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Antimicrobial Susceptibility of *Bacillus* Strains Isolated from Primary Starters for African Traditional Bread Production and Characterization of the Bacitracin Operon and Bacitracin Biosynthesis

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***Bacillus* spp. are widely used as feed additives and probiotics. However, there is limited information on their resistance to various antibiotics, and there is a growing concern over the transfer of antibiotic resistance genes. The MIC for 8 antibiotics was determined for 85 *Bacillus* species strains, *Bacillus subtilis* subsp. *subtilis* ($n = 29$), *Bacillus licheniformis* ($n = 38$), and *Bacillus sonorensis* ($n = 18$), all of which were isolated from starters for Sudanese bread production. All the strains were sensitive to tetracycline (8.0 mg/liter), vancomycin (4.0 mg/liter), and gentamicin (4.0 mg/liter) but resistant to streptomycin. Sensitivity to clindamycin, chloramphenicol, and kanamycin was species specific. The erythromycin resistance genes *ermD* and *ermK* were detected by PCR in all of the erythromycin-resistant (MIC, ≥16.0 mg/liter) *B. licheniformis* strains and one erythromycin-sensitive (MIC, 4.0 mg/liter) *B. licheniformis* strain. Several amino acid changes were present in the translated *ermD* and *ermK* nucleotide sequences of the erythromycin-sensitive strain, which could indicate ErmD and ErmK protein functionalities different from those of the resistance strains. The *ermD* and *ermK* genes were localized on an 11.4-kbp plasmid. All of the *B. sonorensis* strains harbored the bacitracin synthetase gene, *bacA*, and the transporter gene *bcrA*, which correlated with their observed resistance to bacitracin. Bacitracin was produced by all the investigated species strains (28%), as determined by ultra-high-definition quadrupole time-of-flight liquid chromatography-mass spectrometry (UHD-QTOF LC/MS). The present study has revealed species-specific variations in the antimicrobial susceptibilities of *Bacillus* spp. and provides new information on MIC values, as well as the occurrence of resistance genes in *Bacillus* spp., including the newly described species *B. sonorensis*.**

Bacillus spp. are used for a range of different biotechnological applications, such as probiotic dietary supplements for humans and animal feed inoculants, due to their ability to stimulate the immune system (14, 15, 26) and produce antimicrobial compounds inhibitory to pathogenic microorganisms (3, 10, 32). There is, however, a growing public health concern about the possibility of microbial cultures used as dietary supplements or for food production being potential sources for the transfer of antibiotic resistance genes (19, 57). It has been speculated that commensal microorganisms can act as reservoirs of resistance genes (2, 33, 49). This concern is underlined by the fact that *Bacillus* spp. in a number of commercially available probiotic feed supplements for both humans and animals have been shown to be resistant to several antibiotics, such as chloramphenicol, tetracycline, erythromycin, lincomycin, penicillin, and streptomycin (8, 22, 25, 36).

Microbial resistance to antimicrobial agents is due to either (i) intrinsic properties (natural phenotypic traits) or (ii) the acquisition of resistance genes through mobile genetic elements, such as plasmids and transposons, or the mutation of indigenous genes (16, 24). Such acquired or intrinsic properties could make the bacteria capable of rapid inactivation of specific antibiotics through degradation, exportation of the antibiotics out of the cell through the efflux system, or alteration of the antibiotic target site (6, 16, 24, 47). Until now, most studies on bacterial antibiotic resistance have focused mainly on clinically relevant isolates (pathogenic microorganisms) and lactic acid bacteria. Very limited information on the antimicrobial susceptibility profiles of *Bacillus* spp. is available. Even less information is available on the antimicrobial susceptibility profiles of *Bacillus* spp. isolated from African fermented foods. In a study on the antibiotic susceptibility of *Bacillus* spp. isolated from broiler breeds, it was observed that two of the isolated strains, *Bacillus licheniformis* 56 and *Bacillus clausii* 259, were resistant to erythromycin and lincomycin (3). Mazza et al. (36) also observed that *Bacillus subtilis* in the commercially available probiotic product Enterogermina, later reclassified as *B. clausii* by Senesi et al. (51), was resistant to chloramphenicol, tetracycline, rifampin, and streptomycin.

Erythromycin resistance genes conferring resistance through the methylation of the 23S rRNA macrolide binding sites have been reported in *Bacillus* spp., among others, including *B. licheniformis*, *B. subtilis* (*ermD*, *ermK*), *Bacillus anthracis* (*ermJ*), and *B. clausii* (*erm34*) (5, 23, 29, 30). The *ermD*, *ermK*, and *ermJ* genes identified in the different *Bacillus* spp. show remarkably similar amino acid and DNA sequence homologies (97% to 99%) (47).

Bacitracin is a branched cyclic dodecylpeptide antibiotic that is nonribosomally produced by *B. licheniformis* and *B. subtilis* strains (37, 38, 40, 43). In *B. licheniformis*, the biosynthetic operon has been reported to comprise three genes: *bacA*, *bacB*, and *bacC* (37). The ABC transporter gene cluster, *bcrA*, *bcrB*, and *bcrC*, is located in the downstream region of the *bacABC* operon. In between the

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bacABC operon and the transporter genes are the two-component regulatory system genes *bacR* and *bacS* (37). More recently, Bernard et al. (4) reported that a gene with homology to *bcrC* encodes a protein with undecaprenyl pyrophosphate phosphatase activity involved in bacitracin resistance in *B. subtilis*. Bacitracin resistance and the presence of the bacitracin biosynthesis operon have been associated with erythromycin resistance in several studies (28, 36, 41); Ishihara et al. (28), for instance, observed that *B. licheniformis* strains carrying the bacitracin biosynthesis operon genes (*bac*) were also resistant to erythromycin. Furthermore, the upregulation of the undecaprenyl disphosphatase (*uppP*) gene has been associated with increased bacitracin resistance in *Escherichia coli* (21). Bacitracin was used in the past to promote the growth of animals and also prominently used as human medicine in most tropical countries (6, 11, 40, 55).

The aim of the present study was to determine the antimicrobial susceptibility profiles and to characterize bacitracin operon genes and bacitracin biosynthesis in *Bacillus* species strains isolated from African fermented food. Erythromycin resistance determinant genes were analyzed further in order to investigate any link between the presence of the bacitracin synthetase genes and erythromycin resistance among selected *Bacillus* species strains.

MATERIALS AND METHODS

Microorganisms. A total of 85 *Bacillus* species isolates comprising 38 *B. licheniformis*, 29 *B. subtilis*, and 18 *Bacillus sonorensis* strains were investigated. They were previously isolated from milk- and legume-based primary starters used for Gergoush production in Khartoum, Sudan (54). The isolates were grown aerobically in brain heart infusion (BHI) broth at 37°C with shaking (225 rpm). All the strains were maintained in BHI medium containing 20% glycerol at −80°C. The reference strains used in this study are listed in Table 1.

Genomic DNA extraction. Genomic DNA was extracted from a single colony of each strain grown on BHI agar (Sigma-Aldrich, St. Louis, MO) for 24 h at 30°C using the InstaGene extraction kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the instructions of the manufacturer.

Identification of *B. subtilis* strains to subspecies level based on DNA gyrase subunit A gene (*gyrA*) sequencing. The *B. subtilis* strains were identified to the subspecies level by *gyrA* gene sequencing (7). PCR amplification and sequencing were performed as described previously by Roberts and Cohan (48). The purification and sequencing of the PCR-amplified products were performed by a commercial sequencing service provider (Macrogen, Netherlands). The sequences were analyzed further using the CLC Main Workbench v.5 software (CLC, Aarhus, Denmark) and then aligned with publicly available sequences in the GenBank database (1).

Assessments of MICs. The following 8 antibiotics obtained in powdered form from Sigma-Aldrich (St. Louis, MO) were used: chloramphenicol (catalog no. C0378), clindamycin (C5269), erythromycin (E6376), gentamicin (48760), kanamycin (K4000), streptomycin (S6501), and tetracycline (T7660) as inhibitors of protein synthesis and vancomycin (V2002) as an inhibitor of cell wall synthesis. All the selected antibiotics are on the list of those recommended by the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (16), against which microbial strains intended for human use as live culture or probiotic and animal feed additives should be tested. Stock solutions (2.0 g/liter) of the antibiotics were prepared in the appropriate diluents and filter sterilized (0.22-μm Millex-GP syringe-driven filter unit; Millipore, Ireland). The aliquots (1 ml) of the stock solutions were stored at −20°C until use (not exceeding a 4-week storage period). The MICs of antibiotics (mg/liter) for all the bacteria were determined by a modification of the broth microdilution method (13, 35) with different antibiotic concentration ranges depending on the particular antibiotic. In summary, an appropriate amount of the antibiotic stock solution (2.0 g/liter) was added to BHI broth (pH 7.4) followed by a $\log_2$ serial dilution in the same medium until the desired concentrations were obtained. The media (198 μl) were dispensed into the wells of sterile flat-bottom microtiter plates with lids (Fisher Scientific, Biotech Line A/S, Denmark) and stored at −20°C overnight (12 to 18 h). Prior to inoculation, the media were allowed to thaw and attain room temperature and then were inoculated with 2.0 μl of overnight culture of each bacterium to obtain approximately 3×10^6 CFU/ml in a 200-μl total volume. The plates were incubated under aerobic conditions at 37°C for 18 h. The MIC was defined as the lowest concentration of antibiotic which gives a complete inhibition of visible growth in comparison with inoculated and uninoculated antibiotic-free wells. All MIC experiments were performed in duplicate on the same day. The breakpoint values used for categorizing the microorganisms as resistant were 4.0 mg/liter (for clindamycin, erythromycin, gentamicin, and vancomycin) and 8.0 mg/liter (for chloramphenicol, kanamycin, streptomycin, and tetracycline) (16).

TABLE 1 Origin and description of reference strains used in this study

Organism	Source[a]	Reference
Bacitracin resistance control *Bacillus licheniformis* strains (*bac* gene status)		
5A24 (ATCC 11946) (+)	BGSC	28
5A1 (ATCC 8480) (−)	BGSC	28
5A36 (DSM 13^T/ATCC 14580) (−)	BGSC	28
DSM 603 (ATCC 10716) (+)	DSMZ	28
Type strains		
B. subtilis subsp. *subtilis* DSM 10^T	DSMZ	
B. subtilis subsp. *spizizenii* DSM 15029^T	DSMZ	
B. licheniformis DSM 13^T	DSMZ	
B. sonorensis DSM 13779^T	DSMZ	
Erythromycin resistance control strains (*erm* gene type)		
Staphylococcus aureus 1206 Tn*554* (*ermA*)	NFI	47
Enterococcus faecalis JH2-2 Tn*1545* (*ermB*)	NFI	47
Bacillus subtilis pEC 1001 (*ermD* and *ermK*)	NFI	47

[a] BGSC, *Bacillus* Genetic Stock Centre; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; NFI, B. Jensen of National Food Institute, Unit for Antimicrobial Resistance, DTU, Denmark.

Assessment of bacitracin resistance profiles among the *Bacillus* species strains. The bacitracin MIC was determined for all 85 strains in addition to the type strains of *B. licheniformis*, *B. subtilis*, and *B. sonorensis* (Table 1). Bacitracin was obtained from Sigma-Aldrich (product code 11702; potency, ≥60,000 U/g), and the MIC studies were performed by the broth microdilution method as described in "Assessments of MICs." The bacitracin MIC experiment was performed in two independent experiments with BHI broth (MIC, 1.92 to 30.72 U/ml [32 to 512 mg/liter]) and Mueller-Hinton broth (MHB) (MIC, 16 to 256 mg/liter). In both cases the media were supplemented with $ZnSO_4$ (40 mg/liter). Since no breakpoint value is available for bacitracin, the definition of resistance was based upon the MIC value at which a known susceptible strain was completely inhibited, and any strain with a MIC value above that was categorized as resistant.

Molecular detection of erythromycin resistance determinants. All of the *B. licheniformis* strains were tested for the presence of the erythromycin resistance genes *ermA*, *ermB*, *ermC*, *ermD* and *ermK*, and *ermT* and the macrolide efflux genes *msrA* and *msrB*. The positive control strains for the

TABLE 2 Primers used for detection of erythromycin resistance, bacitracin synthetase, bacitracin transporter, *uppP*, and *gyrA* genes

Primer pair	Primer sequence (5′ → 3′)	Gene(s)	Amplicon size (bp)	Annealing temperature (°C)	Source/reference
bacA	Fwd_GGCATCCGATTCTCCGCACGATCG Rev_CCCCGTCATAATGTCTGTGTTCC	*bacA*	825	61	This study
bacB	Fwd_GCGGCGCCGTCTAAAAGCGGAACC Rev_CGGCAATCGGCGAGCCGAC	*bacB*	808	63	This study
bacC	Fwd_CTCCAACAGACAAAACCA Rev_TCTTTTTCGAGCCATTCAC	*bacC*	1,372	63	This study
bcrA	Fwd_ACGGATCTCACGAAAATG Rev_CGCCCCCTATCAACTTCA	*bcrA*	886	55	This study
bcrB	Fwd_AGACCTTTTCCGATACCA Rev_CGGCAACACTGATAAAAGA	*bcrB*	519	55	This study
bcrC	Fwd_GACCCGGACGACAAAAAA Rev_AGAATACAAACAGAGCTGAC	*bcrC*	356	55	This study
uppP	Fwd_CGCGAGAAACCAGGCCAAAG Rev_ATTCAGCTAGGCTCCATTTTAGC	*uppP*	365	51	This study
ermA	Fwd_TCTAAAAAGCATGTAAAAGAA Rev_CTTCGATAGTTTATTAATATTAGT	*ermA*	645	52	53
ermB	Fwd_GAAAAGGTACTCAACCAAATA Rev_AGTAACGGTACTTAAATTGTTTAC	*ermB*	639	52	
ermC	Fwd_TACAAACATAATATAGATAAA Rev_GCTAATATTGTTTAAATCGTCAAT	*ermC*	642	52	
ermD/ermK[1][a]	Fwd_AGGCTCTGTTTGTGTATG Rev_TGGAGGGGGAGAAAAATG	*ermD* and *ermK*	814	52	This study
ermD/ermK[2][a]	Fwd_GCAGACCGCCTGTGATTTTTTATG Rev_CAGGGACATCTGAATCCC	*ermD* and *ermK*	970	55	This study
ermT	Fwd_TCAAAGCATCATATAAATGAA Rev_GCTAATATTGTTTAAATCGTCAAT	*ermT*	642	51	12
msrA/B	Fwd_GCAAATGGTGTAGGTAAGACAACT Rev_ATCATGTGATGTAAACAAAAT	*msrA* and *msrB*	399	52	53
gyrA	Fwd_CAGTCAGGAAATGCGTACGTCCTT Rev_CAAGGTAATGCTCCAGGCATTGCT	*gyrA*	1,025	58	48

[a] The primers were designed to detect the *ermD* and *ermK* genes.

detection of the *ermA*, *ermB*, and *ermD* and *ermK* genes are indicated in Table 1. The primers used, and their corresponding targeted genes and annealing temperatures, are reported in Table 2. All primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). For detection of the *ermA*, *ermB*, *ermC*, and *msrA* and *msrB* genes, the PCR amplification conditions were performed as described by Sutcliffe et al. (53). The *ermT* gene was PCR amplified as described by DiPersio and DiPersio (12). The primers for the *ermD* and *ermK* genes (23, 30) were designed from the published sequences in the GenBank database (M77505.1) using the CLC Main Workbench v.5. The PCR reagents mix used for detection of the *ermD* and *ermK* genes contained 2.5 μl 10× PCR buffer, 0.2 mM deoxynucleoside triphosphate (dNTP) mix, 0.1 μl DreamTaq DNA polymerase (Fermentas GmbH, Germany), 0.1 pmol/μl of each primer pair (ermD/ermK[1]), and 1.0 μl of the DNA. The total volume was adjusted to 25.0 μl using sterile Milli-Q water. The PCR amplification conditions were initial denaturation at 93°C for 3 min, 35 cycles of 93°C for 1 min, annealing at 52°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. After the amplification, 12 μl of the PCR product was separated by electrophoresis in 1% agarose and then visualized by ethidium bromide staining and documented under UV illumination using an AlphaImager HP system (Alpha Innotech).

Determination of chromosomal or plasmid localization of the *ermD* and *ermK* genes. To verify if the detected *ermD* and *ermK* genes among the *B. licheniformis* strains were located on either a plasmid or the chromosome, plasmid DNA was isolated from 8 randomly selected *B. licheniformis* strains harboring the genes. The E.Z.N.A. plasmid miniprep kit (Omega-Bio-Tek, Inc.) was used according to the manufacturer's instructions. The strains were grown in Luria-Bertani medium (5 g/liter yeast extract [catalog no. 212750; BD Becton, NJ], 10 g/liter tryptone [catalog no. 211705; BD, NJ], 10 g/liter NaCl) for 22 h (optical density at 600 nm, 1.3 to 2.0). The isolated plasmid DNA was supplemented with GelRed and separated in a 1% agarose gel by electrophoresis. All observed DNA bands were excised using a sterile scalpel (Swann-Mortion, Sheffield, England) and purified using the NucleoSpin Extract II DNA purification kit (Clontech Laboratories Inc.). Plasmid DNA bands were distinguished further from chromosomal DNA by PCR amplification of the chromosomally located gyrase A gene (*gyrA*) (48) and the undecaprenyl disphosphatase gene (*uppP*) using the purified DNA. The purified plasmid DNA was additionally PCR amplified with the *ermD* and *ermK* gene primer pair (ermD/ermK[1]) as described in "Molecular detection of erythromycin resistance determinants" (above). The total DNA of *B. licheniformis* strain C32 was used as a positive control in the PCRs. The sizes of the plasmids were estimated using the BioNumerics software package version 4.50 (Applied Maths, Sint-Martens-Latem, Belgium).

Sequencing of *ermD* and *ermK* genes of *B. licheniformis* strain C8. A primer pair targeting the highly homologous erythromycin genes *ermD* and *ermK* was designed based on the homologous regions of the published *B. licheniformis ermD* and *ermK* gene sequences (GenBank accession no. M77505.1 and M29832.1). The PCR amplification conditions were the same as described previously except that the annealing temperature for the new primer pair (ermD/ermK[2]) was 55°C and the thermocycling consisted of 40 cycles. The PCR-amplified products were sequenced in both directions by a commercial sequencing service provider (Macrogen, Netherlands). The translated *ermD* and *ermK* nucleotide sequences were compared with the ErmD (AAA22599), ErmJ (AAA22597), and ErmK (AAA22595) amino acid sequences deposited in the GenBank database by using the CLC Main Workbench v.5 software.

Molecular detection of bacitracin biosynthetase (*bacA*, *bacB*, *bacC*), bacitracin transporter (*bcrA*, *bcrB*, *bcrC*), and undecaprenyl-disphosphatase 1 (*uppP*) genes. The specific primers for the detection of the bacitracin synthetase genes (*bacA*, *bacB*, *bacC*) and the transporter genes (*bcrA*, *bcrB*, *bcrC*) were designed from a published sequence of the *B. licheniformis* bacitracin synthetase operon (GenBank accession no. AF007865.2) using the CLC Main Workbench v.5 software. The PCR conditions for amplification of the bacitracin transporter and resistance genes were optimized further for the simultaneous detection of these three genes. In addition, primers (uppP) were designed for specific amplification of the undecaprenyl-disphosphatase 1 gene (*uppP*) (EC 3.6.1.27). All primer sequences and their respective annealing temperatures and PCR product sizes are indicated in Table 2. The PCR amplification conditions were similar to those described in "Molecular detection of erythromycin resistance determinants" except the annealing temperatures were different (Table 2) and 2.0 mM $MgCl_2$ was added to the PCR mix for amplification of the *bacA* gene. The PCR-amplified products were purified, sequenced (Macrogen, Netherlands), and analyzed with the CLC Main Workbench v.5 software in order to verify the PCR products.

Bacitracin analysis by UHD-QTOF LC/MS in a culture supernatant of *Bacillus* species strains. Seventy-four of the 85 *Bacillus* species strains plus 7 reference strains (6 of which are bacitracin positive [bac^{+ve}] and 1 of which is bacitracin negative [bac^{-ve}]) were sampled and tested for bacitracin production. The isolates were grown in 50 ml BHI medium in a shaking water bath incubator (GFL-1086; Gesellschaft für Labortechnik mbH, Burgwedel, Germany) with shaking (140 min^{-1}) at 37°C for 19 h. The culture was centrifuged at 5,000 × *g* at 4°C for 15 min (model 3K10; Sigma Laborzentrifugen GmbH, Germany), and the supernatants were stored at −20°C until analyzed. The bacitracin analysis was performed using an Agilent 1200 Infinity series liquid chromatograph equipped with an Infinity DAD detector and an Agilent 6540 ultra-high-definition (UHD) accurate-mass quadrupole time-of-flight (QTOF) liquid chromatograph/mass spectrometer (LC/MS). The column was an Agilent Poroshell 120 EC-18 (2.1 by 150 mm, 2.7 μm) with an Agilent 1290 Infinity filter house with a 0.3-μm frit. The precolumn was a VanGuard Acquity BEH C_{18} column (2.1-mm inside diameter [i.d.], 5-mm length, 1.7 μm) (Waters, Denmark). The mobile phase A was 0.1% (vol/vol) acetic acid and 0.01% (vol/vol) trifluoroacetic acid in Milli-Q water, and the mobile phase B was 0.1% (vol/vol) acetic acid and 0.008% (vol/vol) trifluoroacetic acid in acetonitrile. The flow rate was 0.3 ml/min, and the injection volume was 10 μl. The column temperature was maintained at 50°C with an initial column pressure of 320 × 10^5 Pa. Bacitracin from *B. licheniformis* (Fluka, catalog no. 11702) with a molecular mass of 1,422.69 g/mol was used as the standard reference sample. Data acquisition and analyses were performed with the Agilent MassHunter Workstation software (Agilent Technologies Inc.).

Accession numbers. The GenBank/EMBL/DDBJ accession numbers for the gene sequences reported in this paper are JQ315396 to JQ315398 (undecaprenyl pyrophosphate phosphatase [*uppP*]), JQ315399 to JQ315401 (bacitracin transporter gene A [*bcrA*]), JQ315402 and JQ315403 (bacitracin transporter gene B [*bcrB*]), JQ315404 (bacitracin transporter gene C [*bcrC*]), JQ315406 to JQ315408 (bacitracin synthetase A gene [*bacA*]), JQ315405 (erythromycin resistance genes *ermD* and *ermK*), and JQ715853 to JQ715880 (gyrase subunit A gene [*gyrA*]).

RESULTS

Phylogenetic analysis of *B. subtilis* strains based on *gyrA* gene sequences. The *gyrA* gene was sequenced for all the *B. subtilis* strains included in the study for unambiguous identification to the subspecies level. A BLAST search of the determined sequences against publicly available sequences in the GenBank database indicated over 99% identity to *B. subtilis* subsp. *subtilis* strain 168 (accession no. AL009126.3) and 94% to *B. subtilis* subsp. *spizizenii* strain $W23^T$ (accession no. CP002183.1). The *B. subtilis* strains were hence identified as *B. subtilis* subsp. *subtilis*. In the following sections, unless otherwise indicated, *B. subtilis* should be read as *B. subtilis* subsp. *subtilis*.

Antimicrobial MICs. The results for the antimicrobial MIC testing are indicated in Table 3 along with the MIC ranges assayed for each antibiotic and the breakpoint values recommended by the European Food Safety Authority (16). As indicated in Table 3, the *B. subtilis* and *B. sonorensis* strains were much more susceptible to clindamycin (MIC_{50}, 4.0 mg/liter) than those of the *B. licheniformis* strains (MIC_{50}, 32.0 mg/liter). All the strains were resistant to streptomycin (MIC, >8.0 mg/liter). The streptomycin MIC_{50} value obtained for all the strains was 64.0 mg/liter, as most of the *B. subtilis* (83%), *B. licheniformis* (87%), and *B. sonorensis* strains (83%) were susceptible to 64.0 mg/liter streptomycin. Twenty-four percent of the *B. licheniformis* strains, 21% of the *B. subtilis* strains, and 6% of the *B. sonorensis* strains were susceptible to 32.0 mg/liter streptomycin. None of the *B. subtilis* strains were resistant to chloramphenicol, while 63% of the *B. licheniformis* strains and 94% of the *B. sonorensis* strains were resistant to this antibiotic. Resistance to kanamycin was not observed among any of the *B. subtilis* or the *B. sonorensis* strains. In contrast, 66% and 18% of the *B. licheniformis* strains were resistant to 8.0 mg/liter and 16.0 mg/liter kanamycin, respectively. Moreover, all the *B. subtilis* and *B. sonorensis* strains were susceptible to erythromycin (4.0 mg/liter), whereas 50% of the *B. licheniformis* strains were resistant to different concentrations (16.0 to 128.0 mg/liter) of this antibiotic. Remarkably, all the strains were susceptible to gentamicin (4.0 mg/liter), tetracycline (8.0 mg/liter), and vancomycin (4.0 mg/liter).

Assessment of bacitracin resistance profiles. When BHI plus 40 mg/liter $ZnSO_4$ was used as the test medium, all of the 85 *Bacillus* spp. and the included reference strains (Table 1) were resistant to 30.72 U/ml bacitracin (512 mg/liter; potency, ≥60,000 U/g). However, when Mueller-Hinton broth (MHB) plus 40 mg/liter $ZnSO_4$ was used as the test medium, different MIC values between 7.68 U/ml and >15.36 U/ml were observed (Table 4). This indicates that certain components of the BHI medium might have inhibited the activity of the bacitracin against the tested *Bacillus* species strains. Thus, MHB plus 40 mg/liter $ZnSO_4$ was used as the test medium. For the control strains, *B. licheniformis* strain 5A36 (bacitracin-susceptible strain) was susceptible to 15.36 U/ml (256 mg/liter) bacitracin, whereas *B. licheniformis* strain DSM 603, *B. licheniformis* strain 5A24, and *B. licheniformis* strain 5A1 (bacitracin-resistant strains) were resistant to 15.36 U/ml bacitracin (Table 4). Thus, 15.36 U/ml (256 mg/liter) was considered the breakpoint value for categorizing the microbial strains as resistant or susceptible. All the 18 *B. sonorensis* strains, 8% of the *B. licheniformis* strains, and 7% of the *B. subtilis* strains were resistant to 15.36 U/ml (256 mg/liter) bacitracin. The resistance of the *B. subtilis* strains to bacitracin was more variable (7.68 to 15.36 U/ml).

Molecular detection of erythromycin resistance genes *ermA*, *ermB*, *ermC*, *ermD* and *ermK*, *ermT*, and *msrA* and *msrB*. Erythromycin resistance was observed among 50% of the *B. licheniformis* strains and with different levels of resistance among the strains. All the 38 strains were therefore additionally investigated for the presence of erythromycin resistance genetic determinants. No *ermA*, *ermB*, *ermC*, or *ermT* genes or the efflux genes *msrA* and *msrB* were detected in any of the strains. However, the *ermD* and *ermK* genes were detected in all of the *B. licheniformis* strains (19

TABLE 3 Antibiotic MICs of *Bacillus* species strains isolated from primary starters used for the production of Gergoush, a Sudanese traditional bread snack[a]

Antibiotic	*Bacillus* sp.	MIC range tested (mg/liter)	EFSA breakpoint values (mg/liter)[b]	Resistance rate (%)[c]	MIC_{50} (mg/liter)	MIC_{90} (mg/liter)
Chloramphenicol	*B. licheniformis*	8–128	8	63	16	32
	B. subtilis subsp. *subtilis*			0	8	8
	B. sonorensis			94	16	32
Clindamycin	*B. licheniformis*	4–128	4	100	32	64
	B. subtilis subsp. *subtilis*			0	4	4
	B. sonorensis			0	4	4
Erythromycin	*B. licheniformis*	4–128	4	50	4	—[d]
	B. subtilis subsp. *subtilis*			0	4	4
	B. sonorensis			0	4	4
Gentamicin	*B. licheniformis*	4–128	4	0	4	4
	B. subtilis subsp. *subtilis*			0	4	4
	B. sonorensis			0	4	4
Kanamycin	*B. licheniformis*	8–256	8	66	16	32
	B. subtilis subsp. *subtilis*			0	8	8
	B. sonorensis			0	8	8
Streptomycin	*B. licheniformis*	8–64	8	100	64	—
	B. subtilis subsp. *subtilis*			100	64	—
	B. sonorensis			100	64	—
Tetracycline	*B. licheniformis*	8	8	0	8	8
	B. subtilis subsp. *subtilis*			0	8	8
	B. sonorensis			0	8	8
Vancomycin	*B. licheniformis*	4	4	0	4	4
	B. subtilis subsp. *subtilis*			0	4	4
	B. sonorensis			0	4	4

[a] Numbers of strains of each species: 38 (*B. licheniformis*), 29 (*B. subtilis* subsp. *subtilis*), and 18 (*B. sonorensis*).
[b] Antibiotic breakpoint values (mg/liter) recommended by the European Food Safety Authority in 2012 (16).
[c] Number (%) of strains with a MIC above the recommended breakpoint value. The MIC_{50} (mg/liter) and MIC_{90} (mg/liter) are the antibiotic concentration values at which 50% and 90% of the strains were susceptible, respectively.
[d] —, no MIC_{90} obtained.

strains) that were phenotypically resistant to erythromycin (MIC, 16 to 128 mg/liter; results partly shown in Fig. 1). In addition, the *ermD* and *ermK* genes were detected in *B. licheniformis* strain C8, which was found to be susceptible to erythromycin (MIC, 4 mg/liter).

Sequencing of *ermD* and *ermK* genes of strain C8. *B. licheniformis* strain C8 was susceptible to 4.0 mg/liter erythromycin. However, PCR analysis of erythromycin resistance determinant genes revealed the presence of the *ermD* and *ermK* genes in this strain, which should have conferred an erythromycin-resistant phenotype (23, 30). Further analysis of the translated *ermD* and *ermK* nucleotide sequences of strain C8 by comparing them with the highly similar reference sequences of ErmD (*B. licheniformis*, GenBank accession no. AAA22599), ErmK (*B. licheniformis*, GenBank accession no. AAA22595), and ErmJ (*B. anthracis*, GenBank accession no. AAA22597) indicated some changes in the amino acid sequence relative to the reference sequences (Fig. 2). In addition, a premature stop codon is observed in the *ermD* and *ermK* nucleotide sequences of *B. licheniformis* strain C8 (indicated as a star to which an arrow points in Fig. 2) in a comparison with the three closely related *erm* genes of the resistant strains. Consequently, this could result in an early termination of ErmD and ErmK protein synthesis.

Localization of the *ermD* and *ermK* genes. Eight *B. licheniformis* strains were screened for the presence of plasmids and PCR amplification of the *ermD* and *ermK* genes in any occurring plasmids. The electrophoresis profiles of the isolated plasmid DNA are shown in Fig. 3. Except for *B. licheniformis* strains C32 and C34, which both harbored one plasmid, all the remaining strains harbored either two or three plasmids. The *ermD* and *ermK* genes were amplified from DNA excised from positions 2, 3, 4, 7, 11, 13, and 15 (size of plasmid, 11.4 kbp), whereas no PCR-amplified product was observed in DNA excised from the remaining positions (results not shown). Except for DNA excised from position 3 on Fig. 3, no PCR-amplified product was obtained for DNA extracted from any of the remaining bands at the indicated positions (results not shown), indicating that the bands at positions 2, 4, 7, 11, 13, and 15 were free of contaminating chromosomal DNA. Taken together, our data strongly indicate that the *ermD* and *ermK* genes that correlate with the erythromycin resistance phenotype among the *B. licheniformis* strains are located on an 11.4-kbp plasmid.

TABLE 4 Characterization of bacitracin operons, bacitracin sensitivities, and bacitracin production in BHI broth by *Bacillus* strains isolated from primary starters used for the production of a Sudanese traditional bread snack, Gergoush, and *Bacillus* type strains

Strain	E^{R}[a]	Bacitracin MIC (U/ml)	*uppP*[b]	Status of indicated bacitracin operon gene				Bacitracin detected by UHD-AM-QTOF LC/MS[c]
				bacA	*bcrA*	*bcrB*	*bcrC*	
B. licheniformis								
C32	R	>15.36	+	−	+	+	+	+
C34	R	>15.36	+	−	+	+	+	+
C18	R	>15.36	+	−	−	−	−	+
C29	S	15.36	+	−	−	−	−	+
L2	S	15.36	+	−	−	−	−	+
L8	S	15.36	+	−	−	−	−	+
L23	S	15.36	+	−	−	−	−	+
L49	S	15.36	+	−	−	−	−	+
B. subtilis subsp. *subtilis*								
C16	S	15.36	+	−	−	−	−	+
C43	S	7.68	+	−	−	−	−	+
C45	S	15.36	+	−	−	−	−	+
F19	S	7.68	+	−	−	−	−	+
L50	S	15.36	+	−	−	−	−	+
W8	S	7.68	+	−	−	−	−	+
B. sonorensis								
C1	S	>15.36	−	+	+	−	−	+
C2	S	>15.36	−	+	+	−	−	+
L12	S	>15.36	−	+	+	−	−	+
L13	S	>15.36	−	+	+	−	−	+
L39	S	>15.36	−	+	+	−	−	+
L41	S	>15.36	−	+	+	−	−	+
W6	S	>15.36		ND	ND	ND	ND	+
Reference and type strains								
B. licheniformis DSM 603	ND	>15.36	ND	+	+	+	+	−
B. licheniformis 5A24	ND	>15.36	ND	+	+	+	+	+
B. licheniformis 5A1	ND	>15.36	ND	+	+	+	+	+
B. licheniformis 5A36	ND	15.36	−	−	−	−	−	ND
B. subtilis subsp. *subtilis* DSM 10^{T}	ND	15.36	+	−	−	−	−	ND
B. subtilis subsp. *spizizenii* DSM 15029^{T}	ND	15.36	+	−	−	−	−	ND
B. licheniformis DSM 13^{T}	ND	15.36	−	−	−	−	−	ND
B. sonorensis DSM 13779^{T}	ND	>15.36	−	+	+	−	−	ND

[a] E^{R}, erythromycin resistance; R, resistant; S, susceptible; ND, not determined.
[b] UPP, undecaprenyl pyrophosphate phosphatase. The upregulation of UPP activity is linked to bacitracin resistance.
[c] UHD-AM-QTOF LC/MS, ultra-high-definition accurate mass quadrupole time-of-flight liquid chromatography/mass spectrometry.

Detection of the bacitracin synthetase genes *bacA*, *bacB*, and *bacC*, the bacitracin transporter genes *bcrA*, *bcrB*, and *bcrC*, and the *uppP* gene. Examples of the PCR-based detection of the bacitracin biosynthetase gene *bacA* and the transporter gene *bcrA* are shown in Fig. 4A. Additionally, an optimized multiplex PCR technique for simultaneous detection of all three bacitracin transporter genes (*bcrA*, *bcrB*, and *bcrC*) was developed (Fig. 4B). The bacitracin biosynthetase gene *bacA* and the transporter gene *bcrA* were both detected in all the *B. sonorensis* strains, including *B. sonorensis* DSM 13779^{T}. However, the bacitracin synthetase genes *bacB* and *bacC*, as well as the transporter genes *bcrB* and *bcrC*, were not detected in any of the *B. sonorensis* strains. None of the bacitracin synthetase genes *bacA*, *bacB*, and *bacC* were detected in any of the *B. licheniformis* and *B. subtilis* strains. The entire bacitracin transporter gene cluster (i.e., *bcrA*, *bcrB*, and *bcrC*) was detected in the *B. licheniformis* strains C32 and C34 (Fig. 4B; Table 4) but not in the remaining *B. licheniformis* and *B. subtilis* strains.

The *uppP* gene was detected in all of the *B. subtilis* and *B. licheniformis* strains of African origin and also in the type strains *B. subtilis* subsp. *subtilis* DSM 10 and *B. subtilis* subsp. *spizizenii* DSM 15029 (results not shown). Contrarily, the *uppP* gene was not detected in any of the *B. sonorensis* strains, including *B. sonorensis* DSM 13779^{T}. It was also not detected in the *B. licheniformis* strain DSM 13^{T}, *B. licheniformis* strain DSM 603, and *B. licheniformis* strain 5A24, while a PCR product with a different size was obtained for *B. licheniformis* strain 5A1.

Bacitracin analysis by UHD accurate-mass QTOF LC/MS in a culture supernatant of the *Bacillus* species strains. The production of bacitracin A was investigated for 74 of the *Bacillus* species strains comprising 35 from *B. licheniformis*, 25 from *B. subtilis*, and 14 from *B. sonorensis*. An example of a chromatogram and mass spectra of bacitracin A produced in a 19-h culture supernatant of *B. sonorensis* strain C2 in comparison with a 20-ppm bacitracin standard spiked in Milli-Q water is shown in Fig. 5. Both double- and triple-charge

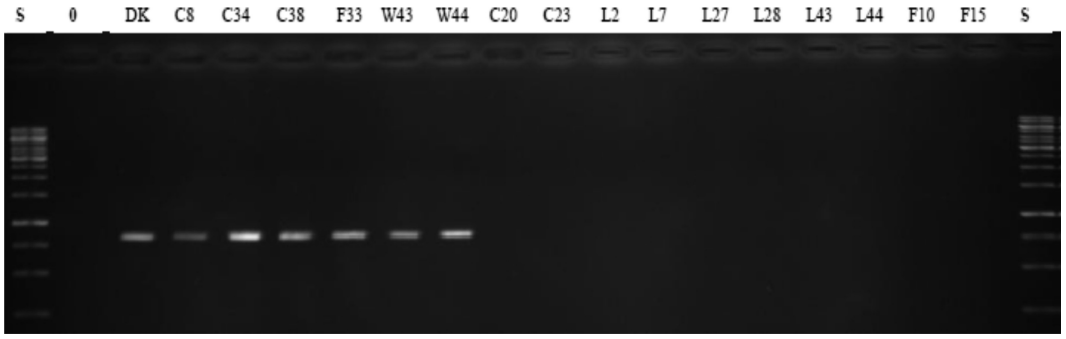

FIG 1 Erythromycin resistance determinant genes (*ermD, ermK*) in *Bacillus licheniformis* and reference strains. Lanes: S, Fermentas 1-kb DNA ladder (SM0313); 0, negative control; DK, *B. subtilis* pEC 1001, *ermD*-positive strain. The *B. licheniformis* strains with an erythromycin MIC of ≥16.0 mg/liter are C34, C38, F33, W43, and W44. *B. licheniformis* strains with an erythromycin MIC of 4.0 mg/liter are C20, C23, L2, L7, L27, L28, L43, L44, F10, and F15.

ions of bacitracin A were detected (Fig. 5B and C). The protonated single-charge parent ion at 1,422.77 U corresponds to the reported molecular mass of bacitracin A (1,421.7727 U). Except for one strain (*B. licheniformis* DSM 603), bacitracin was detected in the culture supernatant of all the bacitracin-positive control strains (Table 4). Bacitracin A production was detected for 11% of the *B. licheniformis* strains, 8% of the *B. subtilis* strains, and 9% of the *B. sonorensis* strains.

DISCUSSION

In the present study, the susceptibilities of 85 *Bacillus* strains comprising three *Bacillus* spp. (*B. licheniformis*, *B. subtilis*, and *B. sonorensis*) against 8 antibiotics were determined.

Species-associated differences in the sensitivities of the strains to different concentrations of kanamycin, chloramphenicol, and clindamycin were observed among the 3 *Bacillus* spp. The *B. li-*

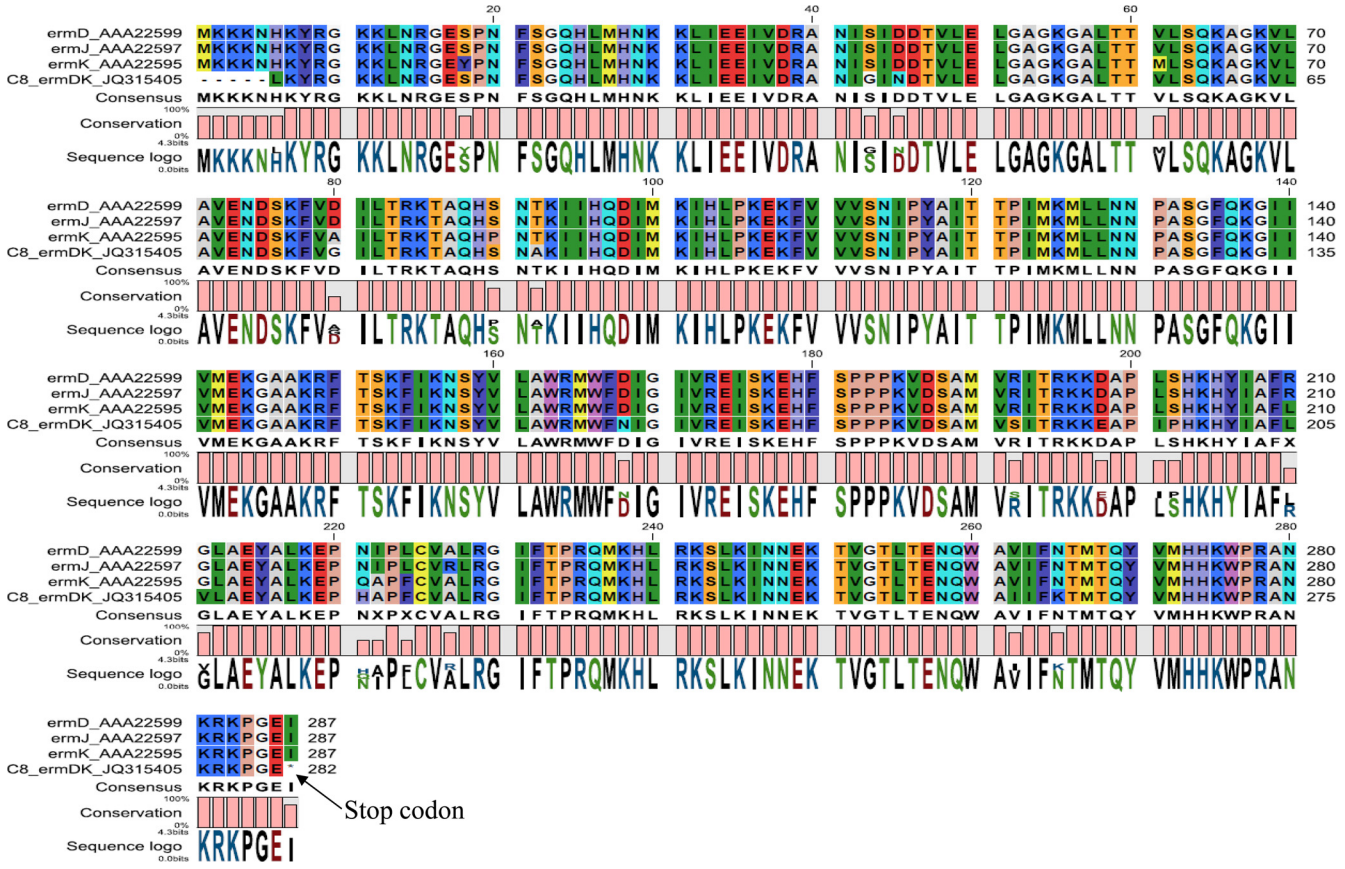

FIG 2 Protein sequence alignments of *Bacillus licheniformis* C8 ErmD and ErmK with ErmD, ErmJ, and ErmK amino acid sequences retrieved from the GenBank database. ErmD and ErmK are the homologous region of the ErmD and ErmK erythromycin resistance proteins. The star with an arrow pointed toward it indicates the position of the termination of protein synthesis in the ErmD and ErmK amino acid sequence.

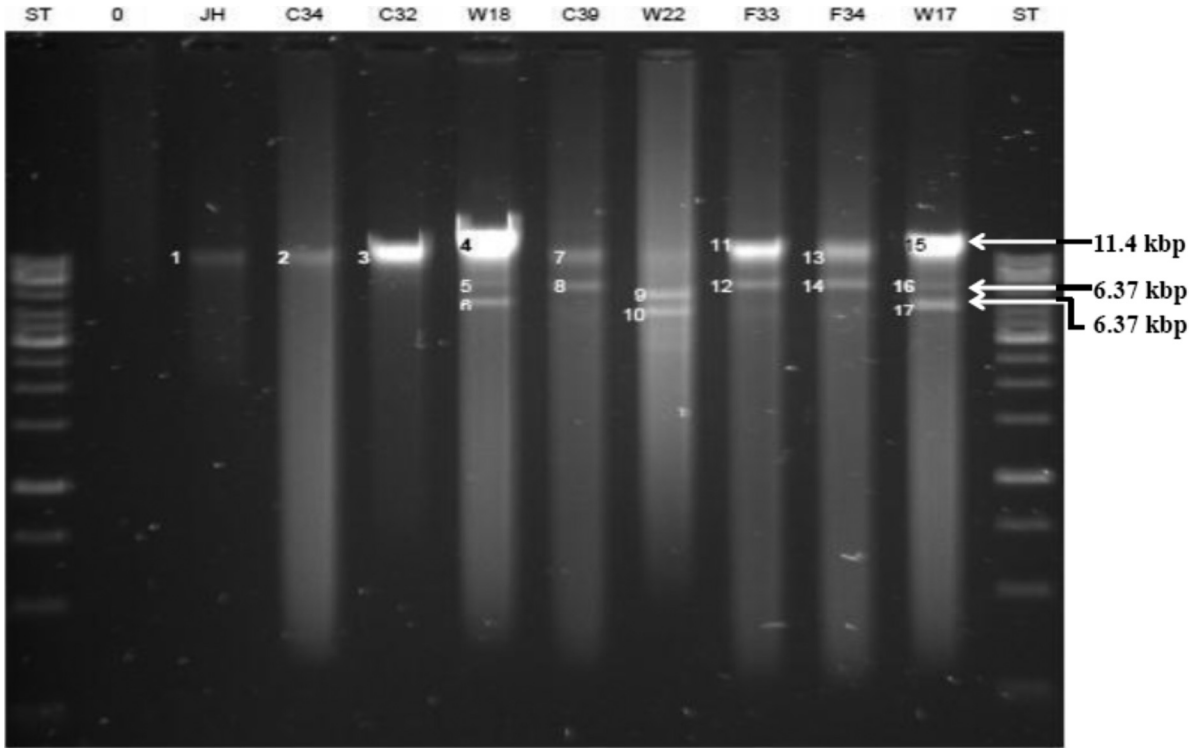

FIG 3 Agarose gel electrophoresis of plasmid DNA isolated from erythromycin-resistant *Bacillus licheniformis* strains. Lanes: ST, Fermentas 1-kb DNA ladder (SM0313); 0, negative control; JH, *Enterococcus faecalis* JH2-2 Tn*1545*. Gel electrophoresis is also shown for *B. licheniformis* strains C34, C32, W18, C39, W22, F33, F34, and W17.

cheniformis strains were predominantly resistant to high levels of erythromycin, clindamycin, and chloramphenicol compared to their resistance profiles to gentamicin, tetracycline, and vancomycin. Generally, high chloramphenicol and clindamycin MIC_{90} values were obtained for the *B. licheniformis* strains. The high resistance rate of the *B. licheniformis* strains to clindamycin and chloramphenicol may be attributed to an intrinsic characteristic of this species due to the uniform distributions of the MIC values. Also, similar resistance profiles among *B. licheniformis* strains from different geographical areas have been reported (39, 52), supporting this assertion. In contrast to the *B. licheniformis* strains, the *B. subtilis* and *B. sonorensis* strains were highly susceptible to erythromycin (4.0 mg/liter), clindamycin (4.0 mg/liter), and kanamycin (8.0 mg/liter). However, species-specific differences in the sensitivities of these two species to chloramphenicol were observed. The *B. subtilis* strains were generally susceptible to chloramphenicol (MIC_{90}, 8.0 mg/liter), whereas the *B. sonorensis* strains were resistant to 8.0 mg/liter chloramphenicol. The MIC_{50} (16.0 mg/liter) and MIC_{90} (32.0 mg/liter) values obtained for the *B. sonorensis* strains to chloramphenicol were similar to those of the *B. licheniformis* strains. These results therefore revealed the existence of species-specific variations in the antimicrobial susceptibilities of *Bacillus* spp., similar to the observations of other authors (18, 46, 56).

Interestingly, all three *Bacillus* spp. were resistant to streptomycin at the recommended breakpoint value (8.0 mg/liter). It is also worth noting that the MIC_{50} of 64.0 mg/liter obtained was similar to the breakpoint value proposed by the European Union Scientific Committee on Animal Nutrition (SCAN) (24), which differs largely from the proposed breakpoint value recommended by the European Food Safety Authority (EFSA) for the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (16). The discrepancy in the breakpoint values of 64.0 mg/liter determined by the European Union Scientific Committee on Animal Nutrition in 2002 (24) and 8.0 mg/liter determined by the European Food Safety Authority in 2012 (16) for the *Bacillus* genus and the fact that limited data on the antibiotic susceptibility profiles of *Bacillus* spp. from different geographical areas are available indicate that more information is needed to enable the harmonization and proposal of new breakpoint values for this genus and also at the species level. Both *B. licheniformis* and *B. subtilis* are listed on the qualified presumption of safety (QPS) of microorganisms list. A generic qualification for all QPS bacterial taxonomic units is the absence of acquired genes for antimicrobial resistance to clinically relevant antibiotics (17, 50), hence the proposal of the antimicrobial breakpoint values for microorganisms by the European Food Safety Authority (16). However, both *B. subtilis* and *B. licheniformis* were given the same antimicrobial breakpoint values for chloramphenicol, clindamycin, and kanamycin (16). Consequently, either of these *Bacillus* species strains with viable commercial applications for human and animal uses or plant protection agents could be wrongly disapproved by the relevant authorities due to the variations in the sensitivities of these species to the antibiotics observed in this study.

The presence of the *ermD* and *ermK* erythromycin resistance genes has been shown to confer an erythromycin-resistant pheno-

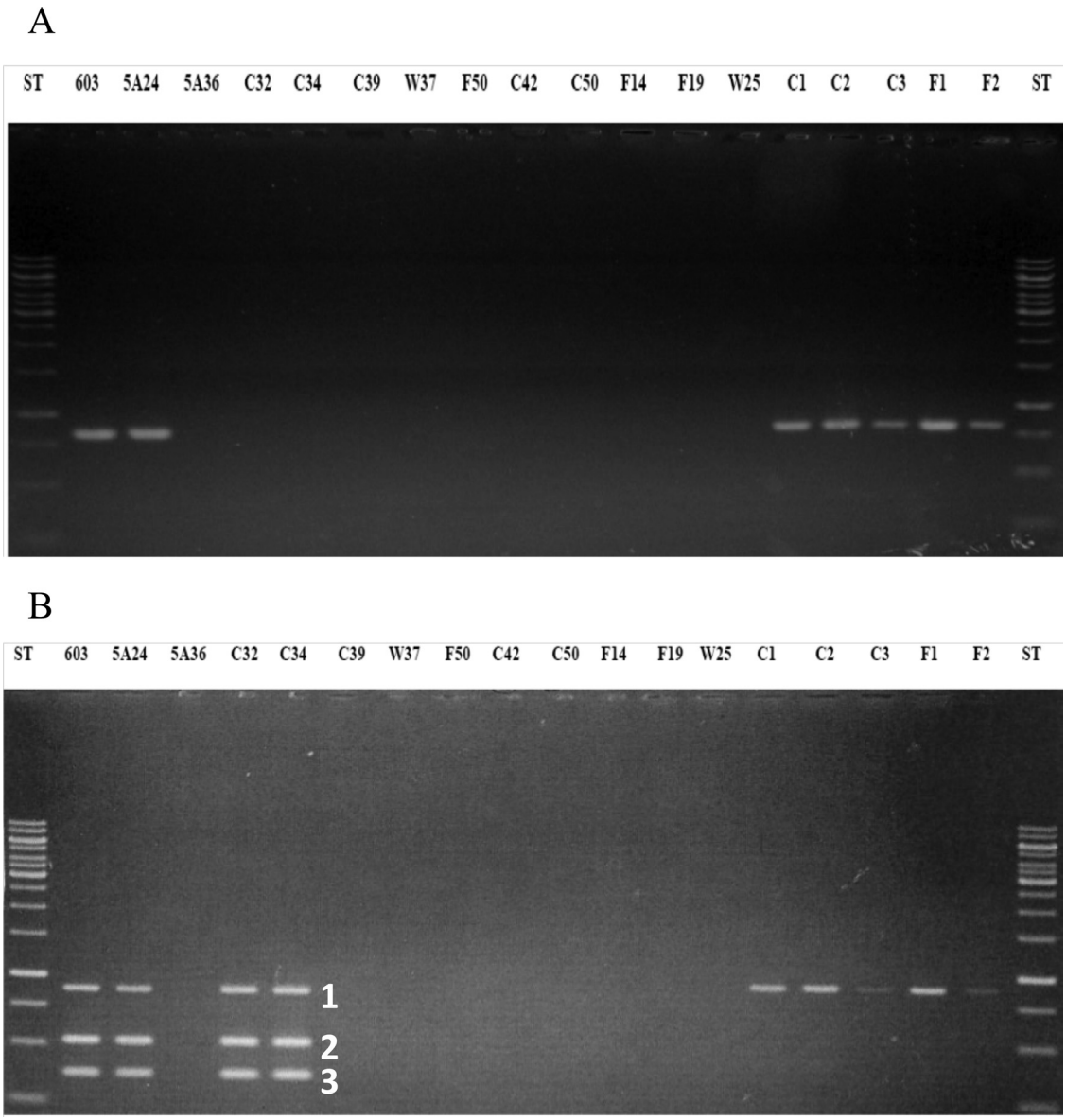

FIG 4 (A) PCR detection of the bacitracin synthetase gene *bacA* in *Bacillus* species strains. (B) Multiplex PCR detection of bacitracin ABC transporter genes. Band 1, *bcrA*; band 2, *bcrB*; band 3, *bcrC* in *Bacillus* species strains. The *bcrABC*-positive reference strains are *B. licheniformis* DSM 603 (ATCC 10716) and *B. licheniformis* 5A24 (ATCC 11946); the *bcrABC*-negative reference strain is *B. licheniformis* 5A36 (ATCC 14580). Some of the strains studied are *B. licheniformis* strains C32, C34, C39, W37, and F50, *B. subtilis* strains C42, C50, F14, F19, and W25, and *B. sonorensis* strains C1, C2, C3, F1, and F2.

type in *B. licheniformis* through the methylation of the 23S rRNA macrolide binding sites (23, 30). In the present study, we also observed a strong correlation between the presence of the *ermD* and *ermK* genes and an erythromycin resistance phenotype among most of the *B. licheniformis* strains. Although the intrinsic or acquired nature of these genes is yet unknown (5), we have observed that the *ermD* and *ermK* genes were localized on an 11.4-kbp plasmid, contrary to the observation of Gryczan et al. (23), who found that the *ermD* gene was located on the chromosome. Barbosa et al. (3) also observed that the *erm34* gene in *B. clausii* strains was located on the chromosome. Plasmids serve a significant role in the dissemination of antibiotic resistance genes as reported from both *in vivo* and *in vitro* studies (9). Thus, plasmid localizations of the observed *ermD* and *ermK* genes indicate the potential risk of spreading to other species. In *B. licheniformis* strain C8, mutations of the *ermD* and *ermK* genes resulted in an erythromycin-susceptible phenotype. Similarly, Hue and Bechhofer (27) also observed that the deletion or insertion of nucleotides in the *ermD* leader region required for the regulation of *ermD* gene expression led to erythromycin-susceptible *Bacillus* species strains. Point mutations in the leader peptide region or codon of the *ermK* methylases have also been indicated to result in the reduced induction of the *ermK* gene in the presence of erythromycin (31).

In the present study, only 3 of the 20 *B. licheniformis* strains resistant to erythromycin were also resistant to bacitracin, indicating no link or association between an erythromycin-resistant phenotype and bacitracin resistance among the strains. This is quite

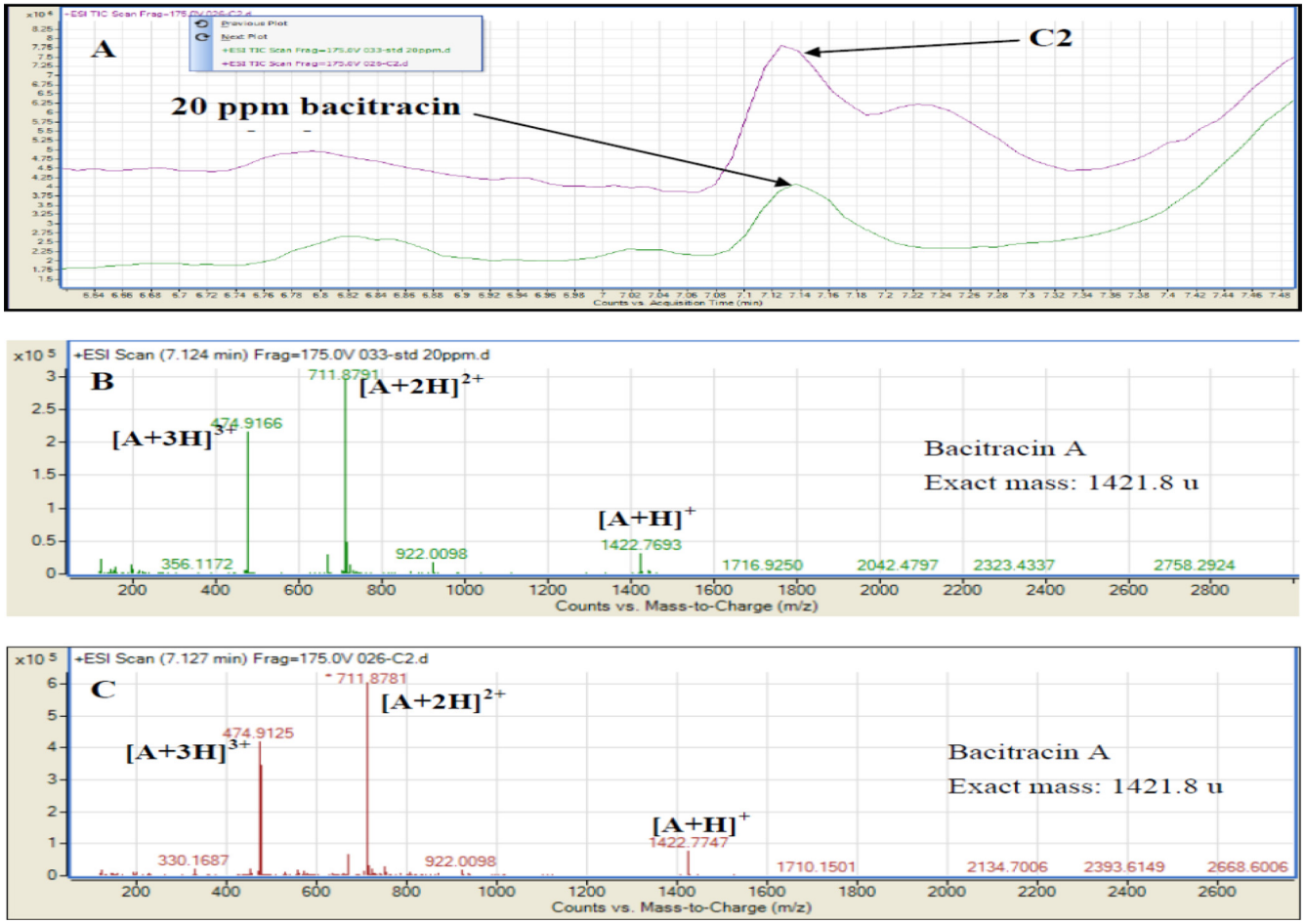

FIG 5 (A) Chromatograms of bacitracin A produced by *B. sonorensis* strain C2 (19-h incubation in BHI broth) in comparison with a 20-ppm bacitracin standard. (B) Full-scan mass spectrum of bacitracin A standard chromatogram showing the molecular mass of bacitracin A (1,421.8 U). (C) Full-scan mass spectrum of bacitracin A produced by *B. sonorensis* strain C2 showing the molecular mass of bacitracin A. Triple-charge ions were obtained for bacitracin A, as indicated in the mass spectrum. The bacitracin was detected by UHD-QTOF LC/MS.

contrary to the observations made by other authors (28, 34, 41). Ishihara et al. (28) observed that *B. licheniformis* strains carrying the bacitracin synthetase genes were also resistant to erythromycin. Of 11 bacitracin-resistant *Streptococcus pyogenes* strains isolated from different geographical areas in Spain, 9 strains were resistant to erythromycin (and harbored the *erm28* gene) and clindamycin (41).

Different mechanisms of bacitracin resistance among bacteria have been reported (4, 20, 21, 44, 45). According to Podlesek et al. (44), bacitracin resistance among *B. licheniformis* strains is mediated by the ABC transporter system: *bcrA*, *bcrB*, and *bcrC*. The overexpression of the undecaprenyl pyrophosphate phosphatase (*uppP*) gene by *E. coli* has been implicated in the increased resistance of *E. coli* to bacitracin (21). The *bcrA* gene was detected in all the *B. sonorensis* strains and in two *B. licheniformis* strains (C32 and C34), all of which were resistant to 15.36 U/ml bacitracin (Table 4). Podlesek et al. (42) observed further that deletion of the *bcrA* or the *bcrC* gene severely impaired bacitracin resistance. Thus, it is possible that the detected *bcrA* gene in all the *B. sonorensis* strains could contribute to the bacitracin-resistant phenotype among the strains due to their uniform resistance to higher concentrations of bacitracin.

The presence of the *bacA* gene without the *bacB* and *bacC* genes in the case of the *B. sonorensis* strains was unexpected, since these genes are normally organized in an operon (37). A similar observation was made in the case of the transporter genes, as only *bcrA* was observed. The nondetection of the remaining bacitracin operon genes in these strains, especially in the case of those producing bacitracin, could be due to the high level of sequence divergence from the reference *B. licheniformis* strain that was used to design the primers.

In conclusion, strains of *B. licheniformis*, *B. subtilis*, and *B. sonorensis* were highly susceptible to tetracycline, vancomycin, and gentamicin but resistant to streptomycin. Species-specific variations in the patterns of resistance to chloramphenicol, clindamycin, erythromycin, and kanamycin were observed. Resistance to multiple antibiotics was particularly common among the *B. licheniformis* strains. The erythromycin-resistant phenotype observed in some of the *B. licheniformis* strains correlated with the presence of the erythromycin resistance genes *ermD* and *ermK*, which were localized on an 11.4-kbp plasmid. The bacitracin synthetase gene *bacA* and the transporter gene *bcrA* were detected in the *B. sonorensis* strains. Even though the *uppP* gene was detected in all the *B. licheniformis* and *B. subtilis*

strains, no correlation was observed between its presence and bacitracin resistance. In addition, no correlation was observed between erythromycin resistance and bacitracin resistance or the presence of the bacitracin biosynthetase genes. The EFSA's antibiotic microbiological breakpoint values for *Bacillus* species strains are currently presented at the genus level due to limited available data on *Bacillus* species antibiotic resistance profiles. Our results highlight the inadequacy of these values and suggest the need for the breakpoint values for *Bacillus* spp. to be given at the species level and not at the current genus level.

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