Infection and Drug Resistance

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ORIGINAL RESEARCH

Genomic Insights into the Pathogenicity and Drug-Resistance of a *Bacillus cereus* Isolated from Human Teeth

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Background: *Bacillus cereus* is a common bacterium found in the environment. Some strains can cause food poisoning, and very few can cause clinically severe infections, leading to death. Here, we characterized the genome sequence of *B. cereus* LIN78 isolated from teeth with deep caries and compared it with those of 25 other related species.

Methods: Third-generation sequencing technology, bacteriological analyses, biochemistry, and mass spectrometry were applied to characterize the drug-resistance genes and virulence factors of *B. cereus* LIN78.

Results: The complete genome sequence of *B. cereus* Lin78 consists of 5647 genes distributed on a circular chromosome, a 393 kbp plasmid, and 928 pseudogenes (37.4% of whole-genome DNA). The LIN78 genome contains 14 sets of 16s, 23s, and 5s ribosomal RNA operons; 106 tRNA genes, one tmRNA, 12 genomic islands, six prophases, 64 repeats; 37 antibiotic-resistant genes; and 1119 putative virulence genes, including enterotoxins and cytolysins. The *B. cereus* LIN78 genome carries multiple copies of non-ribosomal polypeptide synthetase (NRPS) and post-translationally modified peptides (RiPPs). Phylogenetic analysis of the 26 *B. cereus* strains showed that *B. cereus* LIN78 is evolutionarily closely related to *B. thuringiensis* ATCC 10792 and *B. cereus* ATCC 14579.

Conclusion: The newly isolated *B. cereus* carries many virulence genes, including enterotoxins and hemolysins, similar to *B. anthracis*, and multiple antibiotic resistance genes. These findings suggest that the strain has a potential risk of causing disease. Our studies are vital for further exploration of the evolution of *B. cereus*, its pathogenic mechanisms, and the control and treatment of bacterial infections.

Keywords: bacillus cereus, whole-genome DNA sequencing, virulence factor, drug resistance gene, human teeth

Introduction

Bacillus cereus is a common gram-positive bacterium found in the environment that includes several phylogenetically closely related *Bacillus* species. *Bacillus cereus* is usually used as a probiotic; however, some strains are pathogenic.[1] The most extensively studied members of this group are *B. anthracis, B. cereus*, and *B. thuringiensis*, which have strong pathogenicity, and some even serve as biological weapons.[2] Owing to the protection of spores, *B. cereus* can usually survive for a long time in the environment and has been implicated in infections of the eye,[3,4] respiratory tract,[5,6] and wounds.[7] *B. cereus* usually causes food poisoning,[8,9] but more and more cases show that it can cause severe and possibly fatal parenteral infections.[5,10,11]

Bacillus cereus is an important foodborne pathogen. When ingested, it produces and secretes enterotoxins, which cause food poisoning. Common symptoms include diarrhea, vomiting, etc.[2,9] *Bacillus cereus* can cause diarrhea mainly due to enterotoxins, including non-hemolytic enterotoxins (Nhe), hemolysin BL (Hbl), and cytotoxins K (CytK1 and

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CytK2). These enterotoxins can penetrate the host cells, causing them to lose water. Hence, they are also known to be pore-forming toxins.[12–15] Vomiting caused by *B. cereus* results from the secretion of cereulide, which is encoded by the gene cluster of cereulide synthetase (*ces*) and controlled by non-ribosomal peptide synthetase (NRPS).[16–18] Vomiting caused by *B. cereus* can usually self-heal, and recovery generally occurs within a few days.[19] In recent years, drug-resistant *B. cereus* strains have been isolated in the clinic with the widespread use of antibiotics, which increases the complexity and difficulty of treating such bacteria.[20–23]

Whole-genome DNA sequencing provides an essential basis for the molecular epidemiological study of infectious diseases and insights into pathogenic bacterial pathogenesis, antibiotic resistance mechanisms, and treatment options.[24] A previous study analyzed two strains of *B. cereus* isolated from indoor air using whole-genome DNA sequencing and found that they carried both hemolytic (*hblA, hblC*, and *hblD*) and non-hemolytic (*nheA, nheB*, and *nheC*) enterotoxin genes. This suggests that *B. cereus* in an indoor environment may cause diarrhea.[25] Another study also used whole-genome DNA sequencing to analyze the genomes of *B. cereus* in ready-to-eat foods and milk powder and found that they carry toxin genes such as *nheABC, hblCDAB, cytK2, entFM*, and *cesB*.[26] By analyzing the genomes of *B. cereus*, the synthesis and secretion mechanisms of bacterial toxins can be revealed.[8] Through in-depth analysis of the bacterial genome isolated from eye cosmetics, explanations for why these bacteria can survive in high-osmotic pressure cosmetics and their pathogenic mechanisms have been uncovered.[27] However, no study has reported on isolating *B. cereus* from the human oral cavity.

We isolated a strain of *B. cereus* from the dental crevice of a patient for more than 90 days. We used second- and third-generation DNA sequencing to obtain a framework map of the genome of this strain. Based on this, we used comparative genetic analysis to analyze virulence factors, antibiotic resistance genes, interactions between pathogens and hosts, and evolution, and evaluated their pathogenic potential at the genomic level. To our knowledge, no *B. cereus* bacterium is isolated within the oral cavity. Through genome analysis of this strain, we found numerous toxin and antibiotic-resistant genes, including genes that express hemolysin (BL) and enterotoxin (Nhe). We also observed that the flagellar system of this strain was highly developed. These findings suggested that this strain is pathogenic. Additionally, this strain could grow in an aerobic environment. The growth of this strain in the oral cavity may create an anaerobic environment that can enhance the development of anaerobic pathogens in the oral cavity and affect oral health. Through in-depth analysis of the genome of the *B. cereus* LIN78 strain, we provide evidence revealing the pathogenic mechanisms, drug resistance mechanisms, and biological evolutionary trends of this type of bacteria, laying a foundation for developing antibiotics and controlling and treating pathogenic infections.

Materials and Methods

Isolation, Culture, and Identification of Bacterial Strains

A 45-year-old male with tooth decay was admitted to First People's Hospital Affiliated with Huzhou University. *Bacillus cereus* LIN78 was isolated from the crevices of the patient's teeth. The same strain was isolated from the patient's teeth four times over three months. To create bacterial isolation, the material collected from the dental floss was cultivated on sheep blood agar plates and incubated overnight at 37°C under aerobic conditions. After three consecutive selections and purification recultures, a *Bacillus* isolate was obtained. The isolate was identified as *B. cereus* by MALDI-TOF. The patient was discharged without further symptoms.

Whole-Genome DNA Sequencing

The B. cereus strain was grown overnight on sheep blood agar plates at 37°C, and bacteria were scraped from the agar surface. The genomic DNA of the *B. cereus* strain was extracted using a TIANamp Bacteria DNA Kit (TIANGEN Biotech (Beijing) Co., Ltd., Beijing, China), according to the manufacturer's instructions. The purity and quality of the total DNA were verified using agarose gel electrophoresis. According to the manufacturer's protocol, the library was constructed using the NEBNextUltra DNA Library Prep Kit (NEB, USA). After thorough quality inspection, the library was sequenced using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to obtain raw data, yielding 2 × 150-bp paired-end reads, which were filtered using fastp (v0.23.4) to acquire clean data. Raw sequencing data were obtained through quality

control using Porechop and NanoFilt software. *De novo* assembly of sequencing data was performed using Unicycler (0.4.4) software.[28] Genome annotation was performed using Prokka automatic annotation tool (v 1.14.6) software.[29] The tRNAscan-SE, rRNAmmer, and Rfam databases were used to predict tRNA rRNA and sgRNA expressions, respectively. The GIs were predicted as previously described.[30] PhiSpy was used for the prophage prediction (https://github.com/linsalrob/PhiSpy). Seven databases, namely, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Orthologous Clusters (COG), Non Redundant Protein Database (NR), Transport Protein Taxonomy Database), Pfam, Swiss Prot, and TreMBL, were used for whole-genome BLAST searches to predict gene function. Based on the assembled genome sequence and prediction results for coding genes, Proksee generated a genome map (http://proksee.ca/).[31]

Analyses of the Genes Relevant to Risk Assessment

The Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/analyze) was used to predict antimicrobial resistance genes in the bacterial genome.[32] The virulence factors in the *B. cereus* LIN78 strain and plasmid were predicted by blasting protein sequences against the virulence factor database (VFDB; http://www.mgc.ac.cn/VFs/main.htm) with an E-value threshold of ≤1e-5.[33] The obtained data were compared with each of the identified virulence factors. The corresponding functional annotations of virulence factors and their target species were integrated to obtain comprehensive annotation results for the virulence factors of the *B. cereus* LIN78 strain. We used the antibacterial Biocide & Metal Resistance Genes Database (BacMet) (http://bacmet.biomedicine.gu.se/) to predict the presence of metal resistance genes in the *B. cereus* LIN78 strain genome.[34]

Comparative Genomics Analysis

To perform comparative genomic analysis, we downloaded 25 complete genomic DNA sequences closely related to *B. cereus* LIN78 from the GenBank database to create a phylogenetic dendrogram. We aligned the protein sequences using the MUSCLE program and the nucleotide sequences using the MEGA11 alignment function. We constructed a maximum likelihood tree using the MEGA11 software.

Results

Biological and Phylogenetic Characteristics of B. Cereus LIN78

B. cereus LIN78 strain, isolated from a patient's teeth with tooth decay, formed rough, milky-white colonies on sheep blood agar plates at 37°C under aerobic conditions (Figures S1A and S1E). In addition to *Bacillus cereus, Neisseria flava* and *Streptococcus salivarius* were identified from the patient's teeth (Figures S1B-S1D and S1F-S1H). The isolated strain was confirmed to be *B. cereus* by MALDI-TOF (Figure S1I). LIN78 strain possesses peripheral flagella and can produce oval-shaped endospores centrally within the cell (Figures S2A and B). *B. ereus* LIN78 exhibited motility in the LB medium containing 0.3% agar (Figure S2C). We performed PCR against 92 antibiotic-resistance genes for *B. cereus* LIN78 and found that the LIN78 strain carries many antibiotic-resistance genes (Figure S3). Genome analysis revealed that the LIN78 and *B. cereus* ATCC14579 strains were identical. We determined that the average nucleotide identity (ANI), reflecting the degree of evolutionary distance between the compared genomes, between LIN 78 and ATCC 14579 was 98.0985%, identifying the strain as *B. cereus* (Figure S4). This is supported by relevant evidence from the protein annotation. When we used the Non Redundant Protein Database to annotate LIN78 proteins, most proteins belonged to *B. cereus* (Figure S5).

Genome Features of B. Cereus LIN78

The general features of the *B. cereus* LIN78 genome are displayed in Table 1, Figure 1, Figures S4-S11, and Tables S1–S16. *B. cereus* LIN78 has a circular chromosome of 5,322,019 base pairs (bp) long and a plasmid (pLIN78) of 393,129 bp. LIN78 contains 5649 genes, accounting for 83.81% of the genome. The chromosome contained 5257 genes, and the plasmid contained 392 genes. The chromosome contains 14 23S, 5S, and 16S ribosomal RNA operons, and one tmRNA. Interestingly, there is a tmRNA in the genome of *B. cereus* LIN78; to our knowledge, this was first identified in *B. cereus*. *B. cereus* LIN78 possesses 16 genomic islands (GIs), four of which are located on the plasmid (Figure S10 and Table S7).

Table 1 The General Genome Features of Bacillus Cereus LIN78

Category	*Bacillus cereus* LIN78	
	Chromosome	pLIN78
DNA size (bp)	5,322,019	393,129
GC Content (%)	35.3	33.7
Gene Number	5257	392
Gene Length	4,478,166	311,763
Number of tRNAs	106	0
Number of rRNA (16S-23S-5S)	14	0
Number of tmRNA	1	0
Prophage	6	2
Pseudogene number	928	85
Genomics Island	12	4
Repeats	64	4

B. cereus LIN78 possesses 1023 pseudogenes, accounting for 3.8% of the genome (Table S5). *B. cereus* LIN78 also possessed six prophages distributed on the chromosome and plasmids (Table S8), and one CRISPR on the chromosome (Table S6). *B. cereus* LIN78 possessed 68 repeats, accounting for 0.56% of the total genome (Table S9), implying the possibility of genetic recombination between *B. cereus* LIN78 and other species. Artificial modifications were identified in the LIN78 genome (Table S9).

Carbohydrates are essential nutrients for life, and studying carbohydrate-related enzymes will help us to understand vital life processes. In the *B. cereus* LIN78 genome, 183 carbohydrate-related genes were identified (Table S10). We identified 1583 transmembrane proteins (Table S15), 1342 membrane transport proteins (Table S14), and 254 secreted proteins (Table S16).

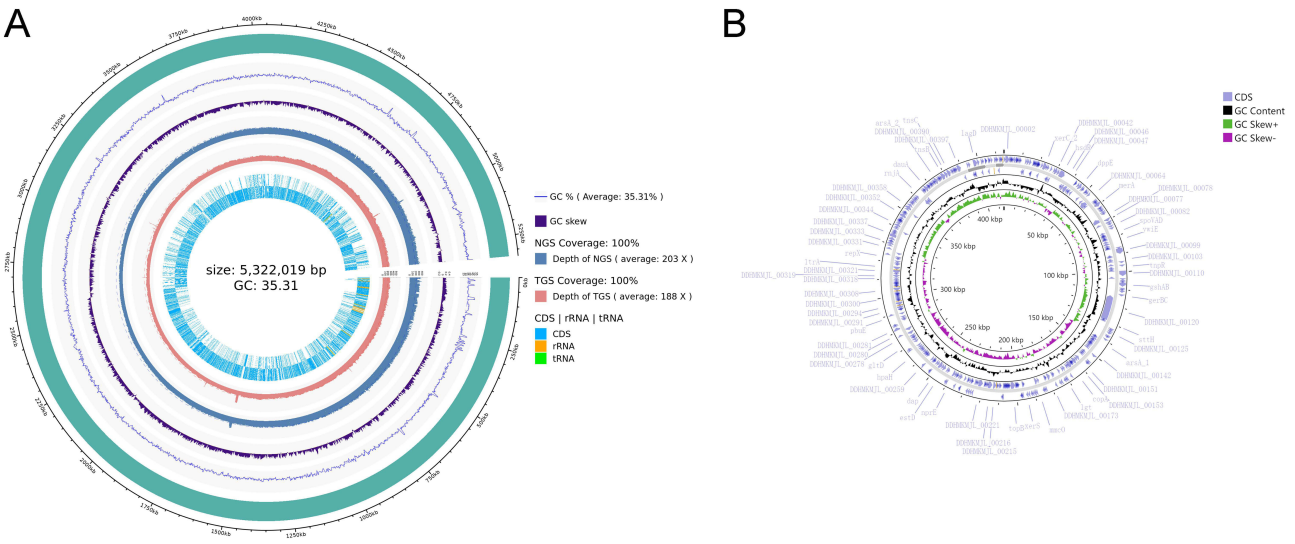

Figure 1 Circular maps of *B. cereus* LIN78. A circular map of the LIN78 genome (**A**) and pLIN78 plasmid (**B**) is shown. (**A**) Circles represent the following characteristics from the outermost circle to the center: (1) contig information, (2) coding sequences on forward strand, (3) GC ratio, (4) GC skew, (5) depth and coverage of genomic sequencing, and (6) CDS, transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs). (**B**) CDS, GC content, GC Skew+, GC Skew-, and coding sequences on the forward strand are shown.

Analysis of the Genome to Identify Virulence Factors and Other Genes Relevant to Risk Assessment

By analyzing the genome of *B. cereus* LIN78 isolates, we identified 1119 genes expressing virulence factors and 1604 genes expressing proteins participating in pathogen-host interactions (Tables 2, S11 and S12). The proteins encoded by these genes function as exoenzymes, exotoxins, motility factors, nutritional/metabolic factors, immune modulators, and transcription regulators. Some genes encode critical virulence factors in the *B. cereus* LIN78 genome that may cause gastrointestinal and non-gastrointestinal diseases. All of these genes are presented in Tables 3 and S13.

Table 2 Genes Relevant to Risk Assessment in B. Cereus LIN78

Gene Category	Genes	Source
Virulence Factor	1119	VFDB
Transporter	1342	TCDB
Antibiotic Resistance	58	CARD
Pathogen Host Interaction	1604	PHI

Table 3 Source and Virulence Genes Found in B. Cereus LIN78

Gene ID	Source Organism	Gene	Identity	Product
DKIALGPF_00693	*B. cereus ATCC 14579*	*inhA*	99.2	Immune inhibitor A metalloprotease
DKIALGPF_00697	*B. cereus ATCC 14579*	*plcA*	99.6	Phospholipase C
DKIALGPF_00698	*B. cereus ATCC 14579*	*sph*	99.1	Sphingomyelin phosphodiesterase
DKIALGPF_01146	*B. cereus ATCC 14579*	*cytK*	99.1	Cytotoxin K
DKIALGPF_01310	*B. cereus ATCC 14579*	*BC_RS06405*	99.9	Immune inhibitor A
DKIALGPF_01675	*B. cereus ATCC 10987*	*BCE_RS08690*	95.3	Flagellar motor protein MotP
DKIALGPF_01676	*B. cereus ATCC 10987*	*BCE_RS08695*	95.3	OmpA family protein
DKIALGPF_01677	*B. cereus ATCC 10987*	*BCE_RSO8700*	95.3	Response regulator
DKIALGPF_01864	*B. cereus ATCC 14579*	*nheA*	95.3	Non-hemolytic enterotoxin A
DKIALGPF_01865	*B. cereus E33L*	*nheB*	95.3	Non-hemolytic enterotoxin B
DKIALGPF_01866	*B. cereus ATCC 14579*	*nheC*	95.3	Non-hemolytic enterotoxin C
DKIALGPF_02367	*B. cereus G9241*	*dhbF*	95.3	Non-ribosomal peptide synthetase, DhbF
DKIALGPF_03003	*B. cereus ATCC 14579*	*BC_RS14930*	95.3	Immune inhibitor A
DKIALGPF_03135	*B. cereus ATCC 14579*	*hblA*	95.3	Hemolysin BL binding component precursor
DKIALGPF_03137	*B. thuringiensis* serovar konkukian str. 97–27	*hblD*	95.3	Hemolysin BL lytic component L1
DKIALGPF_03138	*B. cereus G9241*	*hblC*	95.3	Hemolysin BL lytic component L2
DKIALGPF_03747	*B. cereus ATCC 14579*	*piplc*	95.3	Phosphatidylinositol diacylglycerol-lyase
DKIALGPF_04558	*B. cereus ATCC 10987*	*motA*	95.3	Flagellar motor stator protein MotA
DKIALGPF_04573	*B. anthracis str. Ames Ancestor*	*GBAA_RS23155*	95.3	Iron-siderophore ABC transporter substrate-binding protein
DKIALGPF_04574	*B. anthracis str. Ames Ancestor*	*fhuB*	95.3	Fe(3+)-hydroxamate ABC transporter permease FhuB
DKIALGPF_04588	*B. anthracis str. Ames Ancestor*	*isdG*	95.3	Heme oxygenase
DKIALGPF_04590	*B. anthracis str. Ames Ancestor*	*GBAA_RS23240*	95.3	ABC transporter ATP-binding protein
DKIALGPF_04591	*B. anthracis str. Ames Ancestor*	*GBAA_RS23245*	95.3	Iron ABC transporter permease
DKIALGPF_04592	*B. anthracis str. Ames Ancestor*	*isdE*	95.3	Heme ABC transporter substrate-binding protein IsdE

(*Continued*)

Table 3 (Continued).

Gene ID	Source Organism	Gene	Identity	Product
DKIALGPF_04594	*B. anthracis str. Ames Ancestor*	*GBAA_RS23260*	95.3	NEAT domain-containing protein
DKIALGPF_05320	*B. thuringiensis* str. Al Hakam	*BALH_RS26255*	95.3	Capsular polysaccharide biosynthesis protein
DKIALGPF_05321	*B. cereus ATCC 14579*	*BC_RS26320*	95.3	CpsD/CapB family tyrosine-protein kinase
DKIALGPF_05394	*B. cereus G9241*	*papR*	95.3	Quorum-sensing peptide PapR
DKIALGPF_05493	*B. cereus AH187*	*galE*	95.3	UDP-glucose 4-epimerase GalE
DKIALGPF_05494	*B. cereus ATCC 14579*	*BC_RS27175*	95.3	Hemolysin III family protein

Using the anti-SMASH program (version 7) to predict the genome,[35] ten clusters of secondary metabolic genes were identified in *B. cereus* Lin78 (Figure 2A). These gene clusters mainly focused on non-ribosomal polypeptide synthetase (NRPS), leucine aminopeptidase (LAP), bacteriocin, ribosomally-synthesized and post-translationally modified peptides (RiPP), and terpenes, among which the number of NRPS genes was the highest (Figure 2B). The LIN78 strain carried the complete cereulide synthetase gene cluster sequences (*cesA, cesP, cesT*, and *cesC* (14 copies)) (Table S12). NRPS is a classical regulatory mechanism for emetic toxin synthesis.[36] We hypothesized that *B. cereus* LIN78 synthesizes and secretes emetic toxins via the NRPS system and ABC transporters.

The pairing of PlcR (phospholipase C regulator) and PapR (a small signaling peptide that acts as a quorum-sensing effector) transcription regulators has been shown to play an essential role in the expression of virulence factors in *B. cereus*, including enterotoxins, hemolysins, and proteases.[37,38] PlcR/PapR transcriptional regulators were also in the *B. cereus* LIN78 genome (Table S12).

In addition to transcription factors, the *B. cereus* LIN78 genome contains genes for toxins and enzymes common to *B. cereus*, which were also found in the *B. cereus* LIN78 isolate (Table S12). These genes include three non-hemolytic

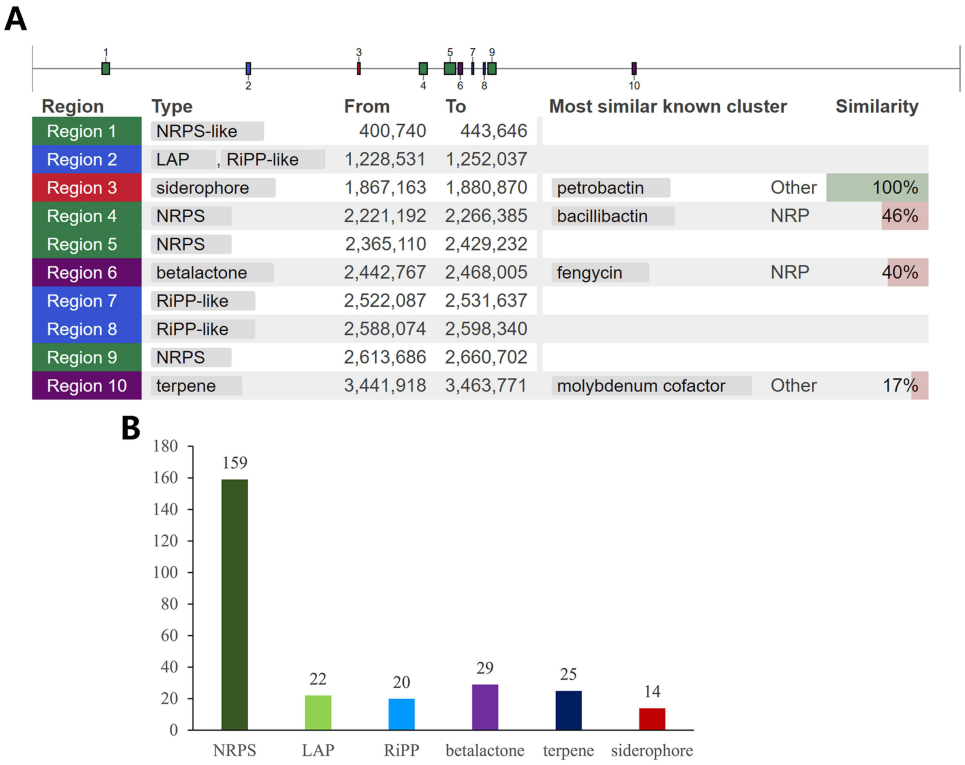

Figure 2 Biosynthetic gene cluster of secondary metabolites within the *B. cereus* LIN78 genome. (**A**) Gene clusters for the biosynthesis of NRPS (green), LAP (blue), RiPP (blue), siderophore (red), beta lactone (purple), and terpene (purple). (**B**) The number of unique genes predicted in each cluster.

enterotoxin genes (*nheA*, six copies; *nheB*, eight copies; *nheC*, five copies), hemolytic enterotoxin genes (*hblA*, eight copies; *hblD*, four copies; *hblC*, four copies), and the gene-encoding cytotoxin K (*cytK*, five copies), which play an essential role in gastrointestinal infection. Genes encoding pore-forming toxins, including thiol-activated cytolysins (*alo*, three copies), hemolysin A (*hlyA*, ten copies), and hemolysin III (*hlyIII*, ten copies), were also found in the *B. cereus* LIN78 genome. Pore-forming toxins play an essential role in the development of non-gastrointestinal infections. *B. cereus* LIN78 also carries genes encoding enzymes, such as phospholipase C (*plcA*, 15 copies) and collagenases (colA, 25 copies). Previous studies indicate that tripartite hemolysin BL, phosphatidylcholine-phospholipase C, and collagenase are essential pathogenic factors for *B. cereus*.[39,40] The immune inhibitor A-type metalloproteases were present in the LIN78 isolate (*inhA*, 23 copies). It has been shown that this type of protein plays an essential role in *B. cereus* to survive and escape macrophage attacks.[41]

Identification of Antimicrobial Resistance Genes in the Genome

Antimicrobial resistance genes in the *B. cereus* LIN78 genome were identified by blasting against the Comprehensive Antibiotic Resistance Database (CARD).[32] Protein sequences of the *B. cereus* strain and plasmid were subjected to BLAST analysis with an E-value threshold of ≤1e-5 against CARD. Interestingly, the *B. cereus* LIN78 genome encodes multiple sigma factors, including SigA, SigW, SigI, YlaC, SigJ, SigW, SigM, SigH, and AlgU (Table S12). The sigma factor binds to RNA polymerase and regulates gene expression in bacteria.[42] Studies have indicated that most sigma factors are essential for antibiotic resistance.[43–46]

The predicted antimicrobial resistance-encoding genes in the *B. cereus* LIN78 genome are listed in Table 4. Specifically, we identified genes encoding proteins involved in resistance to bicyclomycin, bacitracin, tetracycline, macrolides, penicillin,

Table 4 Antimicrobial Resistance Genes Found in B. Cereus LIN78

Genes	Count	Products
mdtG	1	Multidrug resistance protein MdtG
bcr	1	Bicyclomycin resistance protein
bcrB	1	Bacitracin transport permease protein BcrB
bcrA	5	Bacitracin transport ATP-binding protein BcrA
bmr3	6	Multidrug resistance protein 3
mdtL	1	Multidrug resistance protein MdtL
tetA	2	Tetracycline resistance protein, class B
yheI	2	Putative multidrug resistance ABC transporter ATP-binding/permease protein YheI
yheH	2	Putative multidrug resistance ABC transporter ATP-binding/permease protein YheH
macB	1	Macrolide export ATP-binding/permease protein MacB
blaI	1	Penicillinase repressor
mycF	1	Mycinamicin III 3"-O-methyltransferase
norM	1	Multidrug resistance protein NorM
lnrL	2	Linearmycin resistance ATP-binding protein LnrL
lnrN	1	Linearmycin resistance permease protein LnrN
fsr	1	Fosmidomycin resistance protein
bmrA	1	Multidrug resistance ABC transporter ATP-binding/permease protein BmrA
aadK	1	Aminoglycoside 6-adenylyltransferase
oleD	2	Oleandomycin glycosyltransferase
pbp	2	Beta-lactam-inducible penicillin-binding protein
bceA	4	Bacitracin export ATP-binding protein BceA
bceB	3	Bacitracin export permease protein BceB
cat	2	Chloramphenicol acetyltransferase
penPC	1	Beta-lactamase 1
vat	1	Virginiamycin A acetyltransferase
vgb	1	Virginiamycin B lyase

(*Continued*)

Table 4 (Continued).

Genes	Count	Products
rdmC	2	Aclacinomycin methylesterase RdmC
mdtH	2	Multidrug resistance protein MdtH
yokD	1	SPbeta prophage-derived aminoglycoside N(3')-acetyltransferase-like protein
tetO	1	Tetracycline resistance protein TetO
rphC	1	Rifamycin-inactivating phosphotransferase RphC
blaZ	1	Beta-lactamase 3
drrB	1	Daunorubicin/doxorubicin resistance ABC transporter permease protein DrrB
drrA	1	Daunorubicin/doxorubicin resistance ATP-binding protein DrrA
blm	1	Metallo-beta-lactamase type 2
Fluoroquinolones export ATP-binding protein	1	Fluoroquinolones export ATP-binding protein
aadK	1	Aminoglycoside 6-adenylyltransferase

mycinamicin, clindamycin, fosmidomycin, aminoglycoside, oleandomycin, beta-lactam, chloramphenicol, virginiamycin, actinomycin, tetracycline, rifamycin, daunorubicin/doxorubicin, and fluoroquinolones. Furthermore, we found that *B. cereus* LIN78 contains the mercury-methylating gene hgcAB in the pLIN78 plasmid but not in the genome. Through genome analysis, we found that there are also genes that encode proteins related to multiple antibiotic resistance in the genome, such as multidrug resistance proteins, efflux pumps (HrtA, BcrA, BcrB), ABC-type transporters, penicillinase repressors, and beta-lactam-inducible penicillin-binding proteins.

In addition, our analyses revealed genes encoding multiple proteins, including spore coat proteins (CotE, CotX, and CotZ), inner spore coat proteins (CotH), and exosporium protein C, which enhance spore recovery by blocking toxic molecules, thereby enhancing bacterial resistance to oxidants and chemicals.[47] Several genes contributing to arsenic resistance were present in pLIN78 cells (Figure 1B).

Phylogenetic Tree Analysis

We used a whole-genome ML phylogenetic tree to resolve the genetic relationships between the LIN78 isolate and other *Bacillus strains* (Figure 3). Our phylogenetic tree comparing LIN78 to 25 closely related members of *B. cereus* confirmed that *B. anthracis* and *B. cereus* LIN78 evolved from a common ancestor and exhibited a close genetic relationship with *B. thuringiensis* ATCC 10,792 and *B. cereus* ATCC 14579, an established reference strain (Figure 3). This indicated that *B. cereus* LIN78 carried virulence genes similar to ATCC 14,579. Additionally, we noticed that *Bacillus thuringiensis, Bacillus cereus*, and *Bacillus anthracis* were in the same clade. This indicated that these bacteria were genetically similar. Differences between some *B. cereus* and *B. anthracis* genomes were not noticeable. By comparing the genomes of the three Bacillus species, we found they are highly similar. We noted that *Bacillus thuringiensis* is mainly used as an insecticide in the *Bacillus cereus* group. The genomes of *B. cereus* LIN78 and *B. thuringiensis* ATCC 10,792 were very similar, indicating that the extensive use of insecticides may increase the opportunity for gene exchange between these two species.

Interestingly, we found that *B. cereus* LIN78 and *B. cereus* MB1,[48] strains from the Challenger Deep of the Mariana Trench, are in different branches, indicating that the genomes of the two *Bacillus* species are similar. These two bacteria showed less cross-influence during evolution. This result was consistent with our expectations. Since the ocean has become land-based, it has been difficult for bacteria, especially sea ones, to undergo genetic exchange with terrestrial species. By comparing the genomes and proteomes of these two species, powerful clues were provided regarding their origin and evolution.

Discussion

Bacillus cereus is an opportunistic pathogen that is widespread in the environment and often causes food poisoning. It is a quality control bacterium in the food industry.[49] Although there are a few fatal cases caused by *B. cereus*, this type of

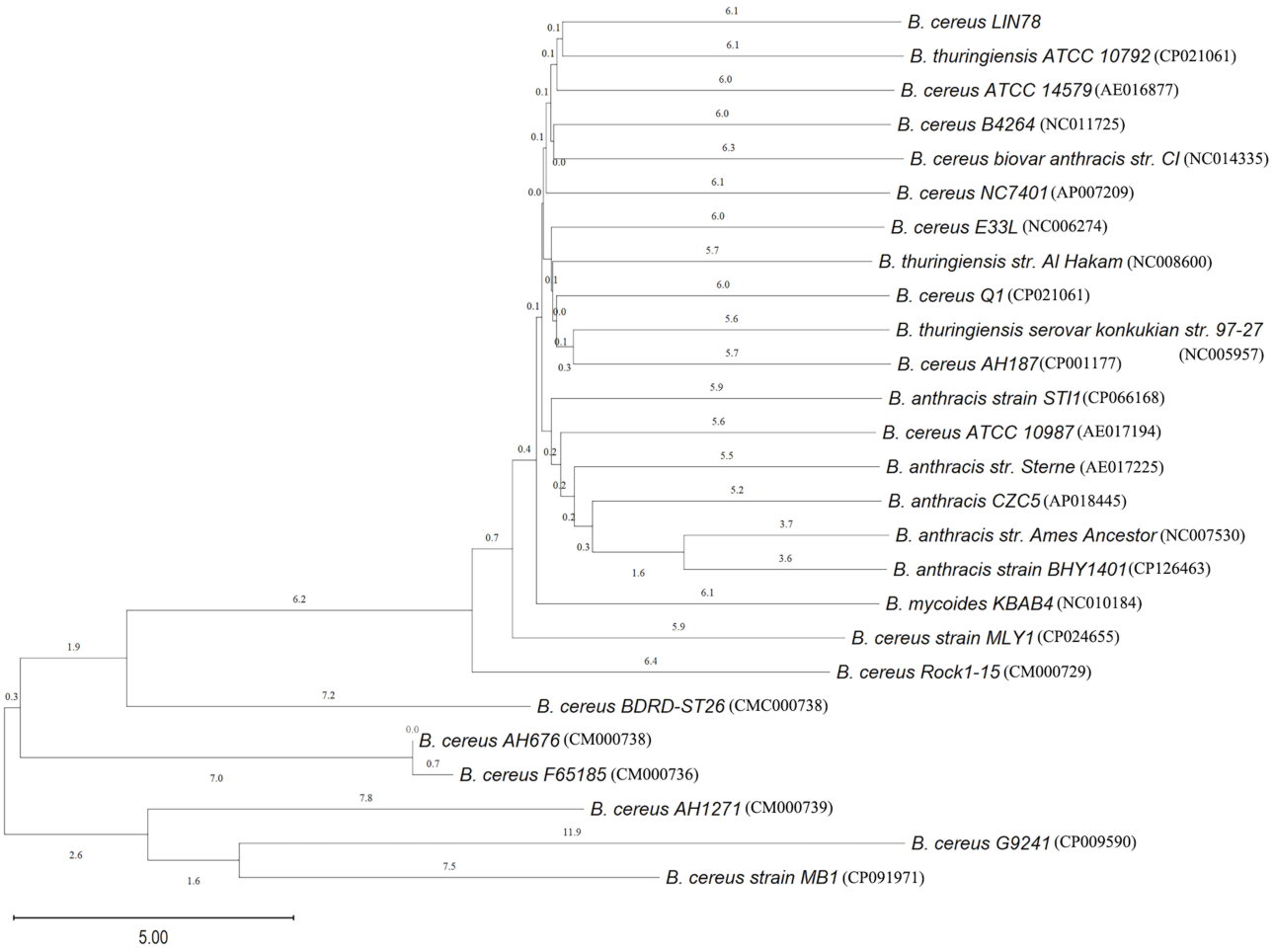

Figure 3 Phylogenetic analysis of *B. cereus* LIN78 and 25 *Bacillus* strains downloaded from databases based on whole genome sequence. Strain names and accessions of the downloaded strains are available in the figure.

bacteria is highly resistant to environmental stress and is widely distributed. Once antibiotic resistance develops, combined with solid virulence factors, it may cause severe disease. We need to monitor these bacteria closely. In this study, *B. cereus* was successfully isolated from human teeth. Whole-genome sequencing was performed using high-throughput sequencing. We found that the genome of this strain is evolutionarily closely related to *Bacillus thuringiensis* and *Bacillus anthracis*. We analyzed the genome of *B. cereus* LIN78 and found that the genome of this strain contained multiple factors that may cause disease in the host and resist environmental stress.

B. cereus is widely distributed in the natural environment and is usually non-pathogenic or conditionally pathogenic. However, this does not mean that these bacteria are non-pathogenic. Food poisoning caused by *B. cereus* often occurs and must be taken seriously.[50,51] The most prominent feature of this type of bacteria is its ability to form spores and resist harsh environmental conditions. Once infected with pathogenic strains, it is difficult to remove them. *B. cereus* is present in many parts of daily life, such as food,[51] cosmetics,[52] and air.[25] Although *B. cereus* is not usually pathogenic, whole-genome analysis has revealed that these bacteria carry several virulence and antibiotic-resistance genes. These virulence genes are similar to those carried by pathogenic bacteria. The *B. cereus* strain discovered in this study did not cause diarrhea or other poisoning symptoms in patients with *B. cereus* infection. However, using whole-genome sequencing, we found that this strain carried 1119 genes expressing virulence factors, including enterotoxins, cytolysins, and cereulide, and had a complete regulatory mechanism required to express these virulence genes. Interestingly, the patient carrying this strain showed no symptoms of food poisoning. We compared this strain with a *Bacillus cereus* food

poisoning strain that broke out in Guizhou in 2021.[8] Many virulence factors coexist in both strains, and which factors lead to disease outbreaks remains unknown and requires further study.

In this study, we report, for the first time, the isolation of *B. cereus* from dental crevices. A search of NCBI found no similar reports. *B. cereus* is often used as a probiotic to treat intestinal flora. Health risks may be associated with consuming large amounts of *Bacillus cereus* as a probiotic.[53] *B. cereus* can form spores that can pass through gastric acid and reach the intestines smoothly. *B. cereus* can consume oxygen in the intestines, maintain an excellent anaerobic environment, and promote the growth of anaerobic bacteria such as lactobacilli. Therefore, *B. cereus* acts as a probiotic. However, the situation in the mouth is different from that in the intestine. Many pathogenic bacteria in the oral cavity are anaerobes. The growth of *B. cereus* in the oral cavity may increase the local anaerobic environment, which is conducive to the growth of oral anaerobic bacteria, and efficiently produces dental calculus, periodontal disease, gingivitis, and other diseases. These symptoms were similar to those observed in our patient, resulting from the *B. cereus* LIN78 strain. *B. cereus* is often used as a probiotic or remains in food and inevitably remains in the mouth during consumption. Our study suggests that attention should be paid to enhancing the detection of *B. cereus* during oral examinations. We noticed that this strain carried 37 antibiotic-resistance genes, indicating it was resistant to multiple antibiotics. Therefore, removing this strain from the mouth is a challenge.

By comparing the genomes of the three Bacillus species, we found they are highly similar. We noted that *Bacillus thuringiensis* is mainly used as an insecticide in the *Bacillus cereus* group. The genomes of *B. cereus* LIN78 and *B. thuringiensis* ATCC 10,792 were very similar, indicating that the extensive use of insecticides may increase the opportunity for gene exchange between these two species. Many countries, particularly in Europe, have warned about using *B. thuringiensis* as a pesticide.[54] This explains why *B. cereus*, which carries several *B. thuringiensis* genes, is present in the human body. It is a complete chain from farmland to water and soil and from food to the human body. Therefore, strictly controlling B. thuringiensis as a pesticide is crucial to reducing its environmental impact.

We noticed the presence of some neurotransmitter-related enzymes and transporters, such as GABA permease, in the isolate, suggesting that *B. cereus* may interact with host neurotransmitters. Previous studies have shown that the long-term use of probiotics may lead to cognitive decline.[55] Gut microbiota affects many essential host functions, including immune responses and the nervous system. Bacteria that regulate GABA levels are present in human intestinal flora.[56] *B. cereus* is often used as a probiotic. Our study provides evidence for the harmful effects of probiotics. Further studies are required to understand how these neurotransmitter-related transporters and enzymes affect the host nervous system. This study provides an essential reference point for future research. When using probiotics in the future, strict normative behaviors must be formulated to reduce blind and long-term use of probiotics.

Through genomic analysis, we identified three groups of antimicrobial peptide synthesis gene cluster[57] In the *B. cereus* LIN78 genome. Antimicrobial peptides are essential antibacterial substances that are commonly found in gram-positive bacteria, gram-negative bacteria, fungi, and parasites.[58] Antimicrobial peptides are essential for combating bacterial antibiotic resistance. Analyzing the antimicrobial peptide synthesis gene cluster in the *B. cereus* genome will help us to understand antimicrobial expression and regulatory mechanisms and provide an essential basis for developing new antimicrobial peptides. This study offers new ideas for the discovery and development of antimicrobial peptides.

Interestingly, we found that *B. cereus* LIN78 and *B. cereus* MB1,[48] strains from the Challenger Deep of the Mariana Trench, are in different branches, indicating that the genomes of the two *Bacillus* species are similar. These two bacteria showed less cross-influence during evolution. This result was consistent with our expectations. Since the ocean has become land-based, it has been difficult for bacteria, especially deep-sea ones, to undergo genetic exchange with terrestrial species. By comparing the genomes and proteomes of these two species, powerful clues were provided regarding their origin and evolution. Further studies will help decipher the origin and evolution of essential virulence genes and the changes in antibiotic-resistant genes and offer ideas and targets for treating pathogens and developing antibiotics.

Although it is not a highly pathogenic pathogen, *B. cereus* exists widely in nature, giving this type of bacteria an excellent opportunity to acquire other traits through gene transfer and recombination. We observed 13 phase-modification sites in the genome of this strain and many repeated sequences. These are traces of the natural modifications of the genome. Interestingly, we noticed that the genome of *the B. cereus* LIN78 isolate had traces of artificial modification. Do

these artificially modified DNAs originate from the probiotics? However, this aspect remains to be studied further. However, interactions between microbial genomes occur frequently in nature. This indicates that genetic exchanges between species, especially between bacteria, are always present. Although bacteria may not be pathogenic, some pathogenic genes may be introduced during gene exchange, causing people to become sick. Therefore, it is necessary to detect changes in the bacterial genomes in the surrounding environment to prevent sudden attacks by pathogenic bacteria.

Conclusions

There are few reports on the isolation of *B. cereus* from human teeth. Although there are no clinical symptoms, the newly isolated *B. cereus* carries many virulence genes, including enterotoxins and hemolysins, as well as multiple antibiotic resistance genes. This strain has many virulence genes similar to *Bacillus anthracis*. These studies suggest that the bacterium has a potential risk of causing disease. Our study provides significant evidence for revealing the evolutionary relationship between *B. cereus* and provides ideas for analyzing the toxin synthesis and secretion mechanisms of *B. cereus*. This study provides a scientific basis for preventing and rapidly diagnosing *B. cereus* infection.

Data Sharing Statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

This study was reviewed and approved by the Medical Ethics Committee of Huzhou University (approval number: 2023-NSFC-04) and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from the patient.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests in this work.

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