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Genome Sequence of *Bacillus cereus* FORC_021, a Food-Borne Pathogen Isolated from a Knife at a Sashimi Restaurant

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Bacillus cereus causes food-borne illness through contaminated foods; therefore, its pathogenicity and genome sequences have been analyzed in several studies. We sequenced and analyzed *B. cereus* strain FORC_021 isolated from a sashimi restaurant. The genome sequence consists of 5,373,294 bp with 35.36% GC contents, 5,350 predicted CDSs, 42 rRNA genes, and 107 tRNA genes. Based on in silico DNA-DNA hybridization values, *B. cereus* ATCC 14579^T was closest to FORC_021 among the complete genome-sequenced strains. Three major enterotoxins were detected in FORC_021. Comparative genomic analysis of FORC_021 with ATCC 14579^T revealed that FORC_021 harbored an additional genomic region encoding virulence factors, such as putative ADP-ribosylating toxin, spore germination protein, internalin, and sortase. Furthermore, in vitro cytotoxicity testing showed that FORC_021 exhibited a high level of cytotoxicity toward INT-407 human epithelial cells. This genomic information of FORC_021 will help us to understand its pathogenesis and assist in managing food contamination.

Keywords: *Bacillus cereus*, food-borne pathogen, virulence factor, enterotoxin, *Listeria* pathogenicity island 1 (LIPI-1)

Introduction

Bacillus cereus is one of the major food-borne pathogens and is an important bacterium in the food industry. The bacteria form biofilms and produce heat-resistant endospores, and can therefore contaminate various foods and survive even after pasteurization and sterilization during food processing [1, 28]. This bacterium produces three most important and well-known enterotoxins, namely, non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK). These toxins are responsible for food-borne illnesses characterized by diarrhea or vomiting, and are often life-threatening in some cases [9, 21, 22]. Various efforts have been made to predict the toxic potential of newly isolated strains. However, differences in the closely related *B. cereus* species are still based on the presence or absence of phenotypic characters. In addition, it is reported that species affiliation of *B. cereus* group strains often does not match phylogenetic relatedness [2, 12]. The trends in prokaryotic species distinction is moving towards the

comparison of entire genomes [12, 17], and therefore, obtaining genome sequences of isolated strains is crucial to identify their characteristics. Several studies have analyzed the *B. cereus* genomes to understand its toxicity [3, 16, 20, 24].

Here, *B. cereus* strain FORC_021 was isolated from a knife used at a sashimi restaurant, and its whole genome was sequenced. Since knives used at sashimi restaurants could come in contact with sashimi and transmit the bacteria to the customer via ingestion, they should be handled carefully. The isolated strain could possibly cause food-borne illness, and its genome information is therefore necessary to understand its characteristics for future pathogen screening.

Materials and Methods

Isolation and Growth Conditions

The *B. cereus* strain FORC_021 was isolated from a knife used at a sashimi restaurant by the Ulsan Institute of Health and Environment, Republic of Korea. The FORC_021 strain was cultivated at 30°C for 12 h in Brain Heart Infusion (Difco, USA) medium. The morphology of the strain was determined using transmission electron microscopy with 2% uranyl acetate staining (Fig. 1). The FORC_021 strain was a gram-positive, rod-shaped, flagellated bacterium that was 2–2.5 μm in length and 0.6–0.8 μm in width.

Cytotoxicity Analysis

The cytotoxicity of FORC_021 was analyzed by lactate dehydrogenase (LDH) assay using human epithelial INT-407 cells. INT-407 cells were cultivated in minimum medium containing 1% (v/v) fetal bovine serum (Gibco-BRL, USA) in 96-well culture dishes (Nunc, Denmark) as described previously [18]. Each well (containing 2×10^4 INT-407 cells) was infected with the FORC_021 or the ATCC 14579^T strain (control) for 2 or 3 h. The LDH activity in the supernatant was analyzed with a cytotoxicity detection kit (Roche, Germany).

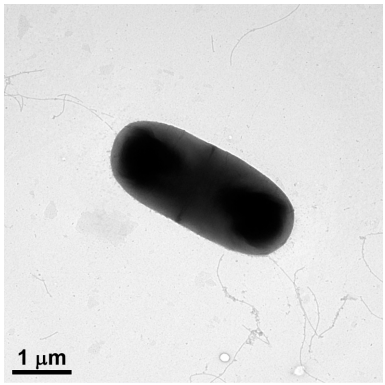

Fig. 1. Transmission electron micrograph image of *B. cereus* FORC_021.
The cells were negatively stained with 2.0% uranyl acetate for 1 min. It was observed using the JEM-2100 transmission electron microscope (JEOL, Japan) at 200 kV.

Genomic DNA Extraction and Identification

Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, USA), and was quantified using a PicoGreen dsDNA Assay Kit (Invitrogen, USA). The 16S rRNA gene was amplified from the extracted genomic DNA and sequenced by an automated ABI3730XL capillary DNA sequencer (Applied Biosystems, USA) for taxonomic identification [19].

Genome Sequencing, Annotation, and Comparison

The genome sequence was determined using the PacBio RS II (Pacific Biosciences, USA) at ChunLab Inc. (South Korea). Raw sequences were assembled with PacBio SMRT Analysis ver. 2.0 software (Pacific Biosciences). Gene prediction was performed using Glimmer 3 [6], and annotations were performed by a homology search against the SEED database, Universal Protein Resource (UniProt) database, and eggNOG database [7, 13, 24, 26]. In silico DNA-DNA hybridization values of the strain FORC_021 with the complete genome sequences of *B. cereus* from a public database were calculated with the Genome-to-Genome Distance Calculator (GGDC; http://ggdc.dsmz.de). A genome tree was constructed based on distance results from GGDC. A comparative genome analysis between FORC_021 and 14579^T was performed using the WebACT (http://www.webcat.org/WebACT/home). The comparative analyses of gene contents were performed using the RAST server (http://rast.nmpdr.org). The obtained genome sequence of *B. cereus* FORC_021 was deposited in NCBI under the accession number CP014486. Genome sequencing of strain FORC_021 was conducted as part of the Food-borne Pathogen Omics Research Center project supported by the Ministry of Food and Drug Safety, South Korea, which aims to collect and construct a database of complete genome sequences of various food-borne pathogens from South Korea.

Results and Discussion

Genome Properties

The genome consisted of one contig with 179.45× coverage and N_{50} contig length of 5,331,694 bp. The genome size was 5,331,694 bp with a 35.36% GC content, 5,350 predicted CDSs, 42 rRNA genes, and 107 tRNA genes. Among the predicted CDSs, 3,994 were annotated to functional proteins and 1,356 CDSs were hypothetical proteins. A total of 4,661 CDSs were assigned to the eggNOG categories, and 3,488 CDSs were assigned to the SEED subsystem categories. Among the eggNOG categories, S (function unknown; 1,207 CDSs) and R (general function prediction only; 513 CDSs) were abundant, followed by E (amino acid transport and metabolism; 360 CDSs) and K (transcription; 342 CDSs). In

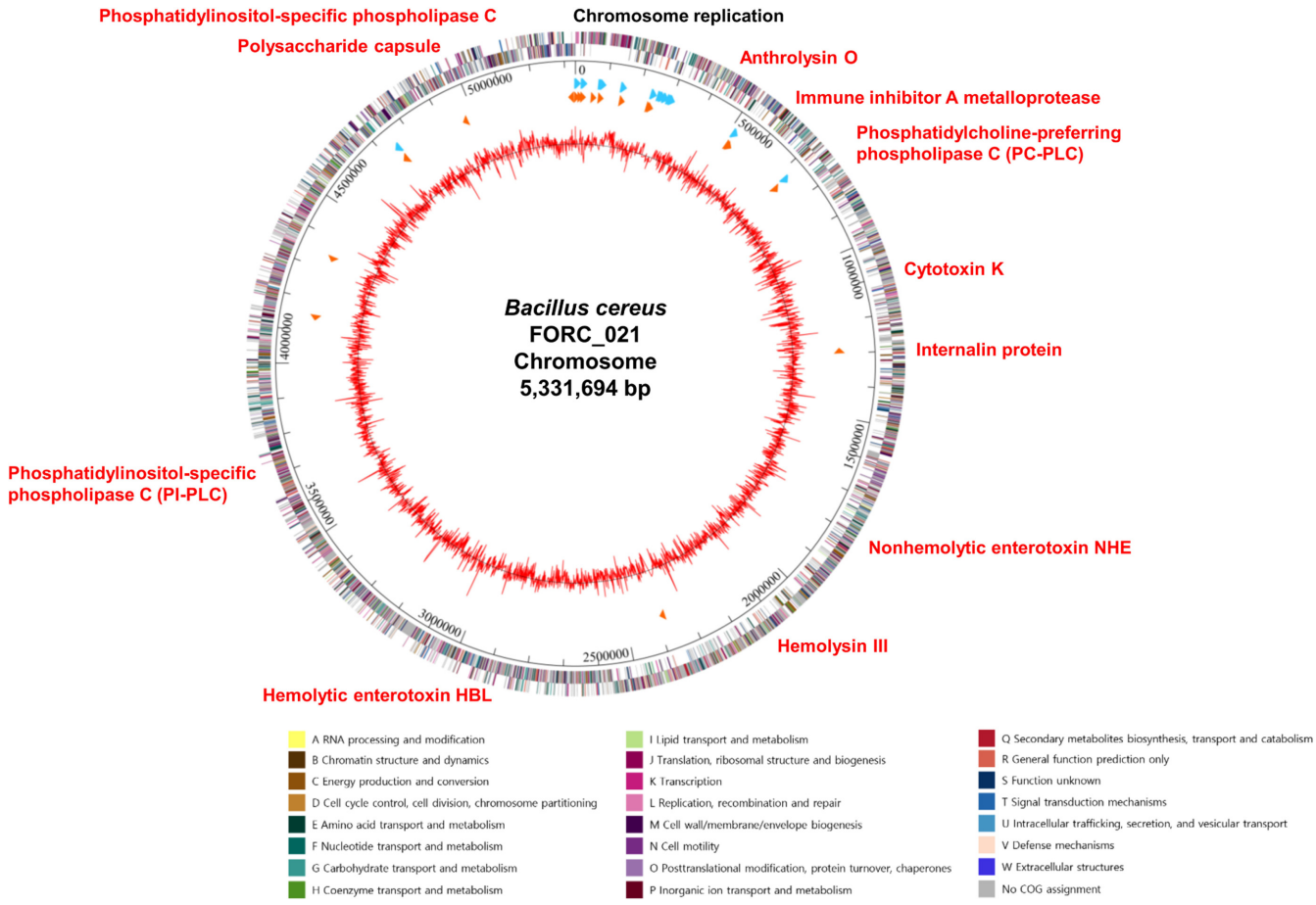

Fig. 2. Genome map of the *B. cereus* FORC_021 chromosome.
The outer circle indicates the locations of all annotated ORFs, and the inner circle with the red peaks indicates GC content. Between these circles, the sky blue arrows indicate the rRNA operons, and the orange arrows indicate tRNAs. All the annotated ORFs are colored differently according to the COG assignments. Genes with specialized functions are labeled with different colors as follows; virulence-related genes, red; prophage-related genes, blue; and other functional genes, black.

the SEED subsystem distribution, the subsystems of amino acid and derivatives (534 CDSs) and carbohydrates (420 CDSs) were predominant. The circular genome maps of the FORC_021 strain, which consisted of predicted ORFs, gene clusters, RNA operons, and GC content, are shown using the GenVision program (DNASTAR, USA; Fig. 2).

Comparative Genome Analysis

The 16S rRNA gene of strain FORC_021 had the highest pairwise similarity with *B. cereus* ATCC 14579^T (99.9%). The genome tree of strain FORC_021 was generated with genome distance values obtained by GGDC (Fig. 3). The genome tree showed that four strains (ATCC 14579^T, B4264, FORC_005, and G9842) were clustered with strain FORC_021 and were separated from 18 other strains. The DNA-DNA hybridization value between FORC_021 and ATCC 14579^T was the highest (90.3 ± 2.1%) among the estimated values of the other strains, whereas the value between FORC_021 and G9842 was the lowest among the clustered strains. The average nucleotide identity value [11] between FORC_021 and ATCC 14579^T was also higher (98.8%) than the calculated values between other strains. The abundances of gene contents in subsystems were similar between strains FORC_021 and ATCC 14579^T (Table 1). *B. cereus* ATCC 14579^T was first isolated from the air of a cow shed in the United Kingdom, and is the type strain of *B. cereus* [10]. *B. cereus* strain ATCC 14579^T expresses several different toxins, such as hemolysin BL, non-hemolytic enterotoxin, hemolysin II, hemolysin III, and phospholipase C, and therefore has pathogenic potential in humans [15]. Strain

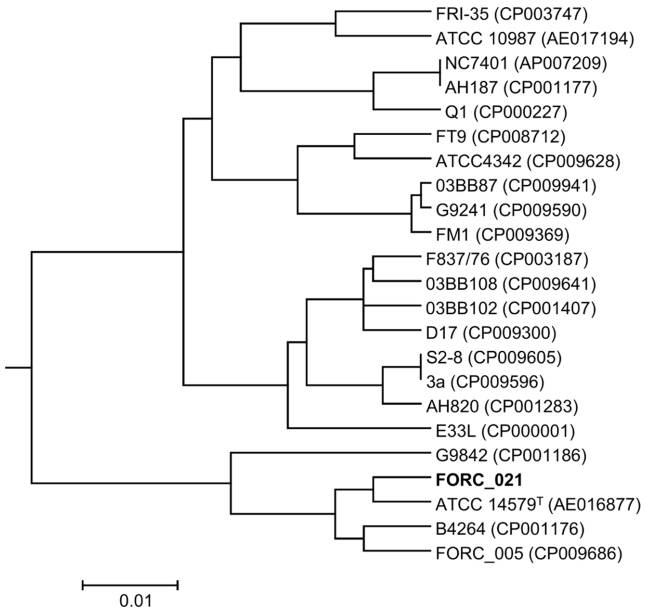

Fig. 3. Genome tree of strain FORC_021 with the completely sequenced *Bacillus cereus* strains, obtained based on the genomic distance using the Genome-to-Genome Distance Calculator.

B4264 was isolated from a male patient with fatal pneumonia in 1969 [14]. The most different feature counts in FORC_021 compared with clustered strains were found in the Phages, Prophages, Transposable Elements, Plasmids subsystem. Strain FORC_021 contained the relatively lowest counts (14) among the clustered strains (19 to 33). Orthologs for *Listeria* pathogenicity island 1 (LIPI-1) were detected in all the clustered strains (Table 2). LIPI-1 consists of six genes and contains virulence genes essential for intracellular parasitism [27]. Three genes for phosphatidylinositol-specific phospholipase (*plcA*), listeriolysin O (*hly*), and zinc metalloproteinase precursor (*mpl*) of LIPI-1 were detected in FORC_021.

A comparative analysis between FORC_021 and 14579^T using the WebACT revealed four different genomic regions. The genomic region ranging from FORC21_2852 to FORC21_2867 (2,864,401–2,875,309 bp) was different between the two strains (Fig. S1A) and contained the putative ADP-ribosylating toxin (FORC21_2854 and 2864), which disrupts the actin cytoskeleton (Fig. S1B) [23]. The second different genomic region (ranging from FORC21_2994 to FORC21_2996; 3,011,004–3,014,935 bp) (Fig. S2A) contained the spore germination proteins (FORC21_2994 and 2995), which are famous for their ability to cause food poisoning (Fig. S2B) [4]. The third different genomic region (ranging from FORC21_3522 to FORC21_3539; 3,534,069–3,550,072 bp) (Fig. S3A) contained internalin-J (FORC21_3525), which can play an important role in host cell invasion (Fig. S3B) [20]. The fourth different genomic region in FORC_021 (ranging from FORC21_5078 to FORC21_5080; 5,036,952–5,042,545 bp) detected in a comparative genomic analysis between FORC_021 and ATCC 14579^T (Fig. S4A) encoded sortase (FORC21_5078 to 5080), which plays a critical role in gram-positive bacterial pathogenesis (Fig. S4B) [5]. These results indicated that compared with the ATCC 14579^T genome, the FORC_021 genome might contain additional virulence factors. Furthermore, the cytotoxic potential of FORC_021 was higher than that of the positive control ATCC 14579^T in LDH release assays (Fig. S5), suggesting that the FORC_021 strain shows potential pathogenicity.

Pathogenesis and Virulence Factors

B. cereus causes food-borne illness through ingestion of contaminated food by producing three major toxins (cytotoxin K, hemolysin BL, and non-hemolytic enterotoxin). A BLAST search in the Virulence Factor Database showed that the FORC_021 strain contained one cytotoxin K gene (FORC21_1054), and a hemolysin BL gene cluster (FORC21_2950 to FORC21_2953), and a non-hemolytic enterotoxin gene cluster (FORC21_1773 to FORC21_1775) (Table S1). Furthermore, hemolysin III homolog genes (FORC21_2117 and FORC21_5314) and four enterotoxin-related regions (FORC21_0722, FORC21_2783, FORC21_3577, and FORC21_5102) were also detected in the genome. Sphingomyelinase, a virulence factor that interacts with non-hemolytic enterotoxin in insects and murine intestinal epithelial cells [8], was encoded by a single CDS (FORC21_0599). Four CDSs (FORC21_0480, FORC21_1291, FORC21_1550, and FORC21_3385) were annotated to the putative internalin, which plays an important role in host cell invasion [20]. A total of 89 CDSs in the contig were homologous to spore-forming proteins, which help survive against heat or acids. These results indicate that strain FORC_021 could cause food-borne illness via ingestion of contaminated food.

In summary, the FORC_021 genome encodes major virulence factors such as cytotoxin K, hemolysin BL, and non-hemolytic enterotoxin. Furthermore, the FORC_021 strain showed a high level of cytotoxicity in LDH release assays compared with the type strain of *B. cereus*, and contained additional virulence factors such as putative ADP-ribosylating toxin, spore germination proteins, internalin-J, and sortase. The genomic information obtained

Table 1. Comparison of subsystem feature counts in strain FORC_021 with those in the clustered strains in the genome tree.

Subsystem category	*B. cereus* strain				
	FORC_021	ATCC 14579^T	B4264	FORC_005	G9842
Cofactors, Vitamins, Prosthetic Groups, and Pigments	285	284	279	274	270
Cell Wall and Capsule	181	184	171	173	181
Virulence, Disease, and Defense	119	123	119	118	114
Potassium Metabolism	19	19	19	19	19
Miscellaneous	58	59	60	59	57
Phages, Prophages, Transposable Elements, and Plasmids	14	24	22	19	33
Membrane Transport	160	159	162	138	135
Iron Acquisition and Metabolism	71	69	62	61	69
RNA Metabolism	184	187	183	186	186
Nucleosides and Nucleotides	134	147	136	133	137
Protein Metabolism	266	275	278	268	272
Cell Division and Cell Cycle	52	55	54	55	59
Motility and Chemotaxis	82	88	82	82	78
Regulation and Cell Signaling	100	104	107	108	112
Secondary Metabolism	8	17	9	9	8
DNA Metabolism	122	125	114	112	129
Fatty Acids, Lipids, and Isoprenoids	145	147	148	149	147
Nitrogen Metabolism	33	32	32	31	41
Dormancy and Sporulation	142	140	153	152	131
Respiration	98	100	98	96	97
Stress Response	114	113	112	110	108
Metabolism of Aromatic Compounds	10	10	14	14	20
Amino Acids and Derivatives	534	543	543	543	534
Sulfur Metabolism	37	37	37	38	35
Phosphorus Metabolism	100	100	104	100	105
Carbohydrates	420	416	418	417	398

Table 2. Comparison of CDS counts of *Listeria* pathogenicity island 1 (LIPI-1) genes in the strain FORC_021 genome with the clustered strains in the genome tree.

B. cereus strain	CDS counts of LIPI-1 gene					
	prfA	*plcA*	*hly*	*mpl*	*actA*	*plcB*
FORC_021	0	1	1	1	0	0
ATCC 14579	0	2	1	1	0	0
B4264	0	1	1	1	0	1
FORC_005	0	2	1	1	0	1
G9842	0	2	1	1	0	1

in this study will help us understand the characteristics of *B. cereus* for future applications and to extend the database of food-borne pathogens.

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