Journal of Tropical Biodiversity and Biotechnology
Volume 09, Issue 01 (2024): jtbb87079
DOI: 10.22146/jtbb.87079

Research Article

Safety Assessment of *Bacillus subtilis* G8 Isolated from Natto for Food Application

Nathania Calista Putri[1#], Hans Victor[1#], Vivian Litanto[1], Reinhard Pinontoan[1], Juandy Jo[1,2]*
1)Department of Biology, Faculty of Science and Technology, Universitas Pelita Harapan. Jl. M.H. Thamrin Boulevard 1100, Tangerang 15811, Banten, Indonesia.
2)Mochtar Riady Institute for Nanotechnology, Jl. Boulevard Jendral Sudirman No.1688, Tangerang 15811, Banten, Indonesia.
* Corresponding author, email: juandy.jo@uph.edu
# Co-first author

Keywords:
Bacillus subtilis G8
natto
antibiotic resistance,
disk diffusion method
whole-genome sequencing.
Submitted:
20 July 2023
Accepted:
09 October 2023
Published:
02 February 2024
Editor:
Miftahul Ilmi

ABSTRACT
Various bacteria are widely used as food-fermenting agents, including *Lactobacillus*, *Bifidobacterium*, and *Bacillus*. Despite they are generally recognized as safe to be consumed by humans, those bacteria could potentially cause antibiotic resistance as they could acquire and transfer antibiotic resistance genes from or to other microbes within the human gastrointestinal tract. Profiling antibiotic resistance pattern in those bacteria is therefore important to control the spread of antibiotic resistance. In this study, antibiotic resistance profile of *Bacillus subtilis* G8 was assessed. *B. subtilis* G8 had been isolated from commercialised Japanese natto in Indonesia and had been previously reported for its fibrinolytic characteristics. The antibiotic resistance phenotype and genotype of *B. subtilis* G8 were assessed through the Kirby-Bauer disk diffusion method and whole-genome analysis, respectively. *B. subtilis G8* exhibited resistance towards Oxacillin, Lincomycin and Tiamulin-Lefamulin. The bioinformatics analysis indicated several responsible genes mediating those resistance, i.e., *ybxI* (for Oxacillin), *lmrB* (for Lincomycin) and *vmlR* (for Lincomycin and Tiamulin-Lefamulin). All identified genes were found in the chromosomal DNA. Further analysis found no mobile genetic elements within the genome, therefore reducing a risk of resistance gene transfer via plasmid and subsequently supporting safety profile of *B. subtilis* G8 in food fermentation usage.

INTRODUCTION
Fermentation converts various food components through controlled microbial growth (Marco et al. 2017). Various bacterial strains have been used to ferment food with a particular criterion of selecting the fermentation agent is the safety concern of using a particular microbial strain. The safety concern could be assessed by detecting the presence of antibiotic resistance genes within the bacteria as well as the risk of resistance transfer (Gueimonde et al. 2013).

The human gastrointestinal tract is the place where daily interaction between environmental bacteria (e.g., from food) and gut-resident indigenous bacteria occurs. Food indeed could be a source of antibiotic-resistant bacteria. The foodborne transmission of antibiotic-resistant *Escherichia coli* as a cause of urinary tract infections among females lived in Montreal, Canada was an example of it (Vincent et al. 2010). The con-

stant interaction between environmental and indigenous bacteria could facilitate the horizontal transfer of antibiotic resistance genes (Penders et al. 2013). For example, various species of *Lactobacillus* and *Bifidobacterium*, the commensal bacteria in human gastrointestinal tract that commonly used as the starter cultures of fermented food, indeed harbour several antibiotic resistance genes (Ammor et al. 2007a, 2007b). Consequently, this potential reservoir of resistance genes among human gut microbiota might transfer those genes to pathogenic bacteria (Rolain 2013).

Among bacterial species used as starters in food fermentation process, *Bacillus subtilis* is commonly found in the soy-based food, including Korean cheonggukjang and Japanese natto (Kamada et al. 2015). Of note, *Bacillus subtilis* G8 had been isolated from a commercially available Japanese natto in Indonesia (Lucy et al. 2019), in which its whole genome and fibrinolytic characteristics had been recently published (Pinontoan et al. 2021; Dikson et al. 2022). Thus, it would be interesting to assess the genotype and phenotype of antibiotic resistance of *B. subtilis* G8 as well as the presence of any transferrable genetic element within its genome to investigate the safety profile of *B. subtilis* G8 as a fermentation agent. The antibiotic testing on *B. subtilis* G8 and the identification of resistance genes within its genome were therefore performed in this study.

MATERIALS AND METHODS

Bacterial Isolate

Bacillus subtilis G8 had been isolated by the Department of Biology of Universitas Pelita Harapan from Japanese fermented soybean natto commercially available in Indonesia (Lucy et al. 2019). *B. subtilis* G8 were cultured on the nutrient agar (Merck, Germany) until further testing.

Antibiotic Susceptibility Test

The Kirby-Bauer disk diffusion method was performed based on the Clinical and Laboratory Standards Institute reference method (Jorgensen & Turnidge 2015). *B. subtilis* G8 was inoculated in the nutrient broth (Merck, Germany) and incubated at 37°C to obtain a turbidity of 0.5 McFarland. The liquid culture was taken using a sterile cotton swab and pressed against the wall of the test tube to remove excess fluid, then streaked evenly on Mueller Hinton agar media (Himedia, India). Up to 15 minutes after bacterial streaking, twenty-four antibiotic discs, including inhibitors of cell wall synthesis (Amoxicillin (2 µg), Ampicillin (10 µg), Bacitracin (10 IU), Cefoxitin (30 µg), Methicillin (5 µg), Oxacillin (1 µg) and Vancomycin (30 µg)), inhibitors of protein synthesis (Chloramphenicol (30 µg), Clindamycin (2 µg), Erythromycin (15 µg), Gentamicin (10 µg), Kanamycin (30 µg), Lefamulin (20 µg), Lincomycin (2 µg), Neomycin (30 µg), Streptomycin (10 µg), Tetracycline (30 µg), Tiamulin (30 µg) and Tylosin (30 µg)), inhibitors of nucleic acid synthesis (Ciprofloxacin (5 µg), Nalidixic Acid (30 µg), Ofloxacin (5 µg)), inhibitor of DNA-dependent RNA Polymerase (Rifampicin (5 µg)) and inhibitor of folate synthesis (Sulfonamide (300 µg)) (Liofilchem, Italy) were aseptically pressed onto the agar surface. All plates were subsequently incubated at 37°C for 24 hours in an aerobic condition. Following the incubation, a ruler was utilized to measure the diameter of the clear zone, including the diameter of respective antibiotic disc (6 mm). All antibiotic discs were tested three times.

Bioinformatic Analysis

The whole genome of *B. subtilis* G8 had been sequenced by the Novogene

Company Limited (Hong Kong) using the Illumina technology platform (USA) and its data had been recently published (Dikson et al. 2022). Briefly, the whole-genome sequence of *B. subtilis* G8 was checked for its quality by FastQC (Andrews 2010). The contig assembly was subsequently performed using SPAdes (Bankevich et al. 2012). The contig coverage was checked using Qualimap 2 (Okonechnikov et al. 2016). Outliers are searched using the formula $z = (X - \mu) / \sigma$ where X is the mean coverage, μ is the mean, and σ is the standard deviation. The value of z above 3 or below -3 was considered as an outlier and removed. The remaining contigs were re-ordered using Mauve (Darling et al. 2004) with *Bacillus subtilis* subspecies *subtilis* strain 168 (RefSeq: NC_000964.3) as the reference genome. The re-ordered contigs were merged into one FASTA sequence using Artemis (Carver et al. 2012). The complete genome was finally submitted into dFAST for annotation (Tanizawa et al. 2016, 2018). The graphical map of *B. subtilis* G8 genome was created using Proksee (Grant et al. 2023).

Antibiotic resistance genes in the genome were examined using CARD and BacAnt (Alcock et al. 2020; Hua et al. 2021). The antibiotic resistance genes were identified using the Resistance Gene Identifier (RGI) application on CARD, in which the RGI could predict the antibiotic resistome, i.e., the collection of all antibiotic resistance genes in pathogenic and non-pathogenic bacteria (Wright 2007), by using a combination of open reading frame with Prodigal (Hyatt et al. 2010), sequence alignment with BLAST (Camacho et al. 2009) or DIAMOND (Buchfink et al. 2014), as well as curated resistance mutations included with the antimicrobial resistance detection model (Alcock et al. 2020). Similarly, the BacAnt was also used to annotate antibiotic resistance genes within the genome of *B. subtilis* G8 (Hua et al. 2021). If the CARD-RGI and BacAnt results were unavailable, a literature search would be performed to identify published antibiotic resistance genes. The identified resistance gene sequences were further examined using BLAST (Camacho et al. 2009). Results from CARD and BacAnt were subsequently cross-checked with results of dFAST. Outlier contigs were examined individually using BLAST, in which contigs known to be plasmids were examined for the presence of antibiotic resistance genes using CARD and BacAnt as well. The presence of mobile genetic elements within the genome was determined with BacAnt (Hua et al. 2021).

RESULTS AND DISCUSSION

Based on the genome assembly and subsequent bioinformatic analysis, total sequence length of *B. subtilis* G8 was 4,017,503 base pairs, which was comparable to other *Bacillus subtilis* strains (Dikson et al. 2022). *B. subtilis* G8 had GC content of 43.4% and predicted CDS of 4,279, suggesting that it was similar to other *B. subtilis* strains isolated from natto (Dikson et al. 2022). A visualization of its genomic map, along with five antibiotic resistance genes found in this study, were shown in Figure 1.

Susceptibility of *B. subtilis* G8 towards 24 types of antibiotics were assessed using the Kirby-Bauer disc diffusion method. The measurement of the clear zone diameters and their interpretation were depicted in Figure 2 and Table 1. The interpretation was based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) definition on susceptibility testing categories (susceptible, standard dosing regimen [S]; susceptible, increased exposure [I] and resistance [R]), in which while a microorganism was described as susceptible if its diameter was within the category of S or I, a microorganism was identified as resistant if its result was within the category of R (2022). Of note, the interpreta-

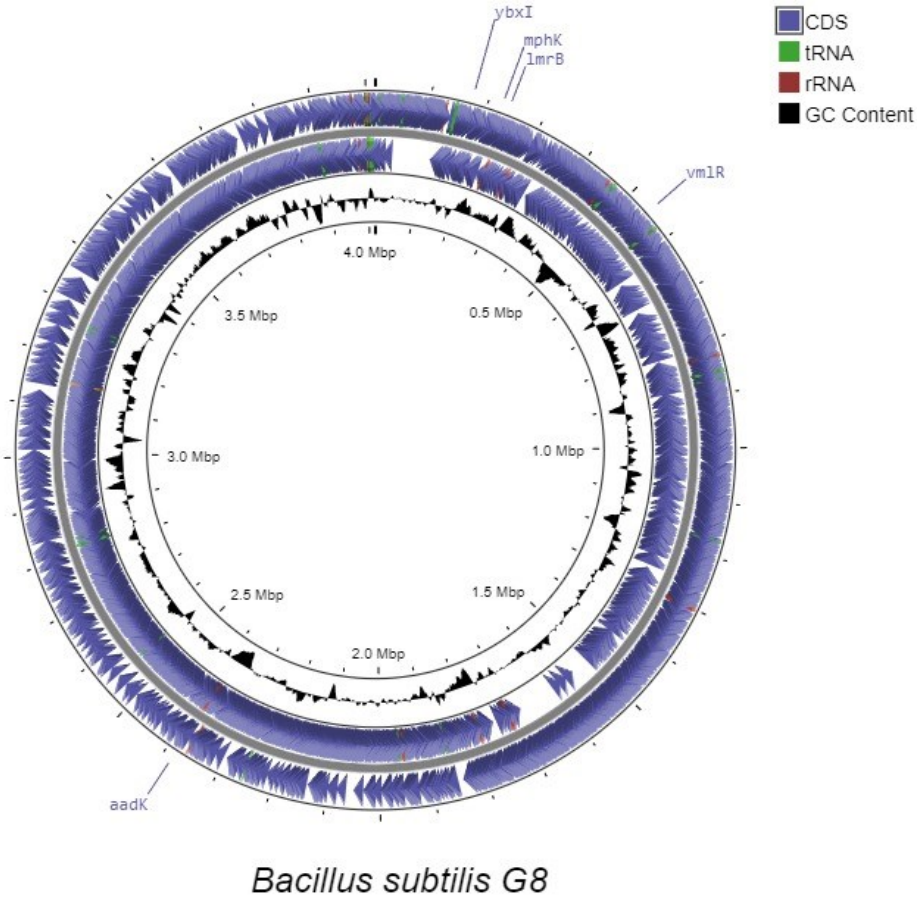

Figure 1. A visualisation of whole genome of *Bacillus subtilis* G8. The outer ring depicted location of predicted CDS and its strand, as well as the location of predicted tRNA and rRNA. The inner ring showed the GC content distribution plot. Genomic location of the identified antibiotic resistance genes was shown outside of the outer ring. The genomic map was constructed using Proksee (Grant et al. 2023).

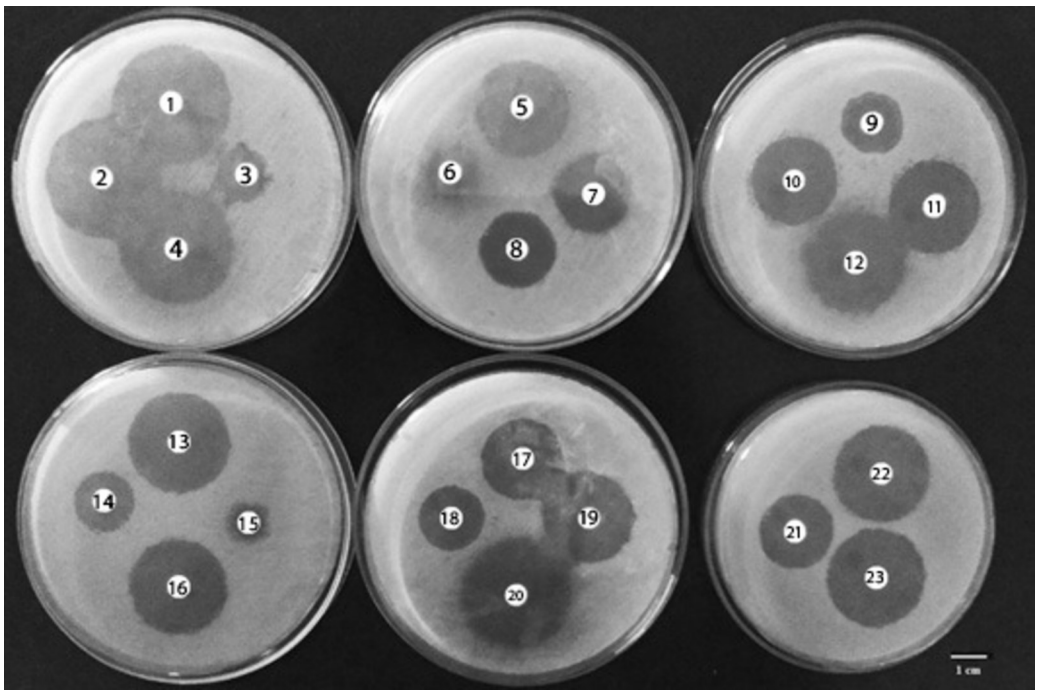

Figure 2. A representative result of the disc diffusion method on *Bacillus subtilis* G8. Each plate contained 3-4 antibiotic discs. The result was obtained after 24-hour incubation at 37°C. *(1)* Amoxicillin 2 µg; *(2)* Ampicillin 10 µg; *(3)* Bacitracin 10 IU; *(4)* Cefoxitin 30 µg; *(5)* Methicillin 5 µg; *(6)* Oxacillin 1 µg; *(7)* Vancomycin 30 µg; *(8)* Neomycin 30 µg; *(9)* Streptomycin 10 µg; *(10)* Gentamicin 10 µg; *(11)* Kanamycin 30 µg; *(12)* Tetracycline 30 µg; *(13)* Chloramphenicol 30 µg; *(14)* Clindamycin 2 µg; *(15)* Lincomycin 2 µg; *(16)* Erythromycin 15 µg; *(17)* Tylosin 30 µg; *(18)* Tiamulin 30 µg; *(19)* Sulfonamide 300 µg; *(20)* Ciprofloxacin 5 µg; *(21)* Nalidixic acid 30 µg; *(22)* Ofloxacin 5 µg; *(23)* Rifampicin 5 µg.

Table 1. Results of antibiotic disc diffusion assay on *Bacillus subtilis* G8.

Class	Antibiotic	Zone Diameter Interpretive Standard (mm)			Result (mm)
		S	I	R	*mean (min – max)*
Aminopenicillins	Amoxicillin 2 µg[B]	≥29	-	≤28	*36.0 (35-38)*
	Ampicillin 10 µg[A]	≥29	-	≤28	*34.7 (33-36)*
Polypeptide	Bacitracin 10 IU[A]	>13	-	-	*13.7 (13-15)*
Cephalosporins	Cefoxitin 30 µg[A]	≥25	-	≤24	*34.7 (32-38)*
Penicillinase-resistant penicillins	Methicillin 5 µg[C]	≥22	-	≤21	*29.3 (27-31)*
	Oxacillin 1 µg[A]	≥18	-	≤17	*12.0 (10-13)*
Glycopeptides	Vancomycin 30 µg[A]	≥17	15-16	≤14	*21.0 (20-22)*
Aminoglycosides	Neomycin 30 µg[A]	≥16	14-15	≤13	*21.3 (21-22)*
	Streptomycin 10 µg[A]	≥15	12-14	≤11	*15.0 (13-17)*
	Gentamicin 10 µg[A]	≥15	13-14	≤12	*24.7 (24-25)*
	Kanamycin 30 µg[A]	≥18	14-17	<13	*27.0 (26-28)*
Tetracyclines	Tetracycline 30 µg[A]	≥19	15-18	≤14	*28.0 (26-30)*
Phenicols	Chloramphenicol 30 µg[A]	≥18	13-17	≤12	*29.3 (26-31)*
Lincosamides	Clindamycin 2 µg[A]	≥21	15-20	≤14	*19.7 (16-22)*
	Lincomycin 2 µg[D]	≥21	-	≤14	*13.3 (8-17)*
Macrolides	Erythromycin 15 µg[A]	≥23	14-22	<13	*27.7 (27-28)*
	Tylosin 30 µg[E]	≥23	14-22	<13	*24.3 (24-25)*
Pleuromutilins	Tiamulin 30 µg[F]	≥23	-	-	*16.0 (15-17)*
	Lefamulin 20 µg[A]	≥23	-	-	*19.3 (19-20)*
Folate antagonists	Sulfonamide 300 µg[A]	≥17	13-16	≤12	*27.0 (26-28)*
Quinolones	Ciprofloxacin 5 µg[A]	≥21	16-20	≤15	*31.3 (31-32)*
	Nalidixic acid 30 µg[A]	≥19	14-18	≤13	*24.0 (21-26)*
	Ofloxacin 5 µg[A]	≥18	15-17	≤14	*29.7 (28-31)*
Ansamycins	Rifampicin 5 µg[A]	≥20	17-19	≤16	*30.3 (29-31)*

[A]The clear zone was primarily interpreted based on the CLSI (2021) standard against *Staphylococcus* spp.
[B]The standard for Amoxicillin 2 µg was unavailable, hence the standard for Ampicillin 10 µg was adopted.
[C]The standard for Methicillin 5 µg was unavailable, hence the standard for Oxacillin 1 µg was adopted.
[D]The standard for Lincomycin 2 µg was adopted from (Chukiatsiri et al. 2012).
[E]The standard for Tylosin 30 µg was unavailable, hence the standard for Erythromycin 15 µg was adopted.
[F]The standard for Tiamulin 30 µg was unavailable, hence the standard for Lefamulin 20 µg was adopted.
The measurement results included the diameter of the antibiotic disc (6 mm). *S*, susceptible, standard dosing regimen; *I*, susceptible, increased exposure; *R*, resistance; -. no available data. Results from triplicate experiments were presented as mean (minimum to maximum).

tive criteria was mainly adopted from those for *Staphylococcus* species (Clinical and Laboratory Standards Institute (CLSI) 2021).

Overall, *B. subtilis* G8 was described to be susceptible to most tested antibiotic discs (⌈S⌉=19 antibiotics and ⌈I⌉=1 antibiotic). These predominant phenotypes of antibiotic susceptibility were observed as well in *B. subtilis* HTI-23 isolated from stingless bees (Amin et al. 2020) and other strains of *B. subtilis* isolated from a hospital in Iraq (Yassin & Ahmad 2012). The isolate of *B. subtilis* G8 was observed to be resistant, however, towards Tiamulin (30 µg), Oxacillin (1 µg) and Lincomycin (2 µg) discs. The variation in clear zone diameters upon Tiamulin (30 µg) or Oxacillin (1 µg) treatment were very small, suggesting a clear resistance phenotype of *B. subtilis* G8 towards both antibiotics. First, the finding of Tiamulin resistance phenotype was in accordance with a published study, reporting that due to the presence of VmlR, an ATP-binding cassette protein of the F type, *B. subtilis* was resistant towards Tiamulin (Crowe-McAuliffe et al. 2018). Tiamulin belongs to the class of pleuromutilins, a natural antibiotic produced by *Pleurotus mutilus* (now known as *Clitopilus scyphoides*), which functions as the protein synthesis inhibitor through

rRNA binding in the ribosome (Islam et al. 2009). It is commonly used in veterinary medicine, hence prompting a question on why *B. subtilis* G8, obtained from human food, developed resistance towards Tiamulin. It is unknown on whether this resistance phenotype was recently acquired. However, it has been reported that the administration of antibiotics to animals could select the antibiotic resistance bacteria, which subsequently could transfer their antibiotic resistance genes to other bacteria (Rolain 2013). The resistance of *B. subtilis* G8 towards Lefamulin, the pleuromutilin used in humans (Paukner & Riedl 2017), was subsequently tested. Indeed, *B. subtilis* G8 was also resistant towards Lefamulin (Table 1), suggesting a similar resistance mechanism for various members of pleuromutilin existed in this bacterial strain.

Second, the finding of Oxacillin resistance phenotype in *B. subtilis* G8 was supported by another study, reporting that the tested strains of *B. subtilis* were highly resistant towards Oxacillin (Irkitova et al. 2019). Of note, Oxacillin and Cefoxitin are used to identify Methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most common antibiotic-resistant pathogenic bacteria (Broekema et al. 2009; Larsen et al. 2022). Interestingly, *B. subtilis* G8 was observed to be sensitive towards Cefoxitin 30 µg and Methicillin 5 µg in this study, suggesting that *B. subtilis* G8 had a unique mechanism that mediated resistance only towards Oxacillin and were likely not having the *mecA* gene that conferred resistance to all beta-lactams in MRSA (Broekema et al. 2009; Ramandinianto et al. 2020).

Third, the variation in clear zone diameter upon Lincomycin (2 µg) treatment was relatively high, in which the individual clear zone diameter was 15 mm, 17 mm and 8 mm obtained from the first, second and third measurement, respectively (individual data not shown). Hence, the Lincomycin resistance phenotype of *B. subtilis* G8 should be interpreted cautiously. Lincomycin acts as a protein synthesis inhibitor that binds to the 50s ribosomal subunit, in which the presence of VmlR protein in *B. subtilis* could confer the resistance phenotype to Lincomycin (Crowe-McAuliffe et al. 2018). This finding was in contrast, however, to published results on *B. subtilis* isolated from food wastes (DET6) and from the soil (BYS2, BQ3, BD17, BG5 and BGY12), which reporting those strains were sensitive to Lincomycin (Patel et al. 2009; Guo et al. 2017). This discrepancy could be due to variation among *B. subtilis* strains as well as differences in the concentration of antibiotic disks and the reference standard used in those previous studies.

A subsequent bioinformatic analysis was performed to correlate the antibiotic resistance phenotypes of *B. subtilis* G8 with its putative genes that could mediate resistance to Oxacillin, Lincomycin and Tiamulin-Lefamulin. As shown in Table 2, three antimicrobial resistance genes of *B. subtilis* G8 were identified *in silico* to mediate resistance towards Oxacillin (*ybxI* gene), Lincomycin (*lmrB* and *vmlR* genes) and Tiamulin-Lefamulin (*vmlR* gene). Of note, all identified antibiotic resistance genes were found in the chromosomal DNA of *B. subtilis* G8. The *ybxI* gene encodes a class D β-lactamase, also known as oxacillinase or OXA-type β -lactamase (Antunes & Fisher 2014), with low activity (Colombo et al. 2004). The *ybxI* gene was suspected to mediate *B. subtilis* G8 resistance to Oxacillin. However, it had been reported that the *ybxI* gene did not encode true β-lactamases, as it did not hydrolyze D-alanyl-D-alanine peptidase (Colombo et al. 2004). Instead, the *ybxI* gene was assumed to encode Penicillin-binding protein (PBP) with low β-lactamase activity. The product of the *ybxI* gene were estimated belonging to the Penicillin-recognition enzyme family, but with an intermediate activity between

Table 2. Antimicrobial resistance genes of *Bacillus subtilis* G8 towards Oxacillin, Lincomycin and Tiamulin.

Gene	AMR gene family	Antibiotic class	Resistance mechanism
ybxI	*Class D β -lactamases*	Penicillinase-resistant penicillins	putative *β*-lactamase
lmrB	*ATP-binding cassette* (ABC) *antibiotic efflux pump*	Lincosamides	Antibiotic efflux
vmlR	*ABC-F ATP-binding cassette ribosomal protection protein*	Macrolides, Lincosamides, Streptogramin, Tetracyclines, Oxazolidinone, Phenicols, Pleuromutilins	Antibiotic target protection

AMR, antimicrobial resistance.

PBP and β-lactamase (Colombo et al. 2004). Thus, it was elusive yet whether the resistance to Oxacillin was mediated by the *ybxI* gene.

Next, the *lmrB* gene is a part of the *lmrAB* operon found in *B. subtilis* genome, in which the *lmrB* gene is a drug efflux transporter, while the *lmrA* gene is a suppressor of its own operon. As *lmrA* suppresses expression of *lmrB*. mutations within the *lmrA* gene causes the *lmrB* gene to be expressed, hence conferring a Lincomycin-resistant phenotype (Yoshida et al. 2004). It is unknown yet, however, whether *B. subtilis* G8 genome had a mutated *lmrA* gene or defect in its expression. Finally, the *vmlR* gene in *B. subtilis* genome had been described to encode a ribosomal protective protein that conferred resistance to Virginiamycin M, Lincomycin and Tiamulin, but not to Chloramphenicol, Linezolid and Erythromycin (Crowe-McAuliffe et al. 2018). The presence of *vmlR* gene in *B. subtilis* G8 genome could therefore mediate resistance towards Lincomycin and Tiamulin-Lefamulin.

Intriguingly, both BacAnt and CARD identified genes within genome of *B. subtilis* G8 that could induce resistance towards Macrolides (*mphK*) and Streptomycin (*aadK*) (Table 3), although *B. subtilis* G8 was observed to be sensitive against discs of Erythromycin, Tylosin and Streptomycin (Table 1). The *mphK* gene observed within *B. subtilis* G8 genome encodes macrolide phosphotransferase (mph). The enzyme mph inactivates macrolides by phosphorylating 2'-OH on essential dimethylamino sugars, thereby preventing macrolides from binding to bacterial ribosomes (Pawlowski et al. 2018). The mphK is a part of the mph enzyme family that targets Erythromycin, although the antibiotic disc diffusion assay in this study demonstrated that *B. subtilis* G8 was sensitive to Erythromycin. The mphK phosphorylated erythromycin poorly resulting in a sensitive phenotype (Pawlowski et al. 2018), which could explain the Erythromycin-sensitive phenotype in *B. subtilis* G8.

The *aadK* gene, originally found within *B. subtilis* 168 genome, contributes to a low-grade resistance to Streptomycin (Noguchi et al. 1993). The *aadK* gene encodes an aminoglycoside 6-adenylyltransferase or AAD (6), which is capable of inactivating Streptomycin through adenylation of the C-6 position on streptomycin (Noguchi et al. 1993). Based on the result of antibiotic disc diffusion assay, *B. subtilis* G8 was considered as sen-

Table 3. Putative antimicrobial resistance genes of *Bacillus subtilis* G8 detected by CARD and BacAnt.

Gene	AMR gene family	Antibiotic class	Resistance mechanism
mphK	*Macrolide phosphotransferase* (mph)	Macrolide	Antibiotic inactivation
aadK	*6-adenyltransferase* (AAD(6))	Aminoglycoside	Antibiotic inactivation

AMR, antimicrobial resistance.

sitive to Streptomycin (Table 1). However, this interpretation was based on the standard against *Staphylococcus* species (Clinical and Laboratory Standards Institute (CLSI) 2021), not against *Bacillus* species. It would be of interest to further investigate Streptomycin resistance phenotype and genotype of *B. subtilis* G8.

Considering the risk of antibiotic resistance gene transfer via mobile genetic elements, genome of *B. subtilis* G8 was also analysed using BacAnt to identify the presence of transposons and integrons. None of the transferrable genetic element was found with BacAnt in the genome of *B. subtilis* G8.

CONCLUSION

The isolate of *B. subtilis G8* exhibited resistance phenotype towards Oxacillin, Lincomycin and Tiamulin-Lefamulin. The antibiotic susceptibility's results were corroborated by the presence of respective antibiotic resistance genes within *B. subtilis* G8 genome, comprising *ybxI*, *lmrB* and *vmlR*. As those genes were present in its chromosomal genome and there were no transferrable genetic elements found, it is unlikely that *B. subtilis* G8 could disseminate those resistance genes via plasmid, supporting its safety to be used as the fermentation agent.

AUTHORS CONTRIBUTION

N.C.P. and H.V. contributed equally. N.C.P. and V.L. performed the experiments and collected the data. N.C.P., H.V. and J.V. analysed dan interpreted the data. N.C.P. and J.J. drafted the article. H.V., V.L., R.P. and J.J. critically reviewed the article. All authors approved the final version.

ACKNOWLEDGMENTS

This work was supported by the Directorate of Higher Education, Indonesian Ministry of Education, Culture, Research and Technology [No. 1218/LL3/PG/2021, 2021]. The authors thank Elvina, S.Si. and Dikson, S.Si., for their technical supports.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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