between closely related species [18]. The circular genome comparison (Fig. 2) of strain CtC72 also showed many dissimilarities against different species as gaps, indicating the uniqueness of this isolate.

Table 2. Genome annotation of the strain CtC72 using RAST server.

Attributes	Values
Genome size	3,413,161 bp
Total number of contigs	99
G+C content (%)	68
Total number of subsystems	322
Total number of coding sequence	3828
tRNAs	49
rRNAs	3

Metabolite analysis

The strain CtC72 was found to degrade and ferment CMC, avicel, and xylan (Table 1). We also compared the fermentation products of strain CtC72 with type strain

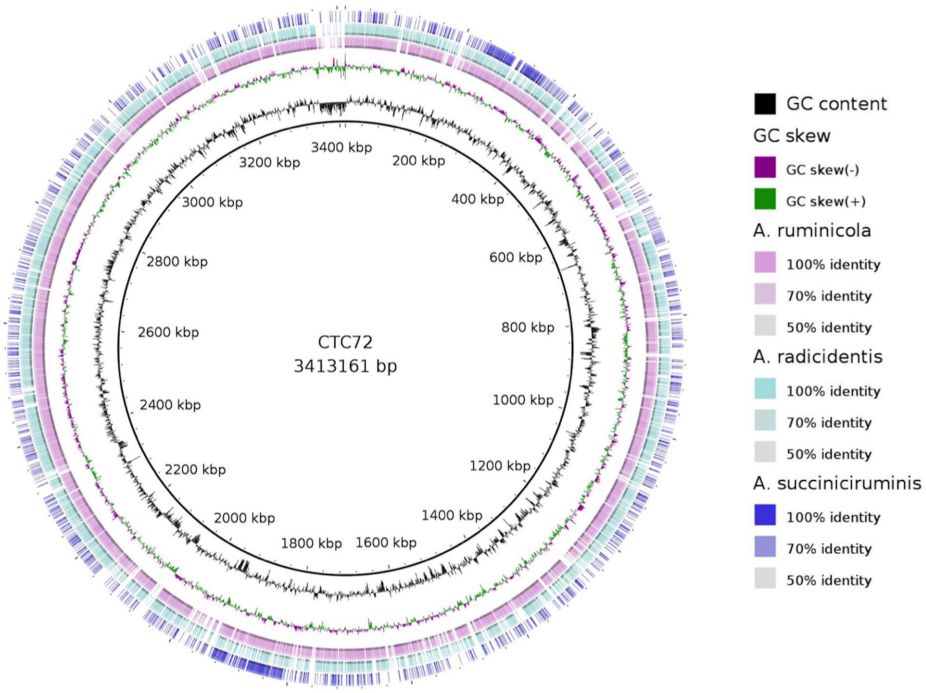

Fig. 2. BRIG analysis of *Actinomyces* sp. CtC72 with the genomes of *A. ruminicola*, *A. succiniciruminis,* and *A. radicidentis*. The innermost Ring 1 represents the GC content, followed by GC skew (- & +), Ring 3 is CtC72 (pink), Ring 4 is *A. ruminicola* (Green), Ring 5 is *A. succiniciruminis* (Dark blue) and Ring 6 is *A. radicidentis* (Light blue). Colors indicate the percentage of sequence identity.

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A. ruminicola DSM 27982. Major fermentation products of both cultures were carbon dioxide, ethanol, and acetate, while propionate and butyrate were produced in minor amounts. In comparison, the strain CtC72 produced more acetate on avicel and xylan and better ethanol on xylan.

Genes involved in carbohydrate utilization

The annotation analysis by dbCAN predicted 191 genes encoding for carbohydrate-active enzymes (CAZymes) including 37 glycosyl transferase (GT), 23 carbohydrate esterase (CE), 18 carbohydrate binding module (CBM), 110 glycosidase hydrolase (GH), two auxiliary activity (AA) and one polysaccharide lyase (PL) family. The presence of different subfamilies of glycosidase hydrolase and carbohydrate esterase suggests that the strain CtC72 can ferment various polysaccharides. For cellulose degradation, we found the genes encoding enzymes like β-glucosidase (GH3 and 5). The complex polysaccharides are broken down into oligosaccharides or monosaccharides by bacterial lyases or glycosidase. The presence of a vast number of CAZymes, including the important GHs and PLs in strain CtC72, suggests the complex mechanism of polysaccharide degradation [25].

Hemicellulose is the second most abundant polysaccharides in nature, major part of which is constituted by xylan. The complete degradation of xylan involves the activity of several xylanolytic enzymes such as β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, and acetyl xylan esterase [26]. A total of 37 genes encoding for enzymes involved in xylan metabolism were identified in strain CtC72, namely three endo-1,4-β-xylanase A genes (FIG00475203), four β-xylosidase genes (FIG00003086), two α-xylosidase (FIG00003174), two acetyl xylan esterase (FIG01955910), five α-L-arabinofuranosidase (FIG00037240), one ABC alpha-xylosidase transporter genes (FIG00089781), three xylose ABC transporter (FIG01276112), a xylose isomerase gene (FIG00019456), three xylulose kinase genes (FIG00000793), three xyloside transporter *Xyn*T genes and three xylose responsive transcription regulation factor ROK family. Of these, the most important enzymes are β-xylosidase and xylose isomerase, which carries out the first and second step in xylan degradation [27]. The

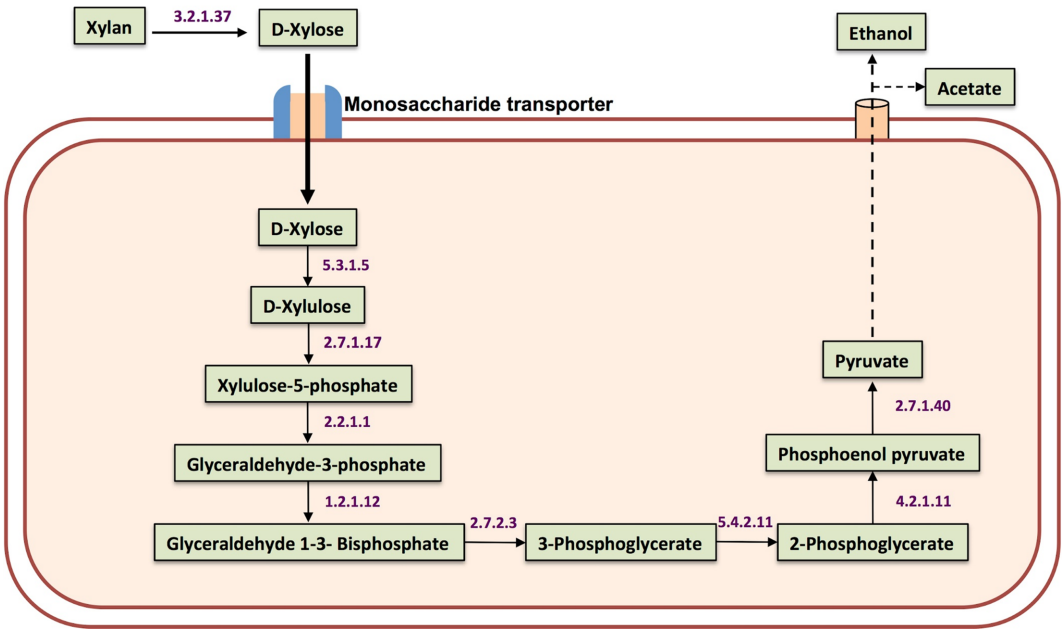

Fig. 3. Xylan degradation pathway elucidated through KEGG annotation from the genome of strain CtC72. The pathways involved in xylan degradation are pentose phosphate pathway, glycolysis, and pyruvate metabolism. Enzymes involved in xylan degradation: 3.2.1.37 - Beta-xylosidase; 5.3.1.5 - xylose isomerase; 2.7.1.17 - xylulokinase; 2.2.1.1. - transketolase; 1.2.1.12 - glyceraldehyde 3-phosphate dehydrogenase; 2.7.2.3. - phosphoglycerate kinase; 5.4.2.11. - 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; 4.2.1.11. -enolase; 2.7.1.40. -pyruvate kinase. Dotted lines indicate that more than one reaction are involved in the formation of the end product.

strain CtC72 contained all the essential enzymes required for efficient xylan utilization making it an efficient xylanolytic organism (Fig. 3).

The genome of strain CtC72 also has the genes necessary for the production of fermentation metabolites like acetate, formate, lactate, and ethanol. The data from KEGG and RAST server identified several enzymes like pyruvate: formate lyase and lactate dehydrogenase required for the production of formate and acetate, and lactate, respectively.

Genes involved in ethanol production

The production of ethanol from pyruvate involves a two-step or three-step fermentation pathway. In two-step pathway, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC), and the resulting acetaldehyde is converted to ethanol by alcohol dehydrogenase (ADH) [28]. In the three-step ethanol fermentation pathway, pyruvate is decarboxylated to acetyl-CoA by pyruvate ferredoxin oxidoreductase (POR) and pyruvate formate lyase (PFL). The resultant acetyl-CoA is converted to acetaldehyde and finally to ethanol through the action of CoA-dependent-acetylating acetaldehyde dehydrogenase (ALDH), and ADH, respectively [29, 30]. The BlastKOALA annotation file of strain CtC72 was mapped to KEGG pathway mapper to detect the genes involved in ethanol fermentation. Interestingly, the PDC and acetaldehyde dehydrogenase gene, which are most commonly involved in ethanol production in bacteria were not detected in strain CtC72 genome. However, five copies of ADH (EC 1.1.1.1) and POR complex (EC 1.2.7.1) were identified. Since ethanol is detected in significant amount when using avicel and xylan as a substrate (Table 1), it suggests that POR carries out the conversion of pyruvate to acetaldehyde with the release of CO_2 and acetaldehyde into ethanol by ADH (Fig. 4), similar to a hyperthermophilic archaeon *Pyrococcus furiosus* [31].

Stress resistance genes

To survive in extreme environmental conditions such as heat, osmotic stress, and initial high sugar concentrations during fermentation, an organism requires stress resistance genes [32–34]. The genome of strain CtC72 showed 44 genes involved in stress resistance (Table S1), including genes for osmotic stress (8), oxidative stress (16), heat shock protein (13) and for detoxification, stress and periplasmic stress (7). The genes for osmotic stress, oxidative stress, and heat shock proteins are necessarily required by microbes to tolerate high concentrations of sugars and ethanol [35–37].

Bacteriocin production and antibiotic resistance genes

Bacteriocins are protein complexes that exhibit antibacterial activity towards closely related species [38]. Genome mining for bacteriocin gene clusters in strain CtC72 showed the presence of gene cluster for a lanthionine-containing bacteriocin gallidermin (Fig. 5). Gallidermin exhibits antimicrobial property by inhibition of peptidoglycan synthesis and pore formation in membranes by interaction with the cell wall precursor lipid II of bacteria [39, 40]. An earlier study has shown improved ethanol production by a nisin producing bacteria through the selective elimination of contaminating *Lactobacillus* [41]. Hence, the bacteriocin-producing ability of strain CtC72 can be helpful in inhibition of

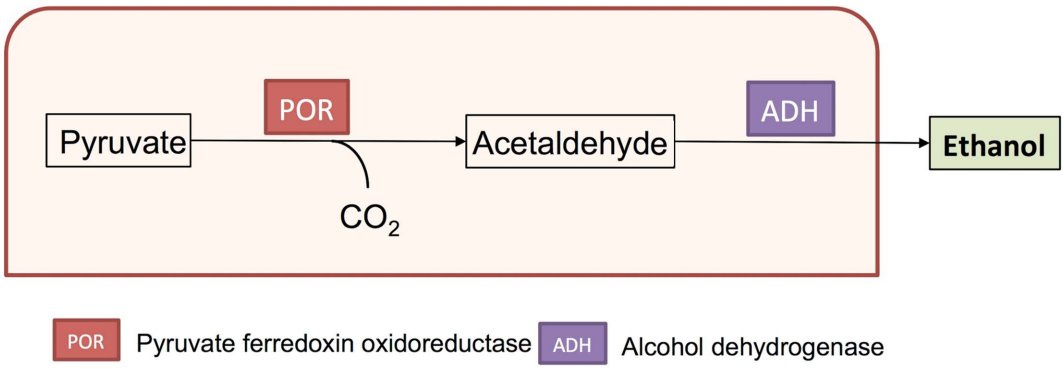

Fig. 4. Ethanol production from pyruvate catalyzed by pyruvate ferredoxin oxidoreductase (POR) and alcohol dehydrogenase (ADH) activity.

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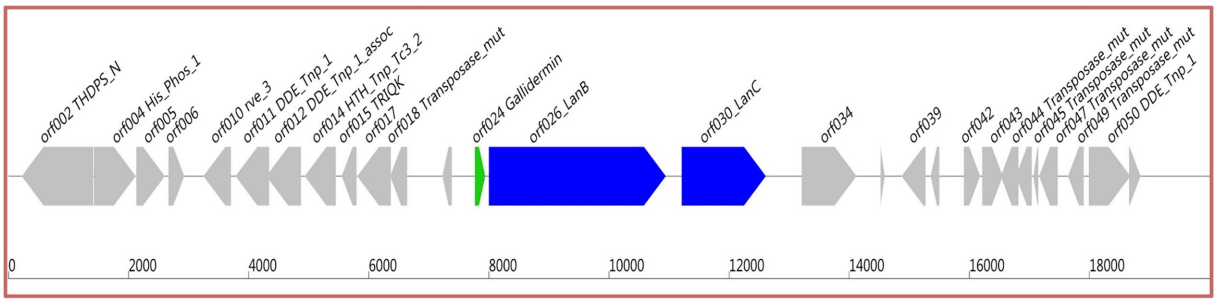

Fig. 5. Bacteriocin predicted in the genome of strain CtC72 using BAGEL showing gene cluster for gallidermin.

Table 3. Antibiotic resistance genes in strain CtC72 identified using CARD.

Antibiotic resistance genes
mfd protein - fluoroquinolone resistance
ileS conferring resistance to mupirocin
cdeA protein multidrug efflux pump complex
murA conferring resistance to fosfomycin
desR protein - macrolide resistance
parY mutant conferring resistance to aminocoumarin
EF-Tu mutants conferring resistance to elfamycin

undesirable bacteria during development of industrial processes for lignocellulose degradation.

The genome analysis also revealed the presence of genes related to antibiotic resistance (Table 4) and efflux pumps (Fig. S2) in strain CtC72. RAST annotation predicted the presence of genes for DNA gyrase, topoisomerase IV subunits A & B which are responsible for resistance to fluoroquinolones. Similarly, genes encoding for resistance to vancomycin, polymyxin, streptothricin, tetracyclin and heavy metals were also discovered. These results indicate that the strain CtC72 is capable of surviving against a wide range of antibiotics, which might be helpful in selective removal of bacterial contaminants.

Unique genes

The strain CtC72 contains 89 unique genes when compared with its nearest phylogenetic affiliate *Actinomyces ruminicola* DSM 27982. Genes for chitin and N-acetylglucosamine utilization, trehalose uptake and utilization, genes for ABC transporter for dipeptides, beta-glucosidase metabolism, D-galacturonate and D-glucuronate utilization, mannose metabolism, L-arabinose utilization, beta-glucoside metabolism, inositol catabolism, bacitracin stress response, lanthionine synthetases, iron transport system, etc. However, the strain CtC72 also lacked 70 genes in comparison to *A. ruminicola* DSM 27982, including genes for exopolysaccharide biosynthesis, denitrifying gene clusters, L-rhamnose utilization, etc. The presence of additional sugar metabolism

Table 4. Genes responsible for chitin utilization in strain CtC72.

Genes	Figfam
Beta-hexosaminidase (EC 3.2.1.52)	FIG00001088
Chitinase (EC 3.2.1.14)	FIG00001347
Glucosamine-6-phosphate deaminase (EC 3.5.99.6); Glucosamine-6-phosphate deaminase [isomerizing], alternative (EC 3.5.99.6)	FIG00000645
N-Acetyl-D-glucosamine ABC transport system, permease protein 1	FIG00008591 FIG01966738
N-Acetyl-D-glucosamine ABC transport system, permease protein 2	FIG00501923
N-Acetyl-D-glucosamine ABC transport system, sugar-binding protein	FIG00013626
N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	FIG00076542
PTS system, N-acetylglucosamine-specific IIB component (EC 2.7.1.69) / PTS system, N-acetylglucosamine-specific IIC component (EC 2.7.1.69)	FIG01962194

genes indicates efficient carbohydrate degradation and sugar utilization in strain CtC72. The presence of 2 chitinase enzymes and numerous ABC transport genes in the strain CtC72 (Table 4) suggests the possible chitinolytic behavior of this organism [42]. All hydrolytic bacteria compete with anaerobic fungi for substrates in the rumen environment. Therefore, the chitinolytic activities might be helpful for strain CtC72 in hydrolyzing the chitin cell wall of anaerobic fungi [43].

Genes encoding value-added by-products

Several genes which encode for enzymes like acetolactate synthase (EC 2.2.1.6), diacetyl reductase (EC 1.1.1.304), butanediol dehydrogenase (EC 1.1.1.76), lactaldehyde reductase (EC 1.1.1.77), xylitol dehydrogenase (EC 1.1.1.9) were also detected in the genome of strain CtC72. These enzymes are responsible for the production of value-added compounds like acetoin, 2, 3-butanediol, 1, 2-propanediol, xylitol, etc. [44–47], highlighting the biotechnological potential of strain CtC72.

In conclusion, an anaerobic bacterium strain CtC72 was isolated from the rumen of cattle and identified as a novel *Actinomyces* species. The strain utilized and fermented avicel, CMC, and xylan to CO_2, acetate, and ethanol. The genome analysis of the strain CtC72 revealed its taxonomic novelty, the genes involved in efficient xylan and cellulose degradation, ethanol production, and ability to tolerate stress conditions. Several key enzymes and transporters involved in chitin degradation were also identified. These findings underscore potential applications of *Actinomyces* sp. CtC72 in the conversion of lignocellulosic biomass into industrially useful compounds, especially bioethanol.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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