

Characterization of *Clostridium novyi* isolated from a sow in a sudden death case in Korea

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Abstract

Background

Multifocal spherical nonstaining cavities and gram-positive, rod-shaped, and endospore-forming bacteria were found in the liver of a sow that died suddenly. *Clostridium novyi* type B was identified and isolated from the sudden death case and the isolate was characterized by molecular analyses and bioassays in the current study.

Results

C. novyi was isolated from the liver and was confirmed as *C. novyi* type B by differential PCR. The *C. novyi* isolate fermented glucose and maltose and demonstrated lecithinase activity, and the cell-free culture supernatant of the *C. novyi* isolate exhibited cytotoxicity toward Vero cells, demonstrating that the isolate produces toxins. In addition, whole-genome sequencing of the *C. novyi* isolate was performed, and the complete sequences of the chromosome (2.29 Mbp) and two plasmids (134 and 68 kbp) were identified for the first time. Based on genome annotation, 7 genes were identified as glycosyltransferases, which are known as alpha toxins; 23 genes were found to be related to sporulation; 12 genes were found to be related to germination; and 20 genes were found to be related to chemotaxis.

Conclusion

C. novyi type B was isolated from a sow in a sudden death case and confirmed by biochemical and molecular characterization. Various virulence-associated genes were identified for the first time based on whole-genome sequencing.

Background

Clostridium novyi (*C. novyi*), originally named *Bacillus oedematis maligni* no. 2, was first isolated in 1894 from guinea pigs by Dr. Frederick Novy (1). *C. novyi* is broadly distributed in soil, water and marine sediments and affects humans and animals worldwide (2–4). *C. novyi* is a gram-positive, noncapsulated, motile obligatory anaerobe that produces endospores to resist unfavorable environments (1, 5).

Based on the toxins they produce, *C. novyi* are classified into four types: A, B, C and D. *C. novyi* type A produces alpha, gamma, delta and epsilon toxins. *C. novyi* type B produces alpha, beta, and zeta toxins, while type C produces gamma toxin (2, 6, 7). *C. novyi* type D is considered a different species, *Clostridium haemolyticum*, because it does not produce alpha toxin and because the disease that it causes is different from those caused by types A and B (8). *C. haemolyticum* produces beta, eta and theta toxins (9). *C. novyi* type A is frequently involved in gas gangrene infections in humans and animals, while type B is the etiological agent of infectious necrotic hepatitis (black disease), which is usually observed in sheep, cattle and swine (10). *C. novyi* type C is not known to induce illness in and is normally considered nonpathogenic to laboratory animals (7). *C. novyi* type D (*C. haemolyticum*) is responsible for

hemoglobinuria in calves (7). *C. novyi* types A and B (producing alpha toxin) cause sudden death in swine, and the carcasses exhibit gross distension and livers with gas bubble infiltration or sponge-like appearances (11, 12).

The 16S rRNA sequence has been used to detect genetic relatedness between different species of bacteria. Currently, next-generation sequencing is utilized as a rapid tool to perform whole-genome sequencing of clinical isolates. Indeed, this method has proved to be of great value for understanding bacterial evolution, outbreaks, toxigenicity, and antimicrobial resistance in a number of studies involving *Vibrio cholera*, *Escherichia coli*, *Clostridium difficile*, and *Mycobacterium tuberculosis* (12, 13). In the present study, *C. novyi* type B was for the first time isolated from a sudden death case in Korea, and the isolate was characterized by molecular analyses and bioassays. To the best of our knowledge, this is the first report of the complete genome sequence of *C. novyi* type B in Korea.

Results

Isolation of the *C. novyi* isolate

After 72 hours of anaerobic incubation, colonies showing irregular shapes with unclear borders appeared on agar media, and gram-positive, rod-shaped, endospore-forming bacteria were identified (Fig. 1a and 1b) in the colonies. Differential PCR was conducted on the DNA extracted from a single colony, which confirmed the isolate as *C. novyi* type B (14). No other bacteria were detected (data not shown).

Biochemical characterization of the *C. novyi* isolate

Biochemical analysis revealed that the *C. novyi* isolate generates gas and exhibits beta hemolysis. The biochemical characteristics of the *C. novyi* isolate are summarized in Table 1. The *C. novyi* isolate was positive for D-glucose, gelatin, D-maltose, salicin, L-rhamnose, D-cellobiose, and lecithinase and negative for L-tryptophan, urea, D-mannitol, D-lactose, D-saccharose, D-xylose, L-arabinose, esculin, glycerol, D-mannose, D-melezitose, D-sorbitol, D-trehalose, and catalase.

Table 1
Biochemical test results for the
C. novyi isolate

Active ingredients	Results
D-Glucose	+
Gelatin	+
Lecithinase	+
L-Tryptophan	-
Urea	-
D-Mannitol	-
D-Lactose	-
D-Saccharose	+
D-Maltose	+
Salicin	+
D-Xylose	-
L-Arabinose	-
Esculin	-
Glycerol	-
D-Cellobiose	+
D-Mannose	-
D-Melezitose	-
D-Sorbitol	-
L-Rhamnose	+
D-Trehalose	-
Catalase	-
Endospore	+
Gram	+
Hemolysis	+
+ positive; - negative	

Phylogenetic analysis of the isolate based on the 16S rRNA sequence

Based on the 16S rRNA sequence analysis, the *C. novyi* isolate showed more than 99% similarity with *C. novyi* types B and C, *C. haemolyticum*, and *C. botulinum* types C and D. The *C. novyi* isolate exhibited 84 to 91% similarity with *C. perfringens*, *C. sporogenes*, and *C. sordellii*. A comparison of the 16S rRNA sequences of the *C. novyi* isolate with those of different *Clostridium* species is shown in Fig. 2.

Histopathological examination of the liver

The liver tissues showed coagulative necrosis throughout the whole field. Multifocal spherical nonstaining cavities were found. The cavities were well demarcated, and the cavity margins were clear (Fig. 3a and 3b) Furthermore, gram-positive, rod-shaped bacteria were observed on the liver tissue (Fig. 3c).

Cytopathic effects of the *C. novyi* isolate on Vero cells

The cytotoxic activity of the *C. novyi* isolate toward Vero 76 cells was observed for 5 days. Cytopathic effects were detected at days 1 and 2 for dilutions of 1:4 and 1:8, respectively. However, the 1:16 and 1:32 dilutions revealed cytopathic effects on day 3 (Fig. 4)

Sequencing and genome features of the *C. novyi* isolate

The complete genome features of the *C. novyi* isolate are summarized in Table 2 and Figure 5. The genome consisted of a single circular chromosome with two circular plasmids. The sizes of the *C. novyi* isolate chromosome and plasmids 1 and 2 were 2,296,219 bp, 134,627 bp, 68,232 bp, respectively. The GC content of the chromosomal DNA was 27.9%, and 33 rRNA genes, 84 tRNA genes, and 54 pseudogenes were identified. The GC content of plasmid 1 was 26.3%, and 13 pseudogenes were identified. The GC content of plasmid 2 was 25.5%, and 13 pseudogenes were identified. The assembled and annotated sequences of the *C. novyi* isolate chromosome and plasmids 1 and 2 were submitted to NCBI (accession numbers: CP029458.1, CP029459.1, and CP029460.1).

	Chromosome	Plasmid 1	Plasmid 2
Genome size (bp)	2,296,219	134,627	68,232
GC content (%)	27.9	26.3	25.5
Protein coding genes	2,009	141	54
rRNAs	33	-	-
tRNAs	84	-	-
Pseudogenes	54	13	13

Table 2

Genomic characteristic of the *C. novyi* isolate

Prediction of genes associated with pathogenicity

All of the identified genes of the *C. novyi* isolate are summarized in Additional file 1. A total of 35 genes related to sporulation and germination were detected in the whole genome of the *C. novyi* isolate. Among these genes, sporulation-related sigma factors (DFH04_RS02880, DFH04_RS08440, DFH04_RS08465, DFH04_RS08470, and DFH04_RS10365) and stage-specific sporulation genes were detected. Additionally, 7 glycosyltransferase genes related to alpha toxin were identified (DFH04_RS05070, DFH04_RS05155, DFH04_RS10400, DFH04_RS10410, DFH04_RS10425, DFH04_RS10435, and DFH04_RS10440). Various genes encoding chemotaxis proteins were also identified in the *C. novyi* isolate genome, including a sensor kinase, the product of DFH04_RS10060; a deamidase, the product of DFH04_RS08070; a methyltransferase, the product of DFH04_RS10065; a docking protein, the product of DFH04_RS08070; and a phosphatase protein, the product of DFH04_RS10055. Additionally, 9 genes were identified as MCPs.

Discussion

Biochemical assays of the isolated colonies showed the presence of lecithinase activity and glucose and maltose fermentation, which are the characteristic features of *C. novyi*. Previously, lecithinase activity and glucose and maltose fermentation have been associated with *C. novyi* types A and B (15). On the basis of biochemical tests, our isolate could also be differentiated from other closely related Clostridia, such as *C. haemolyticum*, which lacks the ability to ferment maltose, while *C. botulinum* types C and D display variable maltose fermentation capabilities and maltose and lecithinase activity (15).

The 16S rRNA sequencing results for the *C. novyi* isolate showed that the isolate shared approximately 98–99% similarity with each type of *C. novyi*, *C. haemolyticum* and *C. botulinum* types C and D. In particular, the 16S rRNA sequence of the *C. novyi* isolate and that of *C. haemolyticum* showed the highest similarity (99.6%). Consistent with this finding, high similarity (> 99.9%) between the 16S rRNA sequence of *C. novyi* type B and that of *C. haemolyticum* has been reported in a previous study (16). Therefore, different methods are needed to distinguish *C. novyi* type B from *C. haemolyticum*. The N- and C-terminal amino acid sequences of FliC are well preserved between *C. novyi* type B and *C. haemolyticum*, but the central region amino acid sequences are not (17). Therefore, the *C. novyi* isolate was distinguished from *C. haemolyticum* using *fliC* gene primers.

In this study, virulence-associated genes were identified by whole-genome sequencing of the *C. novyi* isolate. The other identified genes are summarized in Additional file 1. Endospores are important

contributors to pathogenesis before being the vegetative forms of *C. novyi* type B. Furthermore, the endospores of *C. novyi* are highly resistant to environmental conditions. *Clostridium* spp. initiate the sporulation process when unfavorable conditions are detected. The sporulation process is a carefully orchestrated cascade of events at both the transcriptional and posttranslational levels that involves a multitude of sigma factors, transcription factors, proteases, and phosphatases. *Clostridium* spp. genomes contain genes for all major sporulation-specific transcription and sigma factors. The sporulation process consists of several stages. Sporulation-specific sigma factors affect each sporulation stage (18). Sporulation genes and sigma factor genes were detected in the *C. novyi* isolate; in addition, the *C. novyi* isolate was found to contain germination genes that initiate the germination process when a favorable environment is detected. Although the mechanism has not been completely elucidated, after *C. novyi* endospores are ingested, they are absorbed from the intestine and reach the liver via the portal circulation. Subsequently, the endospores are spread to other organs. The endospores germinate and produce toxins in organs when anaerobic conditions form (19).

The main pathogenic protein of *C. novyi* type B is alpha toxin. Alpha toxin is produced and released by the vegetative forms of *C. novyi* type B, and its monoglycosyltransferase activity inactivates several GTP-binding proteins in cells, resulting in modification and redistribution of the actin cytoskeleton. For these reasons, necrosis occurs in the liver, and the cut surface of the affected liver exhibits a sponge-like appearance (19–24). Glycosyltransferases were detected in the genome of the *C. novyi* isolate, indicating that the *C. novyi* isolate produces alpha toxin. In this study, the effect of the toxins produced by the isolate was tested by observing the effect of the cell-free supernatant of the *C. novyi* isolate on Vero cells. The cytopathic effect produced was similar to the observations in a previous study in which the purified alpha toxin exhibited a strong effect on Vero cells, resulting in rounded cells in addition to lysed cells (25).

Chemotaxis enables bacteria to move according to chemical gradients. Chemotaxis affords key physiological benefits, including enhanced contact with growth substrates. Another important aspect of chemotaxis is that it plays a role in infection and disease, as chemotaxis signaling pathways are widely distributed among diverse pathogenic bacteria (26). In this study, 15 genes related to chemotaxis, namely, DFH04_RS10060 (CheA), DFH04_RS10080 (CheW), DFH04_RS10075 (CheD), DFH04_RS08070 (CheV), DFH04_RS10055 (CheC), DFH04_RS10065 (CheR), and nine MCPs, were detected in the genome of the *C. novyi* isolate. As reported previously (26), the products of these genes transfer signals by phosphorylation and activate the flagellum. The activated flagellum enables the bacterium to move toward an attractant. Chemotaxis-associated genes were also detected in a previous study based on *Clostridium novyi*-NT (27).

Conclusion

This *C. novyi* isolate was first isolated from a sudden death case of sow in Korea and confirmed by biochemical and molecular characterization. Furthermore, various virulence-associated genes were identified in the genome of the isolate, indicating that the isolate might have had a role in the sudden death of the sow. However, more research including animal experiments is needed to determine the

pathogenicity of *C. novyi*. Nonetheless, the complete genomic sequence of *C. novyi* isolate will contribute to a better understanding of the biology of *C. novyi* and related species.

Methods

Sample collection and isolation of *C. novyi*

Diagnostic samples, mainly livers, collected from sows with sudden death within 12 hours after death were submitted to the Jeonbuk National University Veterinary Diagnostic Center in 2015 (case no. 150557) (14). Swab samples taken from affected sites on the livers were inoculated in RCM (BD Biosciences, New Jersey, USA) and incubated anaerobically at 37°C for 72 hours in a gas jar. The incubated broth media were heated at 100 °C for 10 min, reinoculated in 5% sheep blood agar, and again incubated under the same conditions. Suspicious colonies of *C. novyi* that exhibited beta hemolysis on the sheep blood agar plates were previously identified as *C. novyi* type B based on differential PCR, Gram staining and 16S rRNA sequencing. (14). The morphology of the bacteria in the identified colonies was confirmed further using endospore staining. Spores and vegetative cells were identified using 5% malachite green (Thermo Fisher Scientific Inc., Waltham, MA, USA) staining solution and safranin staining solution (Gram stain kit solution), respectively, as described previously (28).

16s Rrna Sequencing Analysis

16S rRNA sequencing was performed by a sequencing facility (Biofact Co., Daejun, Korea) using a pure culture of the *C. novyi* isolate. The 16S rRNA sequence of the *C. novyi* isolate was identified by a BLAST search and compared with other *Clostridium* species sequences downloaded from the database of the NCBI. Multiple alignment of the 16S rRNA sequences was performed using MegAlign (DNASTAR, Madison, Wisconsin, USA). A phylogenetic tree was constructed with the neighbor joining method using MEGA 6 software (29).

Cytopathic effect of the *C. novyi* isolate on Vero cells

Vero 76 cells (ATCC CRL-1587) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Welgene, Korea) supplemented with heat-inactivated 5% fetal bovine serum (FBS, Invitrogen, USA), 2 mM L-glutamine, and 100X antibiotic-antimycotic solution [Anti-Anti, Invitrogen; 1 × solution contains 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone® (amphotericin B)] at 37°C in a humidified 5% CO₂ atmosphere.

The cytopathic effect assay was performed as reported (30, 31). Briefly, *C. novyi* cell-free supernatant after centrifugation was prepared by filtrating the broth through a 0.2 µm cellulose acetate syringe filter (Corning, Germany). Two-fold serial dilutions of the filtered supernatant were prepared with cell medium for up to 8 dilutions. Cytotoxicity assays were performed in 96-well tissue culture plates (Falcon, NY, USA). The supernatant of cultured Vero 76 cells was discarded, and the cells were washed twice with 1X PBS. Then, the diluted cell-free supernatant of the *C. novyi* isolate was added to the Vero 76 cells, and the

cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. The plates were observed for CPE for 5 days.

Whole-genome Sequencing

A sample of high-quality, high-molecular-weight DNA was used to prepare size-selected SMRTbell templates of approximately 20 kb. A NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a Qubit fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used to measure the concentration of gDNA. All samples passed quality control (QC) screening criteria. For PacBio RS sequencing, 8 g of input gDNA was used for 20 kb library preparation. For gDNA where the size range was less than 17 kb, we used a Bioanalyzer 2100 (Agilent Technologies, CA, USA) to determine the actual size distribution. If the apparent size of the gDNA was greater than 40 kb, the gDNA was sheared with a g-TUBE (Covaris Inc., Woburn, MA, USA) to produce library fragments in the optimal size range and purified using AMPure PB magnetic beads (Beckman Coulter Inc., Brea, CA, USA). Then, the concentration of the gDNA was measured using both a NanoDrop spectrophotometer and a Qubit fluorometer, and approximately 200 ng/μl gDNA was run on a field-inversion gel. A library was prepared in a total volume of 10 μl using a PacBio DNA Template Prep Kit 1.0 (for 3–10 kb). SMRTbell templates were annealed using a PacBio DNA/Polymerase Binding Kit P6. A PacBio DNA Sequencing Kit 4.0 and 1 SMRT Cell was used for sequencing. SMRT Cells (Pacific Biosciences, CA, USA) with C4 chemistry were used, and 240 min movies were captured for each SMRT cell using the PacBio RS II (Pacific Biosciences, CA, USA) sequencing platform. The subsequent steps were based on the PacBio Sample Net-Shared Protocol, which is available at <http://pacificbiosciences.com/>.

Bioinformatics Analysis

All runs were carried out with diffusion-based loading and analyzed using standard primary data analysis methods. The source codes for HGAP and Quiver, the data sets and additional documentation are available at <http://www.pacbiodevnet.com/HGAP> and <http://www.pacbiodevnet.com/quiver>. The coding DNA sequences were predicted with Prokaryotic Genome Annotation Pipeline version 4.5 software on the NCBI website (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Additional functional annotation was performed with the Rapid Annotation using Subsystem Technology server (32).

Abbreviations

MCPs: Methyl-accepting chemotaxis proteins; GTP: Guanosine 5'-triphosphate; RCM: Reinforced clostridial medium; NCBI: National Center for Biotechnology Information; CPE: Cytopathic effects; HGAP: Hierarchical Genome Assembly Process; gDNA: genomic DNA

Declarations

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Consent for publication

Not applicable.

Authors' contributions

CGJ conducted the experiments and drafted the manuscript. AK participated in the data analysis and helped in drafting the manuscript. BJS, BKJ and JHS contributed to the data analysis of the whole genome sequences. BK and MSY contributed to the histopathological examination. SHN provided field samples. SCK contributed to the collection and processing of the samples. WIK designed the entire study and prepared the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional file 1. Genome sequences obtained in current study are deposited in GenBank under accession numbers: CP029458.1, CP029459.1, and CP029460.1. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animal materials used in the present study were received as routine diagnostic submissions and were used under the agreement of Jeonbuk National University-Veterinary Diagnostic Center (JBNU-VDC). The authors also confirmed that ethical approval is not essential to use animal materials received for diagnostic purpose in accordance with the guidelines of Jeonbuk National University-Institutional Animal Care and Use Committee (JBNU-IACUC).

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

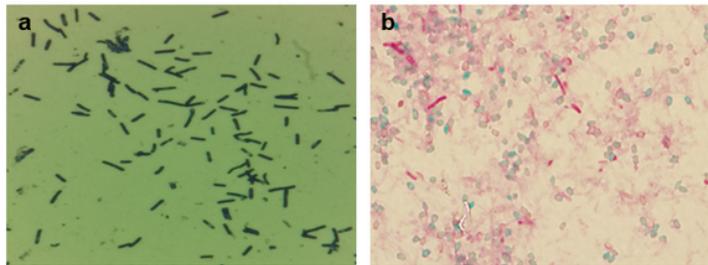


Figure 1

Morphology of Clostridium isolate based on staining. (a) Gram staining was performed on cultured isolate, detecting gram-positive rods. (b) Endospores of the cultured *C. novyi* isolate were observed after staining with 5% malachite green.

Figure 2

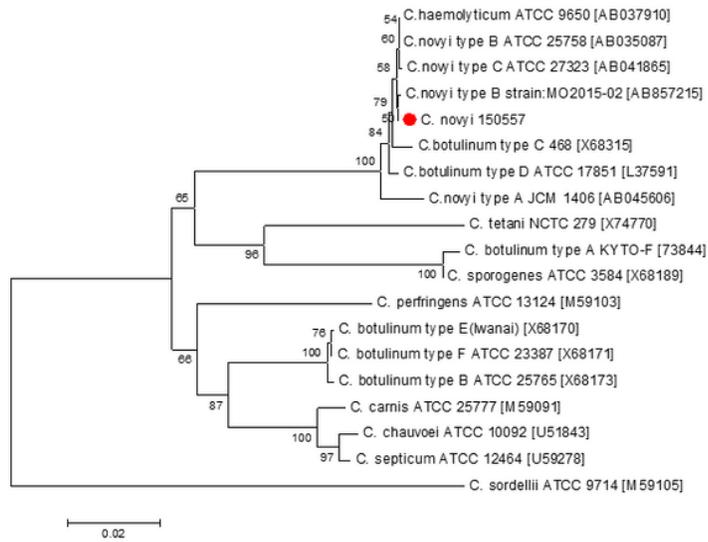


Figure 2

Phylogenetic analysis of the 16S rRNA sequence of *Clostridium* spp. The tree was constructed using the neighbor-joining method with 1000 replicates for bootstrap values with MEGA 6 software. The scale bar represents 0.02 substitutions per nucleotide position. The circle indicates the isolate identified in this study.

Figure 3

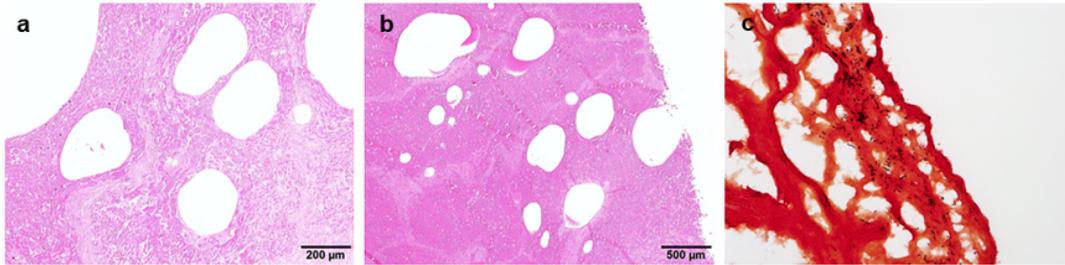


Figure 3

Morphology of Clostridium isolate based on staining. (a) and (b) Coagulation necrosis and multifocal spherical non-staining cavities were found on tissue section. (c) Gram staining was performed on liver tissue (x400).

Figure 4

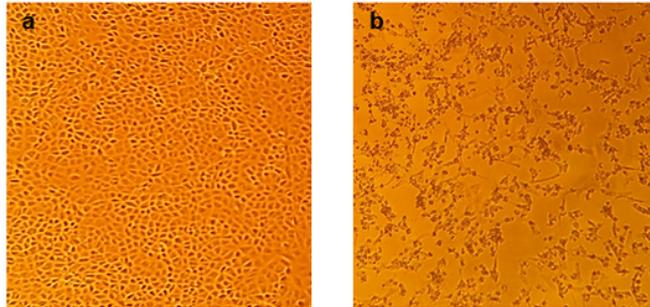


Figure 4

Cytopathic effect of the cell-free supernatant of the *C. novyi* isolate in Vero 76 cells. The cytotoxic activity of the *C. novyi* isolate toward Vero 76 cells was observed for 5 days. (a) Vero 76 cells without treatment. (b) Vero 76 cells inoculated with filtered (0.2 μm) *C. novyi* supernatant (1:32). A cytopathic effect (rounding and disintegration of cells) was observed in cells treated with *C. novyi* supernatant.

Figure 5

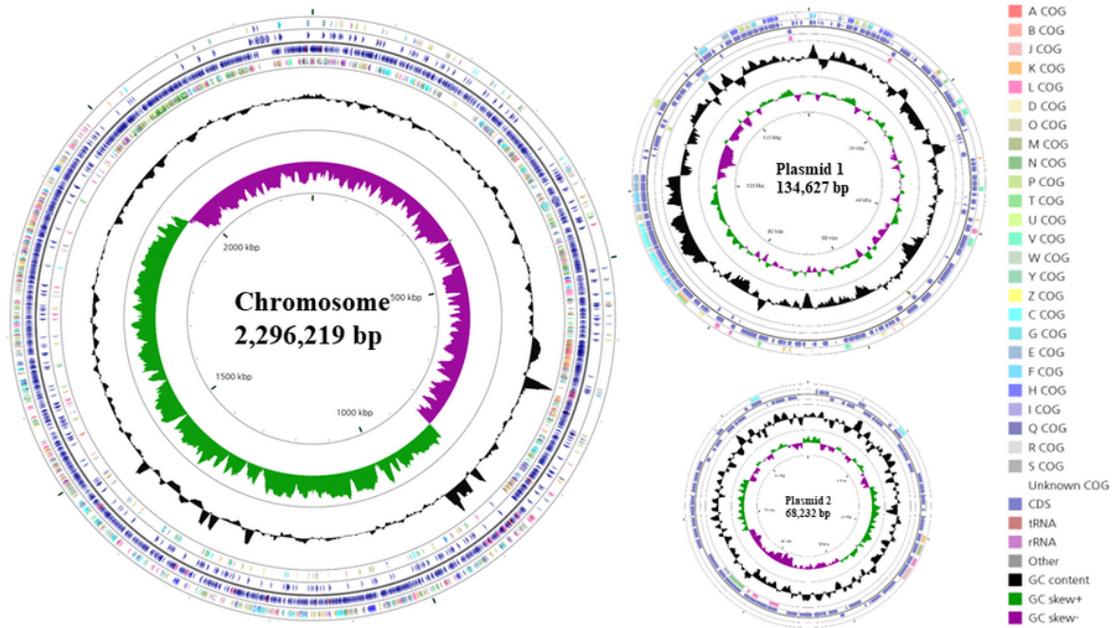


Figure 5

Circular map of the chromosome and two plasmids of the *C. novyi* isolate. From the outside to the center: genes on the forward strand (colored by Clusters of Orthologous Genes (COG) categories), coding DNA sequence (CDS) on the forward strand, CDS on the reverse strand, genes on the reverse strand (colored by COG categories), GC content, and GC skew.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1BMC.xlsx](#)