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Genetic diversity, biofilm formation, and Vancomycin resistance of clinical *Clostridium innocuum* isolates

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Abstract

Background *Clostridium innocuum*, previously considered a commensal microbe, is a spore-forming anaerobic bacterium. *C. innocuum* displays inherent resistance to vancomycin and is associated with extra-intestinal infections, antibiotic-associated diarrhea, and inflammatory bowel disease. This study seeks to establish a multilocus sequence typing (MLST) scheme to explore the correlation between *C. innocuum* genotyping and its potential pathogenic phenotypes.

Methods Fifty-two *C. innocuum* isolates from Linkou Chang Gung Memorial Hospital (CGMH) in Taiwan and 60 sequence-available *C. innocuum* isolates from the National Center for Biotechnolgy Information Genome Database were included. The concentrated sequence of housekeeping genes in *C. innocuum* was determined by amplicon sequencing and used for MLST and phylogenetic analyses. The biofilm production activity of the *C. innocuum* isolates was determined by crystal violet staining.

Results Of the 112 *C. innocuum* isolates, 58 sequence types were identified. Maximum likelihood estimation categorized 52 CGMH isolates into two phylogenetic clades. These isolates were found to be biofilm producers, with isolates in clade I exhibiting significantly higher biofilm production than isolates in clade II. The sub-inhibitory concentration of vancomycin seemed to minimally influence biofilm production by *C. innocuum* isolates. Nevertheless, *C. innocuum* embedded in the biofilm structure demonstrated resistance to vancomycin treatments at a concentration greater than 256 µg/mL.

Conclusions This study suggests that a specific genetic clade of *C. innocuum* produces a substantial amount of biofilm. Furthermore, this phenotype assists *C. innocuum* in resisting high concentrations of vancomycin, which may potentially play undefined roles in *C. innocuum* pathogenesis.

Keywords ***Clostridium innocuum***, MLST, Biofilm, Vancomycin

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Background

Clostridium innocuum, identified as an *Erysipelotrichia* species, is a Gram-positive, anaerobic, spore-forming bacterium [1]. *C. innocuum* was avirulent in intramuscular and intraperitoneal infection models in guinea pigs and did not have known toxin expression; therefore, this anaerobe was defined as the intestinal commensal bacterium [2, 3]. Nonetheless, studies indicate that it is the second most common species that causes extra-intestinal clostridial infections and a potential contributor to antibiotic-associated diarrhea (AAD) [4, 5]. A retrospective case-control study in Taiwan revealed that patients infected with *C. innocuum* experienced more complications related to the gastrointestinal tract than those infected with *Clostridioides difficile* [6]. Furthermore, Ha et al. [7] showed that *C. innocuum* is related to the extra-intestinal manifestation of Crohn's disease, a form of inflammatory bowel disease. These studies suggest that *C. innocuum* may have undefined pathogenic roles within the human intestinal tract. More importantly, David et al. [8] suggested that *C. innocuum* could synthesize the peptidoglycan precursor that terminating in serine to result in low affinity for vancomycin; as a result, *C. innocuum* is intrinsically resistant to vancomycin, with a minimal inhibitory concentration in 8 μg/mL [2].

A phylogenetic analysis using multilocus sequencing indicated that isolates of *C. innocuum* from blood, ascites, and feces could not be differentiated based on their phylogenetic position [5]. Moreover, a cross-sectional study with comparative genomic analysis found no phylogenetic associations between *C. innocuum* isolates from AAD patients and asymptomatic controls [9]. Nonetheless, a recent genomic analysis identified four genetically distinguishable clades among *C. innocuum* isolates based on single nucleotide polymorphisms of the core genome and average nucleotide identity [10]. These results suggest that, although *C. innocuum* is a genetically diverse species, elucidating the genetic variance of *C. innocuum* could help clarify its role in human disease.

The natural habitat of *C. innocuum* is in the human intestine [2, 3]. It has been suggested that the dominant survival niche of colonized bacteria in the gut is in the biofilm [11]. Biofilm can retain water, shield bacteria from antibiotics and immune clearance, and facilitate quorum sensing detection and horizontal gene transfer [12], offering advantages for survival in the intestinal niche. As the intestinal bacterium, the biofilm production activity of *C. innocuum* may contribute to its colonization, persistence, and potential pathogenesis in the intestine. Nonetheless, it remains unclear whether *C. innocuum* can produce biofilms and whether different isolates exhibit varying levels of biofilm formation activities.

Given that *C. innocuum* is a genetically diverse species [10], additional genetic and phenotypical analyses are necessary to establish the molecular epidemiological information for *C. innocuum*. To achieve this objective, there is an urgent need for rapid and cost-effective molecular typing methods for this anaerobic bacterium. This study employed amplicon sequencing, also known as Illumina targeted sequencing, to establish a sequence type for *C. innocuum* isolates. Furthermore, the biofilm production activity of *C. innocuum* isolates was analyzed, and the correlation between the biofilm production activity and the phylogenetic position of these isolates was established.

Methods

Bacterial isolates and culture conditions

Non-repetitive isolates of *Clostridium innocuum* from stool were provided by the Bacteria Bank, Chang Gung Memorial Hospital at Linkou in Taiwan (Supplementary Table S1). All *C. innocuum* isolates included in this study were verified by polymerase chain reaction (PCR) with species-specific primers for *C. innocuum* [13]. It should be noted that clinical history of the patients is currently not available. The *C. innocuum* isolates were cultured on anaerobic blood agar or grew in brain heart infusion (BHI) broth (Becton Dickinson and Company; Sparks, MD, USA) supplemented with 0.1% L-cysteine at 37°C under anaerobic conditions.

DNA manipulations

The total DNA of *C. innocuum* was extracted using the phenol/chloroform method [14]. Briefly, *C. innocuum* pellets (after 12–16 h incubation) were collected (2850 ×*g* at 4°C) and washed with ddH_2O twice. The bacterial pellets were treated with lysozyme (0.8 mg at 37°C for 30 min), protease K (0.1 mg at 56°C for 30 min), and RNase A (0.1 mg at 37°C for 30 min). Bacterial DNAs were extracted by phenol/chloroform (1:1 mix ratio by volume) and precipitated with isopropanol (1:2 mix ratio by volume) at room temperature. The extracted DNAs were air dried, dissolved in ddH_2O and adjusted to a concentration of 100 ng/μL for PCR amplification. Primers were designed using primer3 (https://primer3.ut.ee) according to the *C. innocuum* genome deposited in NCBI [15] and are described in Table 1. The target genes were amplified by PCR in conditions 95°C 30", 57°C/60°C 30", and 72°C 30" for 35 cycles (Table 1). The PCR products used for amplicon sequencing were generated using primers with Nextra transposase adaptors (Illumina, Inc.; San Diego, CA, USA).

Amplicon sequencing

PCR products were checked by 2% agarose gel electrophoresis, mixed, and purified using the Agencourt AMPure XP PCR Purification Kit (Fisher Scientific International Inc.; Pittsburgh, PA, USA). Quantification

Table 1 Primers used for multilocus sequence typing analysis

Target gene	Primer	Sequence (5′-3′) [a]	Amplicon size (bp)	Annealing temperature
atpA	atpA-CD-F	ATTGTATCCTCCACCGCAAG	464	60°C
	atpA-CD-R	TGCTTCATTGCCTTGATCTG		
ddl	ddl-F	GGAAATGGCACATATCGTCATG	587	57°C
	ddl-R	CCTTCATCATAGTCGGATAAC		
dxr	dxr-lab-F	TATCCCAGCCATTCCTTCTG	433	60°C
	dxr-lab-R	TCATCATGGTAGCGGAATCA		
groL	groL-CS-F	GAATACGCAAGCAAGGAAGC	450	60°C
	groL-CS-R	AAAGGTACCACGCAGCTTG		
gyrA	gyrA-CS-F	AAACGGTTCTGTCGGAATTG	491	60°C
	gyrA-CS-R	CACCGAAAGAGGTCTGAAGC		
gyrB	gyrB-F	AGTGCAAAGCAGGGAAGAAA	387	60°C
	gyrB-R	ACCCATAGAAGCACGTACCG		
mdh	mdh-CB-F	AATGTCGTCGGACAGGAAAC	482	60°C
	mdh-CB-R	TAGGTGTTCGGCAGGGTATC		
pgk	pgk-F	GGTGACTTTCGTTCCGGTTA	383	60°C
	pgk-R	CCTTGTCAGCCTTTTTCAGC		
recA	recA-lab-F	AGAGCAGGCGCTGGATATTA	457	60°C
	recA-lab-R	CAGCTCTGCCACATACGAGA		
rpoB	rpoB-F	TCACGGTAACAAGGGTGTCA	388	60°C
	rpoB-R	AGAGTATGGTCCGGTTGCAC		

[a], Nextera transposase adapters, Read1 (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and Read2 (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG); Index for PCR primers, Read 1 (5′ CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG) and Read 2 (5′ AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC) according to the Illumina Adapter Sequences manual

was performed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) according to the manufacturer's instructions. The final purified libraries were applied for cluster generation and sequencing on MiSeq using the V3 600 cycles kit.

Alignment and identify variations of amplicons

The FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) was utilized to process the raw read data files. BWA alignment tools were utilized to align the filtered reads with the reference sequences. The amplicon included 10 targets, *atpA*, *ddl*, *dxr*, *groL*, *gyrA*, *gyrB*, *mdh*, *pgk*, *recA* and *rpoB*. The nucleotide sequence of these 10 genes in CIN141 was amplified by polymerase chain reaction and determined by Sanger sequencing as the reference sequence. The alignment results of paired-end reads were categorized under two conditions: first, read 1 and read 2 were aligned on the same amplicon target, and second, the reads were exclusively aligned to a single amplicon target. Variant calling was done by Clair3 with default options. The qualified variants were defined by fitting with a sequencing depth of over 100× and an allele fraction of over 80%. For the genome sequences fetched from NCBI, the sequences of the selected housekeeping genes were obtained by aligning the fetched genome to the reference sequence by Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

The concentrated sequence of 7 selected targets from each isolate was aligned by Clustal Omega [16], and the phylogenetic distance of the input sequences was calculated by maximum likelihood estimation with the Generalized Time Reversible (GTR) model and bootstrap 1000 (Unipro UGENE v45.0) [17, 18]. The resulting phylogenetic tree was visualized using Figtree (v1.4.4) distributed with Bioconda [19].

Crystal violet biofilm assay

The overnight bacterial cultures in BHI broth supplemented with 0.1% L-cysteine were diluted 100-fold with fresh BHI broth (supplemented with 0.1% L-cysteine and 0.1 M glucose) in a 24-well polystyrene microplate (Costar 3513, Corning Life Sciences) and the biofilm matrix was determined by crystal violet staining as previously described with modifications [20]. Following a 24 h incubation at 37°C, the wells were washed with sterile water to remove non-adherent bacterial cells. The washed wells were air dried, and the biofilm matrix on the well surface was fixed with 95% ethanol at room temperature for 30 min. The biofilm matrix was stained with 0.5% crystal violet at room temperature for 30 min. After three washes with tap water, crystal violet was dissolved with 30% acetic acid and quantified on an ELISA reader at a wavelength of 562 nm (OD_{562}). The well without *C. innocuum* inoculation served as the negative control.

Two biological replicates were performed for biofilm production activity.

Biofilm inhibitory concentration assay

The biofilm susceptibility assay was performed as described in previously established procedures with modifications [21]. *C. innocuum* isolates were grown in BHI broth supplemented with 0.1% L-cysteine for 24 h at 37°C. After diluting these cultures to OD_{600} of 0.1 with BHI broth (supplemented with 0.1 M glucose), 150 µL of bacterial suspension was transferred to a 96-well flat-bottom plate (Nunc™ 269787 MicroWell, Thermo Fisher Scientific Inc.; Waltham, MA, USA) and covered with a modified polystyrene microtiter lid (Nunc™ 445497 Immuno TSP Lids). After incubation, the peg lids were rinsed in sterile water three times and placed on a flat bottom 96-well plate containing 4–256 µg/mL vancomycin and incubated at 37°C for an additional 24 h. After vancomycin treatments, the peg lids were rinsed with sterile water three times and transferred to the BHI broth in the flat bottom 96-well plate. The biofilm on the peg was detached by sonication at room temperature for 5 min (DC150H, Delta Ultrasonic Co., LTD.; New Taipei City, Taiwan), and the peg lids were discarded and replaced by a standard lid. The OD_{600} was measured before and after incubation at 37°C for 24 h. Bacterial growth was defined by the difference in.

OD_{600} (OD_{600} at 24 h – OD_{600} at 0 h) ≥ 0.05. The biofilm minimal inhibitory concentration was defined as the lowest concentration of drugs that inhibited bacterial growth. Three biological replicates were performed to determin the vancomycin BIC.

Statistical analysis

Statistical analyses were performed using Prism software, version 6 (GraphPad Software, Inc.; San Diego, CA, USA). Significant differences between multiple groups were determined using ANOVA and Tukey's multiple comparisons test. Statistical significance was set at $P<0.05$.

Results

Multilocus sequence typing of *Clostridium innocuum* isolates

We selected housekeeping genes used for typing *Clostridium* and *Clostridioides* species from the PubMLST database (https://pubmlst.org/organisms). The homologous of ten house-keeping genes (*atpA*, *ddl*, *dxr*, *groL*, *gyrA*, *gyrB*, *mdh*, *pgk*, *recA*, and *rpoB*) were identified in *C. innocuum*, amplified by polymerase chain reaction, and subjected to amplicon sequencing. Simpson's index of diversity [22, 23], used to evaluate the discriminatory power of the 10 selected genes, was calculated for 52 isolates of *C. innocuum* from Chang Gung Memorial Hospital at Linkou (CGMH, Taiwan) and 60 sequence-available *C. innocuum* isolates from the National Center for Biotechnolgy Information (NCBI) Genome Database (https://www.ncbi.nlm.nih.gov/datasets/taxonomy/1522/), as shown in Table 2. Multilocus sequence typing (MLST) analyses typically use seven loci to type bacterial isolates (https://pubmlst.org). Simpson's index of diversity of *groL*, *pgk*, and *recA* from 112 isolates was less than 0.7 (CGMH+NCBI, Table 2); therefore, these genes were excluded from MLST analysis. Finally, seven genes, including *atpA*, *ddl*, *dxr*, *gyrA*, *gyrB*, *mdh*, and *rpoB*, were utilized to type *C. innocuum* isolates.

Among 52 isolates from CGMH (Taiwan) and 60 isolates from the NCBI Genome Database, 36 and 25 different sequence types (STs) were identified, respectively (Fig. 1A). Among 58 STs, ST1 (5.4%, 6/112), ST6 (11.6%, 13/112) and ST42 (5.4%, 6/112) exhibited a prevalence greater than 5%. Fifty-three STs were exclusive to isolates

Table 2 Simpson's index of the selected genes

Isolates [a]	CGMH (*n* = 52)	NCBI (*n* = 60)	CGMH + NCBI [b] (*n* = 112)
Selected genes			
atpA	0.802	0.748	0.784
ddl	0.778	0.742	0.862
dxr	0.820	0.488	0.960
groL	0.654	0.468	**0.568**
gyrA	0.870	0.648	0.794
gyrB	0.777	0.643	0.731
mdh	0.749	0.740	0.983
pgk	0.739	0.537	**0.646**
recA	0.741	0.621	**0.685**
rpoB	0.723	0.733	0.779
atpA + ddl + dxr + gyrA + gyrB + mdh + ropB	0.998	0.995	0.999

[a]: CGMH: Chang Gung Memorial Hospital at Linkou, Taiwan; NCBI: NCBI Genome Database: https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/2305/

[b]: Simpson's index value lower than 0.7 was indicated by bold text

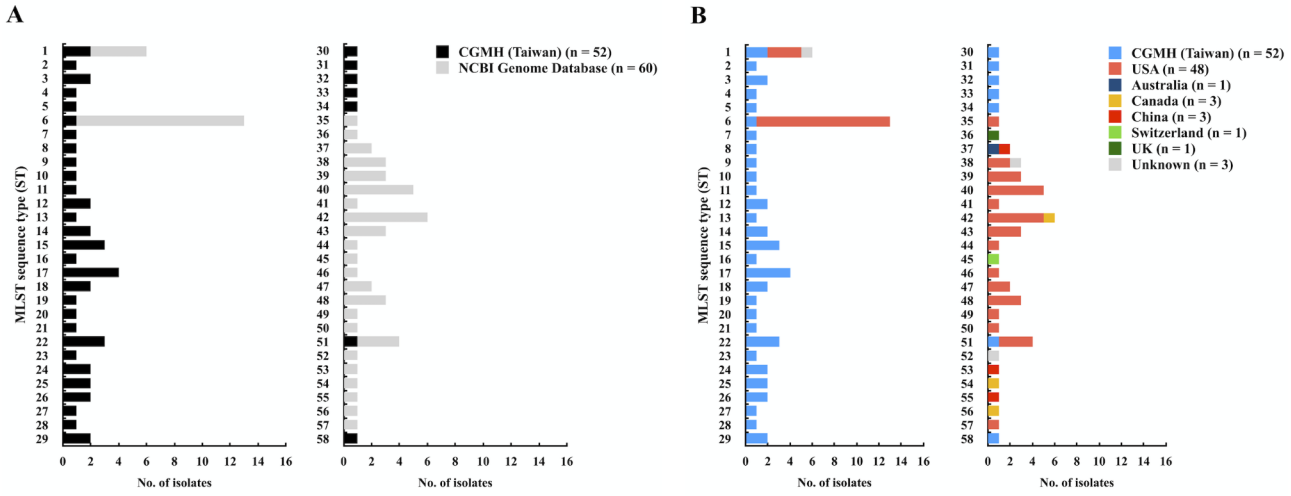

Fig. 1 Multilocus sequence typing for 52 *C. innocuum* isolates in Taiwan (CGMH) and 60 sequence-available *C. innocuum* isolates from the NCBI Genome Database. The housekeeping genes *atpA*, *ddl*, *dxr*, *gyrA*, *gyrB*, *mdh*, and *rpoB* were utilized for typing *C. innocuum* isolates. **(A)** The sequence types (STs) of *C. innocuum* from CGMH and NCBI Genome Database. **(B)** The STs of *C. innocuum* with corresponding geographic distribution information

from specific geographic regions (Fig. 1B). ST1, ST6, and ST51 were found in *C. innocuum* isolates from both CGMH and the NCBI Genome Database, representing isolates from Taiwan and the USA (Fig. 1A and B). Furthermore, ST37 was identified in *C. innocuum* isolates from Australia and China, and ST42 was identified in isolates from the USA and Canada (Fig. 1B). Noticeable, there is only one isolate from Australia, Switzerland and the UK; therefore, these results cannot represent the prevalence of STs in these geographic regions.

Phylogenetic analysis and biofilm production activity of *C. innocuum* isolates

The intestinal microbiota can form a biofilm adhering to the intestinal mucus surface under healthy conditions [11]. To evaluate the biofilm production activity of *C. innocuum*, we cultured 52 isolates from CGMH for 24 h and analyzed their biofilm production using the crystal violet staining assay. All analyzed *C. innocuum* isolates were found to be biofilm producers (OD_{562}>background; acetic acid washes from empty wells). The OD_{562} values ranged from 0.069 to 3.746, with a median value of 0.184 (Fig. 2A). We categorized *C. innocuum* isolates according to biofilm formation levels: isolates with OD_{562}<0.184 (median) exhibited low-level biofilm formation (25/52, 48%), isolates with 0.184<OD_{562}<0.276 (1.5× median) exhibited medium-level biofilm formation (9/52, 17%), isolates with 0.276<OD_{562}<0.552 (3× median) exhibited high-level biofilm formation (7/52, 14%), and isolates with OD_{562}>0.552 exhibited very high-level biofilm formation (11/52, 21%) (Fig. 2B). 52% of *C. innocuum* isolates (27/52) showed medium to very high levels of biofilm production activity. *C. innocuum* isolates with ST2, 4, 15, 18, 21, 26, 28, and 58 exhibited a very high-level biofilm mass (Fig. 2A).

To assess the relationship between the biofilm production phenotype and the genetic phylogenetics of *C. innocuum*, the concentrated sequences of the 7 selected loci were used for the phylogenetic analysis based on maximum likelihood estimation. The results showed that the 52 *C. innocuum* isolates from CGMH could be separated into phylogenetical clade I (13/52, 25%) and clade II (39/52, 75%) (Fig. 2C), with CIN117 as an outgroup isolate (Supplementary Fig. S1). Noticeably, among 11 isolates exhibiting very high-level biofilm formation, 8 isolates were classified as clade I (Fig. 2C), suggesting that the strong biofilm production phenotype was associated with a specific clade of *C. innocuum*. The phylogenetic distance of 52 isolates from CGMH and 60 isolates from the NCBI database was shown in Fig. 2D. Most analyzed isolates showed close phylogenetic distance except 4 outgroup isolates (CIN117, OF1-2LB, DFI.7.33, and DFI.1.206, Fig. 2D).

Vancomycin treatments did not activate the production of biofilm in *C. innocuum*

C. innocuum is intrinsically resistant to vancomycin [4, 5]. As vancomycin is one of the primary drugs used to treat *Clostridioides difficile* infection, it may serve as an external signal or environmental stress for *C. innocuum*. To evaluate whether vancomycin influences the biofilm production activity of *C. innocuum*, we selected isolates from clade I and clade IIa (Fig. 2C) with varying levels of biofilm production activities (Fig. 3A) for analysis. The growth of the selected isolates was inhibited by 8 μg/mL vancomycin (except for CIN152) while remaining unaffected by 2 μg/mL and 4 μg/mL vancomycin treatments

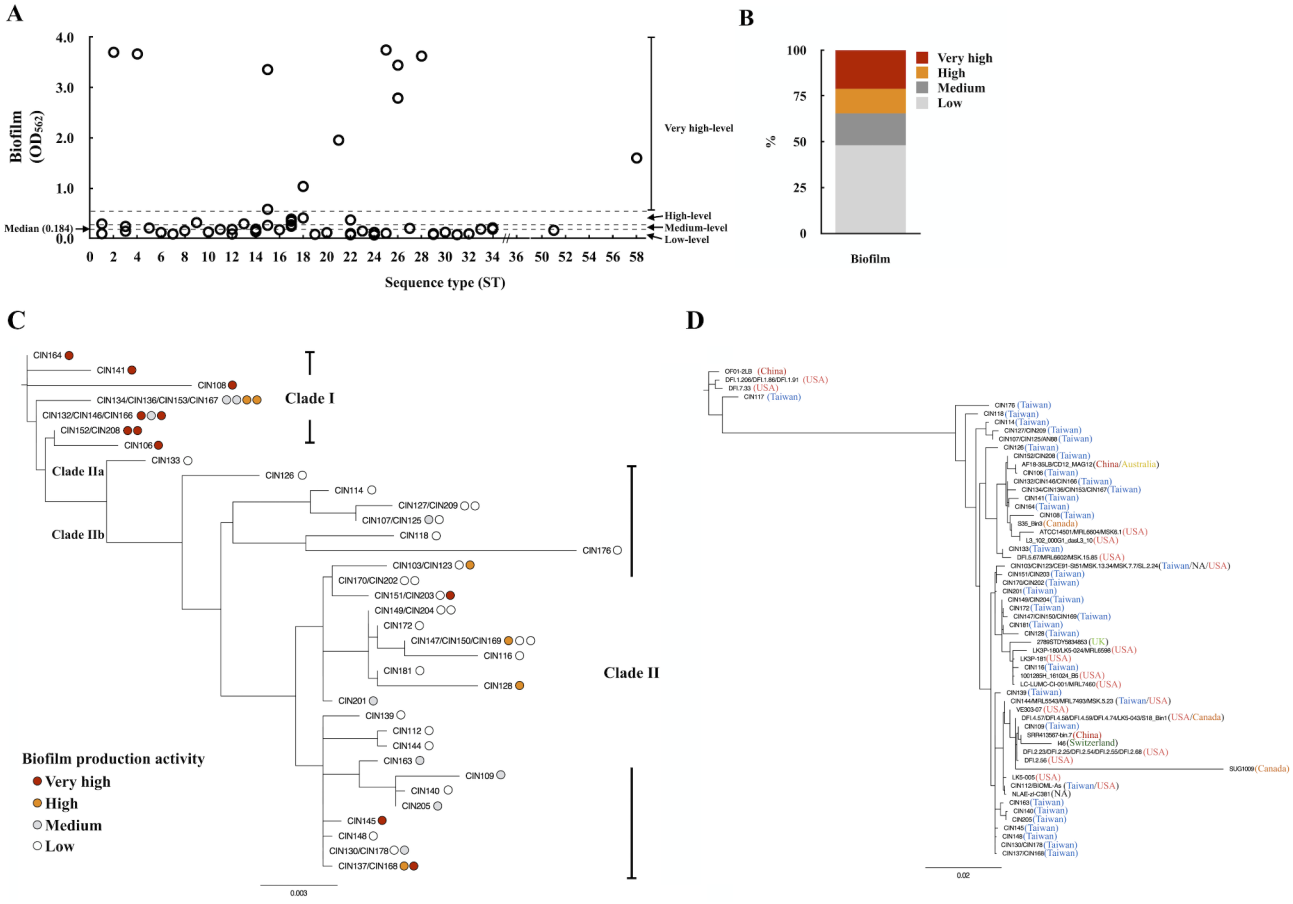

Fig. 2 The phylogenetic distance and biofilm production activity of *C. innocuum* isolates. **(A)** The sequence type (ST) and biofilm production activity of 52 *C. innocuum* isolates from the Chang Gung Memorial Hospital at Linkou (CGMH, Taiwan). **(B)** The percentage distribution of *C. innocuum* isolates exhibited low ($OD_{562} < 0.184$), medium ($0.184 < OD_{562} < 0.276$), high ($0.276 < OD_{562} < 0.552$), and very high ($OD_{562} > 0.552$) levels of biofilm. 0.184 is the median value of the biofilm mass of 52 analyzed *C. innocuum* isolates. The maximum likelihood phylogenetic tree constructed from the sequence of concentrated housekeeping genes of *C. innocuum* isolates **(C)** from the CGMH ($n = 51$; excluding the outgroup isolate CIN117) or **(D)** in both CGMH ($n = 52$) and NCBI Genome Database ($n = 60$). The scale bar represents nucleotide substitutions per site. The phylogenetical clade and biofilm production activity of *C. innocuum* isolates from CGMH are labeled in **(C)**. Two biological replicates were performed for biofilm production activity

(Fig. 3B). The crystal violet staining assay showed that the biofilm production of the selected isolates remained similar under 0 μg/mL and 4 μg/mL vancomycin culture conditions (Fig. 3C). This suggests that vancomycin does not appear to be a factor that affect the biofilm production activity of *C. innocuum* isolates.

Biofilm-embedded *C. innocuum* survived the high concentration of vancomycin treatments

The biofilm provides protection for bacteria against antibiotic elimination. Therefore, this study evaluated whether vancomycin treatments could effectively eliminate *C. innocuum* inside the biofilm. The selected isolates of clade I and IIa were cultured in the 6-well polypropylene plate to form biofilms under vancomycin-free conditions. After 24 h of incubation, planktonic cells were removed and the biofilm mass in the plate was exposed to vancomycin at concentrations ranging from 4 to 16 μg/mL for an additional 24 h. Crystal violet staining showed that only CIN152 and CIN164 exhibited a statistically significant decrease (ANOVA, $p < 0.01$) in biofilm mass under 16 μg/mL vancomycin treatments (Fig. 4A).

To further verify whether the biofilm could protect *C. innocuum* isolates from high concentrations of vancomycin treatments, and whether strong biofilm producers would be more resistant to vancomycin than weak biofilm producers, the vancomycin **b**iofilm **i**nhibitory **c**oncentration (BIC) [21] was compared for the selected *C. innocuum* isolates with different levels of biofilm production. Among the 9 isolates that exhibited low to medium levels of biofilm formation, 8 isolates had vancomycin BIC 8 μg/mL, and one isolate (CIN146) showed vancomycin BIC > 256 μg/mL (Fig. 4B). Among 8 isolates exhibiting high to very-high levels of biofilm formation, 7 isolates had vancomycin BIC > 256 μg/mL (Fig. 4B). These results suggest that strong biofilm producers of *C.*

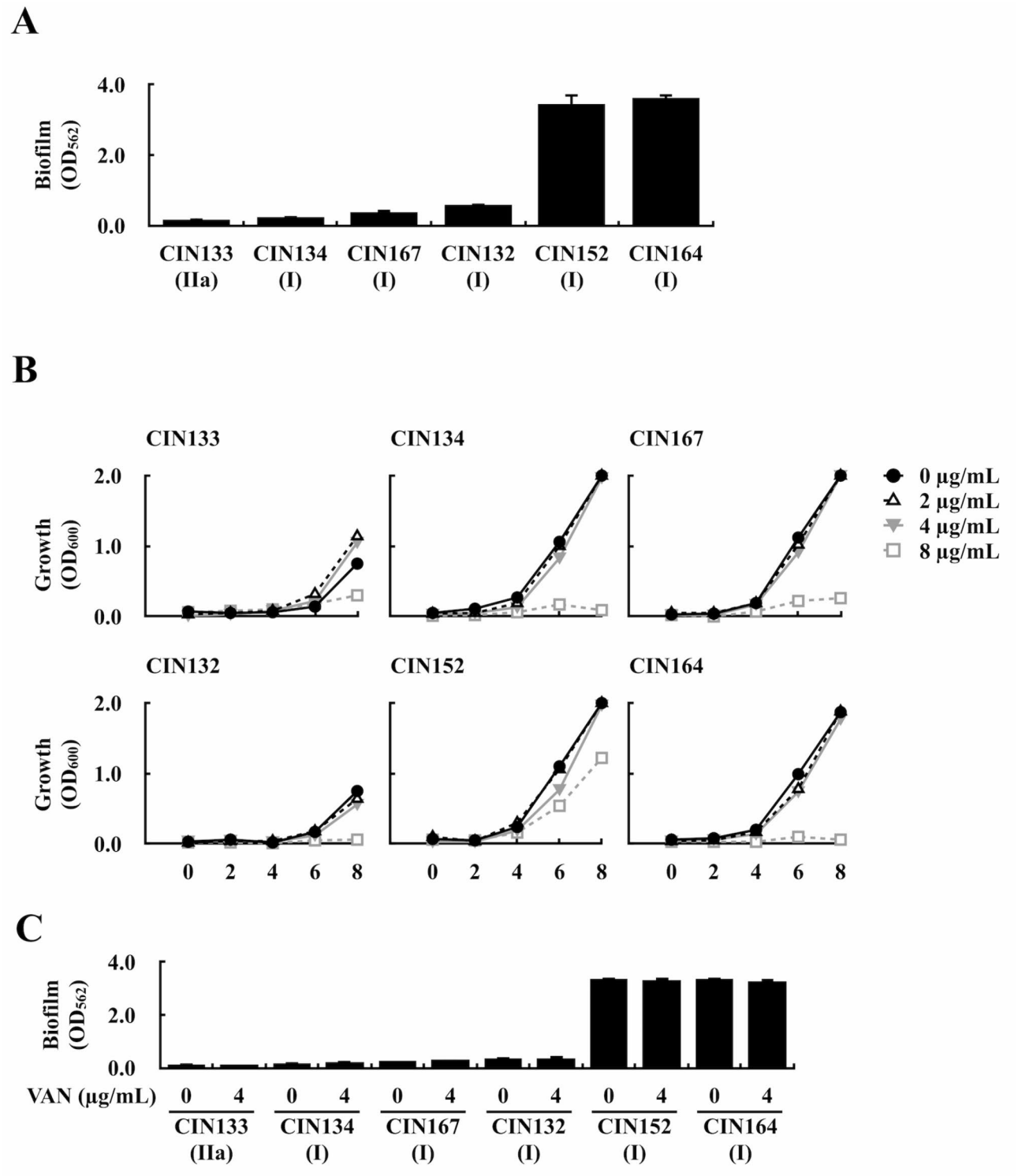

Fig. 3 The sublethal concentration of vancomycin treatments did not induce biofilm production in *C. innocuum* isolates. **(A)** Biofilm production activity of the selected clade I and IIa *C. innocuum* isolates. **(B)** The growth activity of the selected *C. innocuum* isolates under vancomycin concentrations ranged from 0 to 8 µg/mL. **(C)** Biofilm production activity of the selected *C. innocuum* isolates under 0 and 4 µg/mL vancomycin treatments. Two biological replicates were performed for growth activity and biofilm production activity

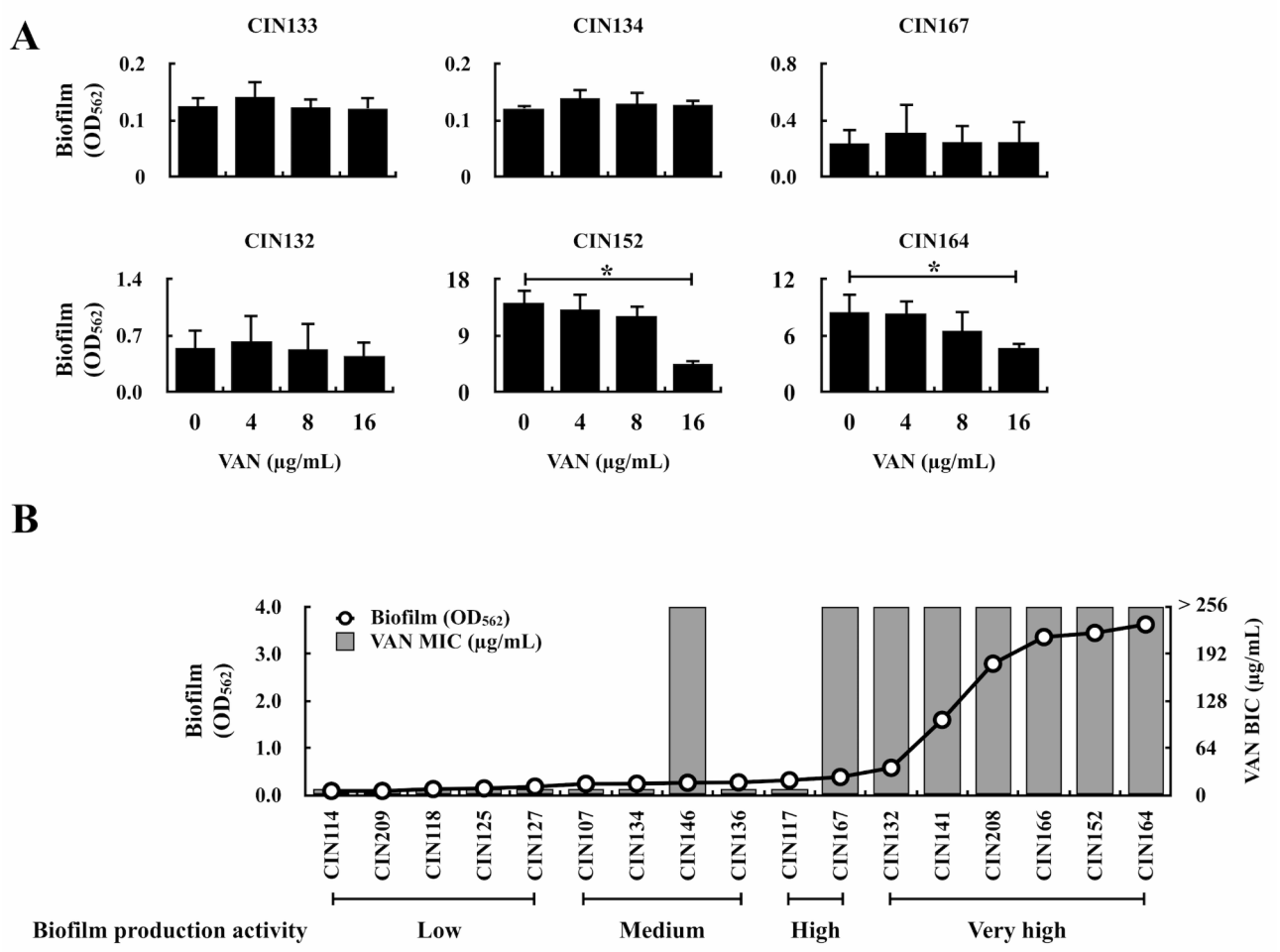

Fig. 4 Biofilm protection by *C. innocuum* isolates against high concentrations of vancomycin treatments. **(A)** Biofilm mass of the *C. innocuum* isolates after exposure to vancomycin treatments at 0, 4, 8, and 16 μg/mL. **(B)** The vancomycin biofilm inhibitory concentration (BIC) of *C. innocuum* isolates, categorized based on their levels of biofilm formation, ranging from low to very high. Three biological replicates were performed for biofilm production and vancomycin BIC. *, $P < 0.05$

innocuum can survive very high concentrations of vancomycin treatments.

Discussion

MLST and sequence-based phylogenetic analyses revealed significant genetic diversity among *C. innocuum* isolates [10]. The extensive genetic variability within the *C. innocuum* isolates suggests a possible natural existence as a commensal organism in the human intestinal tract. The capacity to form spores provides *C. innocuum* a competitive advantage in colonizing the intestine [1]. Furthermore, this study showed that the majority of analyzed *C. innocuum* isolates exhibit a remarkable ability to produce substantial amounts of biofilm. This attribute would enhance the ability of *C. innocuum* to interact with or compete with other commensal bacteria within the intestinal environment and would be related to its pathogenicity.

Biofilm is the structure produced by bacteria that plays a pivotal role in the survival of bacteria under stressful conditions and their ability to establish prolonged colonization [24]. This study showed that all analyzed *C. innocuum* isolates exhibited biofilm-producing capabilities, with 52% (27/52) of the isolates producing medium to very high-levels of biofilm. These results suggest that the biofilm production capacity is a significant and conserved trait within the evolutionary process of *C. innocuum*. In addition to serving as a physical barrier that protects bacteria from the effects of antibiotics, biofilm mass plays crucial roles in interactions among multiple bacterial species [25, 26]. Slater et al. [27] showed that, in mixed-species biofilms, *Bacteroides fragilis* could inhibit *C. difficile* growth; therefore, biofilm and bacteria inside the biofilm may have intricate functions within the intestinal niche. With its intrinsic vancomycin-resistant activity, *C. innocuum* could still produce substantial biofilm masses under 4 μg/mL vancomycin culture conditions.

Additionally, the strong biofilm production activity was related to the high vancomycin biofilm inhibitory concentration in *C. innocuum* isolates. Consequently, the biofilm produced by *C. innocuum* may serve as a protective barrier, not only for *C. innocuum* itself but also for other intestinal commensals and pathogens, such as *C. difficile*, shielding them from vancomycin-induced elimination. Although the potential for *C. innocuum* to form multi-species biofilm communities within the intestine requires further investigation, this possibility expands our understanding of the diverse roles of *C. innocuum* in intestinal and extra-intestinal infections.

Stevens et al. [28] showed that subinhibitory concentrations of the ß-lactam antibiotic nafcillin induce the transcription of toxin genes and increase toxin production in *Staphylococcus aureus*. Furthermore, Chen et al. [29] showed that vancomycin and ampicillin treatments induce α-hemolysin expression and enhance the cytotoxicity of vancomycin-resistant *S. aureus*. In *C. difficile*, Gerber et al. [30] observed that sub-MIC metronidazole and vancomycin treatments were associated with earlier toxin production and increased toxin gene transcription. These results suggest that antibiotics at subinhibitory concentrations may serve as signals to activate bacterial toxin expression. As an intrinsic vancomycin-resistant bacterium, vancomycin would not be able to eliminate *C. innocuum* but act as an external signal to alter the phenotype of *C. innocuum*. This study revealed that biofilm-embedded *C. innocuum* exhibited survival even when exposed to high concentrations of vancomycin. These further suggest that *C. innocuum* within the biofilm can adapt its phenotype in response to vancomycin stimuli.

Conclusions

The pathogenetic mechanism of *C. innocuum* remains largely unknown. The present study showed the correlation between phylogenetical position and biofilm production activity and suggests that its intrinsic resistant to and robust biofilm production activity likely play a significant role, not only in its pathogenesis, but also in its interactions with intestinal commensals and pathogens.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03503-1.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions
CCN and CHC sourced the study funding. CCN, JYH, and CYH designed the methodologies. JYH, CYH, and YCL conducted the experiments. CCN, JYH, and YCL managed the study data and performed the data analysis. YYMC, CHL, and CHC provided study materials, reagents, and instrumentation. CCN wrote the manuscript. All authors read, revised, and approved the final manuscript.

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Data availability
The data that support the findings of this study are uploaded as Supplementary materials.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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