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SHORT COMMUNICATION

Differentiation between *Bacillus amyloliquefaciens* and *Bacillus subtilis* isolated from a South African sugarcane processing factory using ARDRA and *rpoB* gene sequencing

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Abstract
A total of 104 exopolysaccharide (gum)-producing bacteria were isolated from the juice screen and juice tank in a sugarcane processing factory at times of low- and high dextran concentrations in the produced sugar. Dextran is an indicator of cane deterioration and sucrose loss after harvesting of the cane. The isolates were identified as *Bacillus amyloliquefaciens* (96 isolates) and *Bacillus subtilis* (eight isolates) based on restriction enzyme banding patterns of amplified 16S rRNA genes and *rpoB* gene sequence analysis. Exopolysaccharide production in sugarcane is normally associated with dextran produced by *Leuconostoc mesenteroides*. *B. amyloliquefaciens*, and to a lesser extent *B. subtilis*, could, however, also be responsible for exopolysaccharide (slime or gum) production in cane processing factories.

Keywords Sugarcane processing · Sucrose loss · Phylogenetic analysis · ARDRA

Introduction

The limited knowledge and understanding of spoilage microorganisms in sugarcane processing factories is well documented (Kulkarni 1999; Nel 2014; Solomon 2000). Foxon and du Clou (2017) reported that gum production may occur in the factory and that microbial-related sucrose losses are not limited to the field as sugarcane deteriorates post-harvest. The identification of gum-producing bacteria in sugarcane processing is important for developing strategies to prevent post-harvest deterioration and gum production. In a recent study (Nel et al. 2019), we showed that the majority of gum-producing bacteria entering a South African sugarcane factory were different to those isolated from areas in the factory after extraction of the sucrose from the sugarcane in the diffuser. Although treatment of shredded cane above 85 °C in the diffuser would likely kill most bacteria, some species indigenous to the factory may contaminate different areas of the factory and multiply under favorable growth conditions.

The area behind the juice screens appeared to be the most frequent source of microbial contamination in the sugar factory, adding to the increased bacterial levels found in the mixed juice tank (Nel et al. 2019). In many reports, slimy deposits observed on juice screens have been associated with the growth of *Leuconostoc* spp. (Lillehoj et al. 1984; Rein 2007).

Although bacterial identification based on 16S rRNA gene sequencing is the foundation of modern taxonomy (Woese 1987), analysis based on pair-wise alignment of 16S rRNA gene sequences has revealed a high similarity amongst strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Wang et al. 2008). Sequencing of housekeeping genes showed improved differentiation between these two species (Blackwood et al. 2004; Wang et al. 2007). The gene encoding the beta subunit of DNA-directed RNA polymerase in *Bacillus* spp. (*rpoB*) is highly conserved (Mollet et al. 1997), has a single copy in the genome, and is approximately 3.5 kb in length (Ki et al. 2009). Restriction enzyme analyses of genes encoding 16S rRNA (amplified ribosomal DNA restriction analysis; ARDRA) have been used to differentiate

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B. amyloliquefaciens, Bacillus licheniformis, Bacillus pumilis and *B. subtilis* (Jeyaram et al. 2011; Wu et al. 2006).

In the present study, 104 exopolysaccharide-producing bacteria isolated from a South African sugarcane processing factory were identified to species level by ARDRA, using restriction enzymes *Rsa*I, *Hha*I and *Hinf*I, and their identities confirmed with *rpoB* gene sequencing.

Materials and methods

Sampling and isolation of bacteria

Isolation, screening and culturing of the isolates were as described by Nel et al. (2019). A total of 104 exopolysaccharide-producing bacteria were isolated from the juice screen and mixed juice (MJ) tank in a South African sugarcane processing factory when low (< 70 ppm) and high (> 500 ppm) concentrations of dextran in the produced sugar were reported by the South African Sugar Terminals (SAST). Dextran concentrations in the raw sugar were determined by SAST using a modified alcohol haze method (Anon 2015). The method quantifies gums, defined as polysaccharides of high molecular weight precipitated from aqueous solutions in the presence of acidified ethanol (Imrie and Tilbury 1972), against dextran standards. In sugarcane processing streams, these gums may include a collection of polysaccharides, including structural plant polysaccharides, hemicelluloses and starch, as well as polysaccharides from bacterial metabolism such as dextran and levan.

Amplified ribosomal DNA restriction analysis (ARDRA)

Genomic DNA of the gum-producing bacteria was isolated as previously described (Nel et al. 2019). The ARDRA method developed by Jeyaram et al. (2011) was used. Genomic DNA (100 ng) was suspended in 50-µl reaction mixture that contained 10 pmol of each primer, 200 µM of each deoxynucleoside triphosphate (Thermo Scientific), 10 µl of 5 × One Taq Standard Reaction buffer and 1.25 U of One Taq Hot Start DNA polymerase (Thermo Scientific). The primers used to amplify the 16S rRNA gene were fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCCGCA-3′) (Weisburg et al. 1991). The PCR reaction was performed in a programmable thermal cycler (MultiGene OptiMax, Labnet International, Whitehead Scientific, Cape Town, South Africa) with an initial denaturation step (94 °C, 30 s), followed by 30 cycles of denaturation (94 °C, 30 s), primer annealing (65 °C, 30 s) and elongation (68 °C, 90 s). Cycling was completed by a final elongation step (68 °C, 10 min), followed by cooling to 4 °C. Restriction analysis of the amplified 16S rRNA genes was performed using FastDigest *Rsa*I, *Cfo*I (*Hha*I) and *Hinf*I enzymes (Thermo Scientific). Restriction digestion was carried out in 15-µl reaction mixtures containing 1.5 µl of FastDigest Green Buffer, 10-µl template DNA, 3-µl sterile deionised water and 0.5 µl of each restriction enzyme, respectively, and incubated at 37 °C for 10 min. Restriction fragments were separated on a 2% (w/v) agarose gel, stained with ethidium bromide and the DNA fragments visualized under UV light.

PCR amplification, partial sequencing and phylogenetic analysis of the *rpoB* genes

A fragment of the *rpoB* gene (positions 6–585) was amplified by PCR using primers rpoB-f (5′-AGGTCAACTAGTTCAGTATGGAC-3′) and rpoB-r (5′-AAGAACCGTAACCGGCAACTT-3′), according to the method of de Clerck et al. (2004). Reactions were carried out in 50-µl reaction mixtures containing 10 pmol of each primer, 200 µM of each deoxynucleoside triphosphate (Thermo Scientific), 10 µl of 5 × One Taq Standard Reaction buffer, 1.25 U of One Taq Hot Start DNA polymerase (Thermo Scientific) and 100 ng of genomic DNA. DNA amplification was performed in a programmable thermal cycler (MultiGene OptiMax, Labnet International) with an initial denaturation step (94 °C, 30 s), followed by 30 cycles of denaturation (94 °C, 30 s), primer annealing (51 °C, 30 s) and elongation (68 °C, 60 s). Cycling was completed by a final elongation step (68 °C, 10 min), followed by cooling to 4 °C. The resultant amplicons were purified with the DNA Clean and Concentrator™-25 kit (Zymo Research, Inqaba Biotechnical Industries, Hatfield Pretoria, South Africa), according to the manufacturer's instructions. Sequencing was performed using BigDye Cycle Sequencing chemistry (Applied Biosystems, Johannesburg, South Africa) according to the manufacturer's instructions.

Sequence similarity searches were done using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990), and the results indicated that the *rpoB* gene sequences which were amplified from each of the 104 *Bacillus* isolates were most similar to the *rpoB* gene sequences from either *B. amyloliquefaciens* or *B. subtilis*. A data matrix of the representative *rpoB* gene sequences, based on sequence similarities at each sampling location (juice screen and mixed juice tank) for each of the two sampling occasions (when low and high dextran levels were reported in the produced sugar, respectively), was created. Reference strain *rpoB* gene sequences were retrieved from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The data matrix was edited using the BioEdit Sequence Alignment Editor program (Hall 1999) and the sequences aligned using CLUSTAL W (Thompson et al. 1994). A phylogenetic tree was constructed using the Neighbor-Joining (NJ) algorithm with the Tamura

3-parameter method (Tamura 1992) using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar et al. 2016). The strengths of the internal branches of the resultant tree were statistically evaluated by bootstrap analysis (Felsenstein 1985) with 1000 bootstrap replications. The tree was rooted by *B. licheniformis* NRRL NRS-1264^T as the outgroup.

Results and discussion

Amplified ribosomal DNA restriction analysis (ARDRA)

Isolates were grouped according to the areas they were sampled from, i.e., the juice screen and the mixed juice tank, at times when raw sugar with either low or high dextran levels was produced. The number of isolates that displayed identical banding patterns is indicated in brackets, above the line numbers in Fig. 1. Identical banding patterns were obtained for all 104 isolates when amplified 16S rDNA was digested with restriction enzymes *Cfo*I (*Hha*I) and *Hinf*I (Fig. 1). However, two different DNA profiles were obtained when the amplified DNA were digested with *Rsa*I (Fig. 1). The sizes of the DNA fragments corresponded to the fragment sizes reported when amplified 16S rDNA of *B. subtilis* MTCC 2451 and *B. amyloliquefaciens* MTCC 1270 were digested with *Rsa*I, *Cfo*I and *Hinf*I (Jeyaram et al. 2011). Digestion of amplified 16S rDNA of *B. subtilis* MTCC 2451 with these three enzymes yielded 11 well-separated fragments, whereas amplified 16S rDNA of *B. amyloliquefaciens* MTCC 1270 yielded 10 DNA fragments (Jeyaram et al. 2011). Based on the two sets of banding patterns obtained, only eight of the 104 isolates were classified as *B. subtilis* (represented in lanes 4 and 6, Fig. 1). Seven of the isolates (lane 4, Fig. 1) were obtained from a juice screen filtering sugarcane juice, and one isolate (lane 6, Fig. 1) was cultured from the mixed juice tank when sugar with a high dextran content was produced. At this time, most of the bacteria isolated from juice screens (46 isolates, lane 3, Fig. 1) and the mixed juice tank (31 isolates, lane 5, Fig. 1) were identified as *B. amyloliquefaciens*. No *B. subtilis* isolates were found when low dextran raw sugar was produced, and of the 19 *B. amyloliquefaciens* isolates cultured during this time, 14 were from the juice screen (lane 1, Fig. 1) and five from the mixed juice tank (lane 2, Fig. 1).

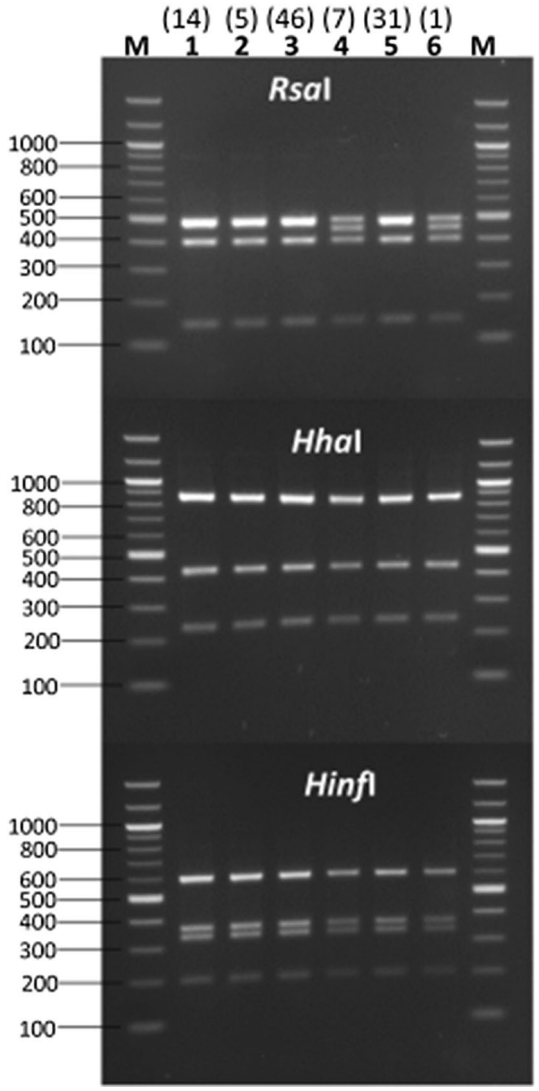

Fig. 1 ARDRA profiles recorded for 104 *Bacillus* isolates digested with *Rsa*I, *Hha*I and *Hinf*I, respectively. Each lane represents a specific sampling point and sampling time. The number of isolates with the same DNA profile is indicated in brackets. Lanes 1 and 2: DNA profiles of isolates sampled from the juice screen and mixed juice tank, respectively, when low dextran raw sugar was produced; lanes 3 and 4: isolates sampled from the juice screen when high dextran raw sugar was produced; lanes 5 and 6: isolates sampled from the mixed juice tank when high dextran raw sugar was produced. A QuickLoad® 100 bp DNA ladder (New England BioLabs, Inqaba Biotechnical Industries, Hatfield Pretoria, South Africa) was used as a size marker (M)

Amplification and partial sequencing of the rpoB gene

The phylogenetic analysis of 104 partial *rpoB* gene sequences and representative *Bacillus* strains (Fig. 2), revealed that most (96) of the isolates clustered with the type strain *B. amyloliquefaciens* B-14393^T (Fig. 2) and confirmed the identifications based on ARDRA. Eight isolates clustered with the type strains of *B. subtilis* subsp. *subtilis* (LMG 7135^T) and *Bacillus subtilis* subsp. *inaquosorum* (NRRL B-2305^T) and are regarded as members of the species *B. subtilis.* Of the eight isolates, B7–19 is phylogenetically more closely related to *B. subtilis* subsp. *inaquosorum*, and strain B7–37 more closely related to *B. subtilis* subsp. *subtilis* (Fig. 2). Results obtained

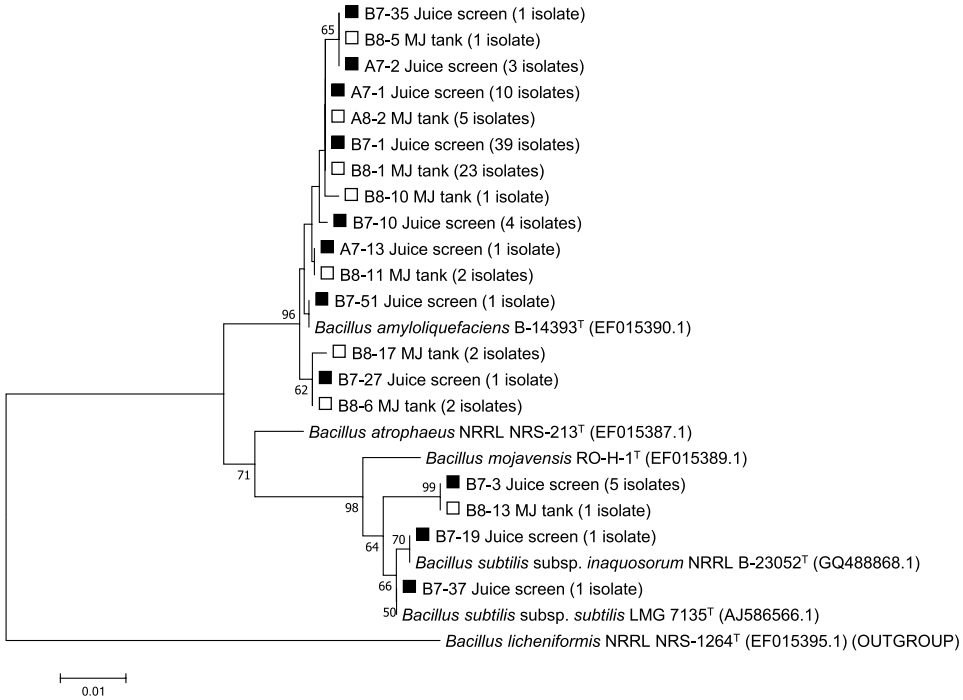

Fig. 2 Phylogenetic tree of the partial *rpoB* gene sequences amplified from *Bacillus* spp. isolated from the juice screen (black square) and mixed juice (MJ) tank (white square) of a South African sugarcane processing factory when low dextran concentrations (representative isolates prefixed by 'A') and high dextran concentrations (representative isolates prefixed by 'B') in the produced sugar were reported. The tree was constructed using the Neighbor-Joining method (Saitou and Nei 1987) with the Tamura 3-parameter model (Tamura 1992). Bootstrap values (> 50%, 1000 replications) are shown at each node. Bar, % estimated substitution per nucleotide position. *Enterococcus faecalis* DSM 20478^T was used as the outgroup

with *rpoB* gene sequence analyses confirmed the separation of the representative isolates from *B. amyloliquefaciens,* as reported with ARDRA.

The majority of *Bacillus* isolates (92%) from cane juice are members of *B. amyloliquefaciens*. Historically, *Leuconostoc mesenteroides* has been associated with slime formation on juice screens and was identified as the main contaminant of cane juice in the mixed juice tank. In this study, we have shown that *B. amyloliquefaciens*, and to a lesser extent *B. subtilis,* could be responsible for some of the exopolysaccharide (slime or gum) production observed in cane processing factories. *B. amyloliquefaciens* and *B. subtilis* are generally not associated with dextran production. However, these bacteria have the ability to produce levan (a fructose-based exopolysaccharide) from sucrose (Marvasi et al. 2010; Tian et al. 2011). Due to the non-specific nature of the modified alcohol haze method used in the sugar industry for dextran quantification (Anon 2015), levan could contribute to what is measured as dextran. Contamination of sugarcane factories with *Bacillus* spp. would, thus, lead to lower sugar production if sucrose is converted into levan, and the build-up of exopolysaccharides may increase the viscosity of the sugarcane syrup and prevent the crystallization of sucrose (Godshall et al. 1996; Jimenez 2005).

Conclusions

Digestion of amplified 16S rRNA genes with *Rsa*I differentiated *B. amyloliquefaciens* from *B. subtilis.* Identification was confirmed by partial sequencing of the *rpoB* gene. The identification of *B. amyloliquefaciens* and *B. subtilis* from a sugar processing factory is significant as these bacteria can contribute to sucrose losses, thus lowering the amount of sugar produced, and possibly producing unwanted metabolites such as exopolysaccharides.

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