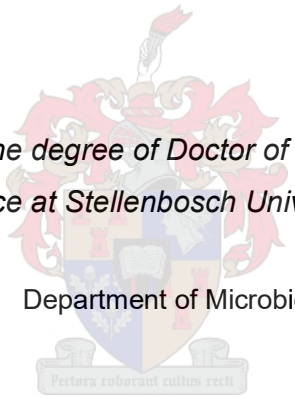
Isolation and identification of polysaccharide (gum)-producing bacteria from a sugarcane factory and strategies to prevent their growth

by

Sanet Nel



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Department of Microbiology

Promoter: Prof. L. M. T. Dicks

Co-promoters: Prof. A. Endo

Mr S. B. Davis

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Declaration

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Sanet Nel

Summary

The biodeterioration (or microbial degradation) of sugarcane is estimated to be costing the South African sugar industry in excess of R 1 billion per year in theoretically avoidable losses. The activity of polysaccharide (gum)-producing bacteria in particular is responsible for substantial economic losses. The South African sugar industry is currently experiencing several challenges that are threatening its sustainability. The focus therefore needs to be on improving sugar quality (to secure preferential markets) and production efficiency to ensure that maximum value can be recovered from the sugarcane crop.

Limited data are available on the identity of gum-producing bacteria in sugarcane processing factories. The first aim of this study was to determine and compare the diversity of gum-producing bacteria in sugarcane processing streams (*viz.* on harvested sugarcane and in various factory streams) during "good" (no gum-related problems reported by the factory) and "bad" (high dextran levels in raw sugar as reported by the factory) conditions. A total of 430 gum-producing bacteria were isolated; 110 strains during spring, when low dextran concentrations were reported in the raw sugar, and 320 strains during summer, when high dextran raw sugar was produced. An unexpected finding was that the same species were isolated at both sampling times. Based on phylogenetic analysis of 16S rRNA gene sequences, the isolates were identified as belonging to the genus *Weissella* (47%), followed by members of *Bacillus* (24%), *Leuconostoc* (19%) and *Lactobacillus* (10%). The incoming sugarcane, sampled as prepared (shredded) cane, was the major source of gum-producing bacteria at times when high dextran raw sugar was produced.

Methods with high discriminatory power were used to identify the isolated bacteria to species level. *Weissella confusa* and *Weissella cibaria* were identified based on phylogenetic analyses of housekeeping genes *pheS* (encoding phenylalanyl t-RNA synthase alpha subunit), *dnaA* (encoding chromosomal replication initiation protein) and *atpA* (encoding alpha subunit of ATP synthase). *Bacillus amyloliquefaciens* and *Bacillus subtilis* were differentiated based on *rpoB* (encoding the beta subunit of DNA-directed RNA polymerase) gene sequencing and amplified ribosomal DNA restriction analysis (ARDRA). Multilocus sequence analyses of housekeeping genes *rpoA* (encoding RNA polymerase alpha subunit), *dnaA*, *pheS* and *tuf* (encoding elongation factor Tu) were used to identify members of the genera *Leuconostoc* and *Lactobacillus*.

Contrary to expectations, *Leuconostoc mesenteroides* was not the major gum-producing bacterium isolated from sugarcane. Instead, high cell numbers of *W. confusa* and *W. cibaria* were recorded. The dominance of *Weissella* spp. on the prepared cane is significant because these bacteria are not usually associated with deteriorated sugarcane and have not previously been isolated from a sugarcane processing factory. This study also report, for the first time, on the isolation of *B. amyloliquefaciens* from a sugarcane processing factory.

The second aim of the study was to evaluate the efficacy of biocides to prevent/limit microbial growth in the factory. Two dithiocarbamate biocides, Busan®1021 and Preventol®Z, each at a 20 ppm dose, were tested against seven gum-producing bacteria. Preventol®Z demonstrated a bactericidal (killing) effect against *Le. mesenteroides* A16-9*, Le. lactis* B9-3*, B. subtilis* B7-19 and *B. amyloliquefaciens* B7-51 after 6 h of contact, but had only a bacteriostatic (growth inhibiting) effect on *W. cibaria* A1-17*, W. confusa* B1-24 and *Lb. fermentum* B19-18 when tested under the same conditions. Busan®1021 had a bactericidal effect on all seven species. *Bacillus subtilis* B7-19 and *B. amyloliquefaciens* B7-51 were susceptible to both biocides, but only for the first 2 h of exposure, after which the killing effect remained constant. Based on results obtained in this study, the concentrations of Preventol®Z and Busan®1021 may need to be increased, or dosage intervals altered, to kill all gum-producing bacteria.

Although sugarcane processing factories have little to no control over the quality of cane entering the factory, poor sanitation in the factory and incorrect process control can also contribute to sucrose loss due to microbial activities and subsequent gum formation. Factories should therefore be mindful of correctly controlling high-temperature processes and reduce the recirculation of sump contents which are not treated with biocides. This study provided valuable knowledge on the identities of gum-producing bacteria in sugarcane processing factories, and their susceptibility to two commercial biocides.

Opsomming

Die biodeteriorasie (of mikrobiese afbraak) van suikerriet kos die Suid-Afrikaanse suikerbedryf na raming meer as R 1 miljard per jaar in teoreties vermybare verliese. Die aktiwiteite van polisakkaried (slym)-produserende bakterieë is veral verantwoordelik vir aansienlike ekonomiese verliese. Die Suid-Afrikaanse suikerbedryf beleef tans 'n hele paar uitdagings wat die volhoubaarheid daarvan bedreig. Die fokus moet dus wees op die verbetering van suikergehalte (om voorkeurmarkte te verseker) en produksie doeltreffendheid te verbeter sodat die maksimum waarde uit die suikerriet oes verhaal kan word.

Beperkte data is beskikbaar aangaande die identiteite van slym-produserende bakterieë in suikerrietverwerkingsfabrieke. Die eerste doel van hierdie studie was om die diversiteit van slym-produserende bakterieë in suikerrietverwerkingstrome (van geoeste suikerriet en in verskeie fabriekstrome) te bepaal en te vergelyk tydens "goeie" (m.a.w geen slym-verwante probleme deur die fabriek ondervind nie) en "slegte" toestande (hoë dekstraanvlakke in rou suiker soos gerapporteer deur die fabriek). ‘n Totaal van 430 slym-produserende bakterieë is geïsoleer; 110 stamme in die lente, tydens die voorkoms van lae dekstraan konsentrasies in onverwerkte suiker en 320 stamme in die somer, tydens die voorkoms van hoë-dekstraan in die onverwerkte suiker. ‘n Overwagte bevinding was dat dieselfde spesies geïsoleer is tydens beide monsternemingsgeleenthede. Die isolate, geïdentifiseer deur filogenetiese analise van 16S rRNS basisopeenvolgingsbepaling, vorm deel van die genus *Weissella* (47%), gevolg deur lede van *Bacillus* (24%), *Leuconostoc* (19%) en *Lactobacillus* (10%). Die inkomende suikerriet was die hoofbron van slym-produserende bakterieë tydens die produksie van hoë-dekstraan onverwerkte suiker.

Taksonomiese metodes met 'n hoë diskriminerende krag is gebruik om die geïsoleerde bakterieë tot op spesie vlak te identifiseer. *Weissella confusa* en *Weissella cibaria* is geïdentifiseer op grond van filogenetiese analise van huishoudingsgene *pheS* (wat kodeer vir die fenielalaniel o-RNS sintase alfa-subeenheid), *dnaA* (wat kodeer vir die chromosomale replikasie inisiasie proteïen) en *atpA* (wat kodeer vir die alfa-subeenheid van adenosientrifosfaatsintase). *Bacillus amyloliquefaciens* en *Bacillus subtilis* is onderskei op grond van die *rpoB* geen (wat kodeer vir die beta-subeenheid van DNS-gerigte RNS-polimerase) en amplifiserende ribosomale DNS beperkingsanalises. Multilokus volgorde ontleding van die huishoudingsgene *rpoA* (wat kodeer vir RNS-polimerase alfa-subeenheid), *dnaA*, *pheS* en *tuf* (wat kodeer vir die verlengingsfaktor Tu) is gebruik om lede van die genera *Leuconostoc* en *Lactobacillus* te identifiseer.

Teenstrydig met verwagtinge was *Leuconostoc mesenteroides* nie die hoof slym-produserende bakterie wat van suikerriet geïsoleer is nie. In plaas daarvan is hoë selgetalle van *W. confusa* en *W. cibaria* aangeteken. Die oorheersing van *Weissella* spp. op die suikerriet is belangrik omdat hierdie bakterieë nie gewoonlik met vrot suikerriet geassosieer word nie en is ook nog nie voorheen uit 'n suikerrietverwerkingsfabriek geïsoleer nie. Hierdie studie doen ook vir die eerste keer verslag oor die isolering van *B. amyloliquefaciens* uit 'n suikerrietverwerkingsfabriek.

Die tweede doel van die studie was om die doeltreffendheid van kommersiële antibakteriële middels wat mikrobiese groei verhoed of beperk te evalueer. Twee ditiokarbamaat middels, Busan®1021 en Preventol®Z, elkeen teen 'n 20 dpm dosis, is getoets teen sewe slym-produserende bakterieë. Preventol®Z het ‘n dodende effek teen *Le. mesenteroides* A16-9, *Le. lactis* B9-3, *B. subtilis* B7-19 en *B. amyloliquefaciens* B7-51 na 6 ure van kontak getoon, maar het net 'n bakteriostatiese (groei inhiberende) effek op *W. cibaria* A1-17, *W. confusa* B1-24 en *Lb. fermentum* B19-18 gehad wanneer onder dieselfde toestande getoets is. Busan®1021 het 'n dodende uitwerking op al sewe spesies gehad. *Bacillus subtilis* B7-19 en *B. amyloliquefaciens* B7-51 was vatbaar vir beide middels, maar net vir die eerste 2 ure van blootstelling, waarna die dodende effek konstant gebly het. Gebaseer op die resultate van hierdie studie moet die konsentrasies van Preventol®Z en Busan®1021 verhoog, of doseringsintervalle verander word, om alle slym-produserende bakterieë dood te maak.

Hoewel suikerrietverwerkingsfabrieke min of geen beheer het oor die kwaliteit van die suikerriet wat hul ontvang nie, kan swak sanitasie in die fabriek en verkeerde prosesbeheer ook bydra tot sukrose verliese as gevolg van mikrobiese aktiwiteite en die geassosieerde slym produksie. Fabrieke moet dus bewus wees van die korrekte beheer, veral van hoë-temperatuur prosesse en die hersirkulasie van onbehandelde stortingstrome verminder. Hierdie studie verskaf waardevolle inligting oor die identiteite van slym-produserende bakterieë in suikerrietverwerkingsfabrieke, en hul vatbaarheid vir twee kommersiële antibakteriële middels.

Preface

This dissertation is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the respective journal. Four articles have been published and two manuscripts have been submitted to ISI-accredited journals for publication.

The literature review provides an overview of the sugar production process in a South African context, with an emphasis on microbial diversity of spoilage bacteria in the field and in the sugarcane processing factories. Microbial identification methods, as well as recent revisions in lactic acid bacterial taxonomy, are also discussed.

The manuscript “Post-harvest biodeterioration of sugarcane: taxonomic history and current perspectives” has been published in the *International Sugar Journal* [2017; CXIX (1424): 632-637] and is presented in Chapter 3 as published. This manuscript was also taken-up in the 2018 *International Sugar Journal* World Sugar Yearbook, pp. 65-71.

The manuscript “Microbial diversity profiling of polysaccharide (gum)-producing bacteria isolated from a South African sugarcane processing factory” has been published in *Current Microbiology* (2019; 76: 527-535) and is presented in Chapter 4 as published.

The manuscript “Phylogenetic analyses of *pheS, dnaA* and *atpA* genes for identification of *Weissella confusa* and *Weissella cibaria* isolated from a South African sugarcane processing factory” has been published in *Current Microbiology* (2019; 76: 1138-1146) and is presented in Chapter 5 as published.

The manuscript “Differentiation between *Bacillus amyloliquefaciens* and *Bacillus subtilis* isolated from a South African sugarcane processing factory using ARDRA and *rpoB* gene sequencing” has been published in *Archives of Microbiology* (2019; 201: 1453-1457) and is presented in Chapter 6 as published.

The manuscript “Identification of *Leuconostoc* and *Lactobacillus* species isolated from shredded sugarcane and different stages of sugarcane processing” has been submitted for publication in *MicrobiologyOpen* (December 2019) and is presented in Chapter 7.

The manuscript “Effect of dithiocarbamate biocides on gum-producing bacteria isolated from a South African sugarcane processing factory” has been published in the *International Sugar Journal* [2019; 121 (1451): 820-825)] and is presented in Chapter 8.

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Contents

CHAPTER 1

Introduction

Introduction

Crystal sugar is the main source of revenue sustaining the agricultural and milling sectors of the South African sugar industry. The sucrose in the standing crop is not all recovered as saleable sugar; losses occur during burning, harvesting, transporting and processing of the sugarcane. The activity of polysaccharide (gum)-producing bacteria in the sugarcane processing industry is responsible for substantial economic losses, both directly and indirectly. The use of recoverable sucrose by bacteria for growth and metabolic activities reduces the amount of sucrose available to be sold as sugar. The average sucrose loss due to cane deterioration has been estimated at 10% of the sucrose that could be delivered to the factory (De Robillard et al., 1990). In South Africa, this could represent a loss of up to 250 000 tons of saleable sugar or R 1.6 billion per year. As much as 93% of sucrose loss prior to milling is thought to be due to microbial action (Eggleston, 2002). These estimates indicate that the biodeterioration (or microbial degradation) of sugarcane may be costing the South African sugar industry in excess of R 1 billion per year in theoretically avoidable losses.

The South African sugar industry is a mature, but declining, industry under severe pressure. An extended drought period from 2014/15 to 2017/18 resulted in 2016/17 recording the lowest annual sugar production since 1993/94, with only 1.54 million tonnes of sugar produced at the time, 80 000 tonnes less than the season before. During 2017/18 approximately 500 000 tonnes of dumped sugar were imported into South Africa due to an inadequate Dollar-Based Reference Price (DBRP), and consequently the domestic sales of South African produced sugar fell to below 1.2 million tonnes of sugar; the lowest it has been since 1999/2000. Sugar imports are subject to the basic economic principles of supply and demand, with the lower the price, the higher the demand. A significant increase of duty free sugar imports from eSwatini (previously known as Swaziland) into South Africa in 2018 had a direct negative impact on the South African share of the domestic market and the competitiveness of South Africa's sugar industry. Each imported tonnage of sugar increases the local sugar surplus, and in turn, that results in increased exports that realise a lower export price, which reduces the size of the income earned by the industry. Despite an increased production in sugar by 10% from 2017/18 (1.99 million tonnes) to 2018/19 (2.19 million tonnes), the local market demand for sugar dropped by 4% in 2018/19. This reduced demand can partially be attributed to product reformulations by the beverage industry in response to the implementation of the Health Promotion Levy (commonly referred to as the sugary beverages tax) which came into effect on 1 April 2018 (Madho et al., 2018; Singels et al., 2019).

At a time when the South African sugar industry is experiencing several challenges which are threatening its sustainability, focus needs to be on improving sugar quality (to secure preferential markets) and production efficiency to ensure that maximum value can be recovered from the sugarcane crop. As such, a theoretical avoidable sucrose loss in excess of R 1 billion due to microbial spoilage of sugarcane should receive urgent attention.

In addition to the direct sucrose loss, microorganisms produce exopolysaccharides (such as dextran), which also contribute to a loss in revenue. Exopolysaccharides are commonly referred to as 'gums' in the sugar industry. The gums enter the factory with the deteriorated sugarcane and greatly reduce factory throughput due to the viscous nature of the gums. High concentrations of gums in the processing streams also interfere with the recovery of sucrose. Furthermore, dextran is transferred from the sugarcane juice to the sugar crystal, and high concentrations of dextran in the produced sugar prevent the sale of raw sugar to financially attractive markets, resulting in tonnes of produced sugar for which the only market is at greatly discounted prices.

Sugarcane deterioration begins after burning and harvesting of the cane. The longer the cane is left in the field, damaged by burning and cutting and exposed to microorganisms in the soil and surrounding environment, the higher the degree of deterioration and sucrose loss. Research in the 1960s to 1980s focussed on identifying the major spoilage bacterium responsible for 'sour' cane, so called due to the distinctive sour smell of the rotting cane. Unfortunately, sugar technologists at the time were limited to bacterial identification methods based on morphological, physiological and biochemical characteristics (phenotypic methods). These methods, when used in isolation, often yield ambiguous results, as the observable characteristics of bacteria may appear different under varying environmental conditions. A few studies reported on the isolation and identification of sugarcane spoilage bacteria, and the presence of *Leuconostoc mesenteroides* was a common outcome of these studies (Bevan and Bond, 1971; Egan and Rehbein, 1963; McNeil and Bond, 1980; Tilbury, 1970). Although other bacteria were also isolated from deteriorated cane and factory processing streams, and it was acknowledged that the differentiation between species of *Leuconostoc* and *Lactobacillus* was difficult (McNeil and Bond, 1980), many sugar technologists, to this day, will confidently declare that *Le. mesenteroides* is the (sole) causative agent of post-harvest cane deterioration. However, this statement is not justified by research using microbial identification methods of high discriminatory power, and scientific literature on microbial diversity in sugarcane processing factories beyond the mid-1980s are severely lacking.

Despite the substantial economic losses caused by gum-producing bacteria in the sugar industry, current methods to prevent these losses, and potential solutions to reduce the undesirable effect of the produced gums on processing, are almost non-existent. Sugarcane processing factories have little to no control over the quality of the cane delivered to the factory, as there is currently no penalty for delivering deteriorated cane for processing. Precise biological data relating to sugarcane deterioration, and in particular, the causative microorganisms, is limited. Recent developments in bacterial taxonomy and related technologies now enable the profiling of gum-producing bacteria in sugarcane processing factories, using methods of high discriminatory power to identify the bacteria.

The objectives of this study were to:

1. Establish a profile consisting of the location and identity of gum-producing bacteria present in, or on, harvested sugarcane and factory processing streams during “good” (low concentrations of dextran in the produced sugar) and “bad” (high concentrations of dextran in the raw sugar) conditions (as reported by the factory).
2. Evaluate the efficacy of commercially available microbicides used by the sugar industry to control bacterial growth.

The final outcome of this study was to gain knowledge on the identities of spoilage bacteria in sugarcane processing factories, to provide a foundation for the development of processes and/or recommendations on how to reduce post-burning/post-harvest deterioration of sugarcane and subsequent processing streams, and the associated revenue losses.

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CHAPTER 2

Literature review

Literature review

Microbial diversity in sugarcane processing

Growing sugarcane in the field (pre-harvest)

Microorganisms naturally associated with healthy sugarcane (*i.e*. pre-harvest) play a vital role in sustaining soil fertility and plant health. These endophytic and plant-associated microorganisms inhabit various tissues of the sugarcane plant, including the roots, stems and leaves, without causing disease and may contribute directly to plant growth by promoting nutrient availability, biological nitrogen fixation and the production of phytohormones (Kumar et al., 2017). Endophytes may also reduce microbial populations that are harmful to the plant through induced resistance (Mehnaz, 2011). Table 1 provides a comprehensive list of bacterial genera and species that have been associated with healthy (non-diseased, pre-harvest) sugarcane.

Table 1 Sugarcane-associated bacteria (Adapted from Mehnaz, 2011)

Bacteria	Source	Country	Reference
Acinetobacter sp.	Rhizosphere, roots, stems	Brazil, China	Beneduzi et al. (2013), Dong et al. (2018)
Acinetobacter baumanii	Apoplast	Cuba	Velázquez et al. (2008)
Achromobacter sp.	Roots, stems	Brazil	Beneduzi et al. (2013)
Agrobacterium sp.	Rhizosphere, roots, stems	Brazil	Beneduzi et al. (2013)
Agrobacterium tumefaciens	Stem, rhizosphere, roots	China, Brazil	Xing et al. (2006), Beneduzi et al. (2013)
Alcaligenes sp.	Stems	Brazil	Beneduzi et al. (2013)
Arthrobacter sp.	Roots, leaves	China, Iran	Dong et al. (2018), Pirhadi et al. (2017)
Asaia bogorensis	Rhizosphere, stems	Brazil	Beneduzi et al. (2013)
Azorhizobium sp.	Rhizosphere, roots	Brazil	Beneduzi et al. (2013)
Azospirillum sp.	Rhizosphere, roots	Egypt, South Africa, Brazil, India	Gangwar and Kuar (2009), Hegazi et al. (1979), Purchase (1980), Ruschel (1981), Beneduzi et al. (2013)
Azospirillum brasilense	Rhizospere, root, stem, leaves	Spain, Pakistan, Brazil	Graciolli et al. (1983), Mehnaz et al. (2010), Reis et al. (2000), Tejera et al. (2005), Beneduzi et al. (2013)
Azospirillum lipoferum	Root, stem, leaves	Brazil	Dobereiner and Day (1976), Reinhardt et al. (2008), Reis et al. (2000), Tejera et al. (2005)
Azospirillum amazonense	Roots, stem	Brazil	Cavalcante and Dobereiner (1988), Reis et al. (2000)
Azotobacter chroococum	Roots	Spain	Tejera et al. (2005)

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Azotobacter vinelandii	Rhizosphere, roots	Egypt, Brazil	Graciolli et al. (1983), Hegazi et al. (1979), Rennie (1980)
Bacillus spp.	Rhizosphere, roots, stem	South Africa, India, Pakistan, Brazil, Iran	Gangwar and Kuar (2009), Hassan et al. (2010), van Antwerpen et al. (2002), Beneduzi et al. (2013), Pirhadi et al. (2017)
Bacillus cereus	Apoplast	Cuba	Velázquez et al. (2008)
Bacillus pumilus	Apoplast	Cuba	Velázquez et al. (2008)
Bacillus subtilis	Apoplast, rhizosphere	Cuba, Pakistan	Hassan et al. (2010), Velázquez et al. (2008)
Beijerinckia sp.	Root	Brazil	Vendruscolo (1995)
Beijerinckia fluminensis	Rhizosphere	Brazil	Dobereiner (1961), Dobereiner and Alvahydo (1959)
Beijerinckia indica	Rhizosphere, roots	Brazil	Dobereiner et al. (1972)
Brevibacillus sp.	Stem, leaves	Brazil	Magnani et al. (2010)
Burkholderia spp.	Stem, leaves, rhizosphere, roots	South Africa, Brazil	Perin et al. (2006b), van Antwerpen et al. (2002), Beneduzi et al. (2013)
Burkholderia ambifaria	Rhizosphere, roots	South Africa	Omarjee et al. (2008)
Burkholderia cepacia	Rhizosphere, roots, stem	Brazil, South Africa	Luzivotto et al. (2010), Mendes et al. (2007), Omarjee et al. (2008), Beneduzi et al. (2013)
Burkholderia cenocepacia	Roots, stem	Brazil	Mendes et al. (2007)
Burkholderia fungorum/ graminis	Rhizosphere, roots	South Africa	Omarjee et al. (2008)
Burkholderia gladioli	Rhizosphere, roots, stem	South Africa	Omarjee et al. (2008), Omarjee et al. (2004)
Burkholderia plantarii/ glumae	Stem	Papua New Guinea	Omarjee et al. (2004)
Burkholderia sacchari	Rhizosphere	Brazil	Bramer et al. (2001)
Burkholderia silvatlantica	Rhizosphere, roots, leaves	Brazil	Omarjee et al. (2008), Perin et al. (2006a)
Burkholderia tropica	Rhizosphere, roots	South Africa, Mexico, Brazil	Omarjee et al. (2008), Perin et al. (2006b), Reis et al. (2004), Beneduzi et al. (2013)
Burkholderia unamae	Stem	Papua New Guinea, Brazil, Mexico	Caballero-Mellado et al. (2004), Omarjee et al. (2004), Perin et al. (2006b)
Burkholderia vietnamiensis	Stem	India	Govindrajan et al. (2007)
Caulobacter crescentus	Roots	Pakistan	Mehnaz et al. (2010)
Citrobacter sp.	Rhizosphere	Brazil	Magnani et al. (2010), Beneduzi et al. (2013)
Clostridium pasteuranum	Rhizosphere	Brazil	Beneduzi et al. (2013)
Comamonas testosteroni	Apoplast	Cuba	Velázquez et al. (2008)
Corynebacterium sp.	Roots	Iran	Pirhadi et al. (2017)
Curtobacterium sp.	Stem	Brazil	Magnani et al. (2010)
Delftia acidovorans	Stem, leaves	Pakistan	Mehnaz et al. (2010)
Derxia gummosa	Rhizosphere	Brazil	Graciolli et al. (1983), Rennie (1980)
Devosia sp.	Roots	China	Dong et al. (2018)
Ensifer sp.	Roots	China	Dong et al. (2018)
Enterobacter sp.	Rhizosphere, roots, stem, leaves	Brazil, Australia, Iran	Li and Macrae (1992), Magnani et al. (2010), Beneduzi et al. (2013), Pirhadi et al. (2017)

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Enterobacter aerogenes	Stem	Pakistan	Mehnaz et al. (2010)
Enterobacter cloacae	Rhizosphere, roots, stem	Pakistan, Brazil	Graciolli et al. (1983), Mehnaz et al. (2010), Mirza et al. (2001), Rennie (1980), Rennie et al. (1982)
Enterobacter oryzae	Stem	Pakistan	Mehnaz et al. (2010)
Erwinia cypripedii (now classified as *Pantoea cypripedii*)	Apoplast	Cuba	Velázquez et al. (2008)
Erwinia herbicola (now classified as *Pantoea agglomerans*)	Stem	Brazil	Graciolli et al. (1983), Rennie et al. (1982)
Gluconacetobacter diazotrophicus	Roots, stem, leaves, apoplast, bud, sugarcane juice	Brazil, Australia, India, Egypt, Cuba, Mexico, Philippines, Argentina	Asis et al. (2000), Bellone et al. (1997), Cavalcante and Dobereiner (1988), Dong et al. (1994), Fuentes-Ramirez et al. (1993), Gillis et al. (1989), Li and Macrae (1992), Muthukumarasamy et al. (1994), Prabudoss and Stella (2009), Reis et al. (1994), Velázquez et al. (2008), Youssef et al. (2004), Beneduzi et al. (2013)
Gluconacetobacter saccharii	Leaf sheath	Australia	Franke et al. (1999)
Herbaspirillum seorpedaceae	Stem, leaves	Brazil, Philippines	Asis et al. (2000), Baldani et al. (1992), Olivares et al. (1996)
Herbaspirillum rubrisubulbicans	Leaves	Brazil, Philippines	Asis et al. (2000), Olivares et al. (1996), Pimental et al. (1991)
Klebsiella sp.	Stem	Brazil, South Africa	Magnani et al. (2010), van Antwerpen et al. (2002), Beneduzi et al. (2013)
Klebsiella oxytoca	Rhizosphere, roots, stem	Pakistan	Mehnaz et al. (2010), Mirza et al. (2001)
Klebsiella pneumoniae	Roots, stem	India, Brazil, Australia	Govindrajan et al. (2007), Graciolli et al. (1983), Li and Macrae (1992), Rennie et al. (1982), Beneduzi et al. (2013)
Klebsiella variicola	Stem	Mexico	Rosenblueth et al. (2004)
Kocuria kristinae	Apoplast	Cuba	Velázquez et al. (2008)
Lactococcus lactis subsp. *lactis*	Leaves, stem	Colombia, Brazil	Cock and de Stauvenel (2006), Beneduzi et al. (2013)
Lentzea sp.	Roots	China	Dong et al. (2018)
Methylobacterium sp.	Rhizosphere	Brazil	Beneduzi et al. (2013)
Microbacterium oleivorans	Apoplast	Cuba	Velázquez et al. (2008)
Microbacterium resistens	Stem	Brazil	Beneduzi et al. (2013)
Microbacterium testaceum	Stem	Brazil	Mendes et al. (2007)
Micrococcus luteus	Apoplast	Cuba	Velázquez et al. (2008)
Ochrobacterium sp.	Rhizosphere, stem	Brazil	Beneduzi et al. (2013)
Ochrobactrum intermedium	Rhizosphere	Pakistan	Hassan et al. (2010)
Ohtaekwangia sp.	Roots	China	Dong et al. (2018)
Paenibacillus sp.	Roots	Iran	Pirhadi et al. (2017)
Paenibacillus azotofixans	Roots	Brazil, Hawaii	Cavalcante and Dobereiner (1988), Seldin and Penido (1986)
Paenibacillus graminis	Roots, stem	Brazil	Beneduzi et al. (2013)
Paenibacillus polymyxa	Roots, stem, rhizosphere	Brazil	Graciolli et al. (1983), Rennie (1980), Rennie et al. (1982), Beneduzi et al. (2013)
Pannonibacter phragmitetus	Root	Pakistan	Mehnaz et al. (2010)

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Pantoea sp.	Stem, leaves	Cuba, Brazil	Loiret et al. (2004), Magnani et al. (2010)
Pantoea ananatis	Stem, rhizosphere	Brazil	Mendes et al. (2007), Beneduzi et al. (2013)
Pantoea agglomerans	Roots	Brazil	Beneduzi et al. (2013)
Pantoea dispersa	Roots	Brazil	Beneduzi et al. (2013)
Pantoea herbicola	Roots, stem, leaves	Brazil	Graciolli et al. (1983)
Pantoea stewartii	Stem	Brazil	Mendes et al. (2007)
Pseudomonas spp.	Rhizosphere, roots, stem, leaves	Brazil, South Africa, Australia, India, China	Gangwar and Kuar (2009), Li and Macrae (1992), Magnani et al. (2010), van Antwerpen et al. (2002), Beneduzi et al. (2013), Li et al. (2017), Dong et al. (2018)
Pseudomonas aeruginosa	Stem	India	Viswanathan et al. (2003)
Pseudomonas aurantiaca	Stem	Pakistan	Mehnaz et al. (2009b)
Pseudomonas fluorescens	Roots, stem	India, Pakistan, Brazil	Mehnaz et al. (2009a), Mendes et al. (2007), Viswanathan and Samiyappan (2002)
Pseudomonas putida	Rhizosphere, roots, stem	India, Pakistan	Mehnaz et al. (2009a), Viswanathan and Samiyappan (2002), Beneduzi et al. (2013)
Pseudomonas reactans	Stem	Pakistan	Mehnaz et al. (2010)
Rahnella aquatilis	Roots	Pakistan	Mehnaz et al. (2010)
Rhizobium sp.	Roots, rhizosphere, stem	Pakistan, Brazil, China	Mehnaz et al. (2010), Beneduzi et al. (2013), Dong et al. (2018)
Rhizobium rhizogenes	Apoplast	Cuba	Velázquez et al. (2008)
Rhodococcus sp.	Leaves	Iran	Pirhadi et al. (2017)
Saccharibacteria sp.	Roots	China	Dong et al. (2018)
Saccharibacillus sacchari	Apoplast	Spain	Rivas et al. (2008)
Serratia spp.	Stem	South Africa	van Antwerpen et al. (2002)
Sinorhizobium sp.	Rhizosphere	Brazil	Beneduzi et al. (2013)
Sphingobacterium sp.	Rhizosphere	Brazil	Beneduzi et al. (2013)
Staphylococcus sp.	Stem, leaves, roots	Brazil, Iran	Magnani et al. (2010), Beneduzi et al. (2013), Pirhadi et al. (2017)
Staphylococcus epidermidis	Apoplast	Cuba	Velázquez et al. (2008)
Staphylococcus saprophyticus	Apoplast	Cuba	Velázquez et al. (2008)
Stenotrophomonas sp.	Rhizosphere, roots, stem	Brazil	Beneduzi et al. (2013)
Stenotrophomonas maltophilia	Rhizosphere, roots, stem	Pakistan, Brazil	Hassan et al. (2010), Mehnaz et al. (2010), Beneduzi et al. (2013)
Stenotrophomonas pavanii	Stem	Brazil	Ramos et al. (2010)
Streptophyta sp.	Roots	China	Dong et al. (2018)
Streptomyces sp.	Roots	China	Dong et al. (2018)
Streptomyces capoamus	Rhizosphere	Brazil	Beneduzi et al. (2013)
Xanthomonas spp.	Stem	South Africa, Pakistan, Brazil	Mehnaz et al. (2010), van Antwerpen et al. (2002), Beneduzi et al. (2013)
Xanthomonas campestris	Apoplast	Cuba	Velázquez et al. (2008)
Xanthomonas oryzae	Apoplast	Cuba	Velázquez et al. (2008)
Zymomonas sp.	Stem	South Africa	van Antwerpen et al. (2002)

Recently, De Souza et al. (2016) used community analysis based on 16S rRNA and internal transcribed spacer (ITS) rRNA profiling to explore the composition and dynamics of bacterial and fungal communities associated with roots, stalks and leaves of sugarcane. Although the study did not trace microbial succession across years, the authors demonstrated that soil communities were the main source of bacteria and fungi colonising the plant. The authors suggested that the microbial diversity present in soil colonised the plant organs at early stages of plant development and that the young shoots budding from the underground ratoon had bacterial and fungal communities very similar to those of roots. The diverse bacterial and fungal communities from the soil invaded the tissues of young plants at an early stage of development. Organs of four-month old plants had enriched microbial communities that remained relatively constant throughout the plant's growth. Surprisingly, De Souza et al. (2016) found that a core microbiome comprised of less than 20% of the total microbial diversity and represented approximately 90% of the relative abundance of bacterial and fungal operational taxonomic units (OTUs) assembled in the different plant organs. Commonly investigated microbial groups usually associated with sugarcane comprised of only a small fraction of the total diversity, and the most abundant microbial groups inhabiting sugarcane are comprised by understudied microorganisms.

Deteriorated sugarcane (post-harvest)

The South African sugar industry harvested 17.38 million tonnes of sugarcane during the 2017/18 season (Singels et al., 2018). The majority of sugarcane harvested annually in South Africa (90%) is burnt prior to harvest to remove the dry brown leaves from the stalk and to ease the working conditions of cane cutters (van Antwerpen et al., 2017). The undulating to steep topography of the majority of areas under sugarcane production in South Africa does not lend itself to mechanical harvesting. Therefore, more than 90% of the crop is cut by hand using cane knives (Meyer and Fenwick, 2003).

Sugarcane starts to deteriorate as soon as the burning and harvesting processes start (Harris, 2017). Cane left in the field after harvest become infected with a variety of microorganisms that occur naturally in the environment. As sucrose is utilised during the metabolic activities of the spoilage microorganisms, less sucrose is recovered, leading to reduced revenue. The severity of infection is dependent on the extent of stalk damage during harvest, environmental conditions post-harvest and the length of time that the cut stalks remain in the field (Watt and Cramer, 2009). Burning of the cane before harvest severely damages the stalk rind which is exposed to temperatures ranging 98 to 400 °C for several seconds, causing the rind to split and juice in the extremities of the stalk to boil (Foster, 1980). As a result, burnt cane is often

partially covered externally with juice that supports extensive microbial growth (Ravnö and Purchase, 2005).

Leuconostoc mesenteroides has been indicated as the main contributor of post-harvest cane deterioration (Eggleston et al., 2009). However, other bacteria have also been associated with deteriorated sugarcane, although to a much lesser extent (Table 2).

Table 2 Bacteria associated with deteriorated sugarcane

Bacteria	Source	Country	Reference
Aerobacter sp. (now classified as *Enterobacter* sp.)	Mechanically-harvested billeted cane	Australia	Bevan and Bond (1971)
Bacillus spp.	Burnt cane	Australia	Bevan and Bond (1971)
Bacillus megaterium	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Bacillus pumilus	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Bacillus subtilis	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Bacterium spp. (now reclassified into several genera)	Burnt cane	Australia	Bevan and Bond (1971)
Corynebacterium sp.	Burnt cane	Australia	Bevan and Bond (1971)
Enterobacter sp.	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Enterobacter cloacae	Long-standing burnt cane	Australia	McNeil and Bond (1980), McNeil and Inkerman (1977)
Enterobacter aerogenes (now classified as *Klebsiella aerogenes*)	Long-standing burnt cane	Australia	McNeil and Bond (1980), McNeil and Inkerman (1977)
Erwinia sp.	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Erwinia herbicola (now classified as *Pantoea agglomerans*)	Long-standing burnt cane	Australia	McNeil and Bond (1980), McNeil and Inkerman (1977)
Erwinia uredovora (now classified as *Pantoea ananas*)	Long-standing burnt cane	Australia	McNeil and Inkerman (1977)
Klebsiella pneumoniae	Long-standing burnt cane	Australia	McNeil and Bond (1980), McNeil and Inkerman (1977)
Lactobacillus spp.	Long-standing burnt cane	Australia	McNeil and Bond (1980)
Lactobacillus casei subsp. *alactosus*	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Lactobacillus casei subsp. *casei*	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Lactobacillus confusus (now classified as *Weissella confusa*)	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Lactobacillus plantarum	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)

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Leuconostoc spp.	Long-standing burnt cane	Australia	McNeil and Bond (1980)
Leuconostoc dextranicum (now classified as *Leuconostoc mesenteroides* subsp. *dextranicum*)	Stored mechanically-harvested billeted cane, stored burnt and manually-harvested whole stalk cane	Australia, Jamaica	Egan and Rehbein (1963), Tilbury (1970)
Leuconostoc mesenteroides	Stalks of frost-damaged cane, stored mechanically-harvested billeted cane, stored burnt and manually-harvested whole stalk cane, burnt cane, long-standing burnt cane	USA, Australia, Jamaica	McCalip and Hall (1938), Egan and Rehbein (1963), Tilbury (1970), Bevan and Bond (1971), McNeil and Inkerman (1977)
Leuconostoc paramesenteroides (now classified as *Weissella paramesenteroides*)	Stored burnt and manually-harvested whole stalk cane	Jamaica	Tilbury (1970)
Xanthomonas sp.	Burnt cane, mechanically-harvested billeted cane	Australia	Bevan and Bond (1971)

Post-harvest deterioration of sugarcane due to microbial activity results in the production of unwanted microbial metabolites such as a variety of polysaccharides (also called gums in the sugar industry, defined as alcohol-precipitable high molecular weight polysaccharides). End-products produced by spoilage bacteria include dextran, levan and sarkaran, oligosaccharides (kestoses, isomaltotriose, isomaltotetraose, leucrose and palatinose), alcohol (ethanol), sugar alcohol (mannitol), lactic acid and acetic acid (Cuddihy Jr et al., 2001; Eggleston and Grisham, 2003; Morel du Boil et al., 2005; Solomon, 2000). Dextran is considered the main problem in the South African sugarcane industry (Eggleston et al., 2008). It is therefore critical that harvested cane reaches the factory and is processed as soon as possible after burning and cutting. However, the average delay between burning/harvesting and processing, or the so-called burn/harvest-to-crush delay (B/HTCD), is often between three and five days in South Africa (Harris, 2017), resulting in substantial sucrose losses and the formation of microbial metabolites which enter the factory with the cane, often severely reducing factory throughput.

Sugarcane processing factory streams

Figure 1 depicts a schematic representation of the various unit operations of a general sugarcane processing factory in South Africa. Upon arrival of sugarcane stalks at the factory from various farms, they are weighed and then either stored in the cane yard until processed, or directly processed by shredding using cane knives and a shredder during the cane preparation stage. Representative samples of prepared (shredded) cane obtained from individual consignments of the sugarcane delivered to the factory are then analysed for pol (*viz.* apparent sucrose), brix and moisture content for cane payment purposes at the South African Sugar Association Cane Testing Service (CTS) station. The prepared cane is washed repeatedly with hot water (75-85 °C) in a diffuser to extract the plant juice, referred to as mixed

juice (MJ). Small fibre particles are removed by pouring the juice over an inclined wedge-wire (Dutch States Mines; DSM) screen. Fibre particles trapped on the screen are returned to the diffuser and the juice transferred to a mixed juice holding tank. The juice is heated to 105 °C and the pH adjusted to approximately 7.5 with calcium hydroxide, followed by removing of solids by adding a polymeric flocculant. The clarified juice is decanted from the clarifier, the settled mud solids filtered in a rotary vacuum filter and the filtrate recycled to the mixed juice tank. Mud solids and added filter aid are collected and removed as filter cake or recycled to the diffuser. The clarified juice is concentrated by evaporation to syrup and with further evaporation under vacuum crystallised in crystallisation pans. Seed crystals are added to the crystallisation pans to facilitate crystal formation. Sugar crystals are separated from the mother liquor (molasses) by centrifugation, dried and packaged as raw (brown) sugar.

Very few studies describe the microbial diversity across the various unit operations in sugarcane processing factories. Available literature (Bevan and Bond, 1971; Lillehoj et al., 1984; McNeil and Bond, 1980; Pederson and Hucker, 1948) covers Australian and American sugarcane factories that use milling trains for extraction and not diffusers. During milling, extraction of sugarcane juice is achieved by squeezing the prepared cane between two or three rollers (milling units), followed by washing with water (imbibition). Several milling units, usually six, are set in tandem to maximise extraction, and is referred to a milling train or milling tandem (Rein, 2007). Extraction of sugarcane juice by milling has historically been the conventional method of processing cane; however, extraction by diffusion became an alternative option in the South African sugar industry in the 1960s and 1970s (Rein, 1995). Currently more than 90% of the sugarcane in southern Africa is processed in diffusers (Rein, 2007). Sugarcane juice is extracted in a diffuser by washing of sucrose from the prepared cane using hot water, followed by diffusion. During this process, sucrose is transferred from plant cells at high sucrose concentration to the surrounding (extracted) juice with a lower sucrose concentration (Rein, 2007). The temperature ranges for the two types of extraction units are quite different. Milling tandems operate from ambient to above 60 °C measured at the final mill if hot imbibition is used. The temperature of juice and cane is above 75 °C in the diffuser (Mackrory et al., 1984). Bacterial activity is thus reduced in sugarcane processing factories that uses diffusers. In factories where milling tandems are used, the temperatures are much lower and bacteria may proliferate (Ravnö and Purchase, 2005). Bacteria isolated from sugarcane processing factories and processing streams are listed in Table 3.

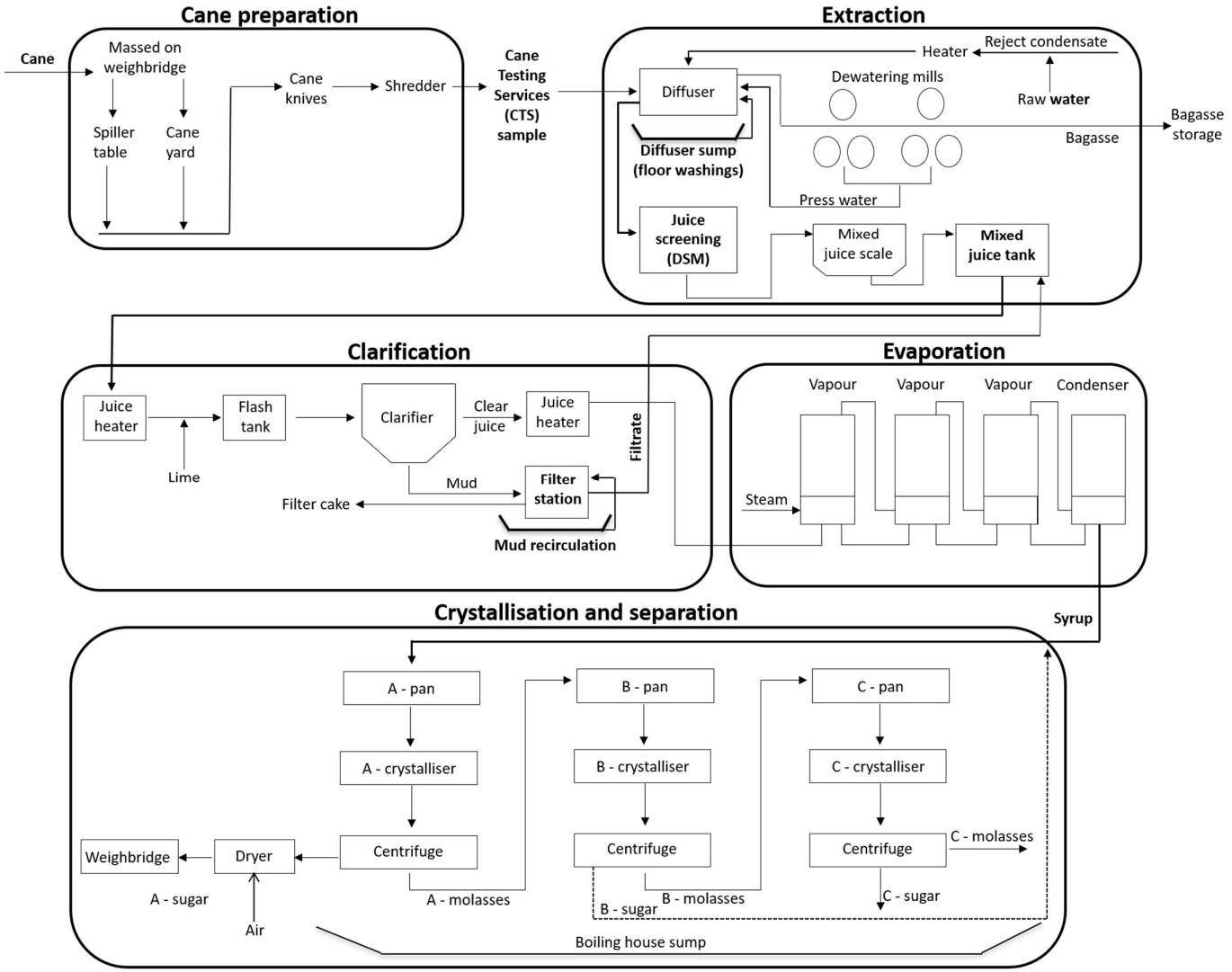

Figure 1 Schematic representation of a general sugarcane processing factory in South Africa

Table 3 Bacteria associated with sugarcane processing factory streams

Bacteria	Source	Country	Reference
Achromobacterium sp. (now classified as *Achromobacter* sp.)	Fresh expressed cane juice	USA*	Pederson and Hucker (1948)
Acinetobacter calcoaceticus	Milling train, mixed juice tank	Australia	McNeil and Bond (1980)
Actinomycetes sp.	Prepared cane after shredding and hammer milling	Australia	Bevan and Bond (1971)
Bacillus spp.	Syrup	Australia	Bevan and Bond (1971)
Bacillus aterrimus (now classified as *B. subtilis* subsp. *aterrimus*)	Fresh expressed cane juice	USA*	Pederson and Hucker (1948)
Bacillus cereus	Fresh expressed cane juice, milling train	USA*, Australia	Pederson and Hucker (1948), McNeil and Bond (1980)
Bacillus coagulans	Milling train	Australia	McNeil and Bond (1980)
Bacillus licheniformis	Milling train, mixed juice tank	Australia	McNeil and Bond (1980)
Bacillus megaterium	Fresh expressed cane juice, clarifier, milling train	USA*, Australia	Pederson and Hucker (1948), Bevan and Bond (1971), McNeil and Bond (1980)
Bacillus mesentericus	Fresh expressed cane juice	USA*	Pederson and Hucker (1948)
Bacillus stearothermophilus	Milling train, mud, clarified juice, diffuser, mixed juice	Australia	McNeil and Bond (1980)
Bacillus subtilis	Fresh expressed cane juice, milling train, diffuser	USA*, Australia	Pederson and Hucker (1948), McNeil and Bond (1980)
Brevibacterium imperiale (now classified as *Microbacterium imperiale*)	Prepared cane after shredding and hammer milling, mixed juice tank	Australia	Bevan and Bond (1971)
Clostridium spp.	Syrup	Australia	Bevan and Bond (1971)
Enterobacter cloacae	Milling train, mixed juice tank	Australia	McNeil and Bond (1980)
Enterobacter aerogenes (now classified as *Klebsiella aerogenes*)	Mixed juice tank	Australia	McNeil and Bond (1980)
Erwinia herbicola (now classified as *Pantoea agglomerans*)	Milling train, mixed juice	Australia	McNeil and Bond (1980)
Escherichia sp.	Fresh expressed cane juice	USA*	Pederson and Hucker (1948)
Flavobacterium sp.	Fresh expressed cane juice	USA*	Pederson and Hucker (1948)
Klebsiella pneumoniae	Milling train, mixed juice tank	Australia	McNeil and Bond (1980)
Lactobacillus spp.	Milling train, mixed juice tank, mixed juice	Australia	McNeil and Bond (1980)
Leuconostoc spp.	Milling train, mixed juice tank, mixed juice, limed juice, rotary vacuum filters	Australia, USA*	McNeil and Bond (1980), Lillehoj et al. (1984)
Leuconostoc mesenteroides	Fresh expressed cane juice, factory cane juice, prepared cane after shredding and hammer milling, milling train, mixed juice tank	USA*, Australia	Pederson and Hucker (1948), McCleskey et al. (1947), Bevan and Bond (1971)
Micrococcus spp.	Fresh expressed cane juice	USA*	Pederson and Hucker (1948)
Pseudomonas spp.	Milling train, mixed juice	Australia	McNeil and Bond (1980)
Serratia marcescens	Milling train, mixed juice tank	Australia	McNeil and Bond (1980)

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*Thermoactinomyces thalpophilus (*now classified as (*Laceyella sacchari*)	Prepared cane after shredding and hammer milling, mixed juice tank	Australia	Bevan and Bond (1971)

*USA = United States of America

The high temperatures in the diffuser and during clarification, as well as the high brix of syrup, are not conducive for microbial activity in sugarcane processing. However, poor factory sanitation and sump management may contribute to microbial contamination in the factory and processing streams. Sumps are holding tanks for wash-outs and overflows, run-offs and leaks from pumps. The sump content, at ambient temperature, is often kept for long retention times before it is pumped back into the diffuser when the liquid reaches a predetermined level. Factory staff occasionally slug-dose biocides into the sumps to limit microbial growth and control contamination (Kalidass et al., 1996). The area behind the juice screens is a frequent source of contamination. Slime deposits, as result of bacterial contamination, have been observed on the screens (Lillehoj et al., 1984; Rein, 2007).

Dextran and other gums increase the viscosity of processing streams, leading to increased boiling times and higher sucrose inversion losses. Other metabolites produced during the degradation of sucrose reduce the purity of the cane juice and thus also sucrose recovery. The impurities reduce evaporation rates and sugar crystals take longer to form (Godshall et al., 1996; Jimenez, 2005). Furthermore, dextran shows high (20%) transfer from juice to crystal, resulting in high carry-over from the sugarcane processing factory to the refinery. Refiners and buyers of raw sugar prefer the product to have low levels of dextran (<100-150 mg/kg), even if the purchase contract does not specify dextran content (Ravnö and Purchase, 2005). Economic losses due to microbial activities are, therefore, not limited to the direct loss of recoverable sucrose and indirect loss due to reduced factory throughput, but also include the difficulty in finding financially attractive markets for high-dextran raw sugar (Moodley and Khomo, 2018).

Methods to mitigate the action of spoilage bacteria in sugarcane processing

Biocides have been applied to sugarcane pre- and post-harvest in the field (field control), as well as in sugarcane processing factories (factory control). The majority of literature on the topic addresses field control of spoilage bacteria, *i.e*. application of biocides prior to, and during, harvesting. This is testament to the understanding that most microbial consumption of sucrose occurs in the field after harvesting.

In-field biocide application

Pre-harvest treatment of sugarcane has involved spraying with solutions of divalent cations (Zn^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, Cu^{2+}) and sodium metasilicate a week prior to harvest (Solomon et al., 1990). Compared to untreated controls, this treatment resulted in less sucrose being lost during harvesting. Further studies have shown that the divalent cations inhibited invertase activity and not microbial growth. Although effective, the method may be difficult to implement on an industrial scale, as pointed out by Solomon et al. (2006).

In South Africa harvesting of cane is mainly done by hand and the application of biocides may not be as practical as spraying of biocides directly onto chopper blades of mechanical harvesters. Biocides have been sprayed directly onto cane stalks using a mist sprayer, followed by covering of the cane bundles with trash (dry cane leaves) and leaving the cane in the field (Solomon, 2000; Solomon, 2009). Fumigation of stored billeted cane with formalin, alcohol and chlorine (Egan, 1968), and dipping and soaking of cane billets in an array of biocide solutions (Egan, 1965) have been tested. The biocides used in these tests were disinfectants (*e.g.* chlorhexidine, polycide, glutaraldehyde and benzalkonium chloride; Singh et al., 2008; Solomon, 2000), chemicals (*e.g.* formaldehyde solution, benzoic acid; Solomon, 2000), bleaching agents (*e.g.* calcium hypochlorite; Egan, 1965), antibiotics (streptomycin sulphate and penicillin; Egan, 1965), commercial biocide formulations (*e.g.* POLMAX ESR, Sucroguard, Kilbact™; Kulkarni, 1999; Kulkarni and Warne, 2004; Solomon, 2000), quaternary ammonium compounds (Solomon et al., 2008), dimethyl benzyl ammonium chloride (Milintawisami et al., 2009), dithiocarbamate formulations (Solomon et al., 2001), and the invertase enzyme inhibitors (potassium permanganate, sodium metasilicate, sodium lauryl sulfate and IFOPOL; Ramos et al., 1992; Solomon, 2000; Solomon et al., 2006).

The success of biocides is normally rated in terms of juice quality parameters and cane deterioration indicators. Not all of the tested biocides inhibited microbial growth, and none have been classified as environmentally safe, practical and economically feasible for sustainable use in sugarcane processing industries. Further research on biocide application and the development of new biocides is thus of paramount importance.

Biocide application in factories

Biocides for application in sugarcane processing factories received less attention than biocides used in preventing spoilage of harvested cane in the field. It is often argued that diffusers are effective in killing microorganisms on the cane that enter the factory. Although high temperatures used in diffusers could effectively kill some of the microorganisms which

enter the factory with the cane, it is imperative to acknowledge that sugarcane processing factories do present locations where environmental conditions (sucrose concentration, temperature and pH) favour microbial growth and proliferation. Foxon and Du Clou (2017) quantified the gums from mixed juice and final molasses for a South African sugarcane processing factory over an entire season to ascertain whether gums may be produced in the factory. Their results suggested that gums were produced in the factory during the last few weeks of the milling season, and that there may be value in considering biocide application in the factory at the end of a season, or under conditions that are conducive to gum production (*i.e.* during short front-end stops).

Learnings from previous factory biocide trials indicated that the sub-lethal dosage of biocides, as recommended by the USA Food and Drug Administration (FDA), may lead to the development of bacterial resistance against a specific biocide formulation, rendering it ineffective (Richards, 1999). Although various biocides have been evaluated for use in both sugar beet and sugarcane processing industries over many years, published papers on the topic is rather limited and shows no agreement on which substance (or formulation of a combination of substances) should be considered the 'go-to' biocide for microbial control in sugarcane fields or factories. Furthermore, published reports on biocide testing are often not clear on the rationale regarding the choice of biocide with respect to target microorganism(s), and often the criteria against which the biocide is judged successful (or not) are not well defined. Problems encountered in factory sanitation, especially selecting an effective biocide, is largely due to a lack of knowledge regarding microorganisms and enzymes present in sugarcane and its processing streams, as pointed out by Kulkarni (1999).

Identification of sugarcane spoilage bacteria – history and future potential

Phenotypic characterisation

Historically, microbial diversity in sugarcane processing was studied using identification techniques based on microbial growth and a few phenotypic characteristics. At the time, the phenotypic identification of bacteria was based on morphological, physiological and biochemical properties. Morphological characteristics include cellular features such as shape, presence of endospores, flagella and inclusion bodies, as well as Gram reaction. This also includes colony characteristics such as colour, dimensions and form. Physiological and biochemical characteristics include the ability to grow at different temperatures, pH values, salt concentrations or atmospheric conditions, as well as growth in the presence of various substances such as antimicrobial compounds, the presence or activity of various enzymes and metabolic activity (Vandamme et al., 1996). Phenotypic identification relies on the expression of genes. The expression of the genes, however, depends on specific

environmental conditions and not all genes are expressed under controlled conditions (Rosello-Mora and Amann, 2001).

An early study on the isolation and identification of *Le. mesenteroides* from sugarcane juice (McCleskey et al., 1947) illustrated the challenges experienced by sugar technologists in identifying sugarcane spoilage bacteria based on phenotypic characterisation. The authors grouped 740 gum-forming isolates into four distinct clusters based on cellular (shape, size and grouping) and colonial (form, elevation, height, diameter, surface, margin and density) features. They noted that the composition of the growth medium and incubation temperature had a pronounced effect on the colonial appearances of the cultures. From the initial 740 cultures, 168 were subjected to further physiological and biochemical analyses. Four distinct groups were described, each with unique fermentation reactions, levels of gum production, acid and gas produced, and optimal growth temperature and pH. Despite these differences, all of the isolates were classified as *Le. mesenteroides*. Although other bacteria have been isolated from deteriorated sugarcane (Table 2), *Le. mesenteroides* continued to be implicated as main causative agent of deteriorated sugarcane and dextran production in sugar industries worldwide for years to come, and few studies proved otherwise. A report by McNeil and Bond (1980) reiterated earlier observations by Sharpe et al. (1966), emphasising the difficulty in distinguishing between dextran-producing species of *Leuconostoc* and *Lactobacillus*. The authors suggested that other species may be responsible for dextran formation by stating that "*Leuconostoc* is not the only species likely to promote the slimes associated with juices and the 'frogs' spawn' effects on mills". Most of the studies on the identification of post-harvest spoilage bacteria were all constrained by the absence of microbial identification methods with high discriminatory power. The challenges experienced could be attributed to the ambiguity of phenotypic characterisation which often causes problems in describing or differentiating species. However, over the years there has been a steady change in the taxonomy and nomenclature of bacteria, driven by technological progress. Genotypic identification methods, based on DNA and RNA studies, now dominate bacterial taxonomy (Vandamme et al., 1996).

Genotypic characterisation

During the 1960s, increasing knowledge on DNA and developing molecular biological techniques raised the awareness that bacteria might be classified by measuring their overall genetic similarity. Subsequently, the technique of DNA-DNA hybridisation (DDH) was developed (Brenner et al., 1969) and became the cornerstone of genotypic characterisation in the 1970s. This technique is based on the fact that, although double-stranded DNA can be denatured at high temperatures, single strands can return to a double helix by lowering the temperature. It is based on three parameters; (i) G+C mol%, (ii) the ionic strength of the

solution and (iii) the melting temperature of the DNA hybrid (Tm), which is the only variable parameter (as ionic strength can be kept constant). Therefore, the higher the similarity between the heteroduplex molecule, the higher the temperature required to separate it (high Tm value). Here, both the gene content and nucleotide similarities of shared genes contribute to a measure of the overall relatedness of their genomes. DDH provided a standardised means for identifying and classifying bacteria which lacked well-defined phenotypic characteristics at times when DNA sequence information was not available. The application of DDH in bacterial classification for delineation of species was later evaluated by a committee on bacterial systematics (Wayne et al., 1987). They recommended that bacterial species generally would include a strain with 70% or greater DDH values, with 5 °C or less ΔTm values, and that both of these values must be considered. Despite its widespread use as standard technique for bacterial classification, DDH has serious limitations. DDH is a time-consuming procedure with a high experimental error and it is not suited for rapid identification of bacteria. Individual strains cannot be analysed and compared with strains in a database, since the method relies on pairwise comparisons of two bacterial genomes (Gevers et al., 2005; Prakash et al., 2007).

The late 1970s yielded a breakthrough in the attempts to determine relationships between distantly related bacteria by the cataloging of rRNA (Stackebrandt et al., 1985) and DNA-RNA hybridisation (De Ley and De Smedt, 1975). In the 1980s, the development of PCR methods and sequencing of the 16S rRNA gene led to major changes in bacterial taxonomy (Woese, 1987). Although already commonly used for the description of new species in the 1990s (Rosello-Mora and Amann, 2001), 16S rRNA sequencing was only considered a key parameter in bacterial taxonomy in 2002 (Stackebrandt et al., 2002). Currently, 16S rRNA sequence analysis is the first-line tool for evaluating the taxonomic status of a bacterial strain at the genus or species level and formed the backbone for the structuring of the second edition of Bergey's Manual of Systematic Bacteriology (Ludwig and Klenk, 2005). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common genetic marker used for the following reasons: (i) 16S rRNA genes are assumed to be least likely to have undergone horizontal gene transfer between species, (ii) 16S rRNA genes are present in all prokaryotes, (iii) the proportion of genetic content to size of 16S rRNA genes (approximately 1500 bp) are sufficient to enable high resolution and well supported phylogenetic trees, (iv) the sequences of 16S rRNA genes are highly conserved due of the fundamental role of the ribosome in protein synthesis and therefore comparable between distantly related species, (v) the 16S rRNA genes also contains variable regions which enable comparisons between closely related species and (vi) some regions of the

16S rRNA gene are completely conserved, enabling the use of “universal primers” for PCR detection or sequencing (Kitahara and Miyazaki, 2013).

The emergence of rapid and cost-effective DNA sequencing technologies has largely circumvented the need for physicochemical determination of genomic similarity. Although the advantages of the sequence-based approaches are clear, classification by 16S rRNA gene sequence analysis alone is not recommended for several reasons (Gevers et al., 2005), the most notable being its insufficient resolution at species level. A higher phylogenetic resolution, especially within genera, can be obtained by performing additional phylogenetic analyses based on protein-coding genes. Housekeeping (metabolic) genes may be used as taxonomic markers due to (i) the presence of slow and fast-evolving regions, (ii) a low rate of horizontal gene transfer and (iii) a single copy per genome (Roux et al., 2011). Since single-based protein-coding genes do not reflect general phylogenetic relationships, multiple gene-based phylogenies were introduced and have been used more frequently in order to overcome the bias caused by single gene sequence-based phylogenies. Gevers et al. (2005) introduced the term “multilocus sequence analysis (MLSA)” for the genotypic characterisation method which considers sequence analysis of internal fragments of several protein-coding genes. Most often, a new strain is identified to genus level on the basis of 16S rRNA gene sequence analysis, followed by MLSA to obtain a higher resolution at species level (Glaeser and Kämpfer, 2015).

Polyphasic approach

The most widely recommended system for bacterial identification relies on a polyphasic approach, which includes phenotypic and genotypic data, as well as phylogenetic information (Vandamme et al., 1996). However, it is possible that established phenotypic classification schemes do not corroborate phylogenetic insights based on rRNA gene sequencing, as in the case of the lactic acid bacteria (Vandamme et al., 1996). As discussed earlier, *Le. mesenteroides* has historically been implicated as the major contributor to post-harvest deterioration of sugarcane (Egan and Rehbein, 1963; Eggleston et al., 2009), with the difficulties in differentiating between species of *Leuconostoc* and *Lactobacillus* well established (Collins et al., 1993; McNeil and Bond, 1980; Sharpe et al., 1966). These genera belong to the group of lactic acid bacteria, which are Gram-positive, non-spore-forming cocci, coccobacilli or rods, with a DNA base composition of less than 50 mol% G+C. Lactic acid bacteria need a fermentable carbohydrate for growth, with glucose converted mainly to lactic acid (homofermentative) or to lactic acid, CO_2 and ethanol or acetic acid, or both (heterofermentative) (Vandamme et al., 1996).

Taxonomic revisions of the lactic acid bacteria

The phylogenetic structure of the lactic acid bacteria, as inferred from rRNA gene sequence analysis, is at present clear but does not always correspond to the phenotypic classification schemes which have been used for almost a century. Most of the taxonomic revisions related to lactic acid bacteria were proposed during the last three decades and are increasingly dependent on rRNA gene sequence information.

Lactic acid bacteria are grouped in Phylum VIII *Firmicutes*, Class I *Bacilli*, Order II *Lactobacillales* and includes the families *Lactobacillaceae* with genera *Lactobacillus, Paralactobacillus* and *Pediococcus*, and *Leuconostocaceae* with genera *Leuconostoc, Weissella* and *Oenococcus* (Ludwig et al., 2009). Phylogenetic relationships among species of the *Lactobacillaceae* and *Leuconostocaceae* have been hotly disputed (Zhang et al., 2011) and some of the recent revisions will be discussed here.

The family *Lactobacillaceae*

The diversity of the *Lactobacillaceae* is greatly influenced by the heterogeneity of its most abundant genus, *Lactobacillus*, which consists of more than 200 species that are phylogenetically and metabolically more diverse than that of a typical bacterial family (Sun et al., 2015). Many classification schemes have been developed since the first description of the genus *Lactobacillus* in 1901 (Beijerinck, 1901). Initial classification of the genus was based on optimum growth temperature and carbohydrate fermentation pathways (Orla-Jensen, 1919). Later revisions categorised the genus according to fermentation characteristics, *viz.* obligate homofermentative, facultative heterofermentative and obligate heterofermentative (Hammes and Vogel, 1995). This phenotype-based classification did not consider the pentose phosphate pathway for pentose conversion to lactate as sole end-product, nor did it reflect the grouping of lactobacilli based on metabolic pathways (Gänzle, 2015). Comparative analysis of 16S rRNA gene sequences of members of the family *Lactobacillaceae* further confirmed the heterogeneity of the genus *Lactobacillus*, which was found intermixed with species of *Pediococcus* (Salvetti et al., 2012). Zheng et al. (2015) suggested a revised classification of lactobacilli which combined metabolic characteristics with phylogenomic analysis and proposed the term *Lactobacillus sensu lato* to include species from the genera *Lactobacillus* and *Pediococcus*. Phylogenomic analysis of lactobacilli revealed that the genus *Lactobacillus* is paraphyletic and that the genera *Pediococcus, Weissella, Leuconostoc, Oenococcus* and *Fructobacillus* are grouped within the lactobacilli as sub-clades. As a result, Sun et al. (2015) proposed the name '*Lactobacillus* Genus Complex' to encompass these six genera.

Salvetti et al. (2018) proposed a novel taxonomic scheme for reclassification of the genus *Lactobacillus* based on two scenarios. The first scenario involved splitting of the genus into two groups based on the presence or absence of the phosphofructokinase gene (*pfk*), which was found present in all homofermentative and facultatively heterofermentative lactobacilli and absent in the obligately heterofermentative lactobacilli and members of the *Leuconostocaceae*. These two groups were found relatively consistent with phylogenetic analyses of ribosomal proteins, housekeeping genes, core genes and were congruent with carbohydrate fermentation profiles. The second scenario involved the proposal of 10 subgroups which emerged from the phylogenetic analysis as nuclei of novel lactobacilli. Both scenarios presented challenges which suggested that genomics-derived thresholds should not be used in isolation for reclassification of species. Instead, the application of a polyphasic approach (Vandamme et al., 1996) was recommended to ensure that the diversity of taxa is coherently described by names at the different taxonomic ranks. Salvetti et al. (2018) advocated an open discussion amongst experts including the lactic acid bacterial scientific and industrial community and members of the Subcommittee of Taxonomy of the Genus *Lactobacillus* (Mattarelli et al., 2014) in order to proceed towards the formal proposal of the reclassification of the genus *Lactobacillus*.

The family *Leuconostocaceae*

The family *Leuconostocaceae* currently consists of four genera, *viz. Leuconostoc, Weissella, Oenococcus* and *Fructobacillus*. These genera were delineated based on phylogenetic analysis of 16S rRNA gene sequences (Lonvaud-Funel, 2014). Differentiation of leuconostocs from atypical lactobacilli using phenotypic characteristics are difficult and often unsuccessful (Collins et al., 1993). Historically, leuconostocs were separated from lactobacilli based primarily on morphological differences (Martinez-Murcia and Collins, 1990). DNA-RNA hybridisation studies by Garvie (1981) showed that *Le. mesenteroides* (and subspecies) formed a group distinct from *Lactobacillus confusus* and *Lactobacillus viridescens*, and that *Leuconostoc oenos* was unrelated to the other leuconostocs and heterofermentative lactobacilli. This prompted a number of investigations based on 16S rRNA gene sequence analysis, resulting in the differentiation of three distinct genetic lines, comprising the genus *Leuconostoc sensu stricto*, the *Leuconostoc paramesenteroides* group (which included the atypical lactobacilli *Lactobacillus confusus, Lactobacillus minor, Lactobacillus kandleri, Lactobacillus halotolerans* and *Lactobacillus viridescens*), and the species *Leuconostoc oenos* (Collins et al., 1991; Martinez-Murcia and Collins, 1990, 1991; Yang and Woese, 1989). In 1993, an in-depth study based on phenotypic, biochemical and 16S rRNA gene analyses by Collins et al. (1993) allowed the differentiation of the new genus, *Weissella*, and the re-assignment of the species previously grouped with *Leuconostoc paramesenteroides* as

Weissella paramesenteroides, Weissella confusa, Weissella halotolerans, Weissella kandleri, Weissella minor and *Weissella viridescens.* Based on 16S and 23S rRNA gene sequencing analyses, Dicks et al. (1995) proposed the reclassification of *Leuconostoc oenos* as a separate genus, *Oenoccocus*, with the type strain being *Oenococcus oeni*. Currently, *Oenococcus* consist of three species, including *Oenococcus kitaharae* (Endo and Okada, 2006) and *Oenococcus alcoholitolerans* (Badotti et al., 2014).

Chelo et al. (2007) analysed 16S rRNA and housekeeping gene sequences, encoding chromosomal replication initiation protein (*dnaA*), DNA gyrase B subunit (*gyrB*), the 70 kDa heat-shock protein (*dnaK*) and the beta subunit of the DNA-dependent RNA polymerase (*rpoC*), to clarify the intra- and intergeneric phylogenetic relationships inside the *Leuconostoc-Oenococcus-Weissella* clade. A well supported and good agreement between the phylogenies of the various genes were obtained, and an almost fully resolved phylogenetic tree was obtained when the combined data were analysed in a Bayesian approach. A rapid basal diversification of the three genera was suggested, with the evolutionary rates of the 16S rRNA gene in these genera to be different and specifically related to the evolution of this group of bacteria.

Endo and Okada (2008) reevaluated the taxonomy of the genus *Leuconostoc* by analysing the 16S rRNA gene sequences and the 16S-23S intergenic spacer region (ISR), as well as the MLSA of two housekeeping genes (*rpoC* and the gene econding a recombinase A protein, *recA*). Results from this study prompted the proposal of a new genus, *Fructobacillus*, and the transfer of *Leuconostoc durionis, Leuconostoc ficulneum, Leuconostoc fructosum* and *Leuconostoc pseudoficulneum* to this new genus. The type species of the genus *Fructobacillus* is *Fructobacillus fructosus*, and currently the genus includes *Fructobacillus durionis, Fructobacillus ficulneus, Fructobacillus pseudoficulneus* and *Fructobacillus tropaeoli* (Endo et al., 2011).

Although genomics of lactic acid bacteria are currently receiving a lot of attention, very few genome sequences for *Leuconostoc, Weissella, Oenococcus* and *Fructobacillus* have been published thus far (Chelo et al., 2010; Endo et al., 2015), and comparative genomic analyses in the Leuconostoc group have been restricted to comparisons with species from other genera (Makarova et al., 2006) or are limited to a single species (Zé-Zé et al., 2000).

The genus *Bacillus*

The genus *Bacillus* does not belong to the group of lactic acid bacteria, as bacilli are noted for their spore-forming ability, a trait which is absent from all ‘true’ lactic acid bacteria. However,

the inclusion of the genus *Bacillus* here is appropriate as some current views generally regard this genus as ancestral to the lactic acid bacteria (Holzapfel and Wood, 2014). The genus *Bacillus* is classified under the Phylum VIII *Firmicutes*, Class I *Bacilli*, Order I *Bacillales* and family *Bacillaceae* (Ludwig et al., 2009). *Bacillus* consist of a large and heterogeneous group of aerobic or facultative anaerobic, rod-shaped endospore-forming bacteria that are widely distributed throughout the environment (Abriouel et al., 2014). It is difficult to differentiate between *Bacillus* species. A large number of phenotypic tests are often required and in some cases only a single property can distinguish a particular species. Molecular methods such as 16S rRNA gene sequence analysis has proved to be a reliable alternative for species identification (Drancourt et al., 2000). The *Bacillus* genus is genetically very heterogeneous, as shown by the wide range of DNA base ratios of approximately 32 to 65 mol% G+C, which is far more extensive than usually considered reasonable for a single genus. This variation in mol% G+C content of the DNA is not only found among species, but also within strains of a single species (Abriouel et al., 2014).

Strains and species within the genus *Bacillus* underwent a number of reclassifications and rearrangements in recent years. The first effective organisation of the genus was carried out by Gordon et al. (1973), identifying *Bacillus subtilis, Bacillus licheniformis* and *Bacillus pumilis* as the 'original' members of the genus. Fukumoto (1943) first isolated *Bacillus amyloliquefaciens*, a bacterium that produced a liquefying amylase enzyme from soil. *B. amyloliquefaciens* was recognised as a distinct species in 1987 (Priest et al., 1987) after an extensive debate about its taxonomical position (Welker and Campbell, 1967). The separation of *B. amyloliquefaciens* from *B. subtilis* was based mainly on low DNA relatedness which was found to be less than 25, 13 and 5% in DNA-DNA hybridisation studies with DNA from *B. subtilis, B. licheniformis* and *B. pumilis*, respectively (Priest et al., 1987). Later, *B. amyloliquefaciens* was combined with the closely related *B. licheniformis, B. pumilis* and *B. subtilis* into the '*B. subtilis* species complex' based on multilocus phylogenetic analysis (Rooney et al., 2009). Based on complete genome analysis, *B. amyloliquefaciens* strains were then divided among the subspecies *B. amyloliquefaciens* subsp. *amyloliquefaciens* and *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al., 2011). Comparative genomic analysis of *B. amyloliquefaciens* subsp. *plantarum* and *Bacillus methylotrophicus* showed that their genomes were highly similar (95%). As a result, *B. amyloliquefaciens* subsp. *plantarum* was synonymised with *B. methylotrophicus* (Dunlap et al., 2015) and subsequently, *B. methylotrophicus* was shown to be a later heterotypic synonym of *Bacillus velezensis* (Dunlap et al., 2016). Recently, the taxonomic status of the *B. subtilis* species complex were assessed by comparing core genome sequences and RNA polymerase beta-subunit (*rpoB*) gene sequences (Fan et al., 2017; Rabbee et al., 2019). The phylogenetic analysis by Fan et

al. (2017) showed that four clades can be distinguished within the *B. subtilis* species complex: clade I, consisting of *B. subtilis* including its three subspecies *subtilis, spizenii* and *inaquosorum*, *Bacillus tequilensis, Bacillus vallismortis, Bacillus mojavensis* and *Bacillus atrophaeus*; clade II, consisting of *Bacillus siamensis, B. amyloliquefaciens* and a conspecific complex consisting of *B. methylotrophicus, B. velezensis* and *B. amyloliquefaciens* subsp. *plantarum*; clade III, consisting of *B. licheniformis* and related species and clade IV consisting of *B. pumilis* and related species. Fan et al. (2017) proposed a novel taxonomic unit above species level but below the *B. subtilis* species complex, namely the 'operational group *B. amyloliquefaciens'* comprising *B. amyloliquefaciens, B. siamensis* and *B. velezensis*, a taxon that includes all the strains previously classified as *B. velezensis, B. methylotrophicus* and *B. amyloliquefaciens* subsp. *plantarum*, as in agreement with the proposal by Dunlap et al. (2016).

Phylogenetic analysis of the complete *rpoB* gene sequences of the type strains of species from the *B. subtilis* species complex showed that the *Bacillus* species synonymous with *B. velezensis* clustered into clades consisting of the operational group *B. amyloliquefaciens, B. amyloliquefaciens* subsp. *plantarum* and *B. methylotropicus* (Rabbee et al., 2019).

Although correct classification and identification of lactic acid bacteria and related genera are difficult without the support of modern genotypic techniques, the large number of species renders an exclusively genotypic approach quite cumbersome. Therefore, phenotypic characteristics (mostly examined *via* commercially available test systems) remain important as tools for identification and classification. The discrepancy between phenotypic data present in traditional microbiology textbooks and information on phylogenetic relationships that have recently become available have not sufficiently been translated into new approaches for future applications. However, for the identification of unknown isolates, it is still recommended that a combination of phenotypic and genotypic techniques may prove to be the most rewarding with respect to accuracy, time and cost (Vandamme et al., 1996).

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CHAPTER 3

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Post-harvest biodeterioration of sugarcane: Taxonomic history and current perspectives

S Nel[1,2*], SB Davis[1], A Endo[3] and LMT Dicks[2]

[1]Sugar Milling Research Institute NPC, c/o University of KwaZulu-Natal, Howard College Campus, Durban 4041, South Africa
[2]Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa
[3]Department of Food and Cosmetic Science, Tokyo University of Agriculture, Hokkaido, Japan

*Corresponding author: S Nel (snel@smri.org)

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Abstract

Direct and indirect losses of saleable sugar are responsible for reduced revenue in the sugarcane processing industry. Sucrose loss due to post-harvest deterioration of sugarcane is a complex issue influenced by a multitude of factors. This paper focuses on the post-harvest biodeterioration of sugarcane; i.e. sucrose loss due to the action of microorganisms, with an emphasis on the isolation and identification of causative bacteria. The research of scientists involved in the first isolation and identification of microorganisms associated with deteriorated sugarcane and processing streams is discussed. The paper gives a scientific perspective on modern day taxonomic techniques that are used in the identification of sugarcane spoilage bacteria.

Introduction

Crystal sugar is the main source of revenue sustaining the agricultural and milling sectors of the South African sugar industry. However, up to 10% of sucrose may be lost during harvesting as juice leaks through cracked stalks of the burnt sugarcane (de Robillard et al., 1990). Further losses occur during transport to the factory, spills in the factory, the conversion of sucrose to glucose and fructose by invertase naturally present in the plant, and the microbial conversion of sucrose to polysaccharides (gums), alcohol and acids (Solomon, 2000). An increase in viscosity of the juice leads to higher sucrose inversion losses in the boiling house and an increase in the quantity of molasses produced (Jimenez, 2005). This paper focuses on the post-harvest biodeterioration of sugarcane, i.e. sucrose loss due to the action of microorganisms, with an emphasis on the isolation and identification of spoilage bacteria.

Research on microbial spoilage of sugarcane dates back to the 1800s when Louis Pasteur first reported slime production by small cocci in sugar-containing liquid (Pasteur, 1861). Members of the genus *Leuconostoc*, and in particular *Leuconostoc mesenteroides*, are responsible for most of the exopolysaccharide production (Geronimos and Greenfield, 1978). Cienkowski (1878) was probably the first to study *Leuconostoc* spp. in sugarcane factories. The isolates were later classified as members of the genus *Leuconostoc* (van Tieghem, 1878). Although other microorganisms have also been isolated from spoiled sugarcane and related processing streams, *L. mesenteroides* had been implicated as the causative agent of biodeteriorated sugarcane for more than a century. Despite all the research conducted on *Leuconostoc* spp. and other spoilage microorganisms, the biodeterioration of sugarcane and the detrimental effects the microbial metabolites have on sugarcane processing, remains a problem. Methods used to prevent microbially-mediated sucrose loss (and potential solutions to reduce the undesirable effects of microbial metabolites on sugarcane processing) are inadequate and do not address the cause of the problem or the effects thereof. A recent paper by Nel (2014) challenged the perception that *L. mesenteroides* is the sole cause of biodeteriorated sugarcane and advocated the concept of microbial diversity profiling of problematic microorganisms in sugarcane processing. This involves the isolation and identification of microorganisms associated with the biodeterioration of sugarcane and its processing streams, and the development of a targeted approach against specific microorganisms present at known locations.

Pioneering contributions are frequently overlooked or even forgotten in our ‘post-modern’ era. This paper acknowledges the pioneers of microbial diversity profiling; researchers who first isolated and identified microorganisms related to the biodeterioration of sugarcane and its processing streams. In addition, the paper provides a current scientific perspective on modern taxonomy, as a means to identify the causative agents of post-harvest biodeteriorated sugarcane.

Historical contributions

In **1938, McCalip and Hall** were the first to report gum-producing bacteria from frost-damaged sugarcane, in particular from stalks with symptoms of ‘splitting freeze’. It is well known that sugarcane is very susceptible to frost and this is the major source of deteriorated cane in the USA (Eggleston et al., 2005). Freezes are known to kill and rupture sugarcane cells. High acidity increases the activity of invertase and the ruptured cells facilitate infection by microorganisms, leading to further sucrose losses. McCalip and Hall (1938) noted that the cells of typical gum-forming cultures were Gram-positive, non-motile spheres of 0.5-1.0 µm in

diameter, occurring singly, in pairs and in short chains. Biochemical analyses revealed that acid was produced from sucrose, xylose and arabinose. Slime (gum) was produced from sucrose but not from glucose. The optimum growth temperature of the isolates was between 20 and 22 °C and they required little oxygen. The authors classified the gum-producing bacteria as *L. mesenteroides*, based on descriptions of morphology, physiology and culture characteristics of an organism described by Bergey et al. (1934). In addition, McCalip and Hall (1938) confirmed that the isolates produced dextran, which led to increased viscosity of the culture medium over time. Mannitol was one of the end products produced from the fermentation of sucrose.

A few years later, **McCleskey and co-workers (1947)** studied the diversity of *Leuconostoc* strains isolated from sugarcane juice at the Experimental Sugar Factory of the Louisiana State University (USA). The authors cited a literature review by Hucker and Pederson (1930) that described the incidence of bacterial cultures which, despite having essential similarities, had been given different identities. The ability of *L. mesenteroides* to produce a polysaccharide (which is often observed as slime) from sucrose is one of the main characteristics of the species. Currently there are three subspecies of the bacteria previously classified as *L. mesenteroides, L. dextranicum* and *L. cremoris* (reclassified as *L. mesenteroides* subsp. *mesenteroides, L. mesenteroides* subsp. *dextranicum* and *L. mesenteroides* subsp. *cremoris*, respectively; Garvie (1983)). From these, only *L. mesenteroides* subsp. *mesenteroides* and *L. mesenteroides* subsp. *dextranicum* produce dextran (slime) from sucrose. However, at the time of the McCleskey et al. (1947) study, sucrose-fermenting and slime-producing bacteria other than *Leuconostoc* spp. had been described (Niven et al., 1941, 1946, Niven and White, 1946, White and Niven, 1946). McCleskey et al. (1947) isolated 740 gum-producing bacteria from sugarcane juice and grouped them into four distinct clusters (A, B, D and F). The colonies differed in size, elevation, topography and optical characteristics. Some correlation was noted between colony type and cell morphology. However, morphology alone could not be used to distinguish between colony types. Isolates were tested for their ability to ferment sucrose, lactose, xylose and arabinose. A considerable number of strains from group D failed to ferment sucrose and lactose, but when tested a few months later, showed a delayed reaction. Most of the strains in the group (97%) fermented xylose, although with a delayed fermentation by some. Only 45% of the D-strains fermented arabinose. Despite the differences in colony characteristics and morphology, all of the gum-producing isolates were identified as *L. mesenteroides* simply because they fermented sucrose and either arabinose, xylose or both. McCleskey et al. (1947) concluded that *L. mesenteroides* isolated from sugarcane juice consisted mainly of four relatively distinct colony types and that these types also differed in

certain fermentation reactions, especially in the levels of gum, acid and gas produced, growth temperature and pH optima.

Pederson and Hucker (1948) studied the incidence of microorganisms, and in particular *Leuconostoc* spp., in various sugar factory juices, syrups and sugars. The isolates belonged to three types, *viz*. slime-producing *Leuconostoc* spp. (although it was not the dominant species isolated from freshly expressed juice), aerobic, spore-forming *Bacillus* spp., including *Bacillus subtilis, Bacillus cereus, Bacillus megaterium* and *Bacillus subtilis* var. *aterrimus*, and aerobic non-spore forming bacteria, including the genera *Micrococcus*, *Flavobacterium*, *Achromobacterium* (now classified as *Achromobacter*) and *Escherichia.*

Egan did pioneering work in the 1960s on sour rot of stored sugarcane in Queensland, Australia (Egan and Rehbein, 1963, Egan, 1964, 1965a, b, c, 1966, 1967, 1968). Egan and Rehbein (1963) isolated bacteria from sour-smelling sugarcane obtained from a mill yard and classified the strains as *Leuconostoc* spp. Two species were distinguished, *viz*. *Leuconostoc dextranicum* (now classified as *Leuconostoc mesenteroides* subsp. *dextranicum*) with raised, jelly-like colonies of 1 mm high and up to 2 mm in diameter, and *L. mesenteroides*, characterised by producing more slimy colonies. A later study by Egan (1965c) detailed the process by which the two *Leuconostoc* species were distinguished. Strains were classified as *L. mesenteroides* and *L. dextranicum* based on their ability to ferment xylose. Egan (1965c) suggested that these colony types corresponded to groups A and D of *L. mesenteroides* reported by McCleskey et al. (1947). Many of the strains in group D were slow in fermenting pentose sugars. The original isolate identified as *L. dextranicum* failed to ferment xylose after nine months, but produced acid slowly from xylose and arabinose after 22 months. Based on this, Egan (1965c) concluded that the *L. dextranicum* strain were the same as group D *L. mesenteroides* strains described by McCleskey et al. (1947). Based on these results, Egan (1965c) concluded that *L. mesenteroides*, defined by Breed et al. (1957), is the only species associated with sour storage rot.

Tilbury (1970) studied the microbiological, physical and chemical changes of stored harvested sugarcane from Jamaica. A total of 206 strains were identified. The lactic acid bacteria comprised *L. mesenteroides* (63 strains), *L. dextranicum* (23 strains; *L. mesenteroides* subsp. *dextranicum*), *Leuconostoc paramesenteroides* (1 strain; now classified as *Weissella paramesenteroides*; Martinez-Murcia and Collins (1990), Martinez-Murcia et al. (1993), Collins et al. (1993)), *Lactobacillus plantarum* (17 strains), *Lactobacillus casei* var. *casei* (5 strains), *Lactobacillus casei* var. *alactosus* (8 strains) and *Lactobacillus confusus* (37 strains, now classified as *Weissella confusa*; Collins et al. (1993)). The genus

Bacillus was represented by *Bacillus pumilus* (8 strains), *Bacillus subtilis* (7 strains) and *Bacillus megaterium* (2 strains). Fourteen strains were characterised as members of the genera *Enterobacter* or *Erwinia*. The yeasts included *Candida pseudotropicalis* (1 strain), *Candida tropicalis* (2 strains), *Torulopsis etchelsii* (1 strain), *Torulopsis dattila* (3 strains), *Torulopsis stellate* (5 strains), *Torulopsis holmii* (5 strains), *Torulopsis sake* (2 strains) and *Torulopsis versitalis* (2 strains). The genus *Torulopsis* has been reclassified as *Candida* (Yarrow and Meyer, 1978). The common orange mould on stored cane was identified as *Monilia sitophila* (now classified as *Chrysonilia sitophila*; von Arx (1981)). Importantly, for the first time a new dextran-producing species was isolated from processed sugarcane and was named *L. confusus* (now *W. confusa*; Martinez-Murcia and Collins (1990), Martinez-Murcia et al. (1993), Collins et al. (1993)).

Bevan and Bond (1971) were among the first researchers to profile the microbial diversity on sugarcane, in a factory in Queensland, Australia. The cell size, shape, motility and Gram reaction of the isolates were noted and classified according to identification guidelines available at the time (Skinner et al., 1947, Gilman, 1956, Breed et al., 1957, Christensen, 1965, Skerman, 1967). Microorganisms were isolated from green, burnt and chopped sugarcane, and, in the factory from prepared cane, the milling train and mixed juice.

A large diversity of microorganisms (approximately 50) were isolated from green sugarcane, and included the bacteria *Leuconostoc*, *Bacillus cereus, Pseudomonas* and *Streptomyces* spp. At least three genera of yeast were identified: *Saccharomyces, Pichia* and *Torula* (*Candida*). Fungi and *Actinomycetes* were also present in large numbers. At least three polysaccharide-producing microorganisms, including *L. mesenteroides*, were isolated. The microflora isolated from the cracks of green sugarcane consisted mostly of cocci, mainly *Leuconostoc*, as well as *Saccharomyces*, a large variety of moulds and some lactic acid producing rods. Seventeen different microorganisms were isolated from the surface of burnt sugarcane, and these were predominantly rods. Some have been tentatively identified as *Xanthomonas* spp., *Bacterium* spp. (now reclassified into several genera), *Corynebacterium* spp. and *Bacillus* spp., much the same as were found on green canes. Fungi such as *Penicillium*, *Rhizopus* and *Aspergillus* were common, especially on canes standing for 24 hours after burning. Yeasts such as *Torula* (*Candida*), *Rhodotorula* and *Candida* were reportedly prevalent on cane 24 hours after burning. Isolates resembling *Leuconostoc* spp. was extremely common, and all swabs taken after burning, even after only 10 minutes, contained members of the genus. Cell numbers of *Leuconostoc* spp. increased markedly with time after burning. When billets of chopped cane were examined, microorganisms such as yeasts, *Leuconostoc* and acid-producing rods were isolated from the interior of the billet

immediately after cutting. Yeast, *Leuconostoc* and mucoid-producing *Xanthomonas* and *Aerobacter* spp. (now classified as *Enterobacter* spp.) were prevalent at these sites. Concluded from these studies, acid and dextran production, and thus loss in sugar purity, starts immediately after cutting and accelerates proportional to the growth rate of the contaminants.

Prepared cane sampled after shredding and hammer-milling were heavily contaminated with the same microorganisms isolated from burnt and chopped sugarcane billets. Yeast, *Leuconostoc*, fungi, *Actinomycetes*, *Brevibacterium* (predominantly the brilliant red *Brevibacterium imperiale* currently reclassified as *Microbacterium imperiale*), and heat-tolerant *Thermoactinomyces thalpophilus* (synonym for *Thermoactinomyces sacchari*, capable of growing at 75 °C) were isolated from prepared cane. All of these isolates metabolised sucrose, glucose and fructose rapidly and produced various organic acids. At this point, the even distribution of microorganisms throughout the hammer-milled cane accelerated the deterioration process.

Samples taken from the milling train contained predominantly *Leuconostoc* spp. This was determined by sub-culturing of isolates on a variety of diagnostic media and the microorganisms were evaluated according to cell size, shape, motility and Gram reaction. Bacteria and yeasts were identified using the guidelines of Breed et al. (1957) and Skerman (1967). A diversity of yeasts was also prevalent, but at noticeably lower numbers at the end of the milling train. Acidophilic thermophiles, mainly spore-producing aerobic bacteria that survived 75 °C, were isolated from the end of the milling train. Areas under the cush-cush screens were particularly prone to microbial activity and were described as visible layers and globules of slimy deposits. Unfortunately, no mention was made of the identity of the causative microorganism(s). Contaminants isolated from mixed juice were characteristic of those coming into the factory with the sugarcane and were mainly yeast and *Leuconostoc* spp.

Bevan and Bond (1971) isolated more than 300 different microorganisms. The authors concluded that the survey on microbial diversity had clearly shown the necessity for further research into microbial-related sugar losses and subsequent production of unwanted microbial by-products in the factory.

McNeil and Bond (1980) studied the microbial diversity of sugarcane and its processing streams in an attempt to determine the degree of biodeterioration in sugarcane processing. The dominant groups of organisms isolated from this study were yeasts, enterobacteria,

mesophilic and thermophilic bacilli and dextran-forming lactic acid bacteria. These groups consisted mainly of *Saccharomyces cerevisiae, Erwinia herbicola* (now classified as *Pantoea agglomerans*; Gavini et al. (1989)), *Enterobacter* spp., *L. mesenteroides, B. subtilis, Bacillus coagulans* and *Bacillus stearothermophilus.* The authors confirmed earlier observations by Sharpe et al. (1966) that it is difficult to distinguish between dextran-producing species of *Leuconostoc* and *Lactobacillus*, and stated that "*Leuconostoc* is not the only species likely to promote the slimes associated with juices and the 'frogs' spawn' effects on mills". Dextran-producing bacteria were identified as *L. mesenteroides*, *L. dextranicum* (*L. mesenteroides* subsp. *dextranicum*) and *Lactobacillus* spp. The yellow pigmented bacterium *Erwinia herbicola* (*P. agglomerans*) appeared to be extremely common on the sugarcane plant and consequently in the cane juice. It was subsequently established that this bacterium produces levan, a fructose-based polysaccharide (Blake et al., 1982). McNeil and Bond (1980) noted that it was not difficult to identify the isolated mesophilic and thermophilic species of *Bacillus*, and that some of these species are known to produce levan. However, the apparent viscosity of these levans in artificial culture media was far less compared to dextran produced by lactic acid bacteria or fructan produced by *E. herbicola* (*P. agglomerans*).

Lillehoj and co-workers (1984) differentiated *Leuconostoc* spp. from *Lactobacillus* spp. by transferring the cultures to a sucrose-based medium. The authors claimed that the sucrose-based medium initiated dextran production in *Leuconostoc* spp., but not in *Lactobacillus* spp. Based on fermentation reactions with arabinose as substrate, most of the isolated *Leuconostoc* spp. were identified as *L. mesenteroides*.

Dextran and levan are not the only microbially-produced polysaccharides related to sugarcane processing. An unidentified polysaccharide described by Nicholson and Lilienthal (1959), suggested by Blake and Clarke (1984) and Morel du Boil et al. (2005) to be the same as the one described by Bruijn (Bruijn, 1966a, b, 1970, 1973), was the first report on sarkaran, a polysaccharide present in deteriorated sugarcane from South Africa. Dextran is usually associated with deteriorated cane. However, partial characterisation of the polysaccharide, and the absence of lactic acid in stale cane juices, suggested that the exopolysaccharide (EPS) was not dextran. This led Bruijn (1966a) to conclude that the EPS was not produced by *Leuconostoc* spp. Structural analyses showed that this polysaccharide had not been described before and the name "sarkaran", derived from the Sanskrit word "Sarkara" (meaning sugar), was proposed (Bruijn, 1973). At that time it was not possible to correlate the formation of the polysaccharide with the occurrence of a specific microorganism and it was suggested that the formation of this polysaccharide was the result of enzymatic reactions in the sugarcane after harvesting. However, decades later Morel du Boil and co-workers (2005)

isolated the causative agent of sarkaran, a filamentous fungal plant pathogen identified as *Phaeocytostroma sacchari* by the South African Sugarcane Research Institute and the Centre for Applied Mycological Studies, South Africa. This report by **Morel du Boil et al. (2005)** was the first published observation linking *P. sacchari* to the production of sarkaran.

Current perspectives

In the early years, *Leuconostoc* spp. were regarded the dominant bacterium associated with biodeteriorated sugarcane. However, over time a larger diversity of EPS-producing microorganisms have been described, including *Lactobacillus* spp. (Tilbury, 1970, McNeil and Bond, 1980). Apart from dextran, levan and sarkaran have also been detected in sugarcane (McNeil and Bond, 1980, Morel du Boil et al., 2005).

The description of microbial diversity has always depended on the available methods used for analysis. Early researchers were limited to the gross morphological differences in microbial cell shapes and colonies and growth characteristics on different culture media. Bacterial taxonomy at the time was solely based on comparative studies of the phenotypic features (*viz.* the observable expression of genotype, including morphological, physiological and biochemical properties) of the organism and this practice was directly applied to pure cultures. As such, it was biased towards aerobic heterotrophic microorganisms (Vandamme et al., 1996, Mora and Amann, 2001). One of the major disadvantages of using phenotypic methods to identify microorganisms is the conditional nature of gene expression wherein the same organism might show different phenotypic characteristics in different environments and under different conditions. This classical approach to bacterial taxonomy, which was based on phenotypical characterisation, was later expanded to include chemotaxonomy, a term referring to the application of analytical methods to collect information on various chemical constituents to classify microorganisms (Vandamme et al., 1996).

Over the years there has been a steady change in the taxonomy and nomenclature of bacteria, driven by technological progress which drastically influenced methodology, and over the past 30 years the changes have been dramatic. Genotypic methods (*viz.* those that are directed towards DNA and RNA molecules) presently dominate modern taxonomy, not only as a consequence of technological progress, but because the view on classification is such that it should reflect the natural relationships as encoded in the DNA (Vandamme et al., 1996).

Another development in microbial taxonomy, the polyphasic approach, refers to the integration of phenotypic, chemotypic and genotypic information of a microbe in order to perform reliable grouping of an organism (Colwell, 1970). A recent trend in the application of a polyphasic

approach to microbial classification includes the application of phylogeny, which represents the evolutionary relationship of microbes. Modern day taxonomy (or biosystematics) therefore consists of four main parts: *classification* (arrangement of organisms based on similarity), *nomenclature* (naming of the organisms), *identification* (determining whether an organism belongs to the group under which it is classified and named) and *phylogeny* (evolutionary relatedness) (Vandamme et al., 1996, Sarethy et al., 2014).

Discussion and Conclusion

The identification of causative microbes of the biodeterioration of sugarcane and its processing streams has historically involved the comparison of phenotypic characteristics of isolated microorganisms; the only method available to researchers at that time. However, the remarkable developments in modern taxonomy present an opportunity to revisit earlier attempts to identity the spoilage organisms. This is particularly significant, given that biodeteriorated sugarcane results in both direct and indirect revenue losses to the sugarcane processing industry, and currently there are no cost-effective solutions to combat microbial infection in the field and contamination in the factory. To date it has been difficult to find reliable indicators for the biodeterioration of sugarcane; this has been exacerbated by the lack of knowledge on the causative agents. Historical assumptions indicated that *Leuconostoc* and the associated production of dextran is the major cause of post-harvest biodeterioration of sugarcane and subsequent processing problems – perhaps it is now time to reconsider. Modern approaches to taxonomy provide enabling technologies for microbial diversity profiling of microorganisms responsible for deterioration of sugarcane and factory processing streams. The information obtained may prove useful to develop strategies to prevent biodeterioration of sugarcane, and/or mitigate the detrimental effects of microbial metabolic products on sugarcane processing, and in this manner reduce revenue losses.

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CHAPTER 4

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Microbial diversity profiling of polysaccharide (gum)-producing bacteria isolated from a South African sugarcane processing factory

Sanet Nel[1,2], Stephen B. Davis[1], Akihito Endo[3] and Leon M. T. Dicks[2#]

[1]Sugar Milling Research Institute NPC, c/o University of KwaZulu-Natal, Durban 4041, South Africa

[2]Department of Microbiology, Stellenbosch University, Stellenbosch 7600, South Africa

[3]Department of Food, Aroma and Cosmetic Chemistry, Tokyo University of Agriculture, Hokkaido, Japan

#Corresponding author: LMT Dicks (LMTD@sun.ac.za), Tel.: +27-21-808 5849, Fax: +27-21-808 5846

Abstract

Polysaccharide (gum)-producing bacteria are responsible for severe economic losses in the sugarcane processing industry. Increased polysaccharide levels in raw sugar are normally an indication that biodeterioration occurred in the cane, soon after harvesting. Once in the sugar processing plant, the cell numbers of gum-producing bacteria escalate and may reach levels difficult to control. We have isolated 430 gum-producing bacteria from sugarcane and different sampling points in a South African sugarcane processing factory. As expected, high cell numbers of gum-producing bacteria were isolated from the factory during a time when sugar with a high dextran content was produced. What we did not expect, was to find the same species in the factory at a time when sugar with a low dextran content was produced. Phylogenetic analyses of the 16S rRNA gene sequences differentiated the gum-producing bacteria into four genera and nine species. The majority of these isolates belonged to the genus *Weissella* (47%), followed by members of *Bacillus* (24%), *Leuconostoc* (19%) and *Lactobacillus* (10%). For the first time we report on the isolation of *Weissella confusa, Weissella cibaria* and *Bacillus amyloliquefaciens* from a sugarcane processing factory.

Introduction

High levels of polysaccharides in sugarcane have a marked effect on the recovery of crystal sugar and cause severe losses in the sugar industry. Deterioration of sugarcane in the field and after harvesting is a complex phenomenon that occurs through a number of interacting processes. A large percentage of the sucrose is lost with bursting of the cane stalks when fields are burned. The cane stalks, many damaged, are then transported to the sugarcane

processing factory. This may take long (several days) and is referred to as “burn/harvest-to-crush-delays”. During this period, the cane is exposed to chemical, microbial and enzymatic deterioration [21, 36].

In South Africa, the sugarcane stalks are shredded and washed repeatedly with hot water (75-85 °C) in a diffuser to extract the plant juice, referred to as mixed juice. Small fibre particles are removed by pouring the juice over an inclined wedge-wire (DSM) screen. Fibre particles trapped on the screen are returned to the diffuser and the juice transferred to a mixed juice holding tank. The juice is heated to 105 °C and the pH adjusted to approximately 7.5 with calcium hydroxide, flocculated with a polymeric flocculant and the suspended solids are allowed to settle. The clarified juice is decanted from the clarifier, the settled mud solids filtered in a rotary vacuum filter and the filtrate recycled to the mixed juice tank. Mud solids together with added filter aid are collected and removed as filter cake. The clarified juice is concentrated by evaporation to syrup and with further evaporation under vacuum crystallised in crystallisation pans into which seed crystals are added to facilitate crystal formation. Sugar crystals are separated from the mother liquor (molasses) by centrifugation, dried and packaged as raw sugar.

Although several papers have been published on post-harvest sugarcane deterioration [4, 13, 19, 21, 37, 55, 59, 66], only a few studies [14, 50] reported on microbial degradation, or biodeterioration, of sugarcane before harvesting or during the sugar production process under actual factory conditions. Hector et al [25] described the microbial diversity found on cut sugarcane which were stored for three days, simulating post-harvest cane deterioration followed by crushing as representation of the milling unit operation of sugarcane processing. There is currently no reliable, rapid, easy and inexpensive method to measure cane deterioration [16].

The rate at which sugarcane deteriorates depends on environmental factors, harvesting conditions and the harvesting technique used. In South Africa, sugarcane is usually burnt before being hand-cut and the tops (15-20% of the aerial part of the plant) removed from the stalks. The damage caused by burning and cutting, as well as delays in delivering harvested sugarcane to the factory, in particular during hot, humid and wet conditions as generally experienced in the South African summer, enhances the biodeterioration of sugarcane [19]. During biodegradation, a variety of polysaccharides (e.g. dextran, levan and sarkaran), oligosaccharides (kestoses, isomaltotriose, isomaltotetraose, leucrose and palatinose), alcohol (ethanol), sugar alcohol (mannitol) and acids (lactic and acetic) are produced [11, 15,

47, 56]. Of these, dextran has generally been considered the main problem in the South African sugarcane industry [19].

Although South Africa does not incorporate a deterioration quality parameter in its sugarcane payment formulae [19], the maximum specification for dextran in raw sugar intended for export, according to the South African Sugar Terminals (SAST), is 150 mg/kg [44] as determined by a modified alcohol haze method [2]. Currently dextran formation in the sugar industry cannot be determined by any method which is specific, quantitative and rapid. The modified alcohol haze method [2] quantifies gums against commercial dextran standards. Gums are defined as polysaccharides of high molecular weight precipitated from aqueous solutions by acidified ethanol [27]. In sugarcane processing streams these gums may include the structural plant polysaccharides hemicelluloses, pentosans, pectins and starch, as well as polysaccharides from bacterial metabolism such as dextran and levan.

Dextran, consisting of D-glucose units linked with α-1,6 glycosidic bonds, and α-1,2, α-1,3 or α-1,4 bonds [31], is produced from sucrose by *Leuconostoc, Weissella, Lactobacillus, Streptococcus* and *Pediococcus* spp. with dextransucrase activity [35, 60]. Dextran increases the viscosity of sugar processing streams, leading to higher sucrose inversion losses due to extended boiling times caused by evaporation difficulties and reduction in crystallisation rates [22, 29]. Oligosaccharides such as 1-, 6-, and neo-kestoses (GF_2), nystose (GF_3) and kestopentaose (GF_4), as well as isomaltotriose, isomaltotetraose, leucrose and palatinose, form as result of glucan- and fructansucrase activity [15]. Similar to dextran, these oligosaccharides affect the rate of sucrose crystallisation by attaching to different crystal faces. Some oligosaccharides (*e.g.* 6-kestose, neo-kestose and theanderose) are transferred more readily to the sugar crystal than 1-kestose, leading to crystal elongation and retarded crystal growth rates [45]. Further concentration of these oligosaccharides during the refining process of raw sugar with elevated oligosaccharide concentrations leads to reduced throughput and slow crystallisation rates [45].

Microorganisms present on sugarcane do not all survive the production process and do not always contribute to the production of polysaccharides in the factory [3]. A limited number of studies have examined the microbial diversity in different unit operations of sugarcane processing factories [7, 40, 43]. These studies were all constrained by the absence of microbial identification methods with high discriminatory power. Modern approaches to microbial taxonomy provide for much more accurate and reliable identification of spoilage microorganisms and offer the opportunity to revisit the findings of other researchers. Nucleic acid-based methods are frequently used to identify bacteria because of the high throughput

potential provided by PCR amplification. For well documented reasons, the most extensively studied genetic marker for bacterial phylogeny and taxonomy has been the 16S rRNA gene [5, 9, 28]. In most cases, the initial 500 bp sequence of the 16S rRNA gene provides adequate data to determine similarities at species level [9, 26, 49].

In this study we explored the comparative diversity of polysaccharide-producing bacteria in a South African sugarcane processing factory. Differences in bacterial populations present in the sugarcane processing streams were observed at times when high and low levels of dextran in the raw sugar were observed. Identification was made by comparing partial 16S rRNA gene sequences with sequences in GenBank.

Materials and Methods

Sampling of bacteria

Samples were collected from different stages in the sugarcane production process (Table 1) at a South African sugarcane processing factory. Dextran concentrations in the raw sugar, as determined by SAST using a modified alcohol haze method [2], served as an indicator of cane deterioration and loss of sucrose. The first sampling was in September 2013, during spring (temperatures ranged from 10 to 27 °C, with a daily mean relative humidity of 70%). At the time, the raw sugar contained low levels of dextran (<70 mg/kg). The second sampling was in November 2013, during summer (temperatures ranged from 13 to 30 °C, with a daily mean relative humidity of 78%), at which time high dextran concentrations (>500 mg/kg) were reported in raw sugar.

Cane samples (10 g each) were added to 100 ml phosphate buffered saline (PBS; [23]) and incubated on a rotary shaker (30 °C, 150 rpm) for 1 h. Liquid samples collected from each of the sampling points and the PBS-cane suspension were serially diluted in PBS. Serial dilutions were streaked onto modified dextransucrase-inducing agar with the following composition: sucrose 100 g/l, peptone 20 g/l, KH_2PO_4 20 g/l, agar 15 g/l and R-salts (4% $MgSO_4 \cdot 7H_2O$, 4% NaCl, 0.2% $FeSO_4 \cdot 7H_2O$ and 0.2% $MnSO_4 \cdot H_2O$) 5 ml [61]. Plates were incubated at 30 °C for 14 to 18 h. Colonies with a glistening and slimy appearance were selected and streaked to purity on modified dextransucrase-inducing agar. From these plates a single colony was inoculated into 5 ml MRS broth (Biolab, Merck South Africa) and the cultures incubated on a shaking incubator (150 rpm) for 14 to 18 h at 30 °C. Cells were harvested by centrifugation (16 000x*g*, 25 °C, 2 min), re-suspended in sterile glycerol (200 µl; 50%, v/v) and stored at -70°C.

Table 1 Sampling point, temperature, pH and Brix content of samples

Sampling points	Low dextran concentration in raw sugar (1st sampling)			High dextran concentration in raw sugar (2nd sampling)		
	Temp (°C)	pH	Brix (°Bx)	Temp (°C)	pH	Brix (°Bx)
Cane Testing Service (CTS) station[a]	21	N/A	N/A	30	N/A	N/A
Diffuser sump	26	4.81	0.3	30	4.24	1.4
DSM screen (juice screen)	66	5.14	16.0	78	5.40	12.2
Mixed juice (MJ) tank	67	5.32	15.1	73	5.18	12.6
Filtrate	58	6.34	14.0	29	6.64	13.8
Mud trough[b]	35	5.92	9.4	64	6.77	11.0
Syrup tank	58	5.50	69.9	51	5.79	69.4

N/A, not applicable

[a]Representative samples of prepared (shredded cane) obtained from individual consignments of the sugarcane delivered to the factory were analysed for cane payment purposes at the CTS station

[b]The mud trough is part of the filter station that holds the mud through which the filter screen rotates and picks up and filters the mud

Genomic DNA extraction

Ten microliter aliquots of frozen culture were inoculated into 5 ml sterile MRS broth (Biolab) and incubated for 16 h at 30 °C on a rotational shaker (150 rpm). Cells were harvested (16 000x*g*, 25 °C, 2 min) and genomic DNA extracted using the GeneJET Genomic DNA Purification kit (Thermo Scientific), according to the manufacturer's instructions. Purified DNA was suspended in 50 µl elution buffer and used as template in amplification reactions.

Amplification of the 16S rRNA gene and sequencing

Genomic DNA was used as template to amplify DNA fragments of approximately 1500 bp encoding part of the 16S rRNA gene. The DNA primers used were 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') [34, 62]. The 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 5x One Taq Standard Reaction buffer, 1.25 U One Taq Hot Start DNA polymerase (Thermo Scientific) and 100 ng template genomic DNA. PCR reactions were 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 (50 °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. The resultant amplicons were purified using the DNA Clean and Concentrator™-25 kit (Zymo Research) according to the manufacturer's instructions. 16S rRNA gene sequencing was performed using BigDye Cycle Sequencing chemistry (Applied Biosystems) according to the manufacturer's instructions. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm [1]. Reference 16S rRNA sequences were retrieved from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Sequences were aligned with ClustalW [57], as implemented in the BioEdit Sequence Alignment Editor program [24]. A data matrix was created for representative sequences of species at each sampling location and sampling time. Phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 software [33]. The evolutionary histories were inferred using the Neighbor-Joining method [54] with the Kimura 2-parameter model [32]. The strengths of the internal branches of the resultant trees were statistically evaluated by bootstrap analysis [20] with 1000 bootstrap replications. *Bifidobacterium bifidum* ATCC 29521^T was used as the outgroup.

Results and Discussion

A total of 430 gum-producing bacteria were isolated from seven locations in a South African sugarcane processing factory. Of these, 110 isolates were obtained from spring samples when low concentrations of dextran (<70 mg/kg) were observed in the produced sugar, and 320 isolates from summer samples when high concentrations of dextran (>500 mg/kg) in raw sugar were reported.

A phylogenetic tree constructed from partial 16S rRNA gene sequences of gum-producing bacteria is shown in Fig. 1. A total of 430 isolates were grouped into four distinct clusters with high (100%) bootstrap values (Fig. 1). Nine species were identified. Cluster 1 contained strains of *Leuconostoc mesenteroides* (subsp. *dextranicum, cremoris* and *mesenteroides*), *Leuconostoc pseudomesenteroides, Leuconostoc citreum* and *Leuconostoc lactis*. Cluster 2 represented strains of *Weissella confusa* and *Weissella cibaria*. Cluster 3 consisted of *Lactobacillus fermentum*, and Cluster 4 *Bacillus amyloliquefaciens* and *Bacillus subtilis*. The majority of gum-producing bacteria belonged to the genus *Weissella* (47%), followed by *Bacillus* (24%), *Leuconostoc* (19%) and *Lactobacillus* (10%).

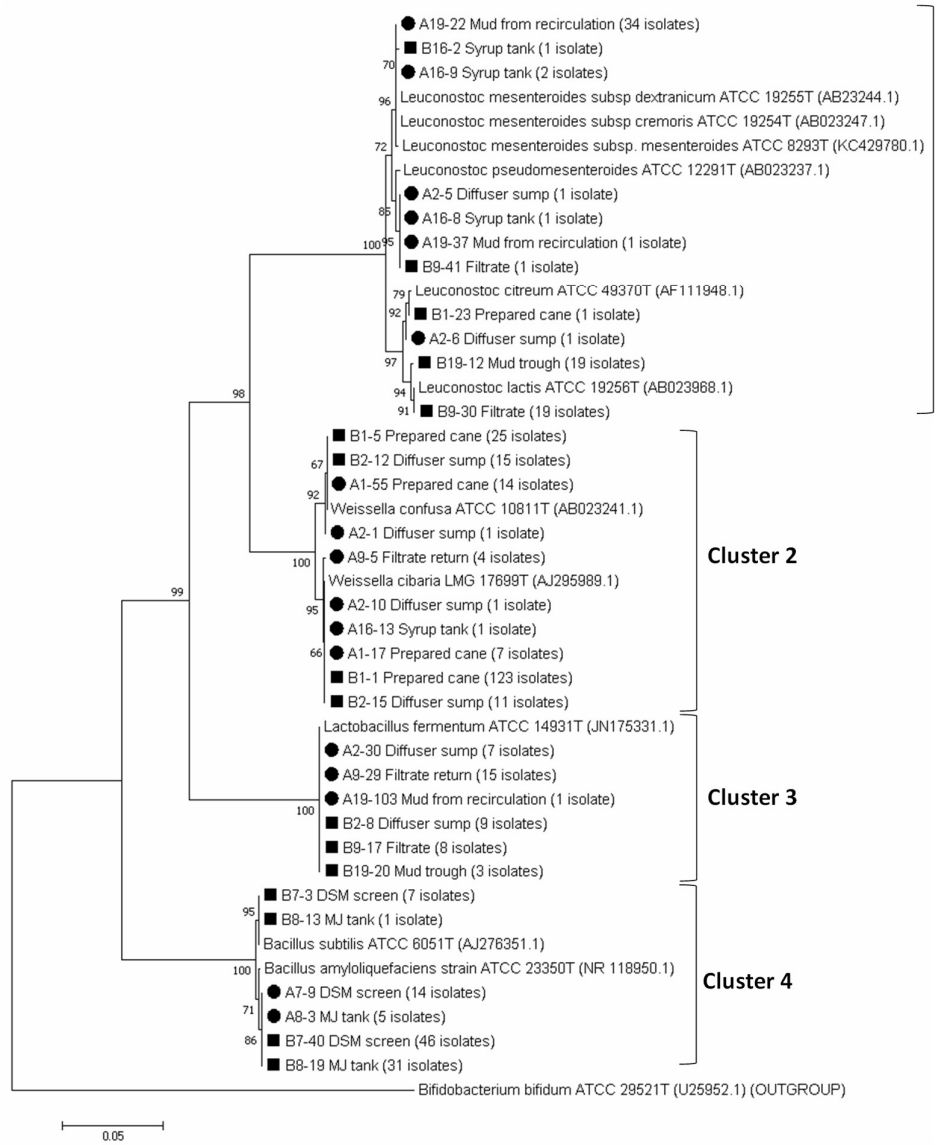

Fig. 1 Phylogenetic tree based on partial 16S rRNA gene sequences of gum-producing bacteria isolated from seven sampling points in a South African sugarcane processing factory. Isolates from the sampling time when low dextran content was observed in raw sugar are labelled with a circle (●) and those when high dextran in raw sugar was reported with a square (■). The tree was constructed using the Neighbor-Joining method with MEGA 7.0 software and included representative isolates from each sampling point. The number of isolates is indicated in brackets. Sequence data of reference strains were from GenBank. Genetic distances were computed by Kimura's two-parameter model [32]. The final dataset had a total of 830 positions. Bootstrap values over 50% (based on 1000 replications) are shown at each node. Bar, % estimated substitution per nucleotide position. *Bifidobacterium bifidum* ATCC 29521^T was used as the outgroup

Fewer gum-producing bacteria were isolated from factory samples when a low dextran concentration in the produced sugar was observed (110 isolates) compared to the sampling time when high dextran concentrations in sugar were reported (320 isolates). However, the phylogenetic diversities of isolates from these two sampling groups were very similar (Fig. 1). *Leuconostoc mesenteroides* was the dominant gum-producing species isolated at a time of low dextran concentration in raw sugar. *Weissella cibaria* dominated in samples when a high dextran concentration in the produced sugar was observed, with a single strain of *Le. mesenteroides* isolated from these samples.

The major contributor of gum-producing bacteria in summer, when high dextran concentrations were reported in the produced raw sugar, was the incoming sugarcane sampled as prepared (shredded) cane at the Cane Testing Service (CTS) station (Table 2). Hot and humid conditions favour rapid accumulation of dextran in harvested cane which is exacerbated by delays in delivering of the cane to the factory [19]. However, contrary to reports from literature, *Leuconostoc* spp. were not the major gum-producing bacteria isolated from the sugarcane. Instead, we recorded high cell numbers of *W. confusa* and *W. cibaria* (Table 2). Only two reports have associated the genus *Weissella* with cane deterioration. Tilbury [59] described the isolation of a new dextran-producing species, *Lactobacillus confusus*, from deteriorated sugarcane. *Lb. confusus* has since been reclassified as *W. confusa* [10]. Recently, Hector et al. [25] isolated *W. confusa* and *W. cibaria* from sugarcane stalks that were stored for three days post-harvest. These authors identified *Leuconostoc, Weissella, Lactobacillus* and *Salmonella* as the gum-producing bacterial genera isolated from crushed sugarcane. The dominance of *Weissella* spp. on prepared cane is significant, because these bacteria are not usually associated with deteriorated sugarcane and have not previously been isolated from a sugarcane processing factory. *Weissella* spp. have the ability to synthesise a variety of polysaccharides and oligosaccharides, which includes high molecular weight, low-branched dextran, as well as levan and inulin, in addition to gluco- and fructo-oligosaccharides and cell-associated ropy polymers [41]. These poly- and oligosaccharides may have a severe impact on the quality and quantity of produced sugar.

Table 2 Distribution of gum-producing species isolated from a South African sugarcane processing factory

Sampling point	Number and species of gum-producing isolates	
	Low dextran content	High dextran content
CTS station	*W. confusa* (14) *W. cibaria* (7)	*W. confusa* (25) *W. cibaria* (123) *Le. citreum* (1)
Diffuser sump	*W. confusa* (1) *W. cibaria* (1) *Le. pseudomesenteroides* (1) *Le. citreum* (1) *Lb. fermentum* (7)	*W. confusa* (15) *W. cibaria* (11) *Lb. fermentum* (8)
DSM screen	*B. amyloliquefaciens* (14)	*B. amyloliquefaciens* (46) *B. subtilis* (7)
Mixed juice tank	*B. amyloliquefaciens* (5)	*B. amyloliquefaciens* (31) *B. subtilis* (1)
Filtrate	*W. cibaria* (4) *Lb. fermentum* (15)	*Le. pseudomesenteroides* (1) *Le. lactis* (19) *Lb. fermentum* (9)
Mud trough	*Le. mesenteroides* (34) *Le. pseudomesenteroides* (1) *Lb. fermentum* (1)	*Le. lactis* (19) *Lb. fermentum* (3)
Syrup tank	*W. cibaria* (1) *Le. mesenteroides* (2) *Le. pseudomesenteroides* (1)	*Le. mesenteroides* (1)

The diffuser sump contained between 10 and 11% of the total number of gum-producing bacteria, as isolated during spring and summer, respectively. Cane diffusers generally operate at an average of 85 °C with a cane retention time of approximately one hour. At these temperatures the growth of most bacteria is repressed if the cells are not killed [40, 53]. However, the diffuser sump, which is a holding tank for diffuser wash-outs and overflows, run-offs and leaks from pumps, is at ambient temperature. The contents of the sump are periodically pumped back into the diffuser when the liquid reaches a predetermined level and can be prone to microbial contamination due to the moderate temperatures and possible long retention times. Factory staff occasionally slug-dose biocides into the diffuser sump to limit microbial growth and control contamination [30]. In this study, *W. confusa and W. cibaria*, isolated from incoming cane at both sampling times, were also present in the diffuser sump. This suggests that these bacteria were transferred from the incoming cane to the sump. In

addition, *Le. pseudomesenteroides, Le. citreum* and *Lb. fermentum* were isolated from the sump contents.

Previous reports [36, 52] have indicated that the area behind the juice screens is the most frequent source of contamination, adding to increased bacterial levels in the mixed juice tank. Slime deposits, as result of bacterial contamination, have been observed on the juice screens and previous studies have indicated *Leuconostoc* spp. as the major contaminants [36, 52]. However, in our study, *Bacillus* spp., and not *Leuconostoc* spp., were isolated from the DSM screen and mixed juice tank. During the first sampling, only *B. amyloliquefaciens* was isolated (17% of the total number of isolates). *Bacillus amyloliquefaciens* (24%) and *B. subtilis* (3%) were isolated during the period of high dextran-containing sugar. This is the first report of *B. amyloliquefaciens* in sugarcane processing. The presence of *B. amyloliquefaciens* and *B. subtilis* on the DSM screen and in the mixed juice tank is important as these bacteria have the potential to (i) produce resistant endospores [42], (ii) form biofilms [12, 48] and (iii) produce levansucrase that form levan (a fructose-based polysaccharide) and fructooligosaccharides (kestoses) from sucrose [58].

Filtration is used to recover sucrose from the mud which is a mixture of juice and settled solids from the clarification process. The filtrate quality often does not receive the attention that it deserves. Severe sucrose losses may occur in filter stations, particularly through microbiological activity [38]. The mud feed for filtration and wash water should be above 80 °C to avoid the risk of sugarcane waxes blocking the filter screens and to prevent microbial growth [51], resulting in filtrate temperatures of around 60 °C. In this study, filtrate temperatures of 58 °C and 29 °C were recorded when sampled at times of low and high dextran concentrations in raw sugar, respectively. Factory staff acknowledged that the low filtrate temperature recorded during the second sampling was due to a processing error. At this time, strains of *Le. lactis* and *Lb. fermentum* were isolated. *Lactobacillus fermentum* dominated the filtrate sample taken at the first sampling. The temperature of the mud in the mud trough at this time (35 °C) was much lower compared to the second sampling (64 °C), possibly due to stoppages and longer retention times of the mud in the trough, resulting in cooling of the mud. A considerable number of *Le. mesenteroides* strains (31% of the total number of strains isolated during the first sampling) were from mud at 35 °C. On the contrary, *Le. lactis* was the major gum-producer in the mud during the second sampling when the temperature was higher (64°C). *Le. lactis* has a higher heat resistance than *Le. mesenteroides* [39]. Although the filtrate is recirculated to the mixed juice tank, none of the gum-producing bacteria isolated in the filtrate were detected in the juice sampled from the mixed juice tank. This is presumably due to the high temperatures (67 °C and 73 °C, respectively) recorded for juice samples, which

allowed growth of endospore-forming *Bacillus* species, but not *Leuconostoc* and *Lactobacillus* spp. [6, 39, 64].

Very few gum-producing bacteria (1% of total) were isolated from the syrup tank. The high sucrose concentrations (approximately 70 °Brix) in the syrup would have limited bacterial growth due to the low water activity and high osmotic pressure [8, 65].

A major challenge for sugar industries worldwide is to identify consignments of deteriorated sugarcane before they are processed, since there is currently no rapid, reliable, easy and inexpensive indicator of cane deterioration available [16]. In this study, high dextran concentration in the produced sugar was used as indicator of cane deterioration and sucrose loss, although the modified haze method [2], which was used to measure dextran, does not selectively quantify dextran, but all gums (polysaccharides) which are able to precipitate in alcohol. *Weissella, Leuconostoc* and *Lactobacillus* are lactic acid bacteria with the ability to produce a variety of polysaccharides (*e.g.* dextran, alternan, mutan, reuteran, levan and inulin) synthesised by glucan- and fructansucrase using sucrose as substrate [63]. Bacteria from the genus *Bacillus* can synthesise levan from sucrose by the action of levansucrase [63]. The impact of dextran as high viscosity polymer in sugarcane processing streams has been well described and, although currently deemed uneconomical, the application of dextranases has been considered to reduce dextran-related processing problems [18, 46]. However, the possible contributions of alternan, mutan, reuteran, levan and inulin to high viscosities in sugar production have not yet been recognised [17].

An indicator of cane deterioration will be useful only if it could be directly related to a processing problem in the factory. The viscosity of syrup is known to increase upon processing of deteriorated cane. This increased viscosity has been attributed to dextran, produced by *Leuconostoc* spp. [17]. Our study has identified gum-producing bacteria previously not recognised by the sugar industry as potential contributors to high viscosity, as well as bacteria which can produce gums other than dextran. This is an important finding that should be taken into consideration by sugar industries when investigating cane deterioration and indicators thereof, as well as developing strategies to reduce the adverse effects of gums on processing.

This is the first report on the isolation of gum-producing bacteria and their phylogenetic identification from various unit operations in a sugarcane processing factory. The authors acknowledge that the limited extent of sampling, i.e., at one factory on two separate occasions within one season only, may not be representative of all sugarcane processing factories at

any given time. However, this exploratory study yielded valuable information regarding microbial diversity in a factory sampled at times when different dextran concentrations, as an indication of cane deterioration and sucrose loss, were reported. The high number of gum-producing bacteria isolated from prepared cane in summer when high dextran concentrations in the produced sugar were observed confirms the prevalence of post-harvest biodeterioration of sugarcane during hot, humid conditions. The sugarcane processing conditions in the factory appear to eliminate most of the bacteria which enter the factory with the cane. Therefore, the fact that dextran is found in the produced sugar indicates that this is formed somewhat earlier in the supply chain and that the polymer potentially enters the factory with the cane. It is therefore critical that the time between burning and harvesting to crushing should be kept to a minimum to limit microbial growth and dextran production. Factory control in maintaining high temperature process streams at the correct temperature and limiting the retention times in low temperature sumps is equally important to prevent microbial growth inside the factory.

This study showed that *Le. mesenteroides* is not the only cause of biodeteriorated sugarcane. The presence of *Weissella* spp. on the prepared cane and inside the factory was not expected and is a significant finding. Similarly, *Bacillus* spp. are usually not associated with contaminated juice screens and mixed juice tanks, and no previous reports have shown an association between *B. amyloliquefaciens* and sugarcane processing. Care should be taken not to mistake the presence and metabolic products of *Bacillus* with those of *Leuconostoc*, in particular when biocide application and other sanitation strategies are considered. The unanticipated presence of *Weissella* and *Bacillus* bacteria on the prepared cane and in the factory reiterates the importance of accurately identifying the spoilage microbes in sugarcane processing at the various unit operations for effective microbial control and development of cane deterioration indicators.

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Supplementary material

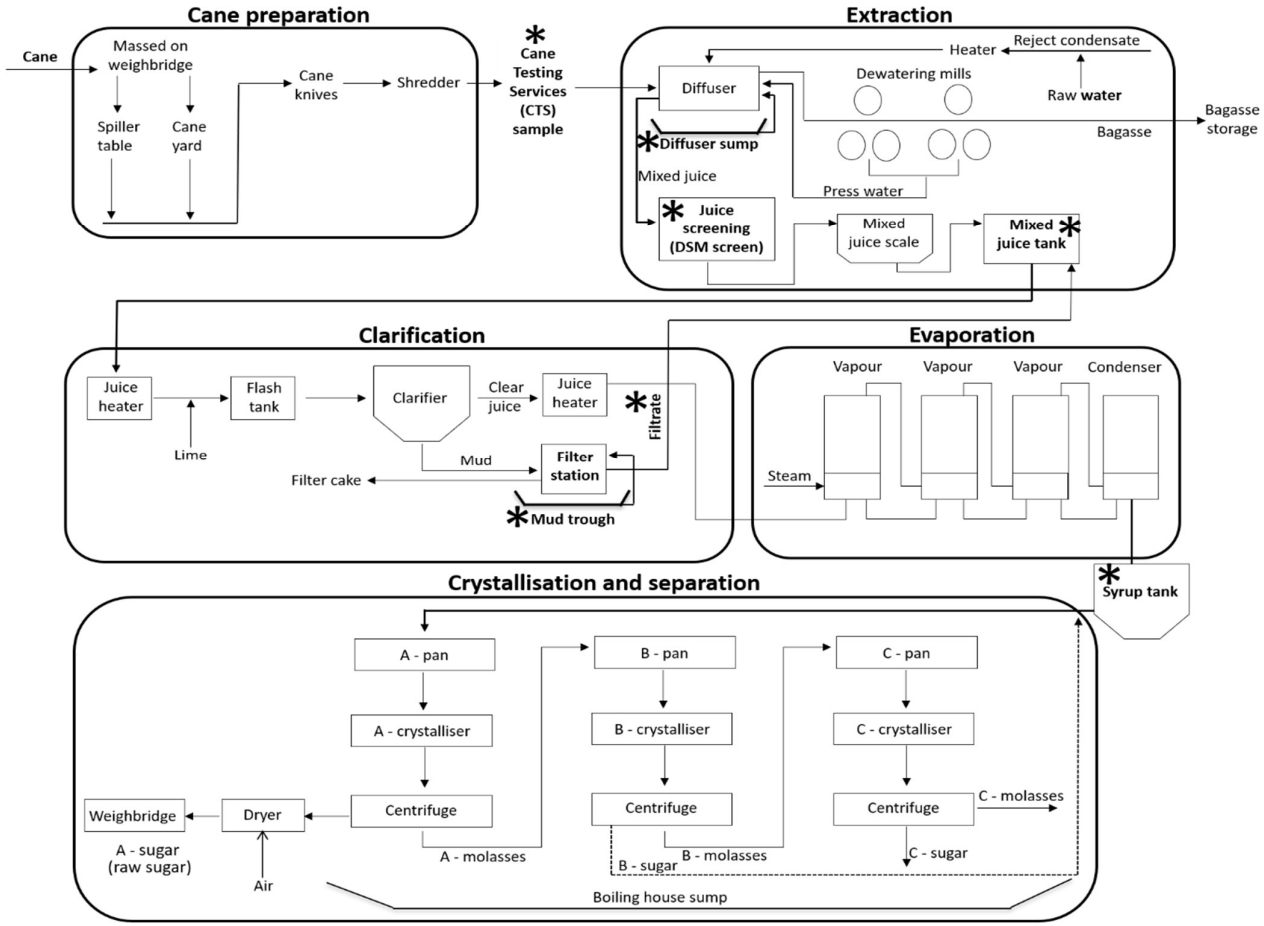
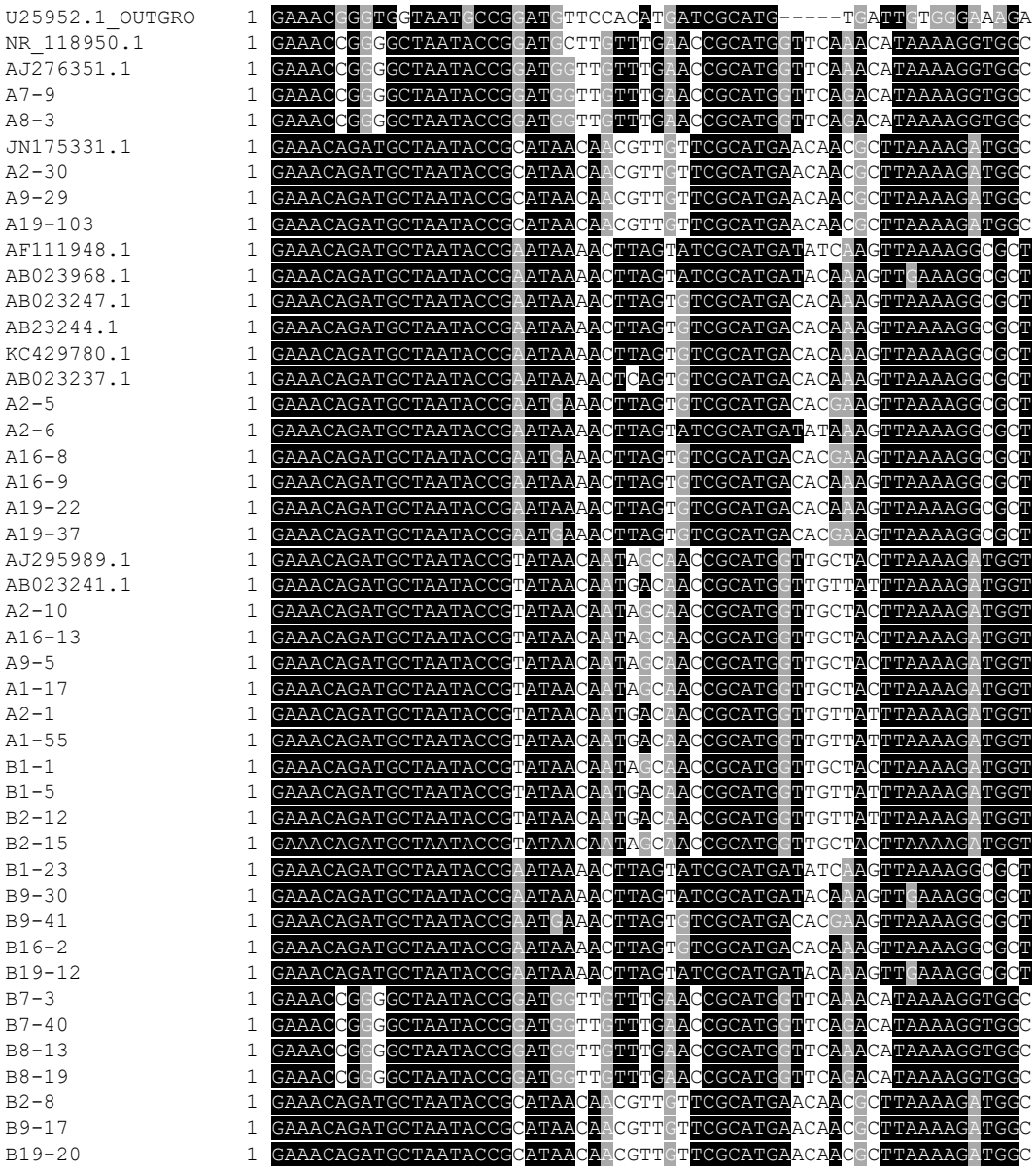


Fig. S1 Schematic representation of the various unit operations of a general South African sugarcane processing factory, with sampling points indicated by asterisks



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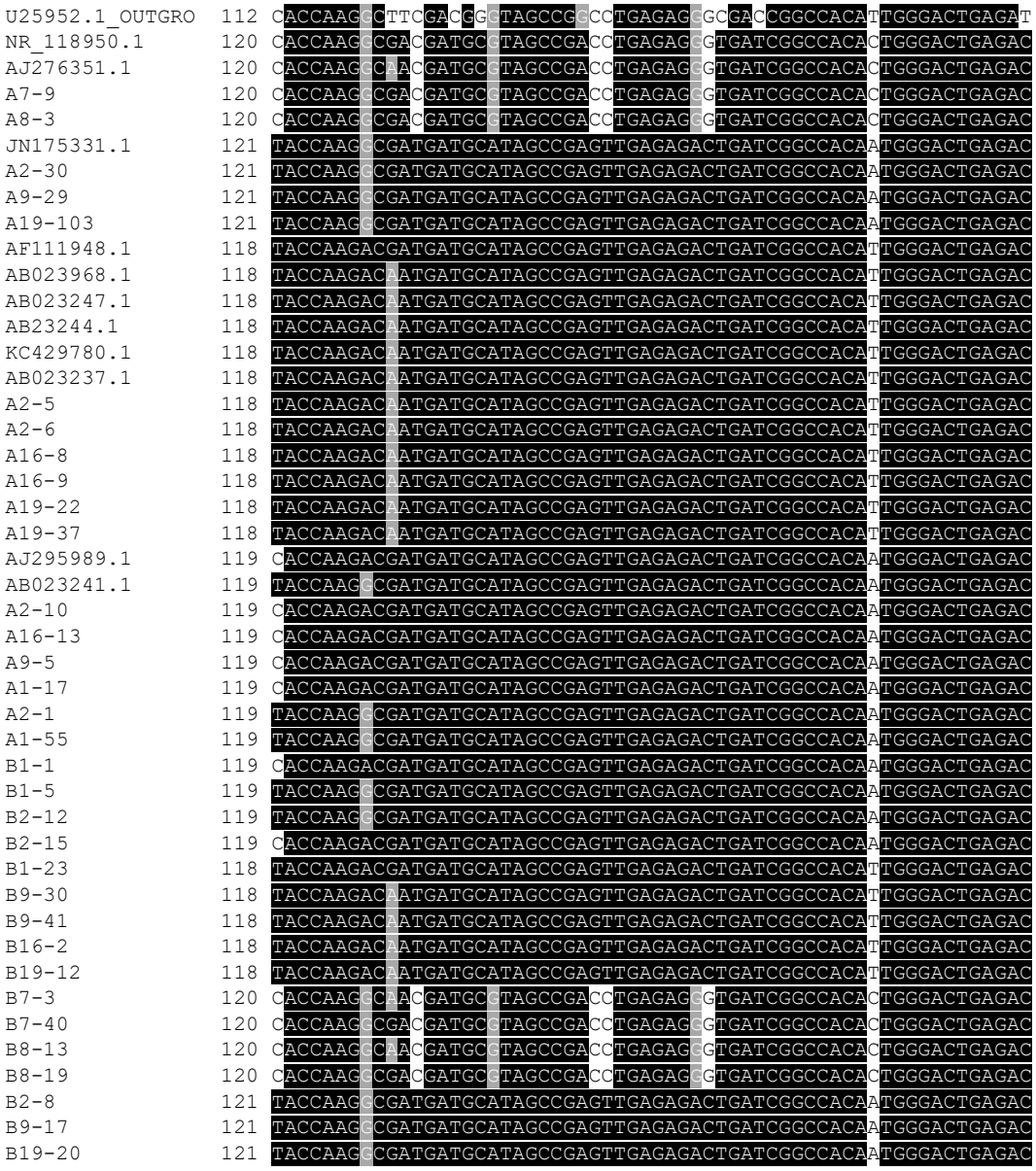

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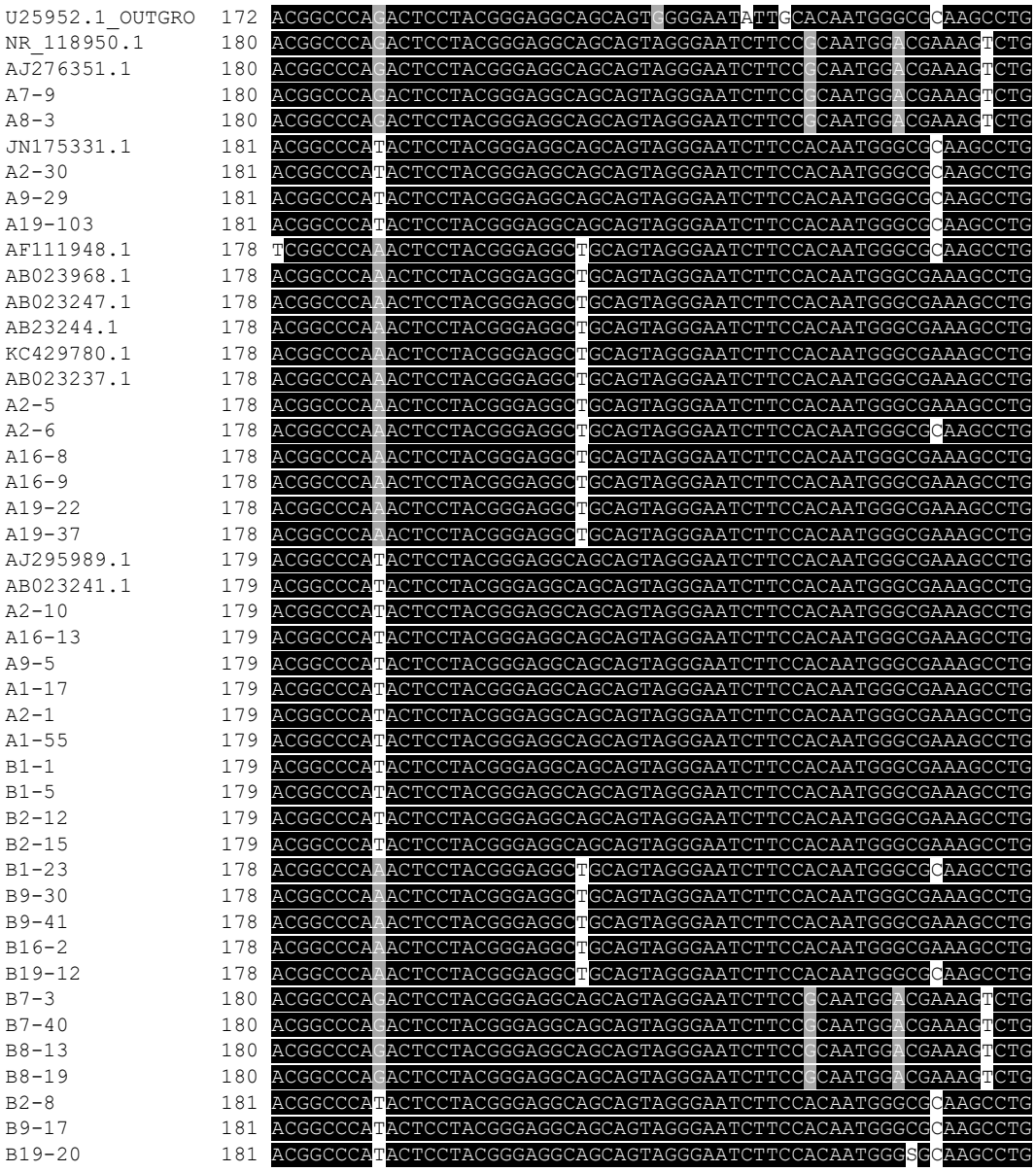

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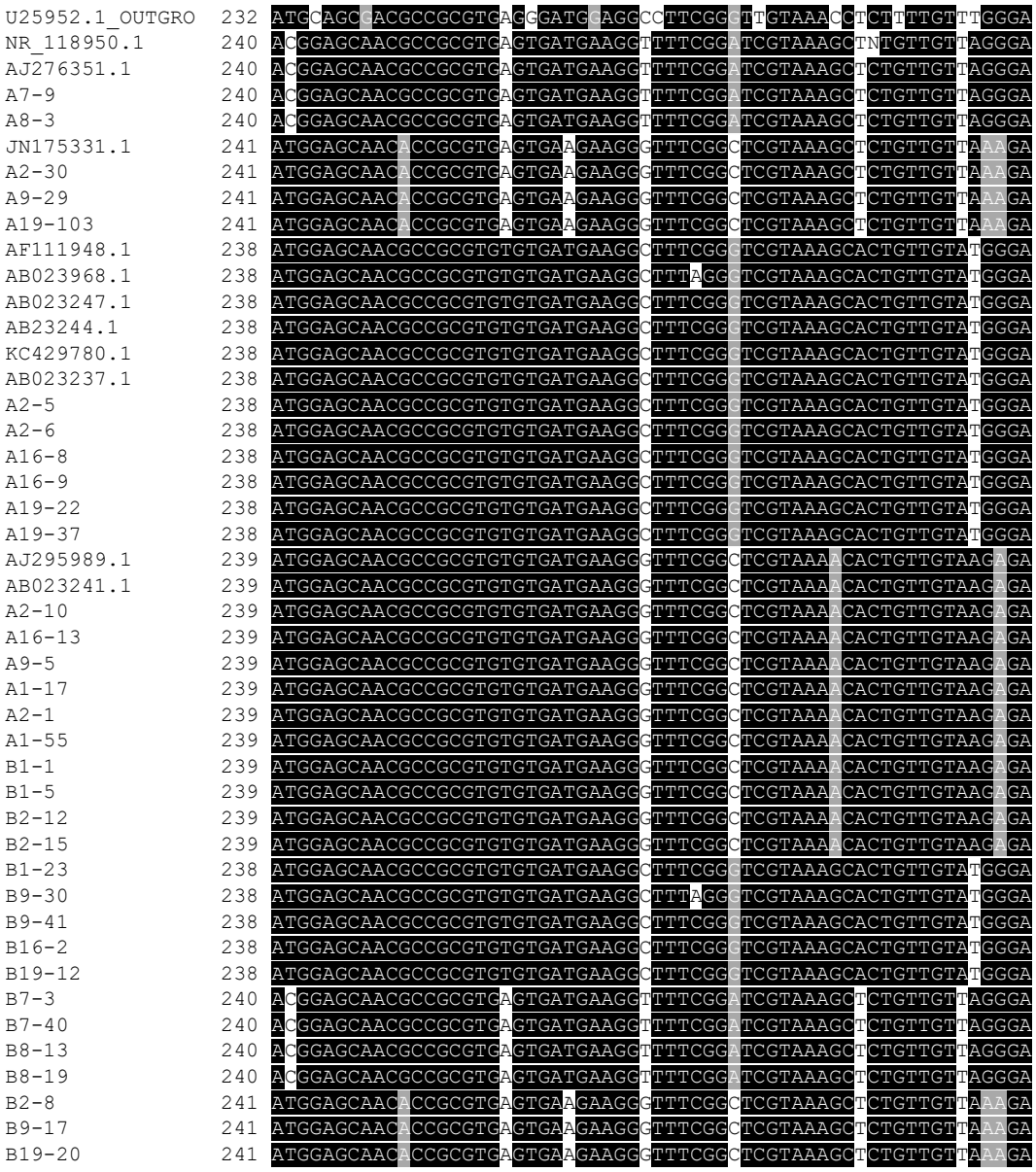

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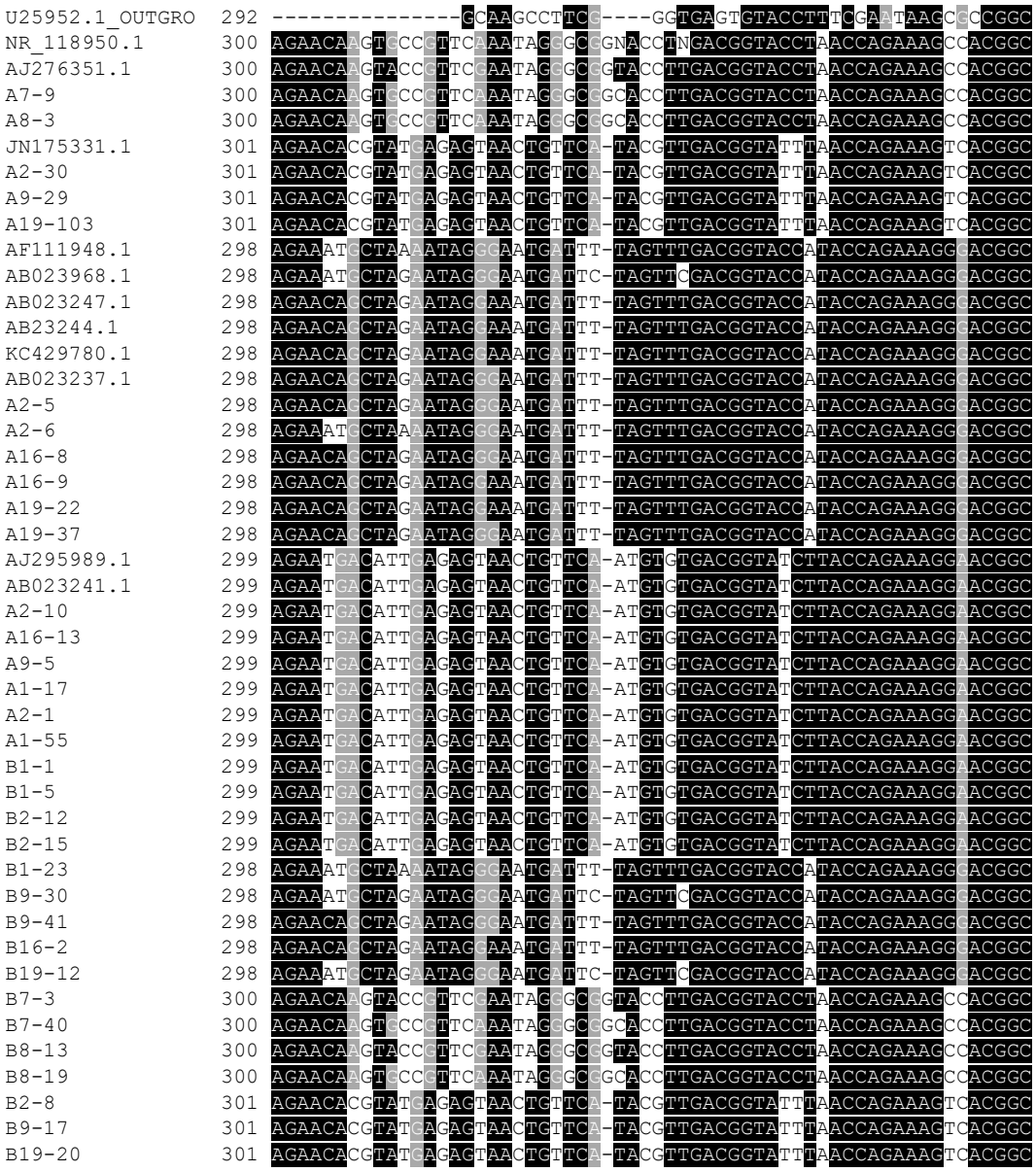

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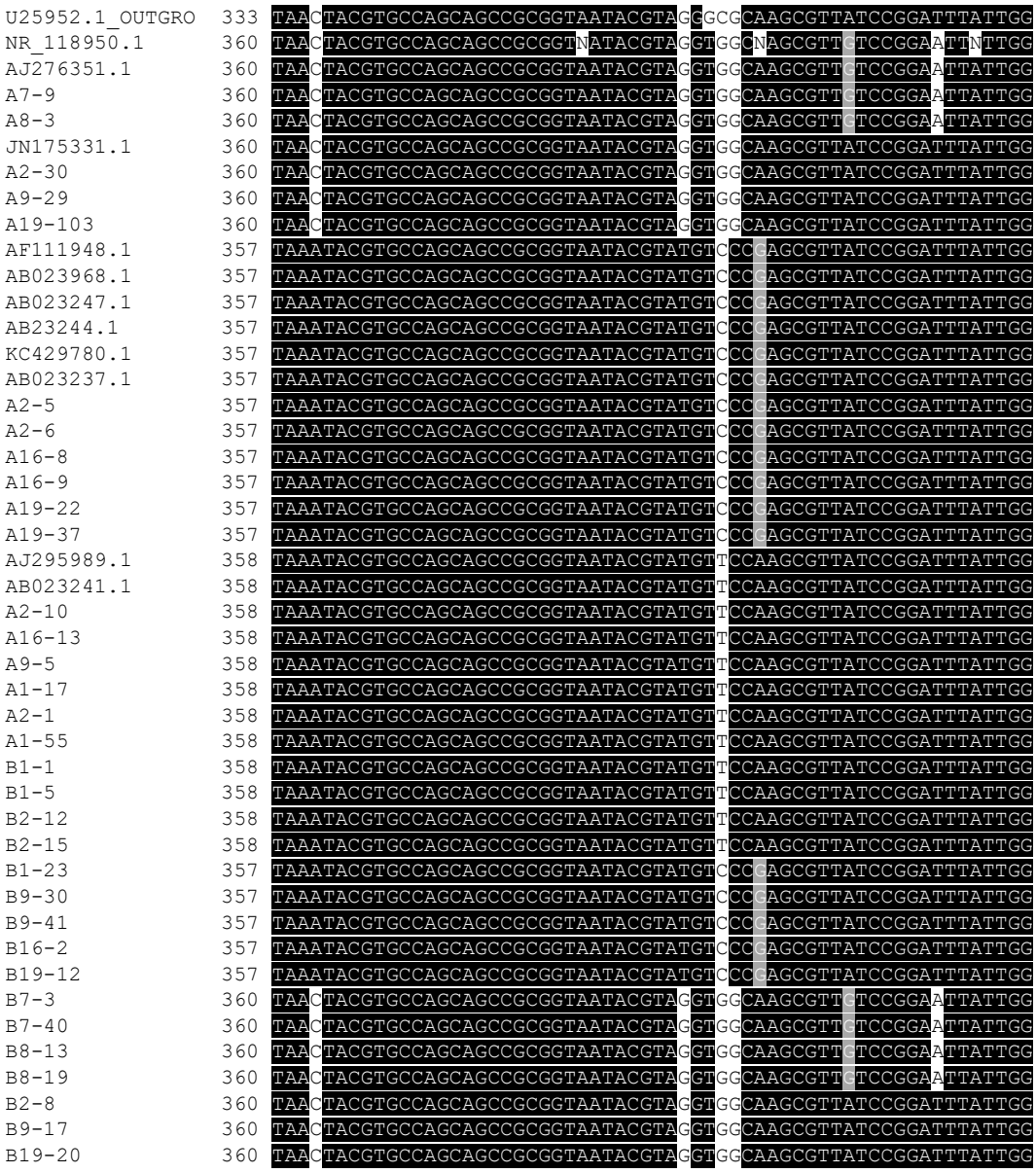

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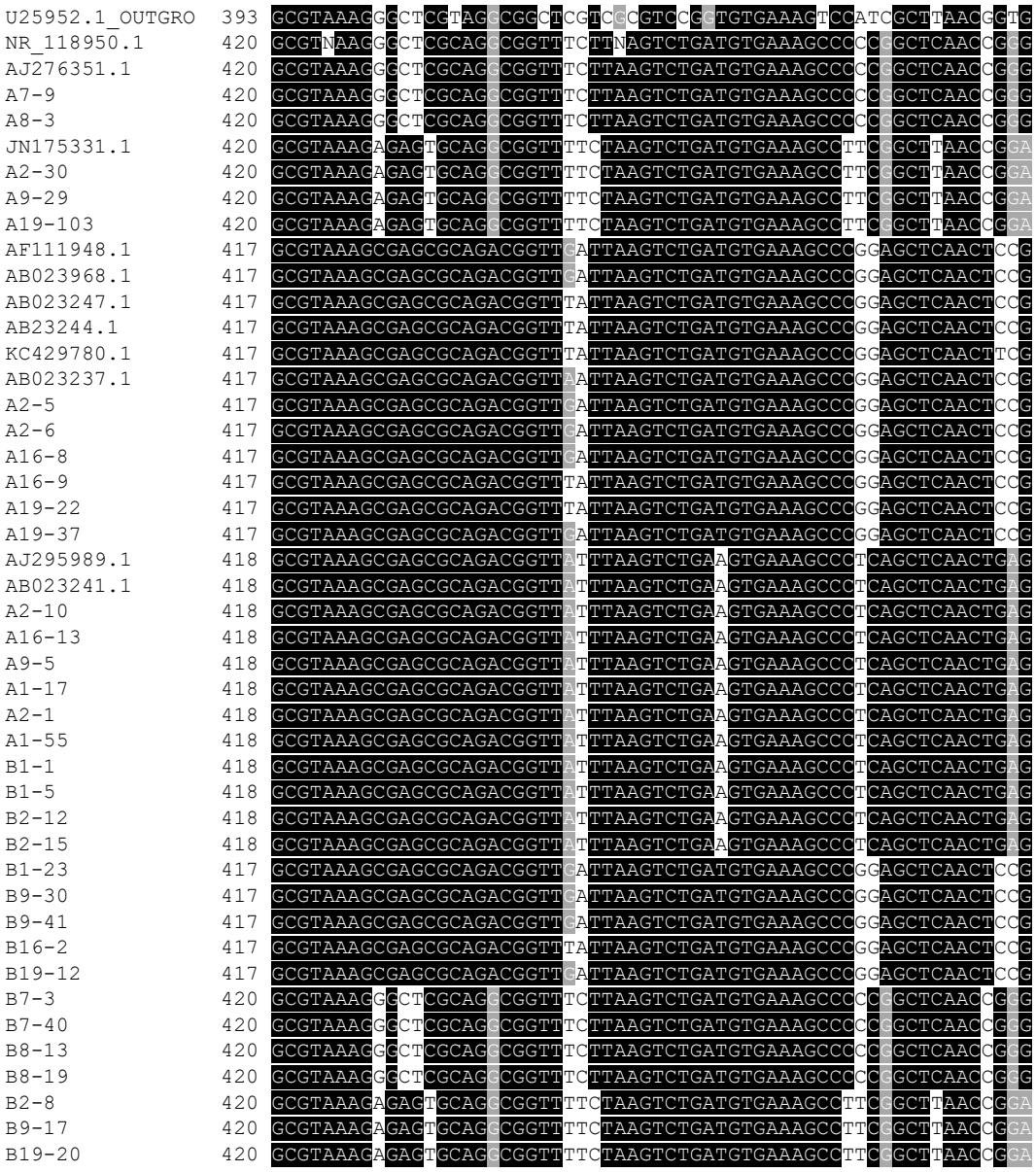

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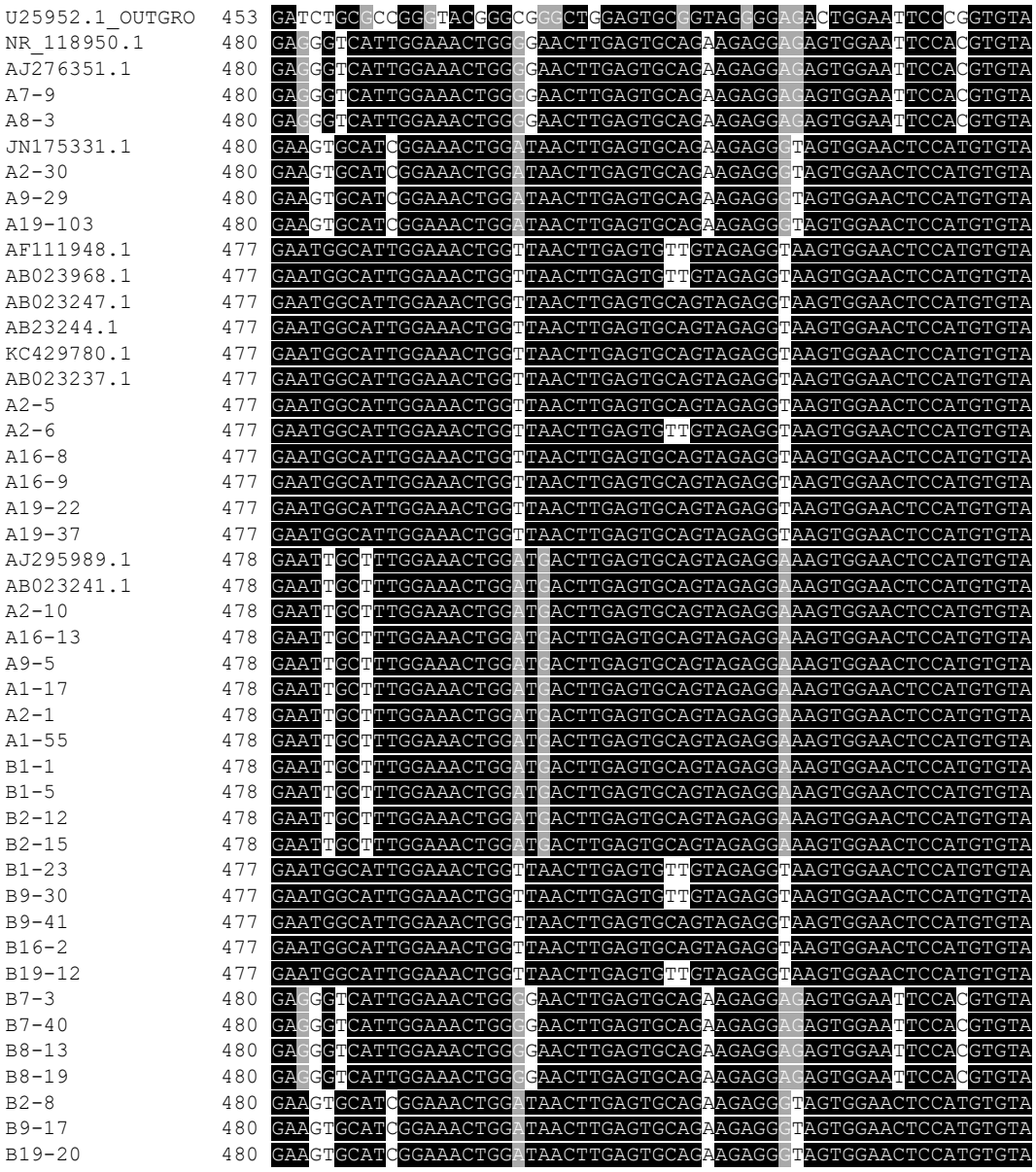

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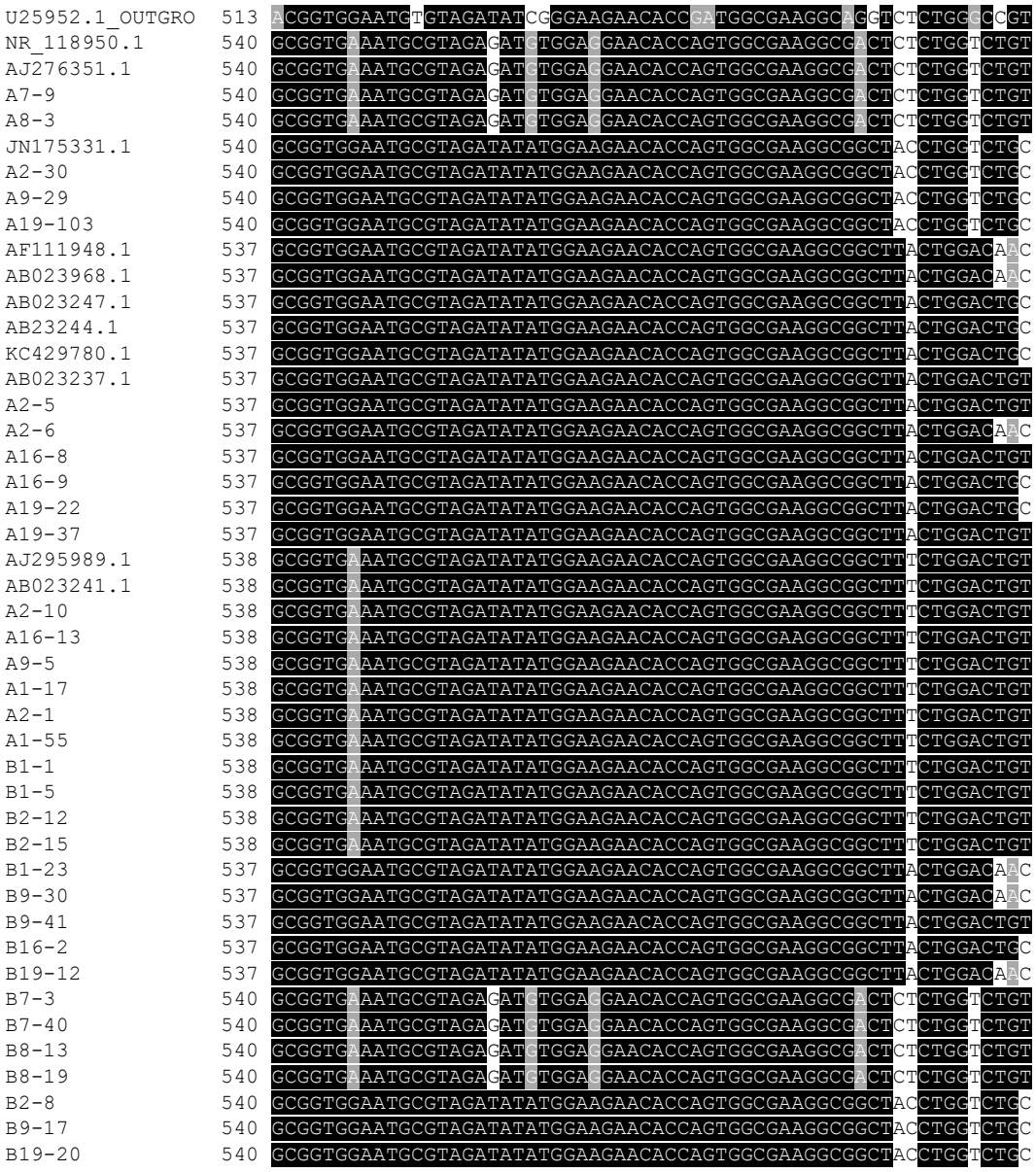

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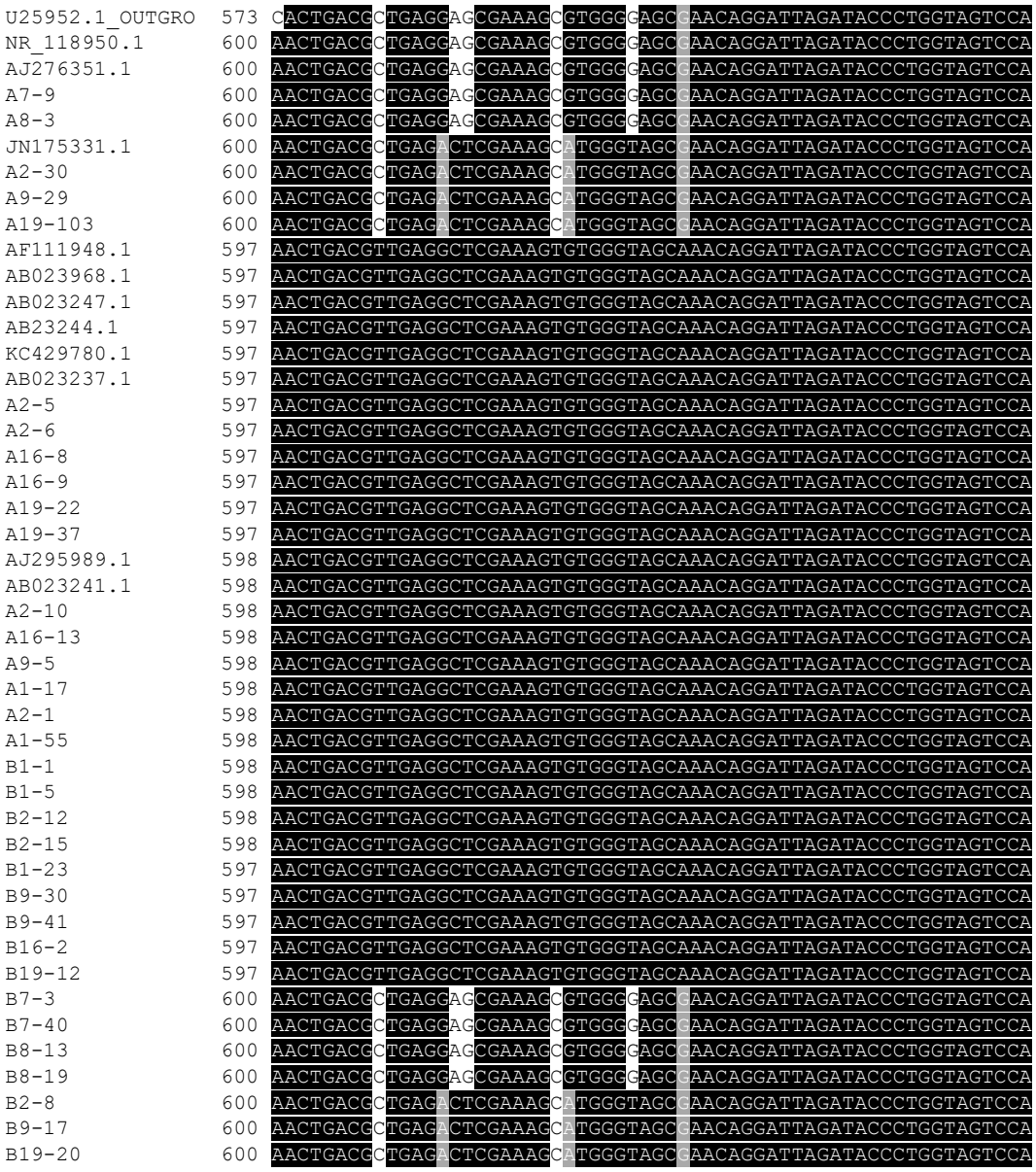

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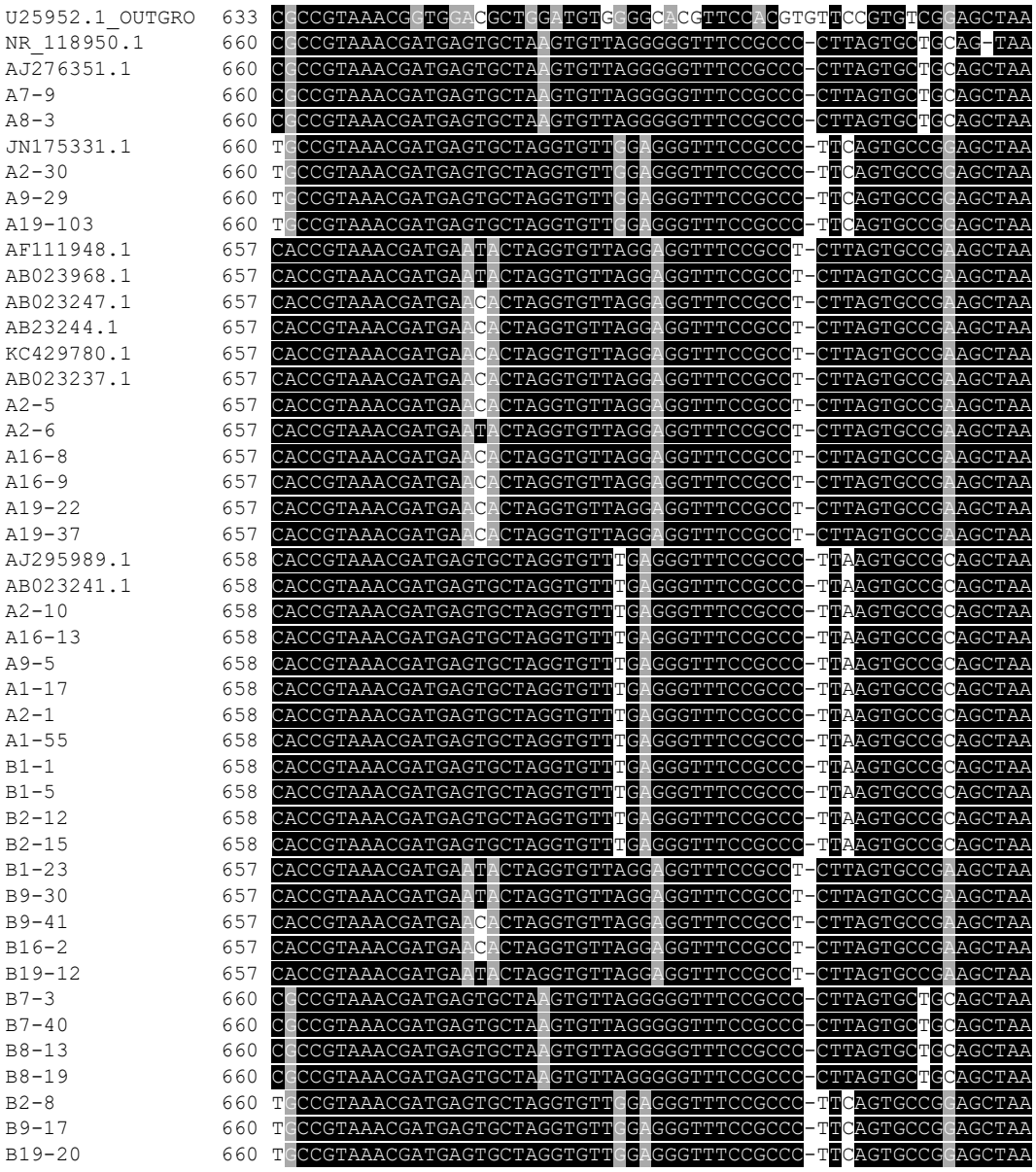

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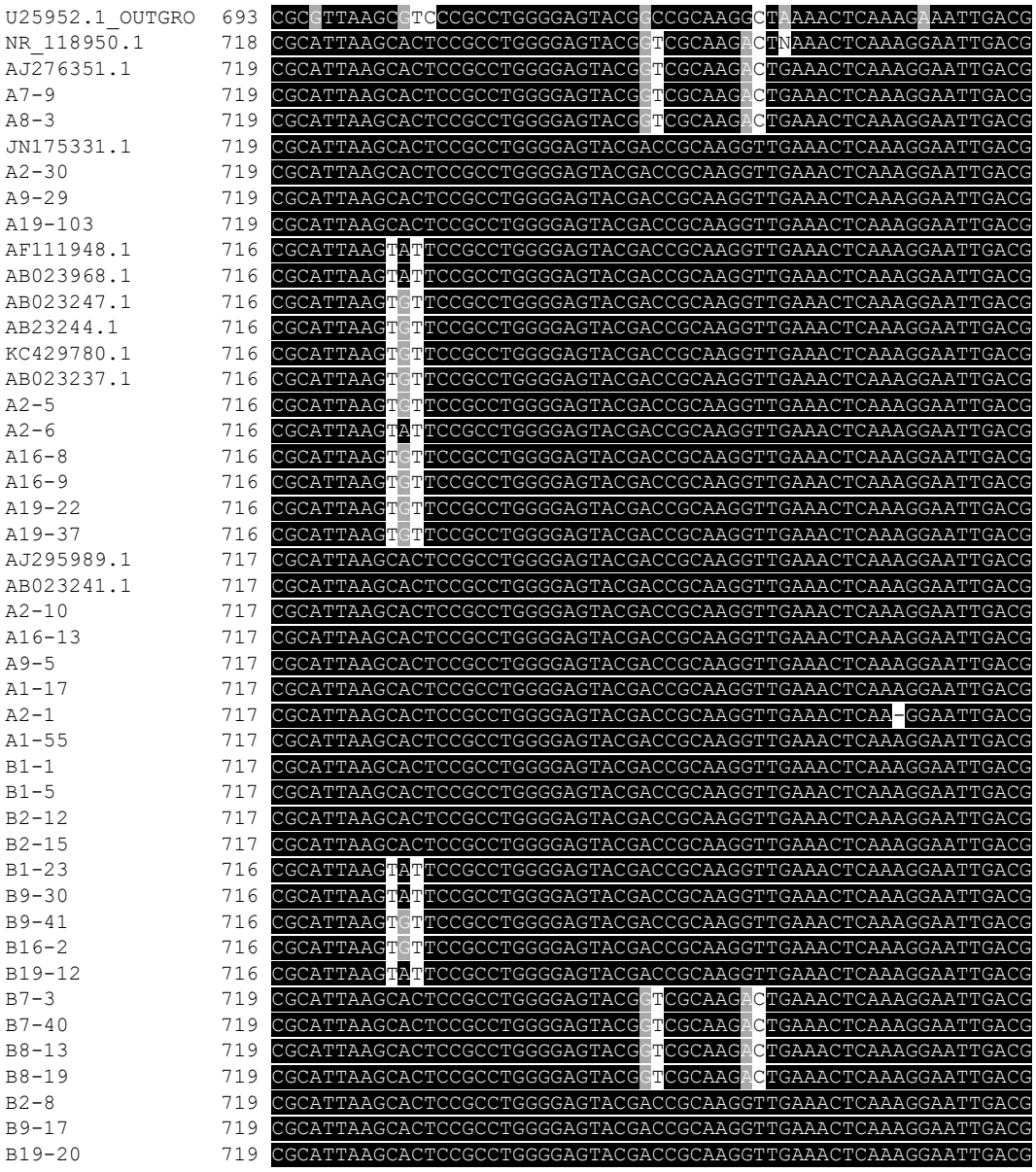

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B1-23            776 GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC-TTAC
B9-30            776 GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC-TTAC
B9-41            776 GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC-TTAC
B16-2            776 GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACC-TTAC
B19-12           776 GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC-TTAC
B7-3              779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA-CCTTAC
B7-40             779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA-CCTTAC
B8-13             779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA-CCTTAC
B8-19             779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA-CCTTAC
B2-8              779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAA-CCTTAC
B9-17             779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAA-CCTTAC
B19-20            779 GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAA-CCTTAC
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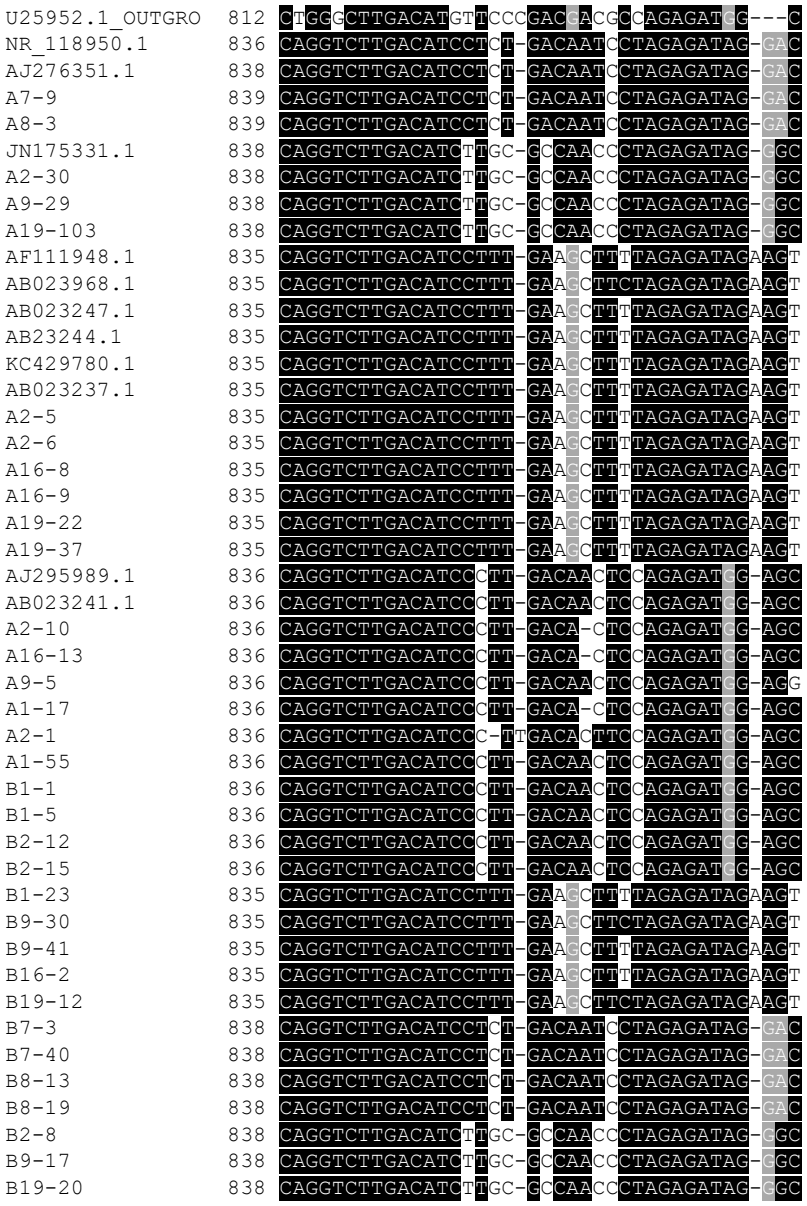

Fig. S2 Multiple DNA sequence alignments of partial 16S rRNA genes of representative and reference strains used in this study. Sequences were aligned with ClustalW [57] as implemented in the BioEdit Sequence Alignment Editor program [24] followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)

CHAPTER 5

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Phylogenetic analyses of the *pheS, dnaA* and *atpA* genes for the identification of *Weissella confusa* and *Weissella cibaria* isolated from a South African sugarcane processing factory

Sanet Nel[1,2], Stephen B. Davis[1], Akihito Endo[3] and Leon M. T. Dicks[2#]

[1]Sugar Milling Research Institute NPC, c/o University of KwaZulu-Natal, Durban 4041, South Africa

[2]Department of Microbiology, Stellenbosch University, Stellenbosch 7600, South Africa

[3]Department of Food, Aroma and Cosmetic Chemistry, Tokyo University of Agriculture, Hokkaido, Japan

#Corresponding author: LMT Dicks (LMTD@sun.ac.za), Tel.: +27-21-808 5849, Fax: +27-21-808 5846

Keywords: Phylogenetic analysis, *pheS, dnaA, atpA*, *Weissella*

Abstract

A previous study reported on the isolation of 430 polysaccharide (gum)-producing bacteria from a South African sugarcane processing factory and the identification of isolates by comparative 16S rRNA gene sequencing. A large number of isolates (202) belonged to the genus *Weissella* and clustered with reference strains of *Weissella cibaria* and *Weissella confusa*. In this study, we identified 147 strains as *W. cibaria* and 55 as *W. confusa* based on the phylogenetic analyses of the *pheS* and *dnaA* gene sequences of representative isolates. We also included the *atpA* gene sequencing analysis of the *Weissella* isolates as potential future phylogenetic marker to differentiate amongst strains of *W. cibaria* and *W. confusa*.

Introduction

High levels of polysaccharides in sugarcane have a marked effect on the recovery of crystal sugar and cause severe losses in the sugar industry [1]. These polysaccharides, of which dextran has generally been considered the main problem in the South African sugarcane industry [2, 3], arise from microbial degradation, or biodeterioration of the cane after harvesting and during the sugar production process. Dextran, consisting of D-glucose units linked with α-1,6 glycosidic bonds, and α-1,2, α-1,3 or α-1,4 bonds [4], is produced from sucrose by *Leuconostoc, Weissella, Lactobacillus, Streptococcus* and *Pediococcus* spp. with dextransucrase activity [5, 6]. Dextran increases the viscosity of sugar processing streams,

leading to higher sucrose inversion losses due to extended boiling times caused by evaporation difficulties and reduction in crystallisation rates [7, 8].

There is currently no reliable, rapid, easy and inexpensive method to measure cane deterioration [9]. Microorganisms present on sugarcane do not all survive the production process and do not always contribute to the production of exopolysaccharides in the factory [10]. A limited number of studies have examined the microbial diversity in different unit operations of sugarcane processing factories [11-13]. These studies were all constrained by the absence of microbial identification methods with high discriminatory power. Modern approaches to microbial taxonomy provide for a much more accurate and reliable identification of spoilage microorganisms and offer the opportunity to revisit the findings of other researchers.

We have isolated 430 exopolysaccharide (gum)-producing bacteria from seven locations in a South African sugarcane processing factory [3]. Of these, 110 isolates were obtained from samples taken during spring (September 2103; temperatures ranged from 10 to 27 °C, with a daily mean relative humidity of 70%) when low concentrations of dextran (<70 mg/kg) were observed in the produced sugar, and 320 isolates from samples during summer (November 2013; temperatures ranged from 13 to 30 °C, with a daily mean relative humidity of 78%) when high concentrations of dextran (>500 mg/kg) were reported in raw sugar. Phylogenetic analysis of the partial 16S rRNA gene sequences differentiated the gum-producing bacteria into four genera and nine species [3]. A large number of isolates (202) belonged to the genus *Weissella* and representative strains clustered with reference strains of *Weissella cibaria* and *Weissella confusa*. A significant finding of this study was that *Leuconostoc mesenteroides* was not the only spoilage bacterium isolated from sugarcane and sugarcane processing streams. Historically, *L. mesenteroides* had been implicated as the main causative agent of biodegraded sugarcane [14]. Nel [15] challenged the perception that *L. mesenteroides* is the sole cause of biodegraded sugarcane and advocated the concept of microbial diversity profiling of spoilage bacteria in sugarcane processing to develop strategies to prevent biodeterioration of sugarcane, and/or mitigate the detrimental effects of microbial metabolic products (such as dextran) on sugarcane processing. Bacterial spoilage of sugarcane and dextran production during sugarcane processing remains a problem. Current methods used to prevent microbially-mediated sucrose loss and solutions to reduce the effects of dextran on sugarcane processing are inadequate and do not address the cause of the problem. The unanticipated presence of *Weissella* spp. on prepared cane and in the factory emphasises the importance of accurately identifying spoilage bacteria in sugarcane processing at the various unit operations for effective microbial control and development of cane deterioration indicators.

Although 16S rRNA gene sequence analysis is still considered important for bacterial identification [16], the main disadvantage of the method is its often-insufficient resolution at species level, especially between strains of closely related species. *W. confusa* and *W. cibaria* share 99.2 % 16S rRNA sequence similarity [17]. Hong and Farrance [18] showed that the overall performance of the first 500 bp sequence of the 16S rRNA gene, compared to the entire 1500 bp sequence for bacterial identification is very high (>90%). The authors suggested that, for bacterial identification, the generation of full-length sequence data for the 16S rRNA gene is inefficient and impractical, and that a higher phylogenetic resolution can be obtained by sequence analysis of the first 500 bp of the 16S rRNA gene in combination with additional phylogenetic analyses of housekeeping or other protein-coding genes.

Gene sequence analyses of the phenylalanyl t-RNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) have been used to differentiate amongst closely related lactic acid bacteria of the genera *Lactobacillus* [19-22] and *Enterococcus* [23]. Gene sequence analysis of the alpha subunit of ATP synthase (*atpA*) were used to differentiate amongst species of the genera *Leuconostoc* [24] and *Pediococcus* [25]. However, *atpA* as molecular marker has not previously been used to differentiate *Weissella* species. Chelo et al. [26] assessed the congruence of evolutionary relationships within the *Leuconostoc-Oenococcus-Weissella* clade by comparison of 16S rRNA gene, *dnaA* (encoding chromosomal replication initiation protein), *gyrB* (encoding DNA gyrase B subunit), *rpoC* (encoding the beta subunit of the DNA-dependent RNA polymerase) and *dnaK* (encoding the 70 kDa heat-shock protein) sequence analyses.

The identification of the 202 *Weissella* strains isolated from a South African sugarcane processing factory involved two steps as suggested by Hong and Farrance [18]. Previously, partial 16S rRNA gene sequencing was used to determine the genera of the unknown strains [3]. In this study, the 16S rRNA sequencing data are compared to the phylogenetic analyses of the housekeeping genes *pheS* and *dnaA* for the identification of the *Weissella* isolates. We also included the *atpA* sequence analysis of the representative *Weissella* isolates as potential future phylogenetic marker to differentiate between strains of *W. cibaria* and *W. confusa.*

Materials and Methods

Isolation of gum-producing bacteria

Samples of crushed sugarcane, and samples from the diffuser sump, juice screen (Dutch State Mines; DSM screen), mixed juice tank (MJ tank), filtrate, mud trough and syrup tank from a South African sugarcane processing factory were collected for the isolation of gum-

(polysaccharide) producing bacteria [3]. Crushed sugarcane samples (10 g each) were added to 100 ml phosphate buffered saline (PBS, [27]) and incubated on a rotary shaker (30 °C, 150 rpm) for 1 h. Liquid samples collected from each of the sampling points and PBS-cane suspensions were serially diluted in PBS and streaked onto modified dextransucrase-inducing agar with the following composition: sucrose 100 g/l, peptone 20 g/l, KH_2PO_4 20 g/l, agar 15 g/l and R-salts (4% $MgSO_4 \cdot 7H_2O$, 4% NaCl, 0.2% $FeSO_4 \cdot 7H_2O$ and 0.2% $MnSO_4 \cdot H_2O$) 5 ml [28]. Plates were incubated at 30 °C for 14 to 18 h. Colonies with a glistening and slimy appearance were selected and streaked to purity on modified dextransucrase-inducing agar. From these plates a single colony was inoculated into 5 ml MRS broth (Biolab, Merck South Africa) and the cultures incubated on a shaking incubator (150 rpm) for 14 to 18 h at 30 °C. Cells were harvested (16 000 x g, 25 °C, 2 min), re-suspended in sterile glycerol (200 µl; 50%, v/v) and stored at -70°C.

Genomic DNA extraction

Ten µl aliquots of stock culture was inoculated into 5 ml sterile MRS broth (Biolab, Merck, Modderfontein, South Africa) and incubated for 16 h at 30 °C on a rotary shaker (150 rpm). Cells were harvested (16 000xg, 25 °C, 2 min) and genomic DNA extracted using the GeneJET Genomic DNA Purification kit (Thermo Scientific, Inqaba Biotechnical Industries, Hatfield Pretoria, South Africa) according to the manufacturer's instructions. Purified DNA was suspended in 50 µl elution buffer and used as template in amplification reactions.

Amplification of 16S rRNA, *pheS*, *dnaA* and *atpA* genes

Genomic DNA was used as template to amplify partial sequences of the 16S rDNA, *pheS*, *dnaA* and *atpA* genes, respectively, for all isolates using the primers listed in Table 1. The 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 5x One Taq Standard Reaction buffer, 1.25 U One Taq Hot Start DNA polymerase (Thermo Scientific) and 100 ng template genomic DNA. PCR reactions were 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 and elongation (see Table 1). Cycling was completed by a final elongation step (68 °C, 10 min), followed by cooling to 4 °C. The resultant amplicons were purified using the DNA Clean and Concentrator™-25 kit (Zymo Research, Inqaba Biotechnical Industries, Hatfield Pretoria, South Africa) according to the manufacturer's instructions.

Table 1 Primer sequences and PCR conditions for the partial amplification of the 16S rRNA gene and the housekeeping genes *pheS, dnaA* and *atpA*

Gene	Primer name	Primer sequence (5'→3') with position in brackets	Annealing temp. (°C)	Elongation time (s)	Reference
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG (27)	50	90	[43, 44]
	1492R	GGTTACCTTGTTACGACTT (1492)			
pheS	pheS-21-F	CAYCCNGCHCGYGAYATGC (557)	56	30	[39]
	pheS-23-R	GGRTGRACCATVCCNGCHCC (968)			
dnaA	dnaA445-F	GGTGGCGTTGGTCTAGGWAA AACMCAYYTRATG (445)	55	60	[26]
	dnaA1253-R	TGCATCACAGTTGTATGATCY YKMCCRCCAAA (1253)			
	dnaA445-Fs*	GGTGGCGTTGGTCTAGG (445)			
atpA	atpA-20-F	TAYRTYGGKGAYGGDATYGC (97)	55	60	[39]
	atpA-26-R	TTCATBGCYTTRATYTGNGC (1108)			

*Sequencing primer only

Gene sequencing and phylogenetic analyses

16S rRNA, *pheS*, *dnaA* and *atpA* gene sequencing were performed by the South African Sugarcane Research Institute (Mount Edgecombe, South Africa) using BigDye Cycle Sequencing chemistry (Applied Biosystems, Johannesburg, South Africa), according to the manufacturer's instructions. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm [29]. Reference 16S rRNA, *pheS*, *atpA* and *dnaA* gene sequences were retrieved from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Where gene sequences were identical for a group of isolates from the respective sampling times and locations, only one isolate was chosen as representative of the group. GenBank accession numbers for 16S rRNA, *pheS, dnaA* and *atpA* gene sequences of representative strains for each sampling time and location, as determined in this study, are listed in Table 2. For phylogenetic inference, five different alignments were created; one corresponding to the 16S rRNA gene sequences of *Weissella* type strains as obtained from GenBank and four alignments corresponding to the single locus analysis of the 16S rRNA, *pheS, dnaA* and *atpA* genes of strains representing different groups of isolates. The 16S rRNA gene sequences were aligned using the SILVA Incremental Aligner software version 1.2.11 [30], and the *pheS, dnaA* and *aptA* gene sequences were aligned with ClustalW [31] as implemented in the BioEdit Sequence Alignment Editor program [32]. A data matrix for each alignment was created for the representative sequences of strains at each sampling location and sampling time. Phylogenetic analyses were conducted using the

Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 software [33]. The evolutionary histories were inferred using the Neighbor-Joining method [34] with the Kimura 2-parameter model [35] for the 16S rRNA gene sequence analyses, and the Tamura 3-parameter model [36] for the respective housekeeping genes. The strengths of the internal branches of the resultant trees were statistically evaluated by bootstrap analysis [37] with 1000 bootstrap replications.

Table 2 GenBank accession numbers of the sequences as determined in this study for representative *Weissella* strains for each sampling location

Representative strain ID	Sampling location	16S rRNA	*pheS*	*dnaA*	*atpA*
A1-11	Prepared cane	MK402146	MK419121	MK419131	MK419141
A1-17	prepared cane	MK402147	MK419122	MK419132	MK419142
A2-1	Diffuser sump	MK402148	MK419123	MK419133	MK419143
A2-10	Diffuser sump	MK402149	MK419124	MK419134	MK419144
A9-5	Filtrate	MK402150	MK419125	MK419135	MK419145
A16-13	Syrup tank	MK402151	MK419126	MK419136	MK419146
B1-4	Prepared cane	MK402152	MK419127	MK419137	MK419147
B1-24	Prepared cane	MK402153	MK419128	MK419138	MK419148
B2-31	Diffuser sump	MK402154	MK419129	MK419139	MK419149
B2-47	Diffuser sump	MK402155	MK419130	MK419140	MK419150

Results and Discussion

A phylogenetic tree constructed from near full-length 16S rRNA gene sequences (1286 bp) of *Weissella* type strains is shown in Fig. 1. The phylogenetic relationship of strains representing different groups of isolates, based on partial 16S rRNA gene sequences (752 bp), is shown in Fig. 2. The tree topology of the partial 16S rRNA gene sequences of representative strains (Fig. 2) is in agreement with that obtained for the near full-length 16S rRNA gene sequences of *Weissella* reference strains (Fig. 1) and as reported by Lee et al. [38].

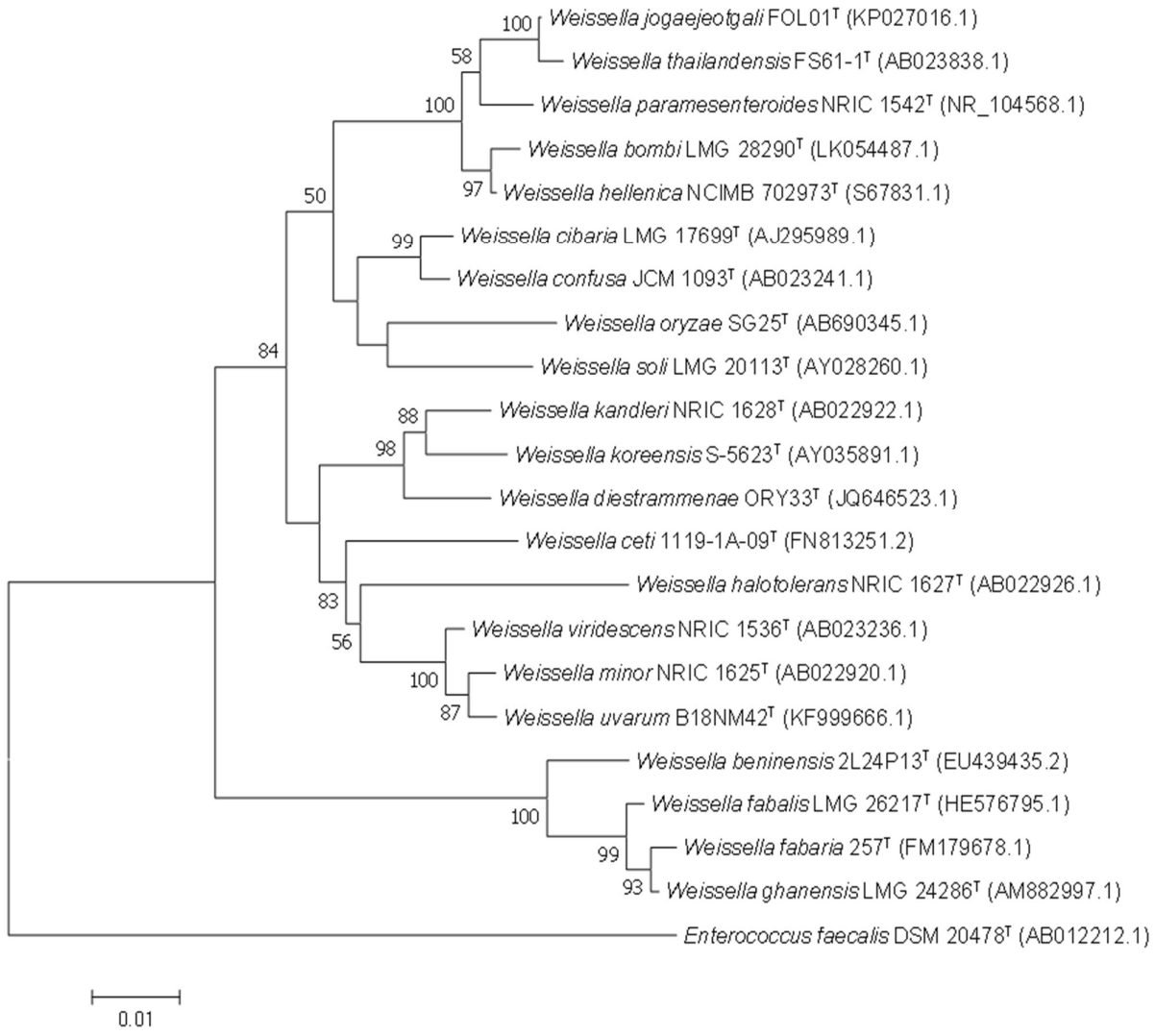

Fig. 1 Phylogenetic analysis of 16S rRNA gene sequences of *Weissella* type strains, using Neighbor-Joining method and Kimura's 2-parameter model [35]. Each dataset had 1285 bp. Bootstrap values (>50 %, 1000 replications) are shown. The bar indicates % estimated substitution per nucleotide position. *Enterococcus faecalis* DSM 20478^T was used as the outgroup

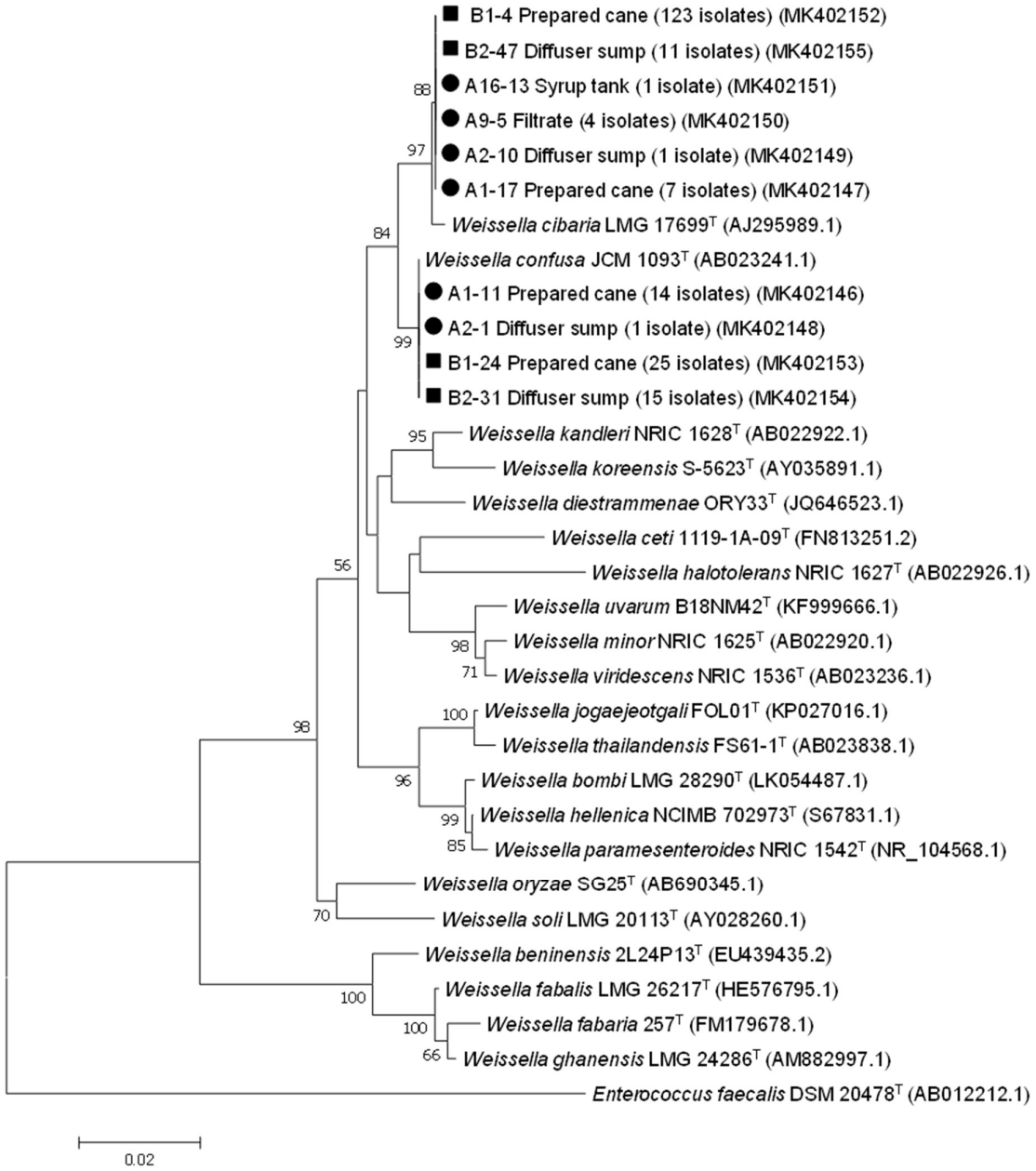

Fig. 2 Phylogenetic analysis of partial 16S rRNA gene sequences of representative *Weissella* strains isolated from a South African sugarcane processing factory at times when low (filled circle) and high (filled square) dextran concentrations were observed in the produced sugar. The number of isolates from each sampling time and location is indicated in brackets. The tree was constructed using the Neighbor-Joining method and Kimura's 2-parameter model [35]. Each dataset had 752 bp. Bootstrap values (>50 %, 1000 replications) are shown. The bar indicates % estimated substitution per nucleotide position. *Enterococcus faecalis* DSM 20478^T was used as the outgroup

A total of 202 strains, representative of the different isolates, grouped into two distinct clusters (Fig. 2), supported by high bootstrap values (97 and 99 %, respectively). The two clusters represent *W. cibaria* (147 isolates) and *W. confusa* (55 isolates). 16S rRNA gene sequence similarities from the *Weissella* type strains (Fig. 1) ranged from 90.2% (*Weissella halotolerans/Weissella beninensis*) to 99.2% (*W. cibaria*/*W. confusa*). Of all the locations sampled, *Weissella* spp. were isolated from the prepared (shredded) sugarcane, diffuser sump, filtrate and syrup tank.

De Bruyne et al. [39] reported on the high discriminatory power of *pheS* gene sequences in comparison to 16S rRNA gene sequences amongst species of *Weissella, Leuconostoc* and *Oenococcus*. A phylogenetic tree constructed from partial *pheS* gene sequences (352 bp) of representative *Weissella* isolates is shown in Fig. 3. The discriminatory power of *pheS* as phylogenetic marker for *W. cibaria* and *W. confusa* is confirmed by the results obtained, with high bootstrap values (100% in both cases). *Weissella* isolates from the sugarcane processing factory clustered with *W. cibaria* (six strains representing 147 isolates) and *W. confusa* (4 strains representing 55 isolates). The *pheS* sequence similarities of the type strains ranged from 70.7% (*W. cibaria/W. beninensis*) to 89.6% (*Weisella fabaria/Weissella ghanensis*), with *W. cibaria/W. confusa* sharing an 88.5% *pheS* sequence similarity.

Contrary to the widespread use of *pheS* as phylogenetic marker to differentiate *Weissella* species, *dnaA* gene sequence analysis has only been used by Chelo et al. [26] to evaluate six *Weissella* type strains as part of a study clarifying the intra- and intergeneric phylogenetic relationships of the *Leuconostoc-Oenococcus-Weissella* clade. Despite its limited use, the phylogenetic tree constructed from partial *dnaA* gene sequences of representative strains of *Weissella* (574 bp, Fig. 4) displayed high bootstrap support (100 and 99%, respectively) for the differentiation of *W. cibaria* (six strains representing 147 isolates) and *W. confusa* (four strains representing 55 isolates). The *dnaA* sequence similarities of the type strains ranged from 59.6% (*Weissella kandleri/W. halotolerans*) to 100% (*Weissella thailandensis/Weissella jogaejeotgali*), with 81.4% *dnaA* sequence similarity between *W. cibaria* and *W. confusa.*

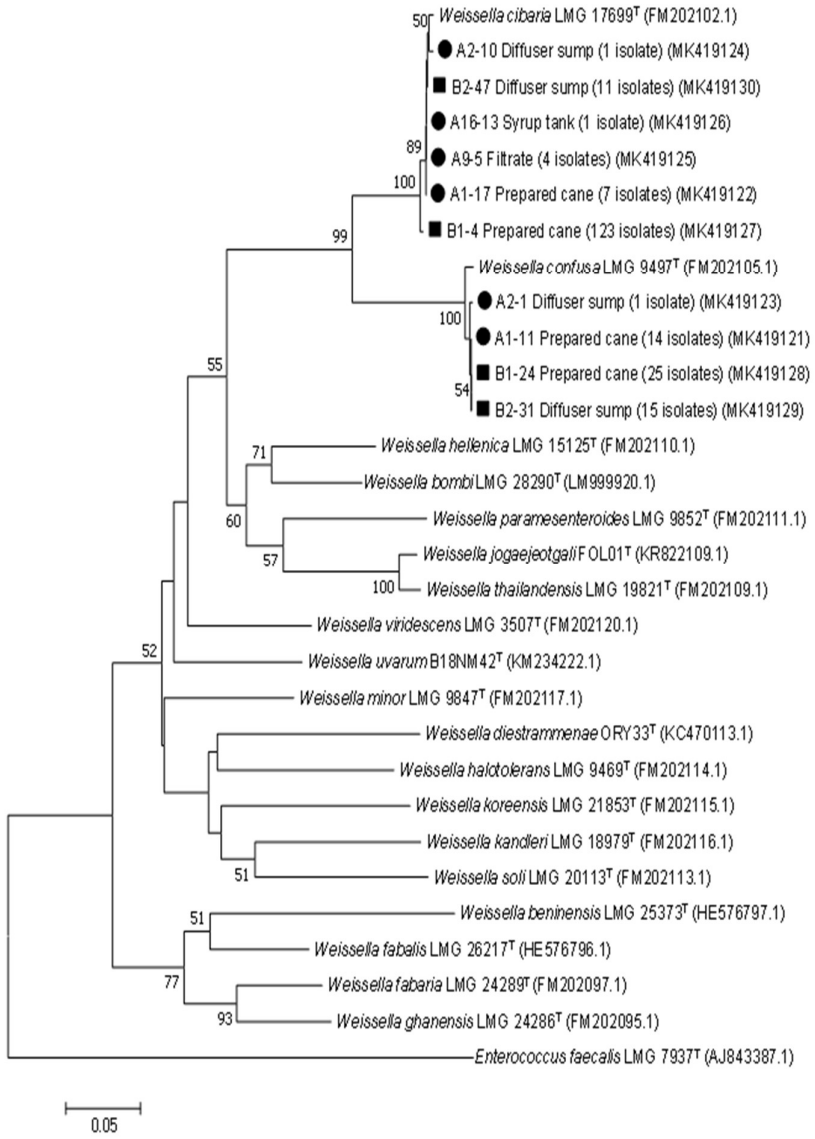

Fig. 3 Phylogenetic analysis of partial *pheS* gene sequences of representative *Weissella* strains isolated from a South African sugarcane processing factory at times when low (filled circle) and high (filled square) dextran concentrations were observed in the produced sugar. The number of isolates from each sampling time and location is shown in brackets. The tree was constructed using the Neighbor-Joining method using Tamura's 3-parameter model [36]. Each dataset had 352 bp. Bootstrap values (>50 %, 1000 replications) are shown. The bar indicates % estimated substitution per nucleotide position. *Enterococcus faecalis* LMG 7937^T was used as the outgroup

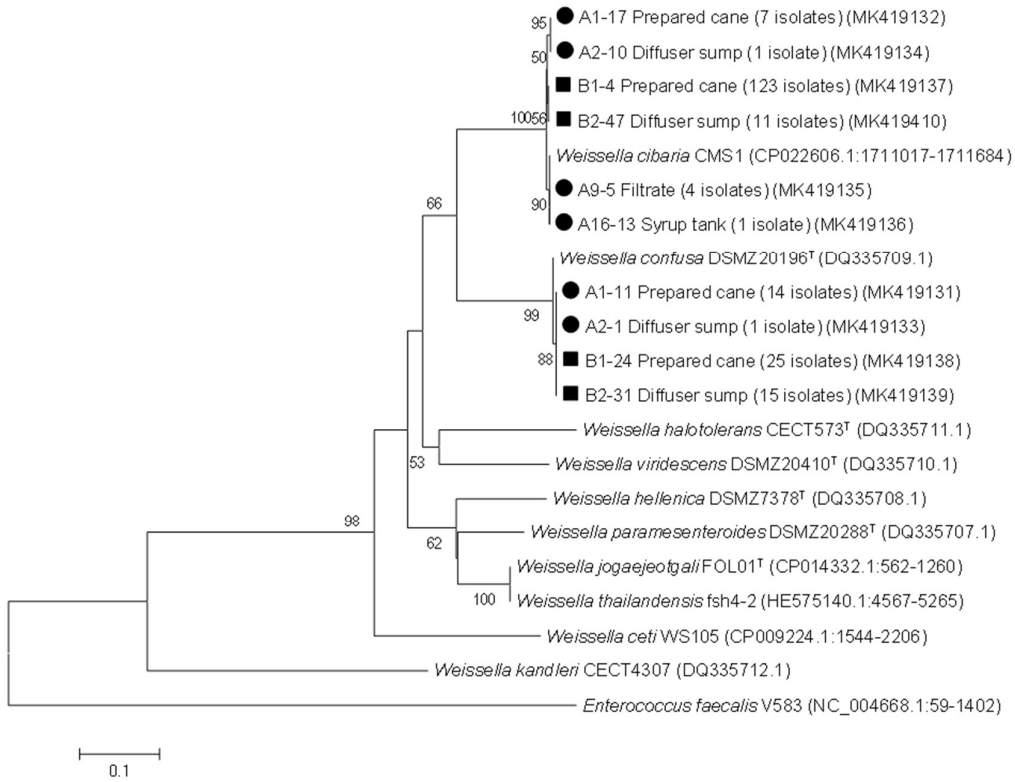

Fig. 4 Phylogenetic analysis of partial *dnaA* gene sequences of representative *Weissella* strains isolated from a South African sugarcane processing factory at times when low (filled circle) and high (filled square) dextran concentrations were observed in the produced sugar. The number of isolates from each sampling time and location is shown in brackets. The tree was constructed using the Neighbor-Joining method using Tamura's 3-parameter model [36]. Each dataset had 574 bp. Bootstrap values (>50 %, 1000 replications) are shown. The bar indicates % estimated substitution per nucleotide position. *Enterococcus faecalis* V583, chromosomal location number NC_004668.1:59-1402, was used as the outgroup

The *atpA* gene has not been previously used as phylogenetic marker for the identification of *Weissella* species. De Bruyne et al [24, 40] used *atpA* gene sequence analysis for the identification of *Pediococcus* and *Leuconostoc* species. Currently, the only available nucleotide sequence for the *atpA* gene for *Weissella* species is that of the *W. viridescens* type strain. The phylogenetic tree inferred from partial *atpA* gene sequences (820 bp) from the isolated *Weissella* strains (Fig. 5) showed the clustering of six strains representing 147 isolates (Cluster 1) and four strains representing 55 isolates (Cluster 2) with high bootstrap support.

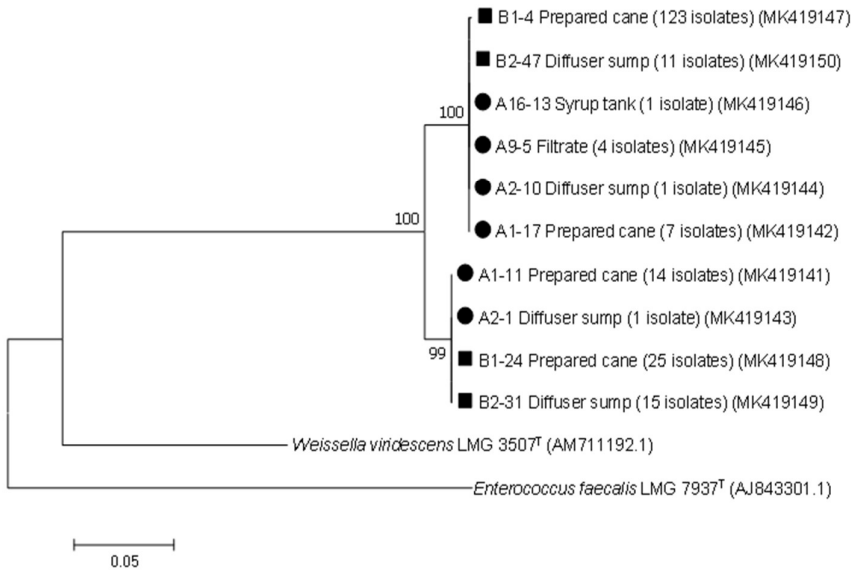

Fig. 5 Phylogenetic analysis of partial *atpA* gene sequences of representative *Weissella* strains isolated from a South African sugarcane processing factory at times when low (filled circle) and high (filled square) dextran concentrations were observed in the produced sugar. The number of isolates from each sampling time and location is shown in brackets. The tree was constructed using the Neighbor-Joining method using Tamura's 3-parameter model [36]. Each dataset had 820 bp. Bootstrap values (>50 %, 1000 replications) are shown. The bar indicates % estimated substitution per nucleotide position. The only *Weissella atpA* gene sequence available was from *Weissella viridescens* LMG 3507^T. *Enterococcus faecalis* LMG 7937^T was used as the outgroup

This study identified *W. cibaria* as major contributor of gum-producing bacteria isolated from the prepared (shredded) sugarcane in summer, when high dextran concentrations were reported in the produced sugar. Hot and humid conditions favour rapid accumulation of dextran in harvested cane which is exacerbated by delays in delivering the cane to the factory [2]. The dominance of *Weissella* spp. on prepared cane is significant because these bacteria are not usually associated with deteriorated sugarcane and sugarcane processing factories. *Weissella* bacteria have been isolated from a diversity of ecological niches, including soil, plants, a variety of fermented foods, as well as from humans and animals [41]. The risk of contamination of *Weissella* bacteria in sugarcane processing factories is therefore quite high. *Weissella* spp. have the ability to synthesise a variety of polysaccharides and oligosaccharides, which includes high molecular weight, low-branched dextran, as well as levan and inulin, in addition to gluco- and fructo-oligosaccharides and cell-associated ropy polymers [42]. These poly- and oligosaccharides may have a severe impact on the quality and quantity of produced sugar, and accurate identification of the spoilage bacteria in

sugarcane processing is key for effective microbial control and development of cane deterioration indicators.

Previous studies have shown that the housekeeping genes *pheS, dnaA* and *atpA* have high discriminatory power in differentiating various lactic acid bacteria. In this study, single locus analyses of *pheS* and *dnaA* gene sequences revealed the clustering of representative *Weissella* isolates with the type strains of *W. cibaria* (147 strains) and *W. confusa* (55 strains) (Fig. 3 and 4). Phylogenetic analysis of the *atpA* gene showed the same grouping of representative *Weissella* isolates into two clusters. Previous studies which examined the microbial diversity in sugarcane processing factories [11-13] were constrained by the absence of microbial identification methods with high discriminatory power. We have shown the potential of phylogenetic analyses of housekeeping genes as alternative identification method for sugarcane processing spoilage microbes.

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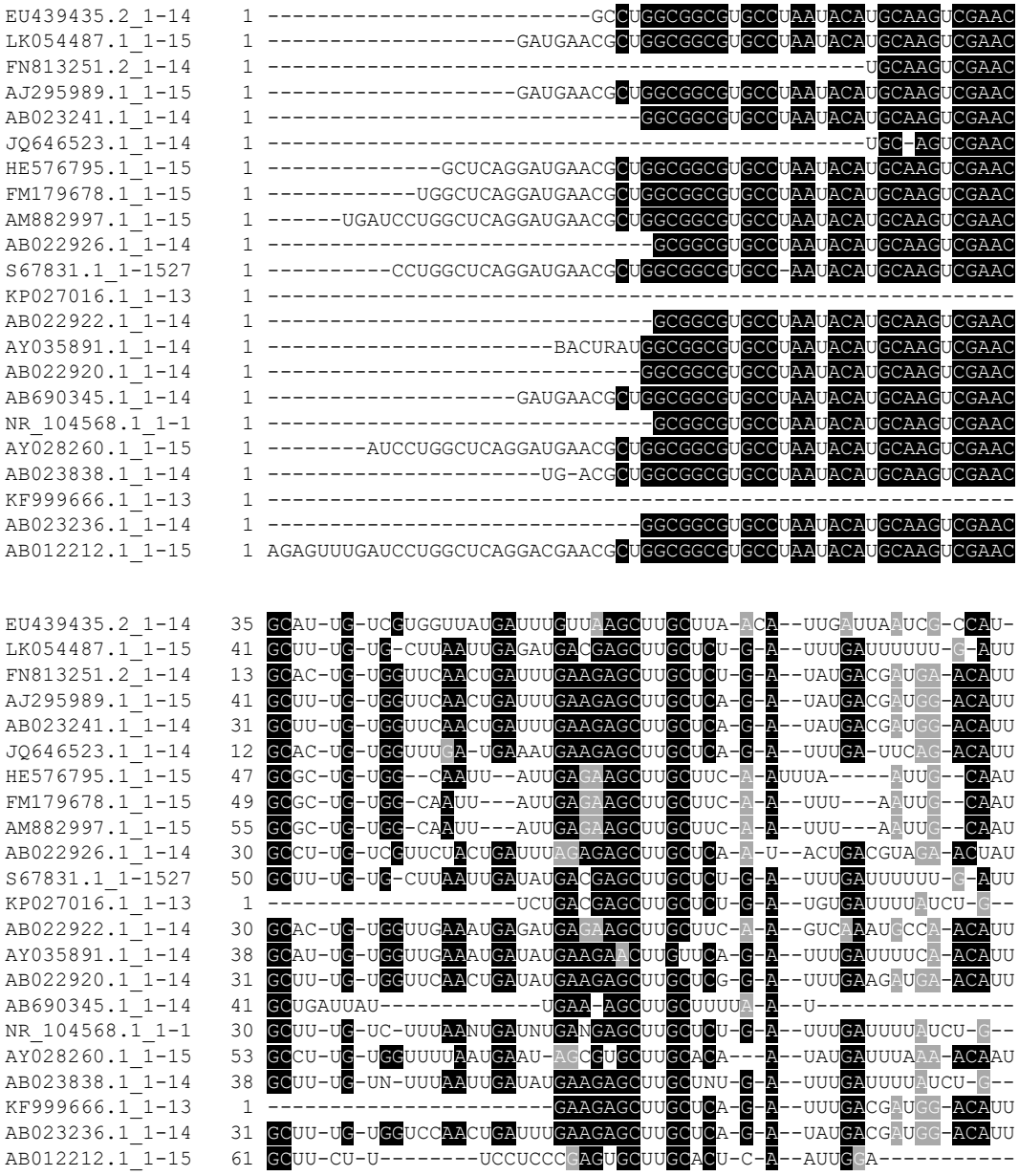
Supplementary material



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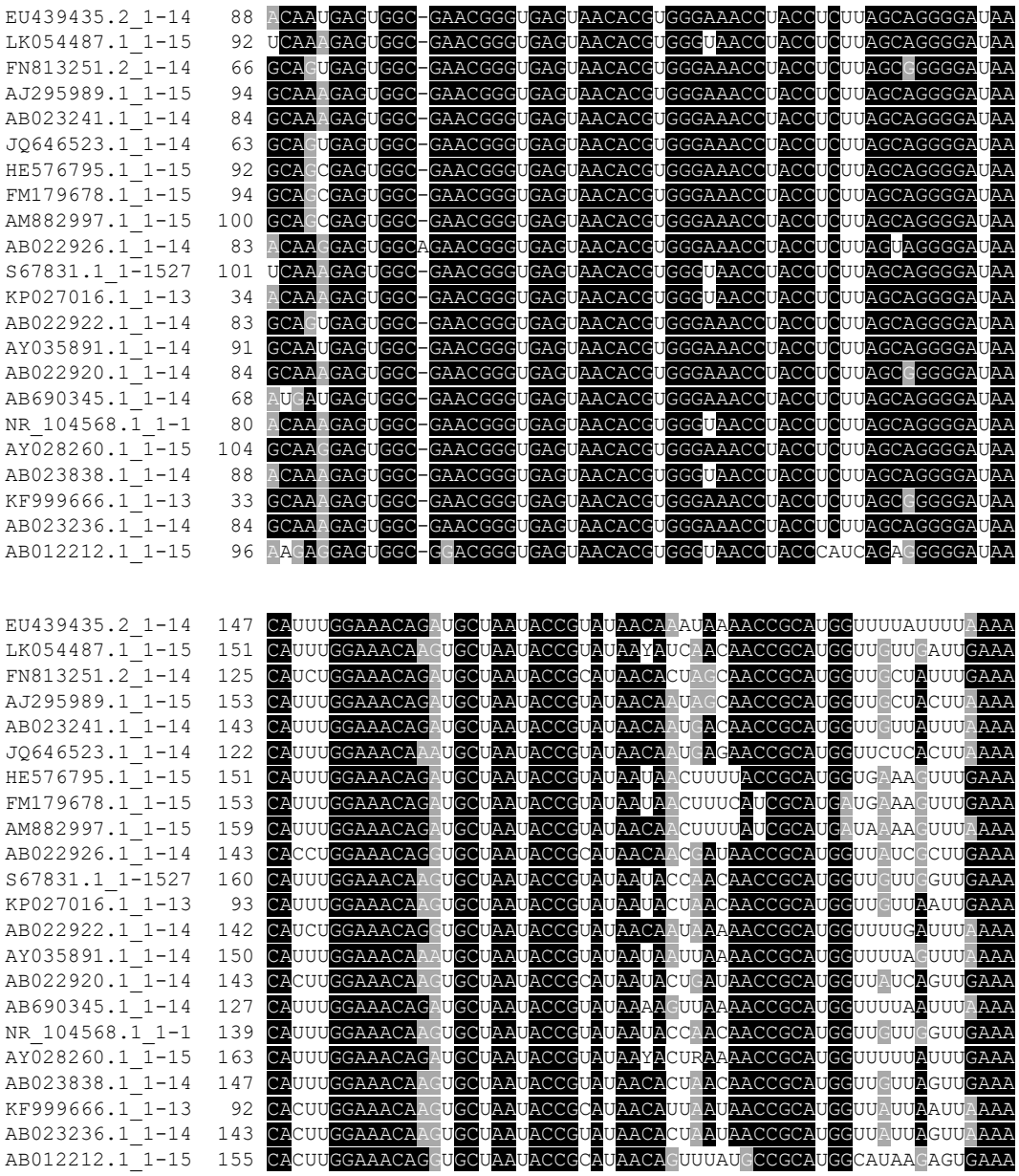

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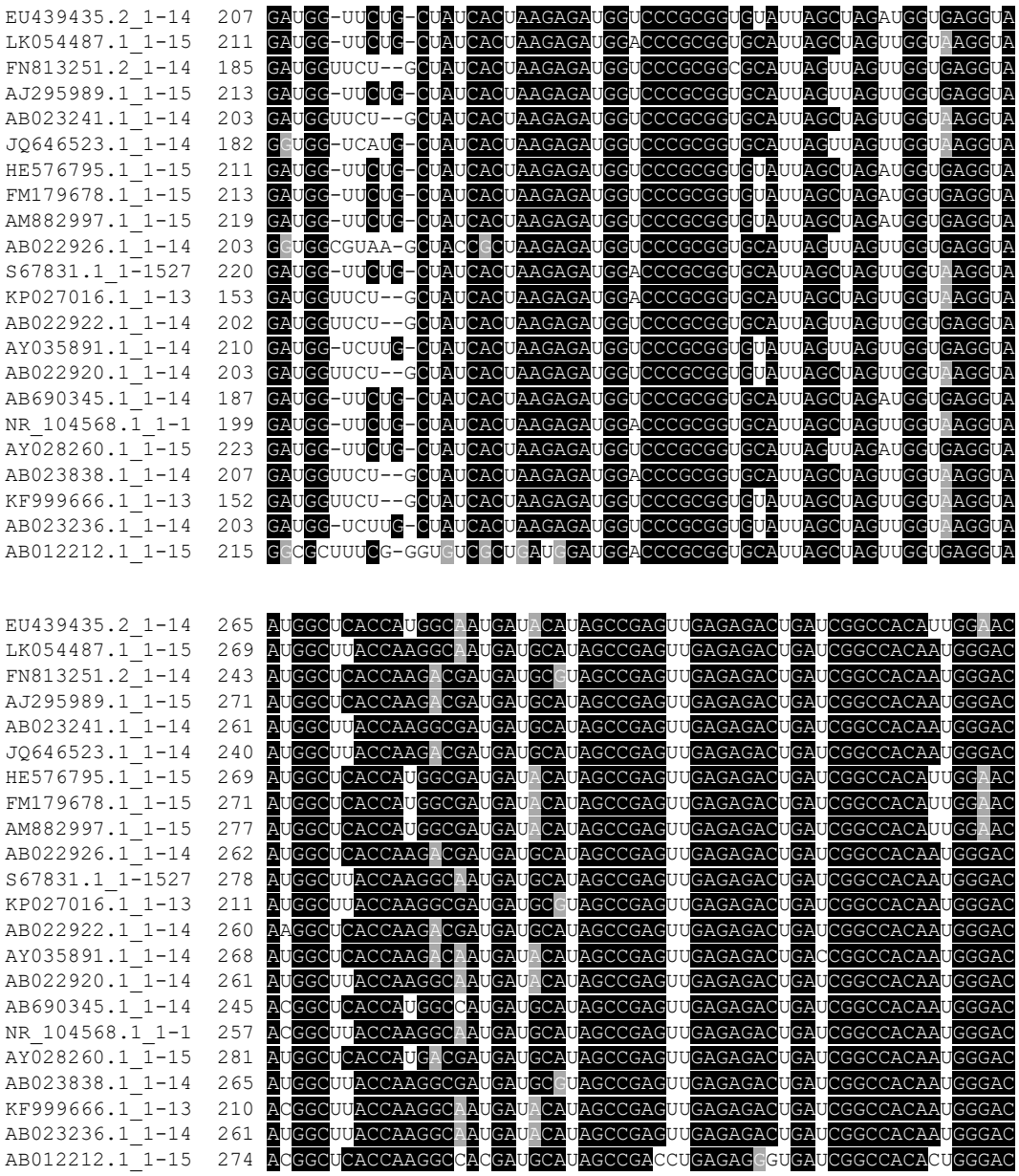

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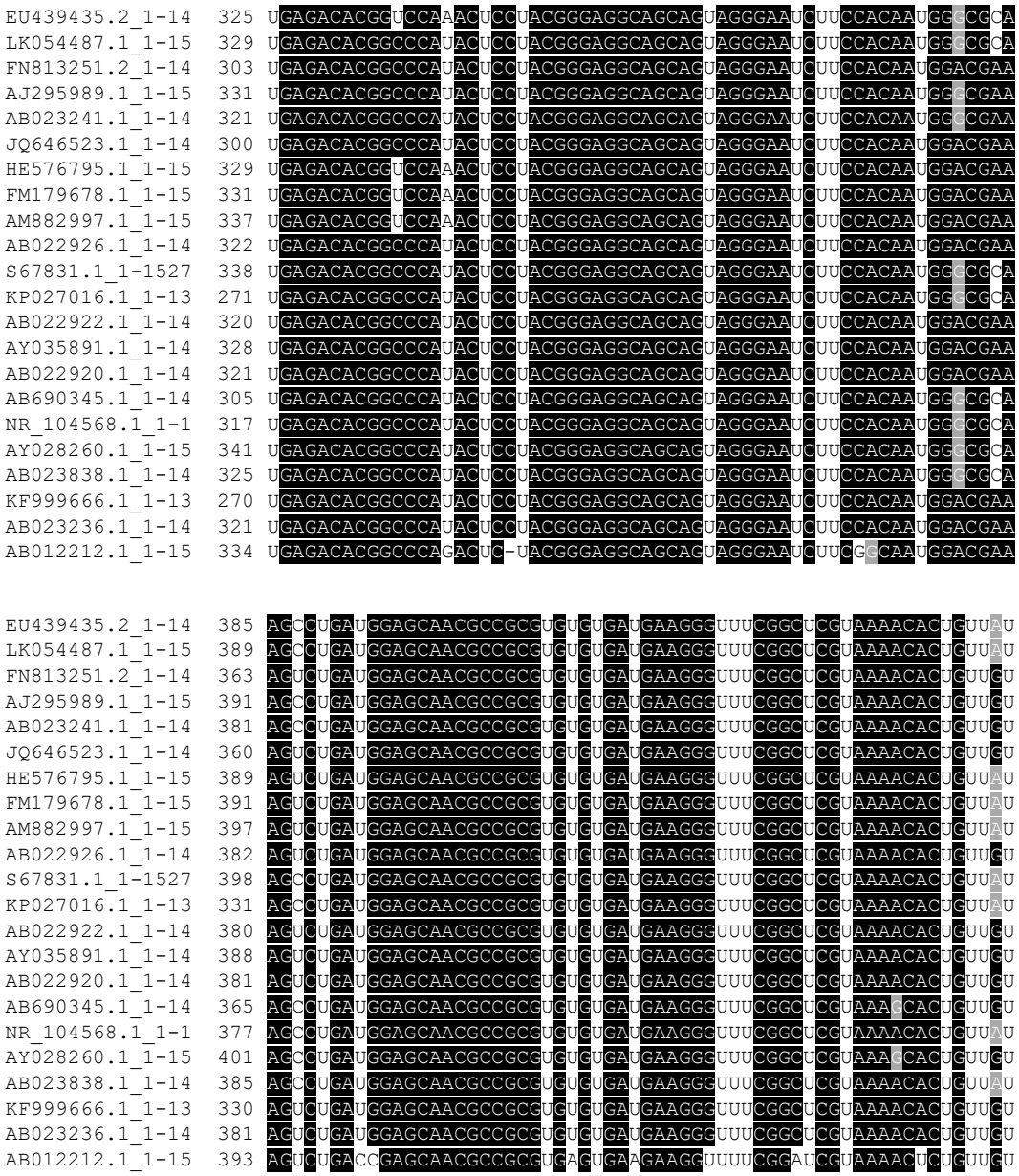

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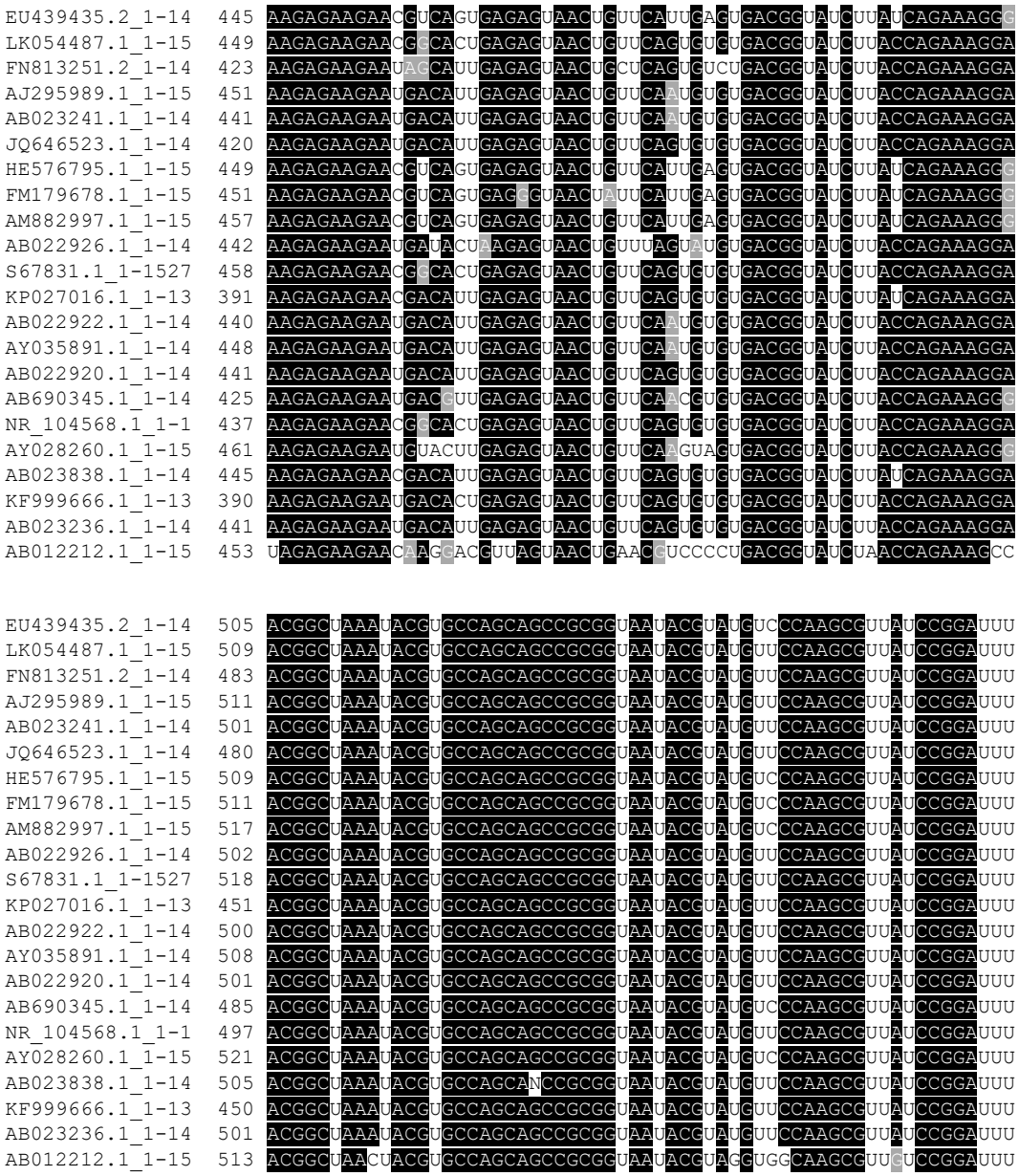

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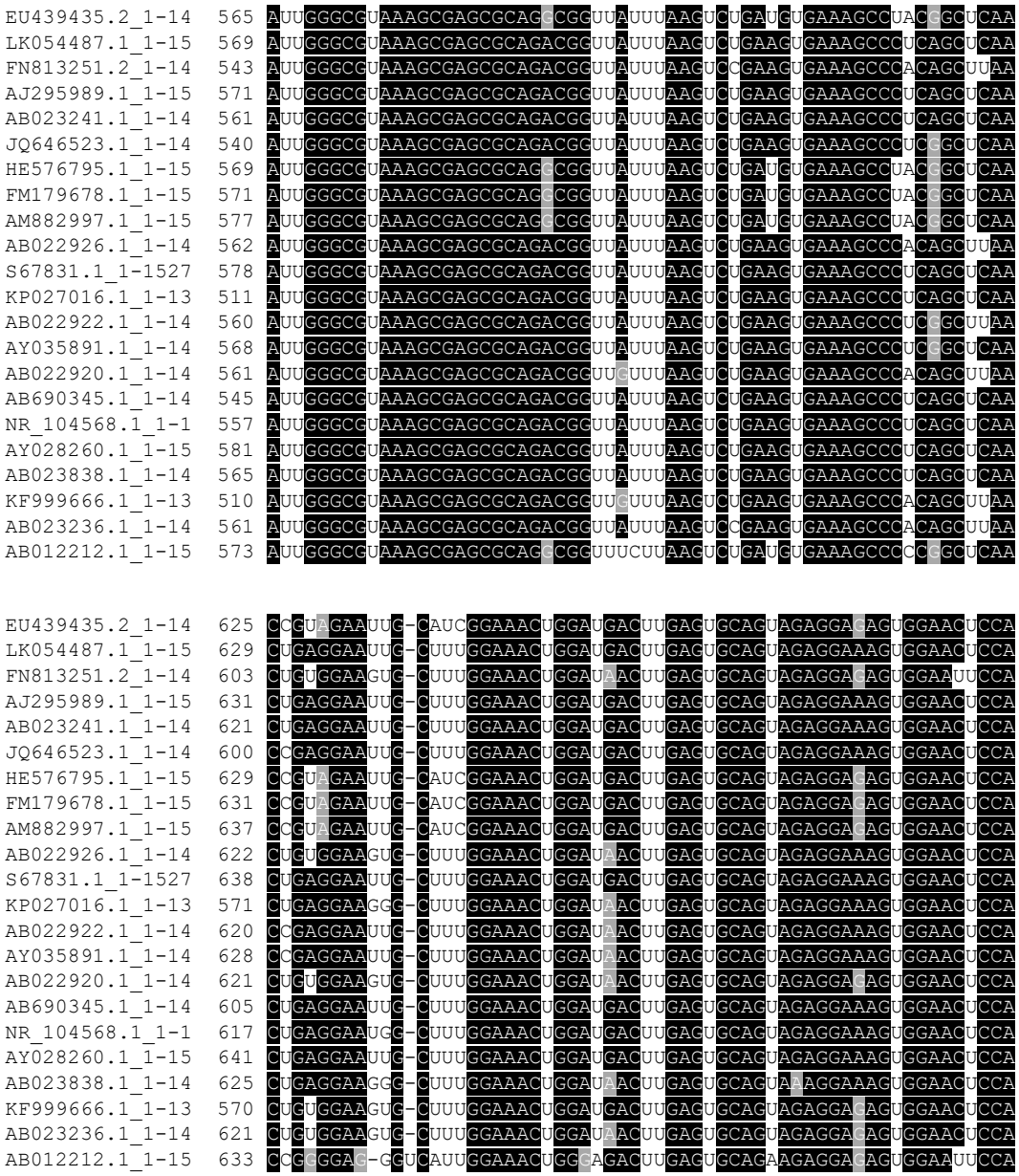

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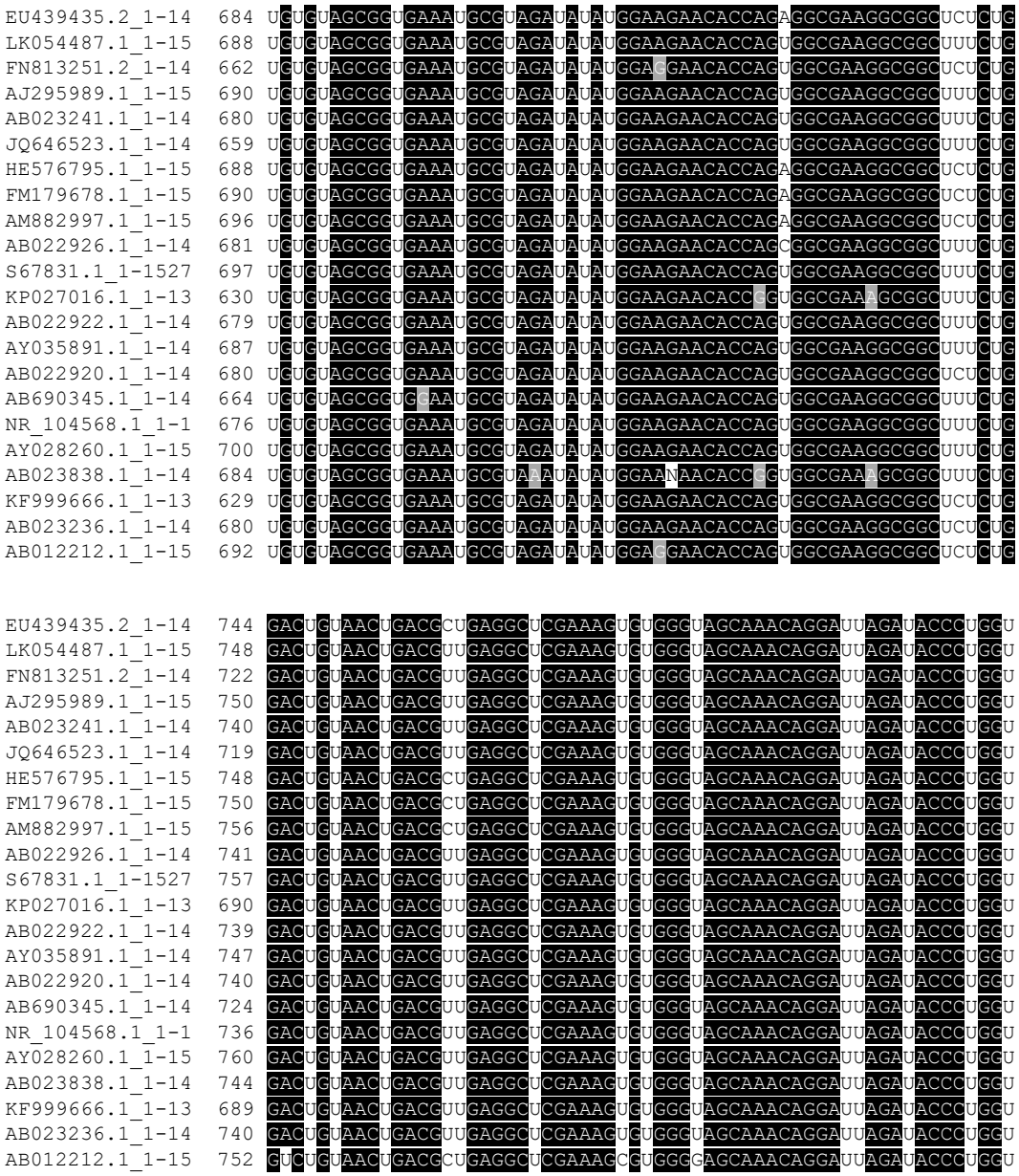

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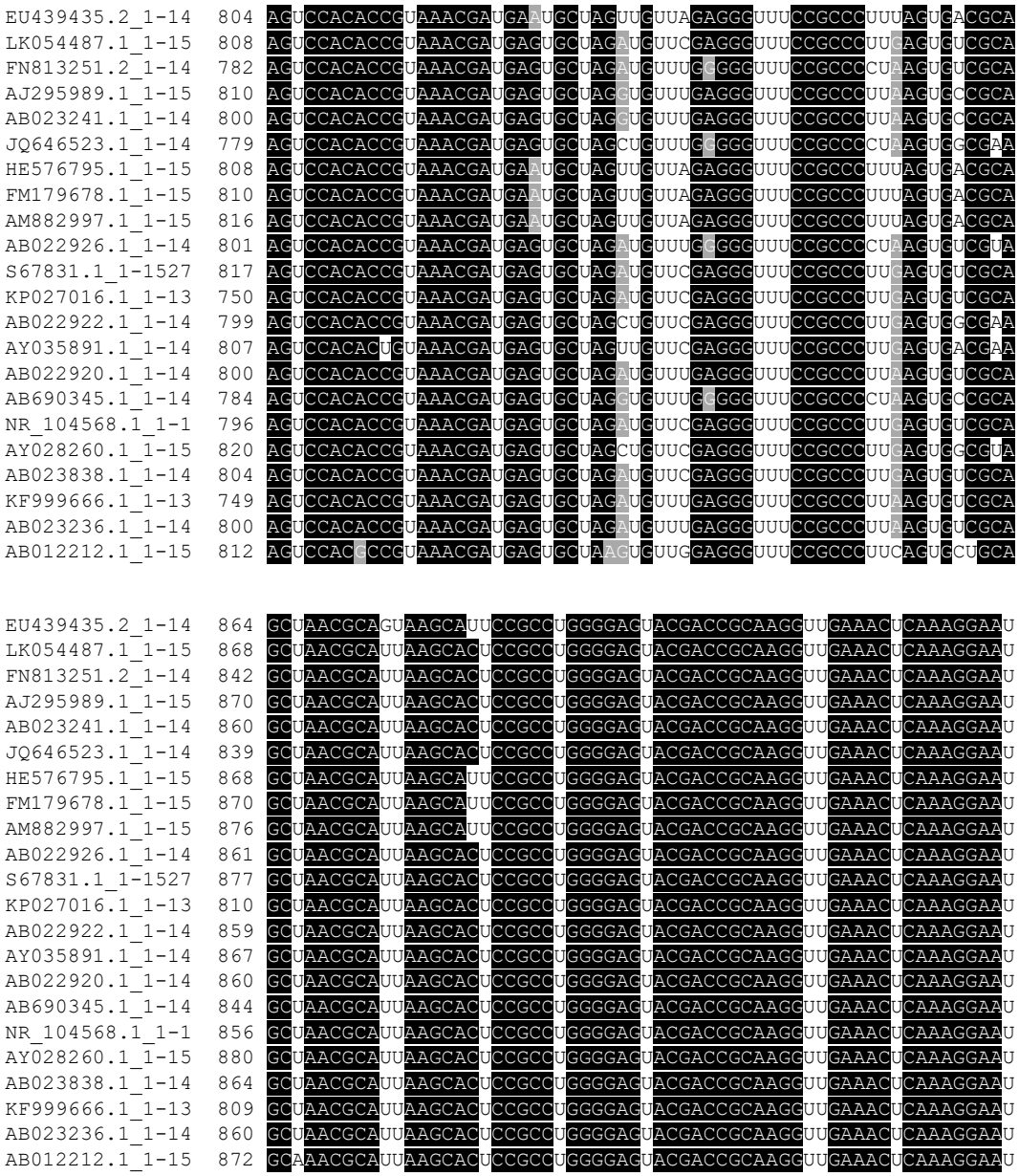

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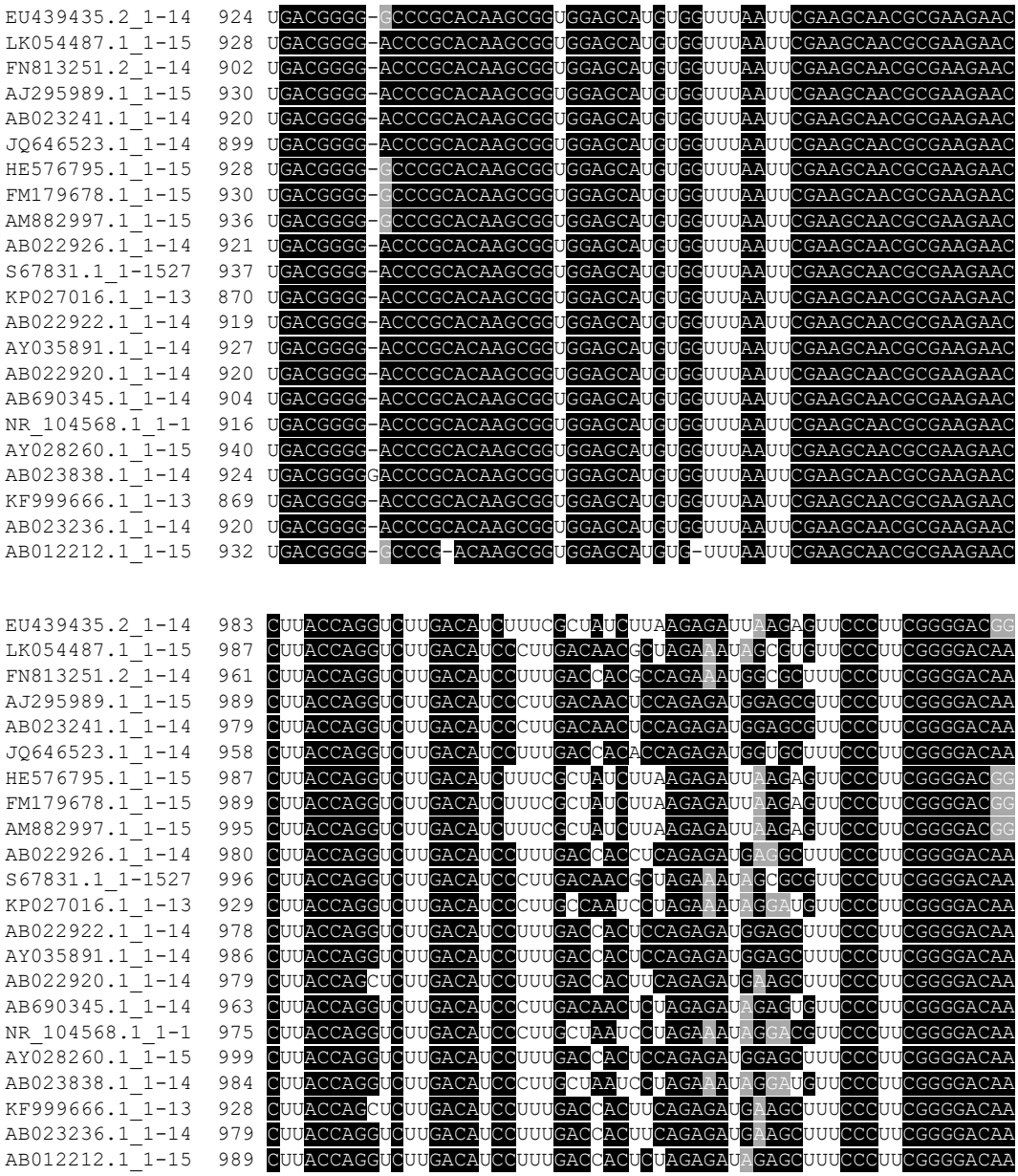

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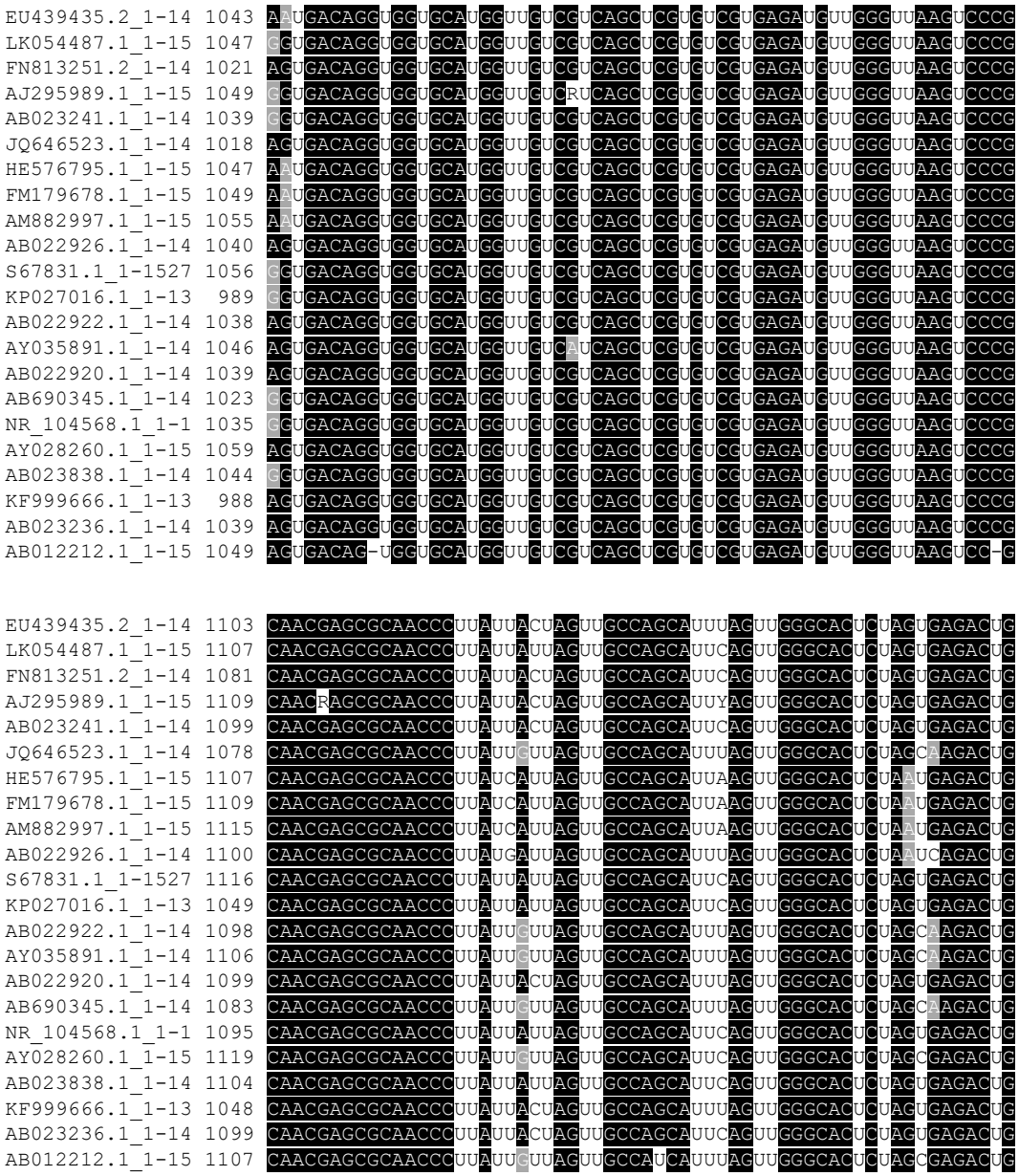

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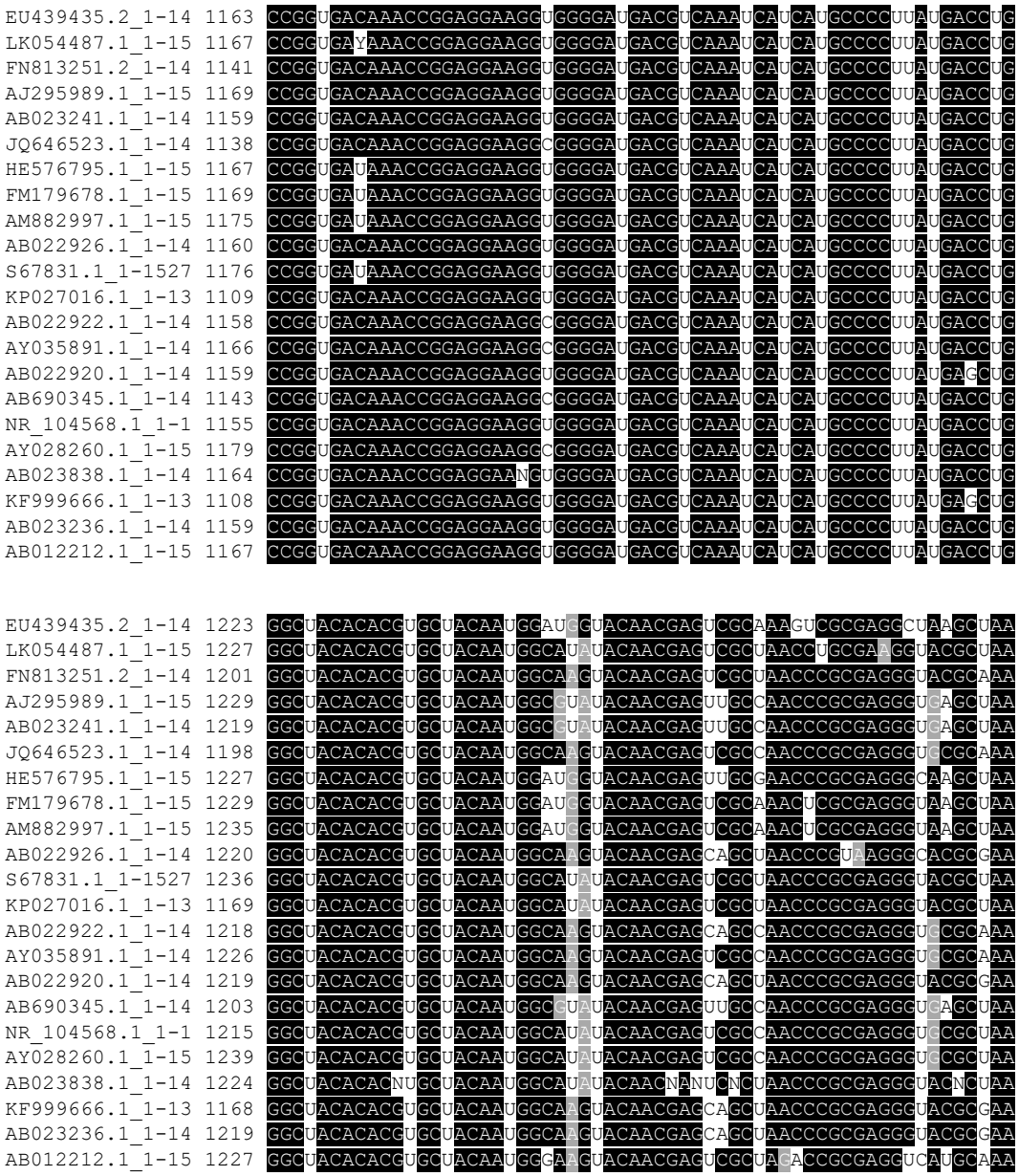

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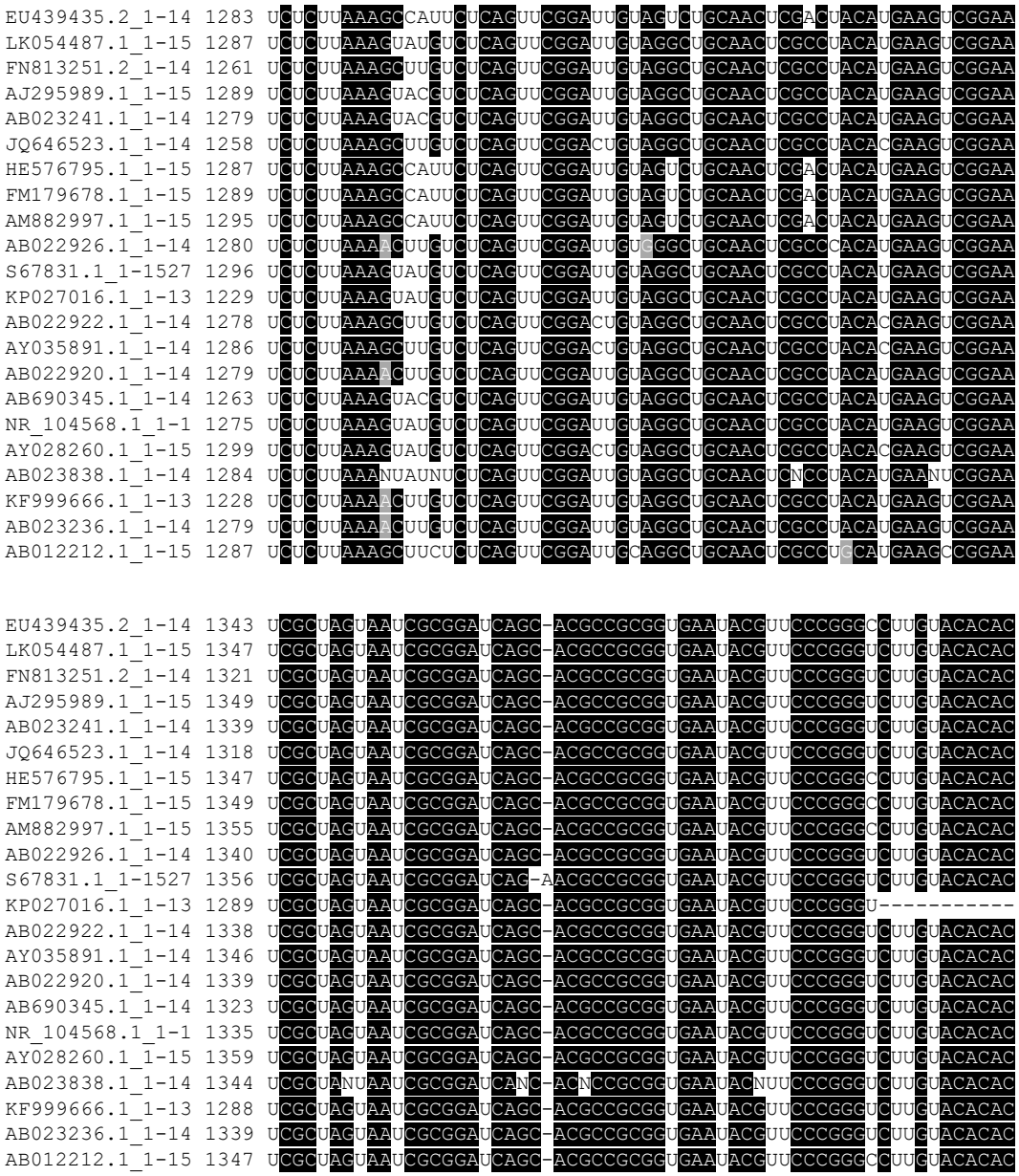

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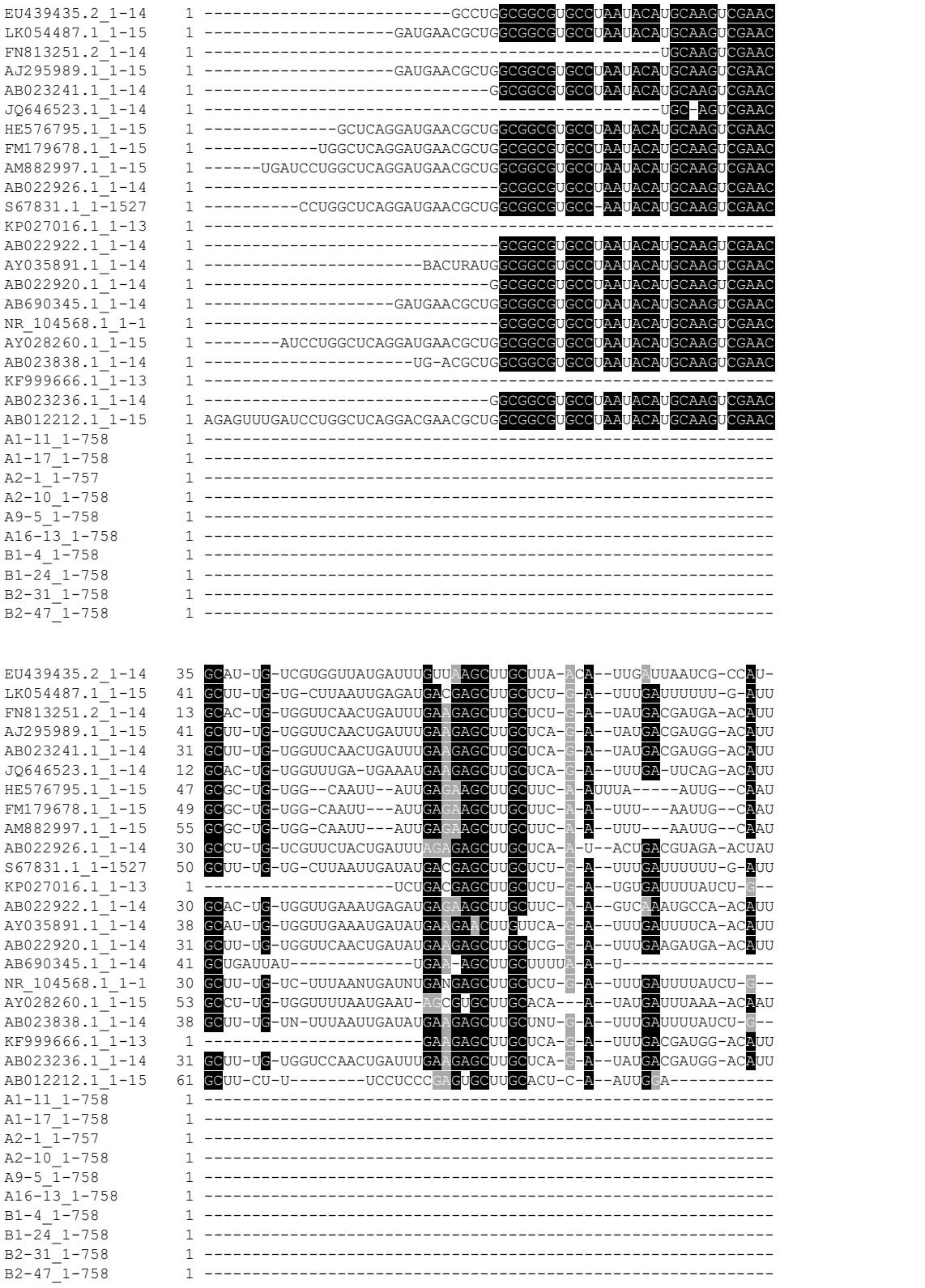
Fig. S1 Multiple sequence alignments of partial 16S rRNA genes of reference *Weissella* strains used in this study. Sequences were aligned using the SILVA Incremental Aligner software version 1.2.11 [30] followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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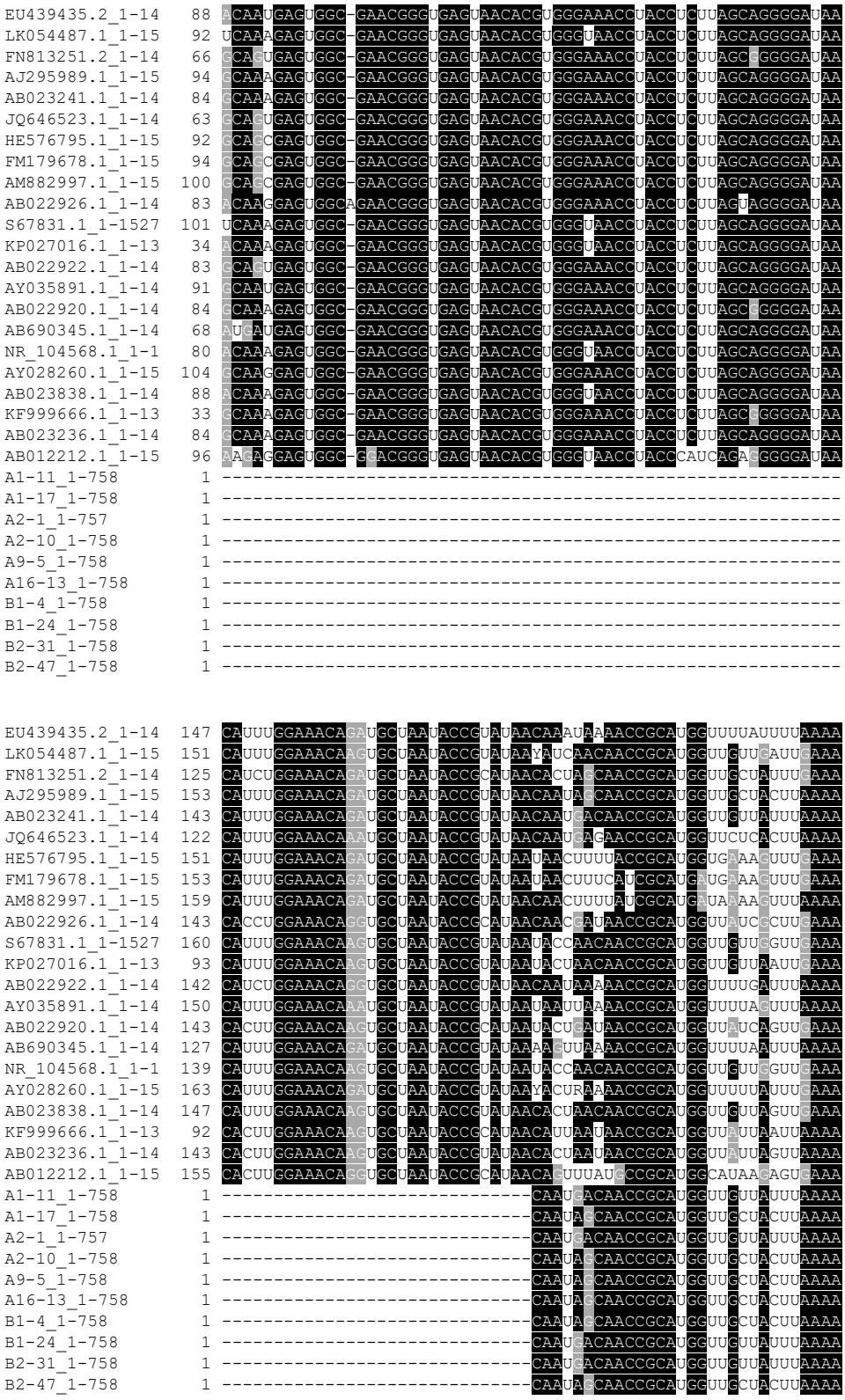

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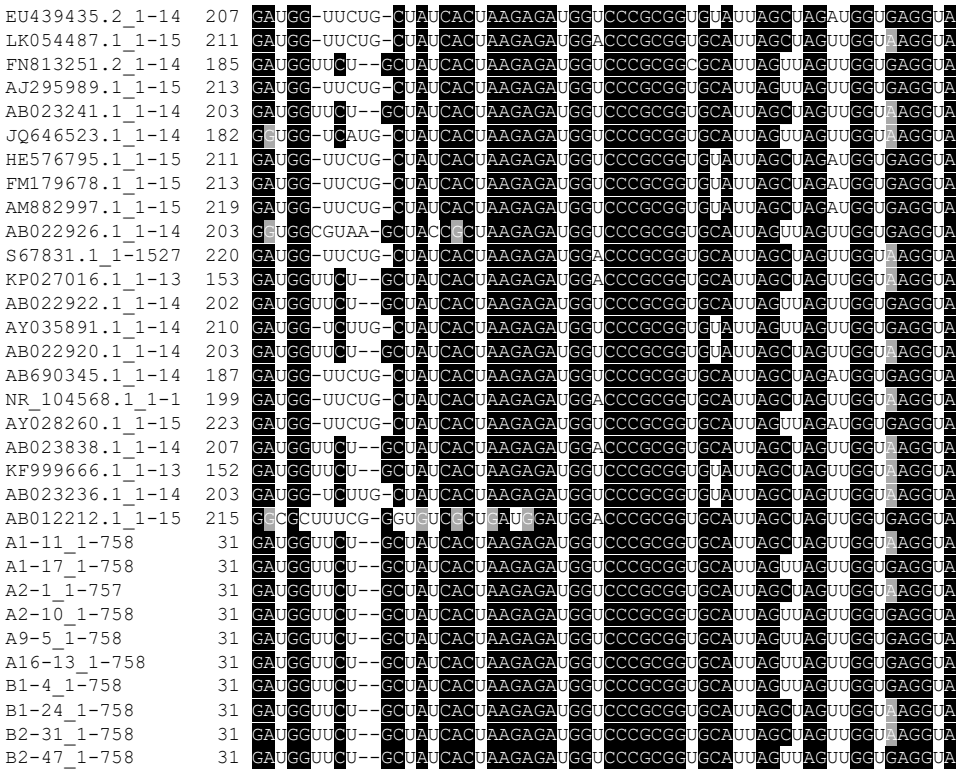

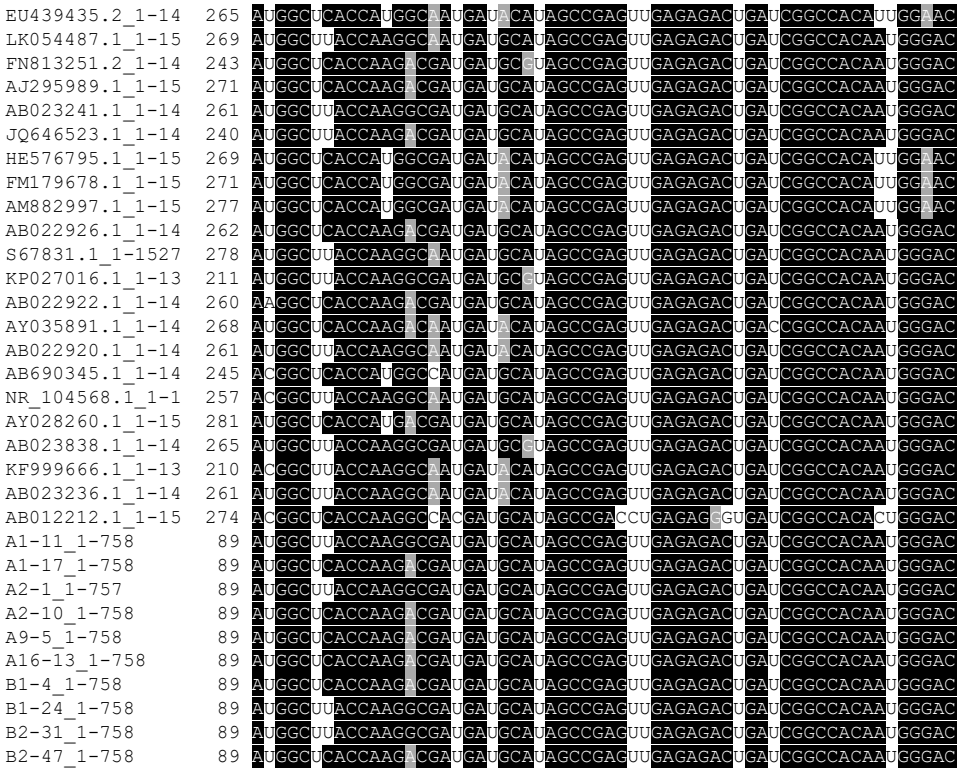

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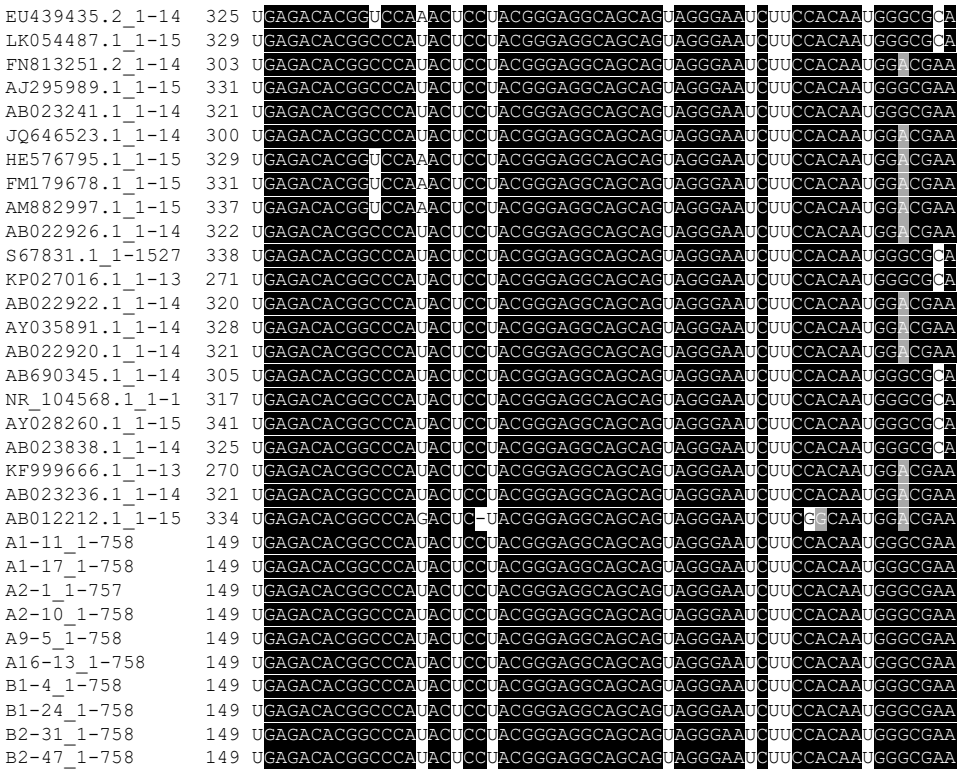

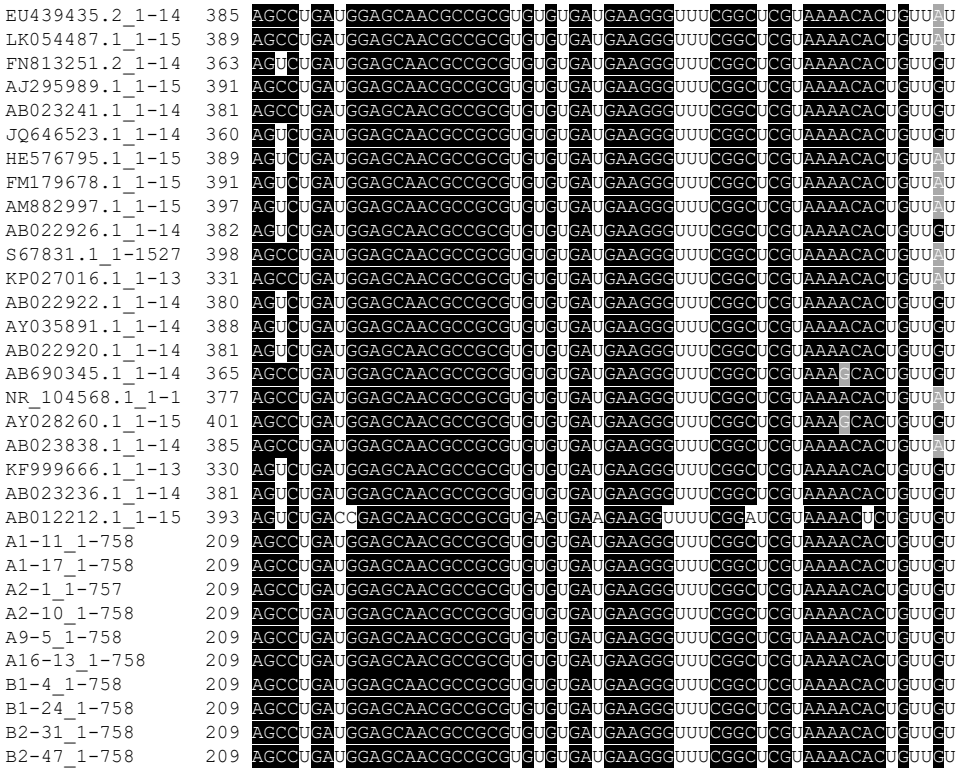

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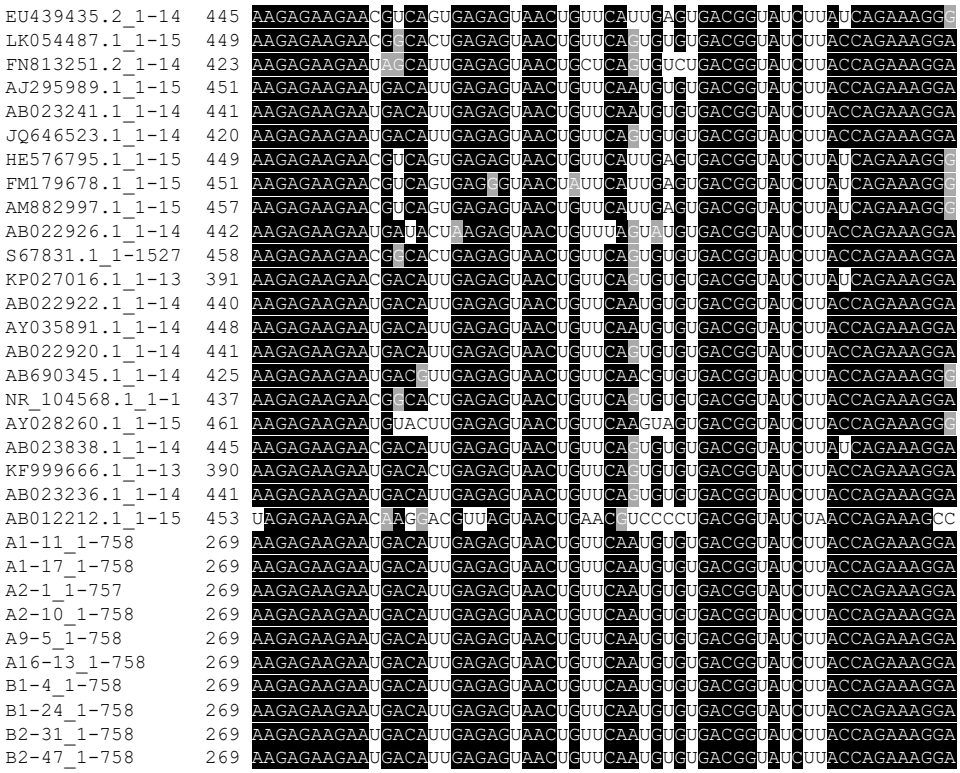

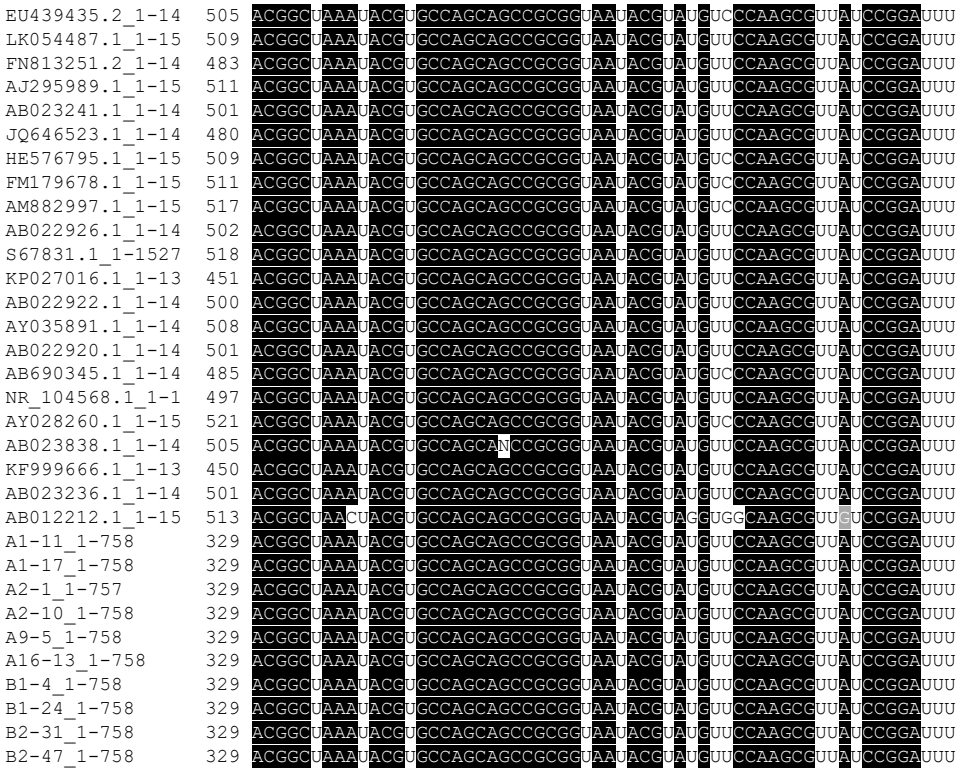

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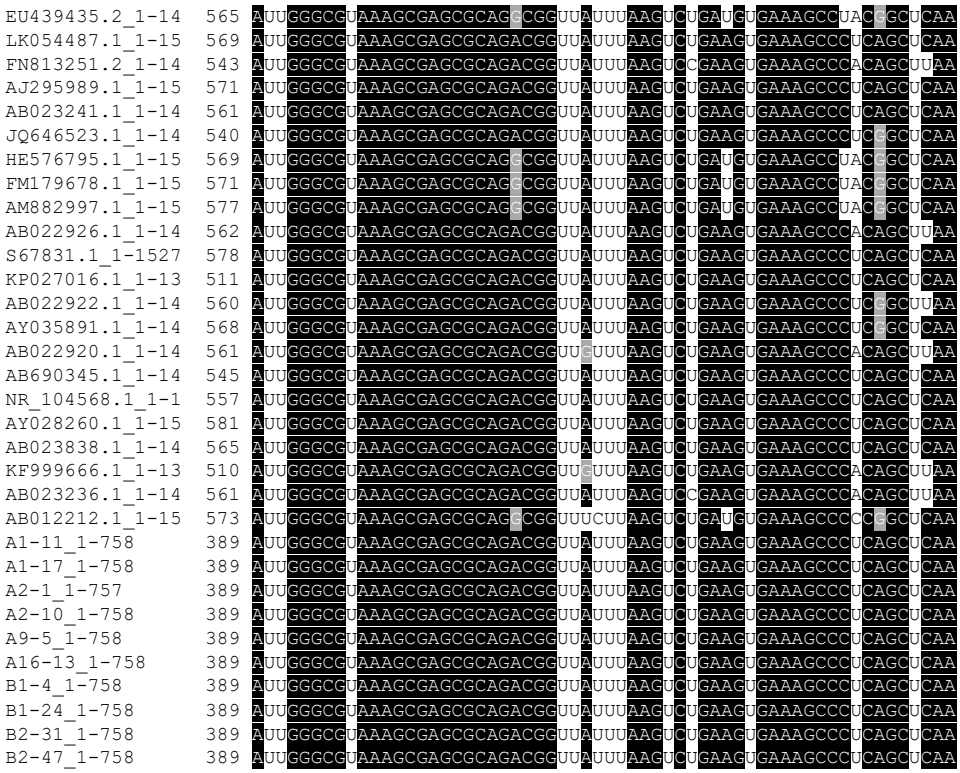

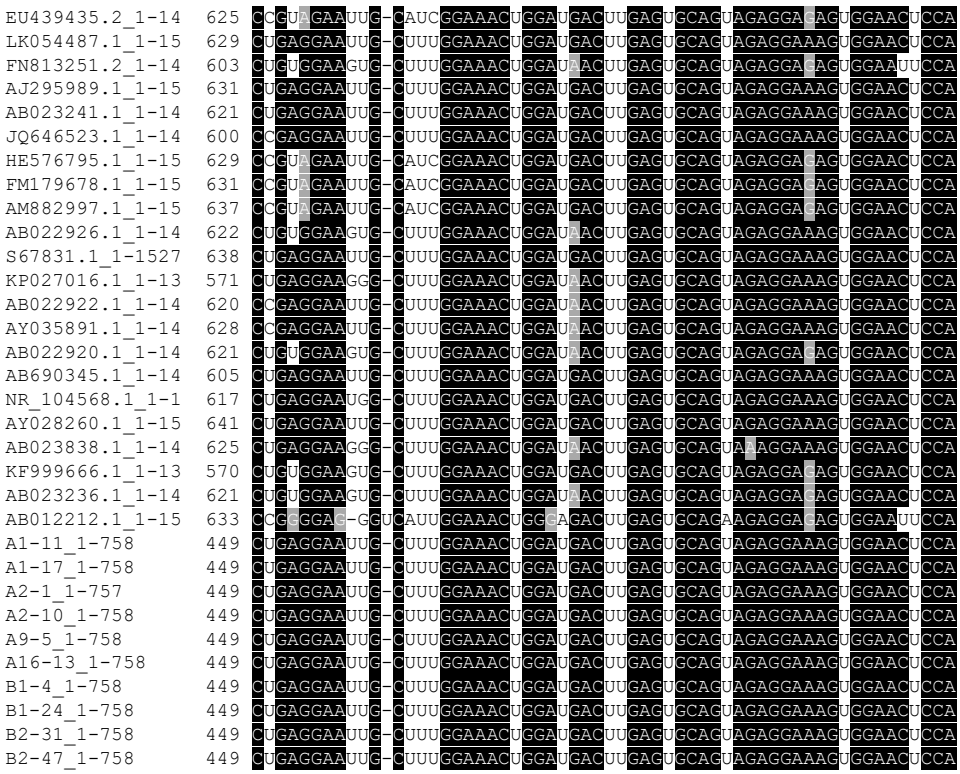

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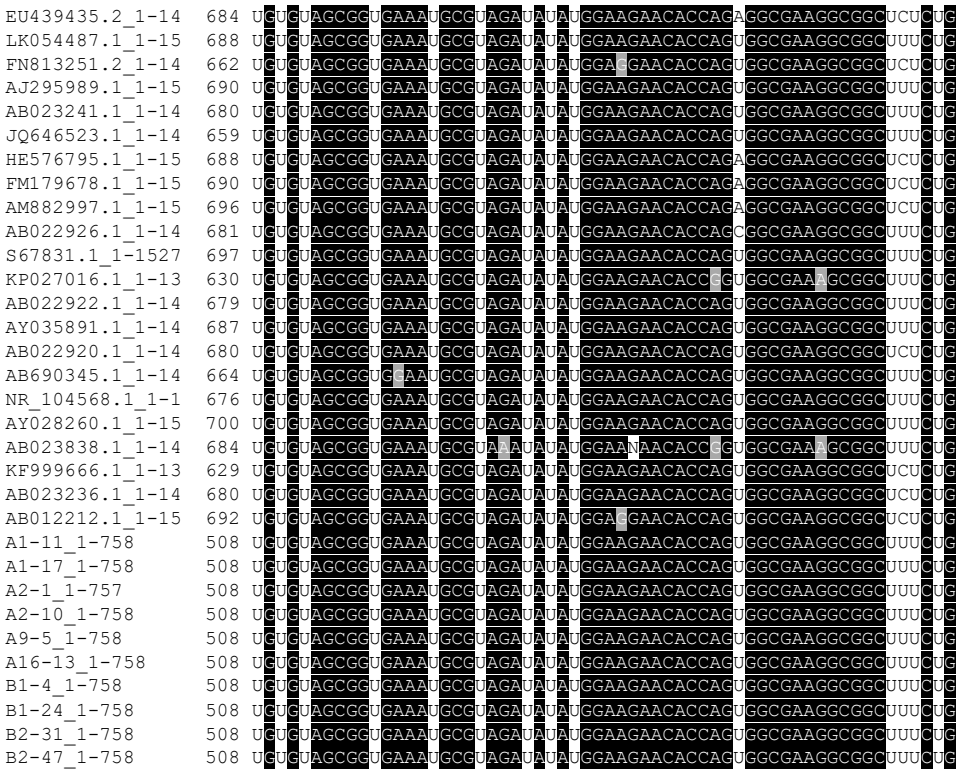

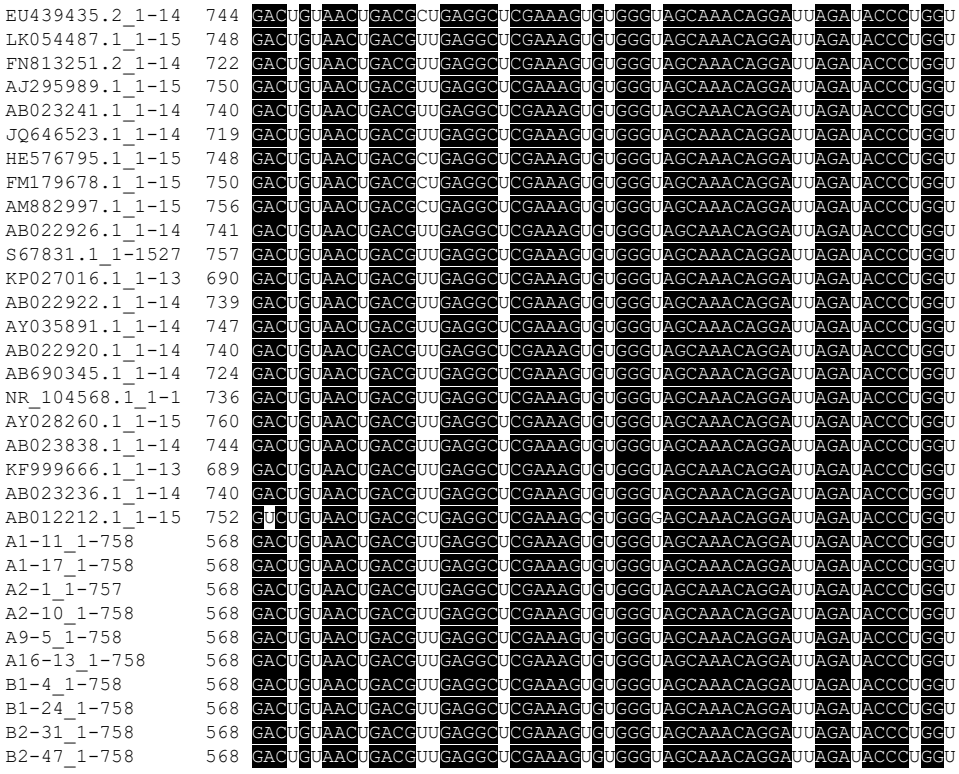

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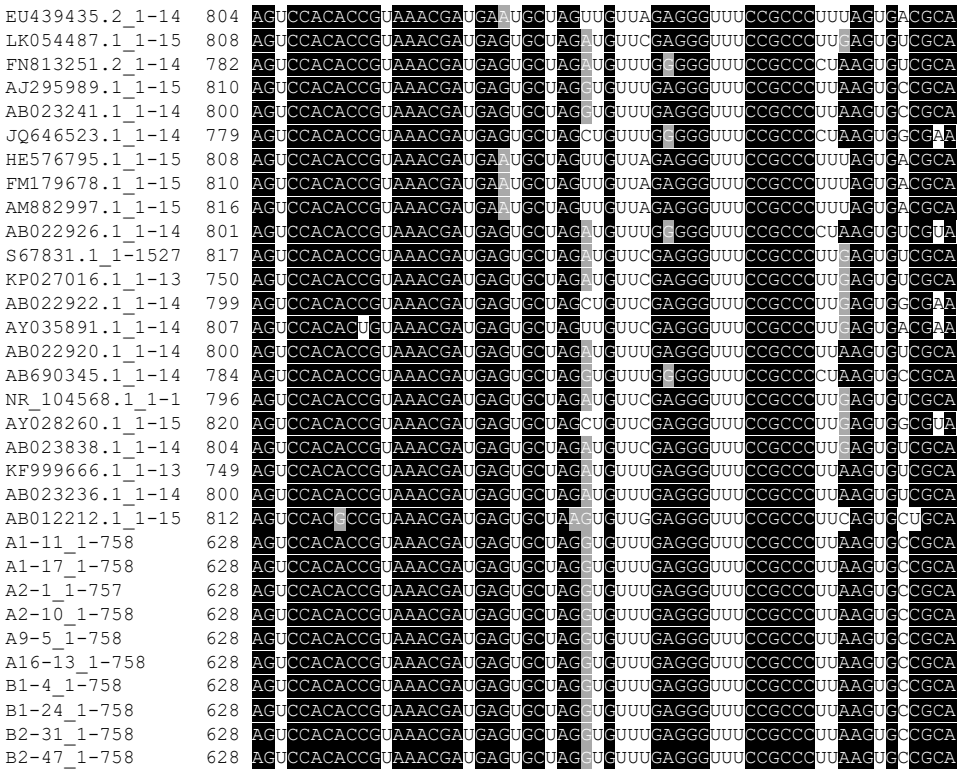

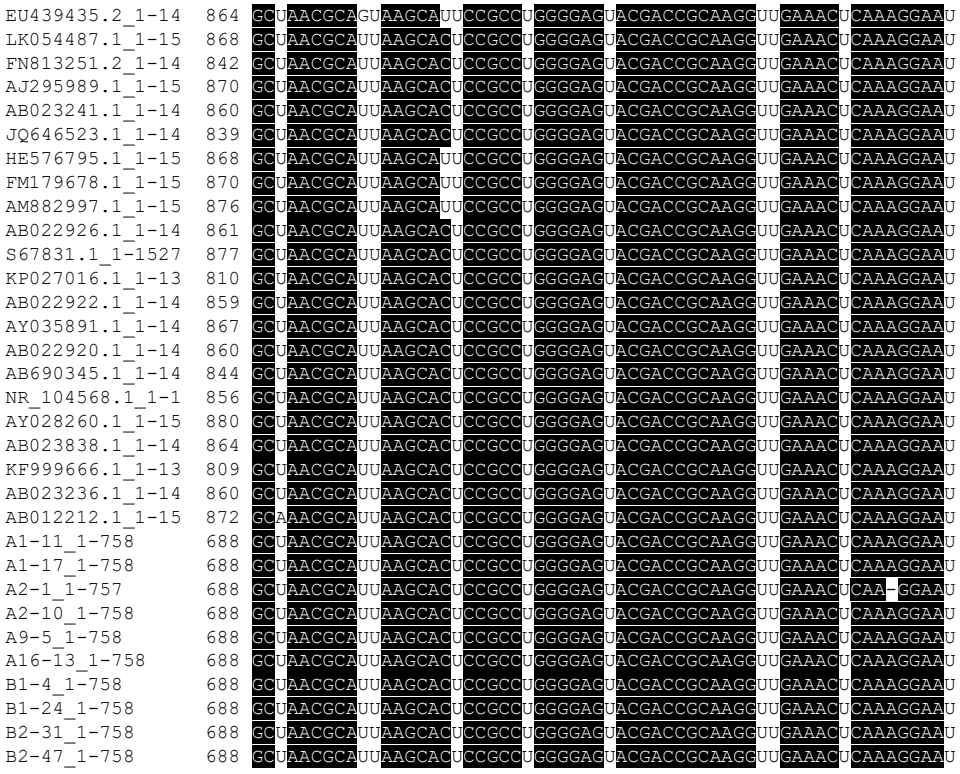

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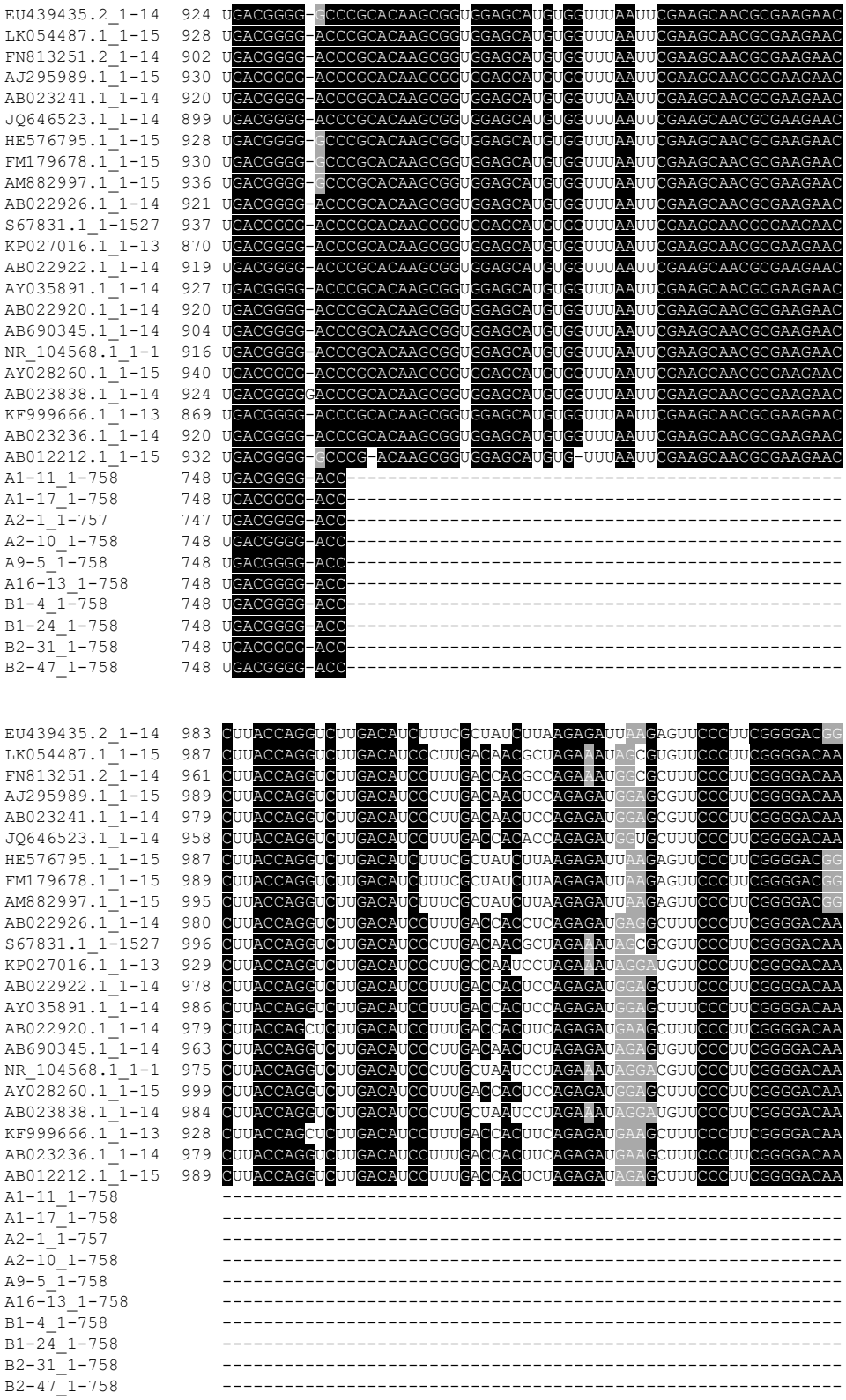

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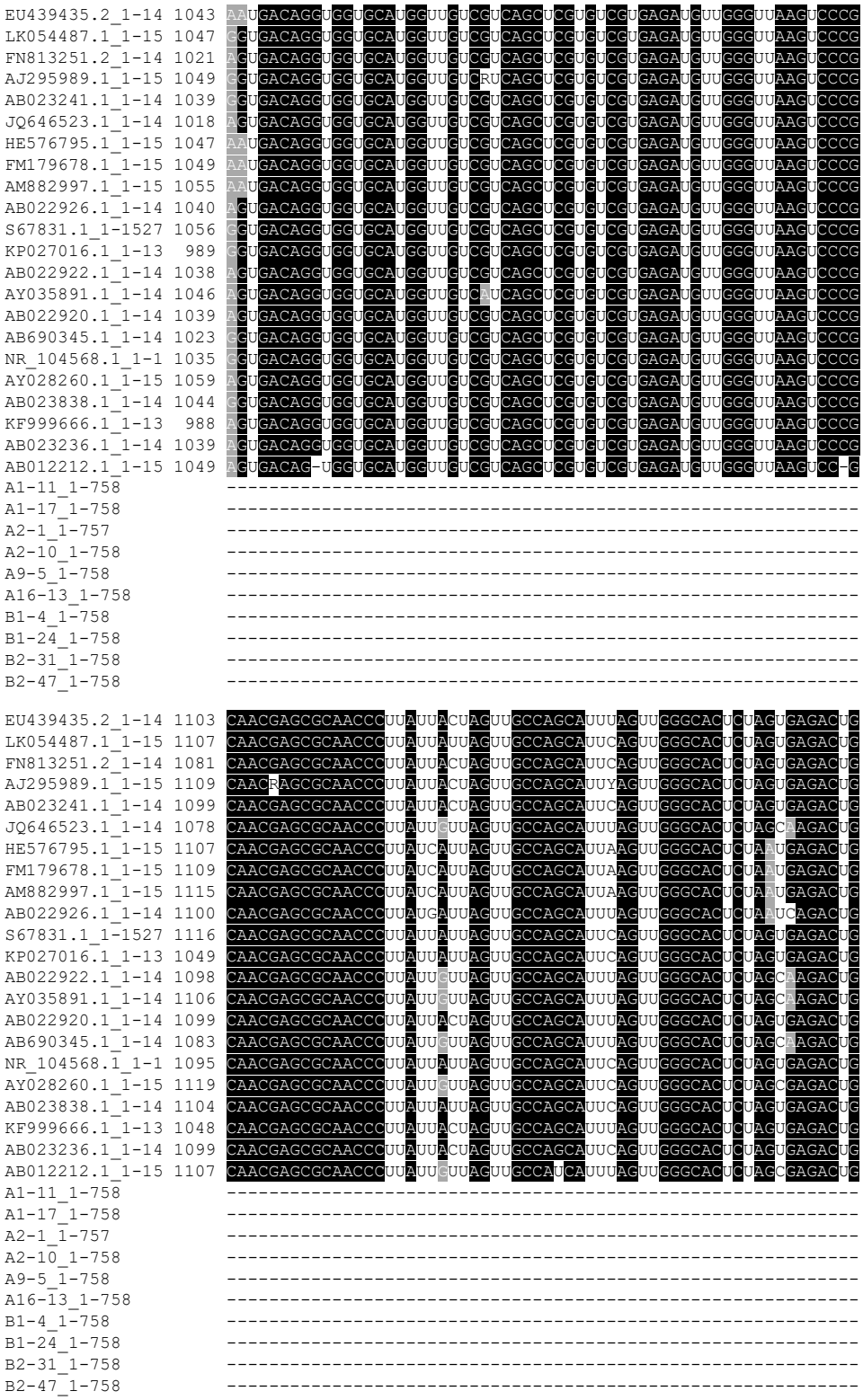

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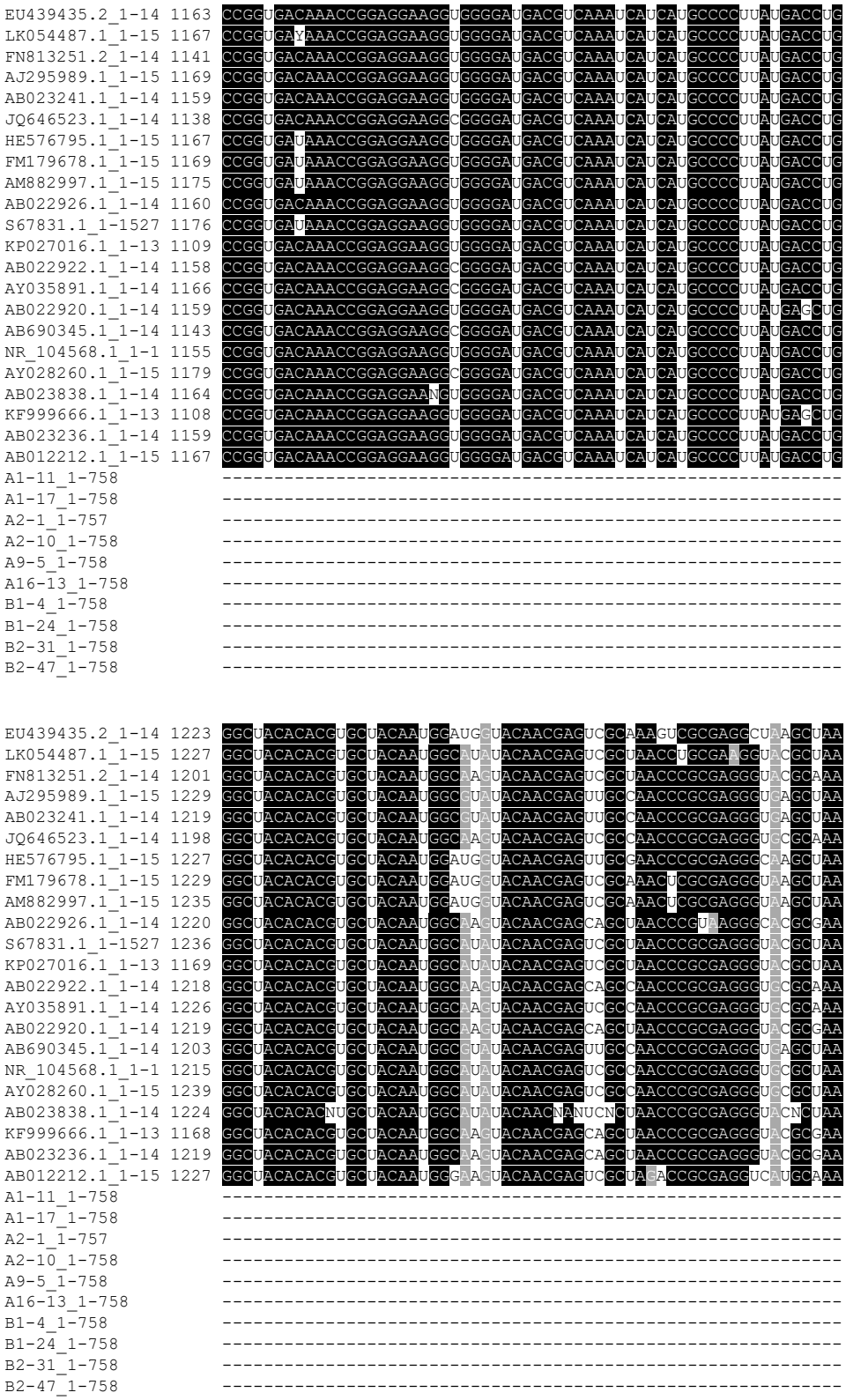

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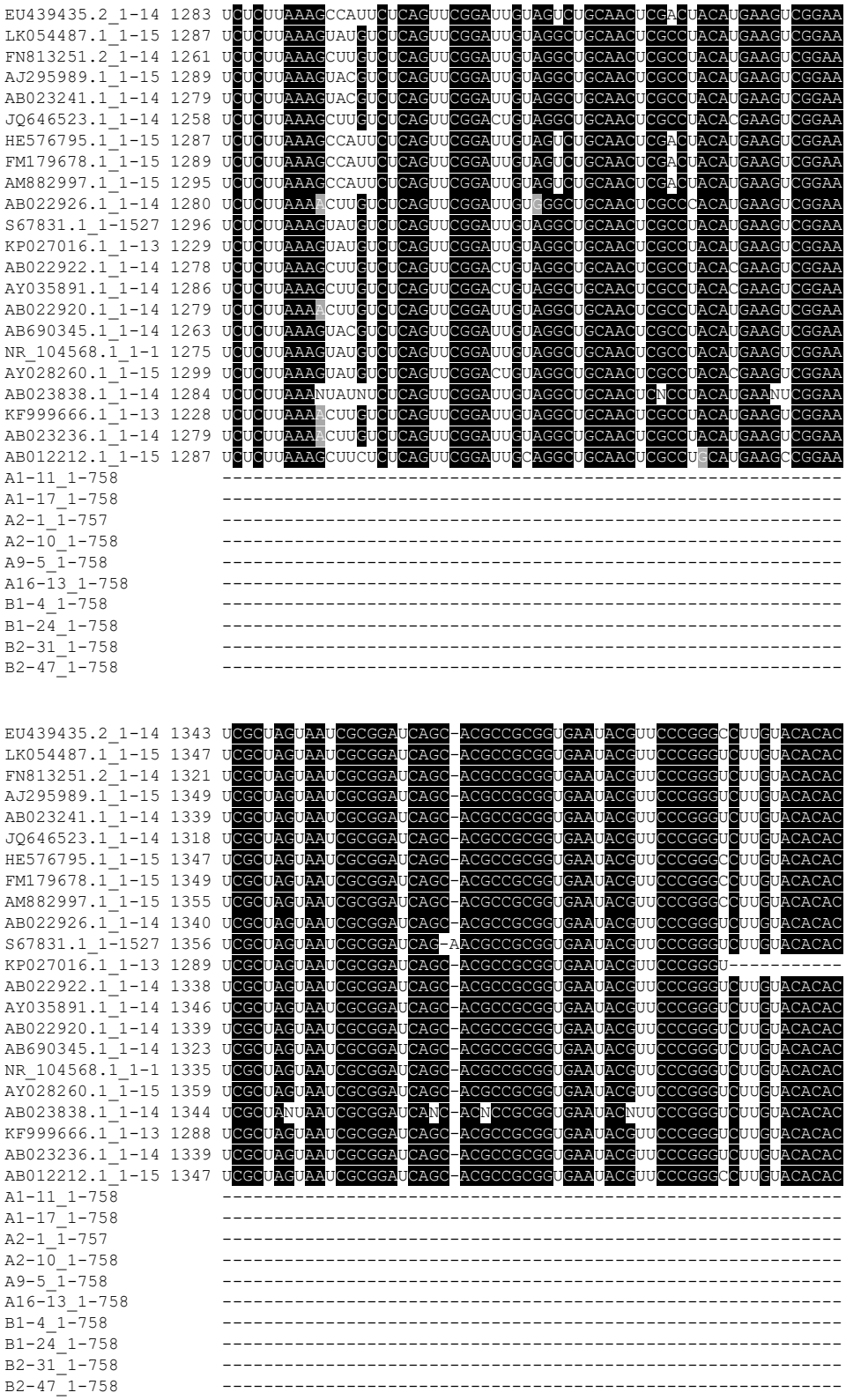

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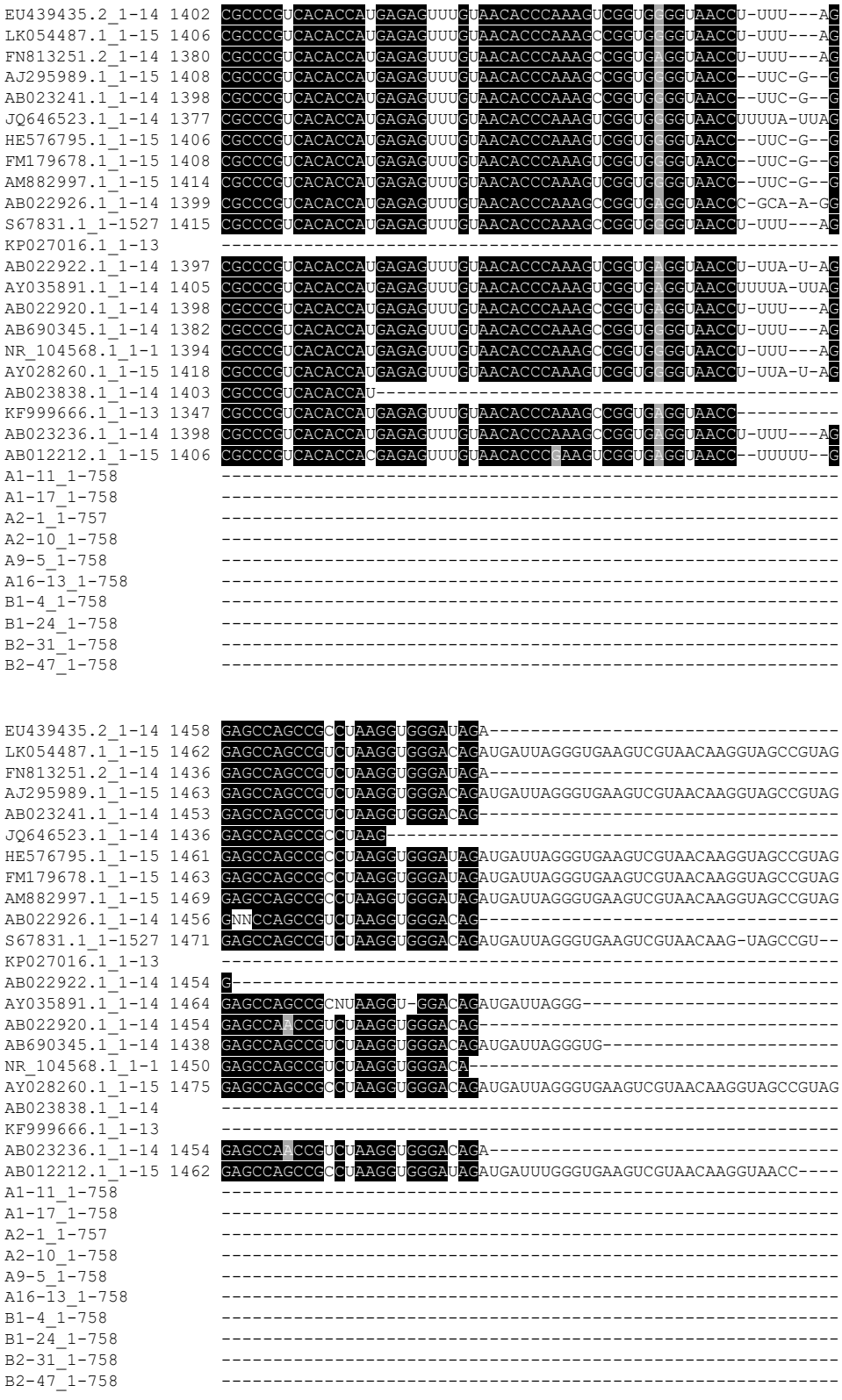

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A2-1_1-757           ------------------------
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A9-5_1-758           ------------------------
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B1-4_1-758           ------------------------
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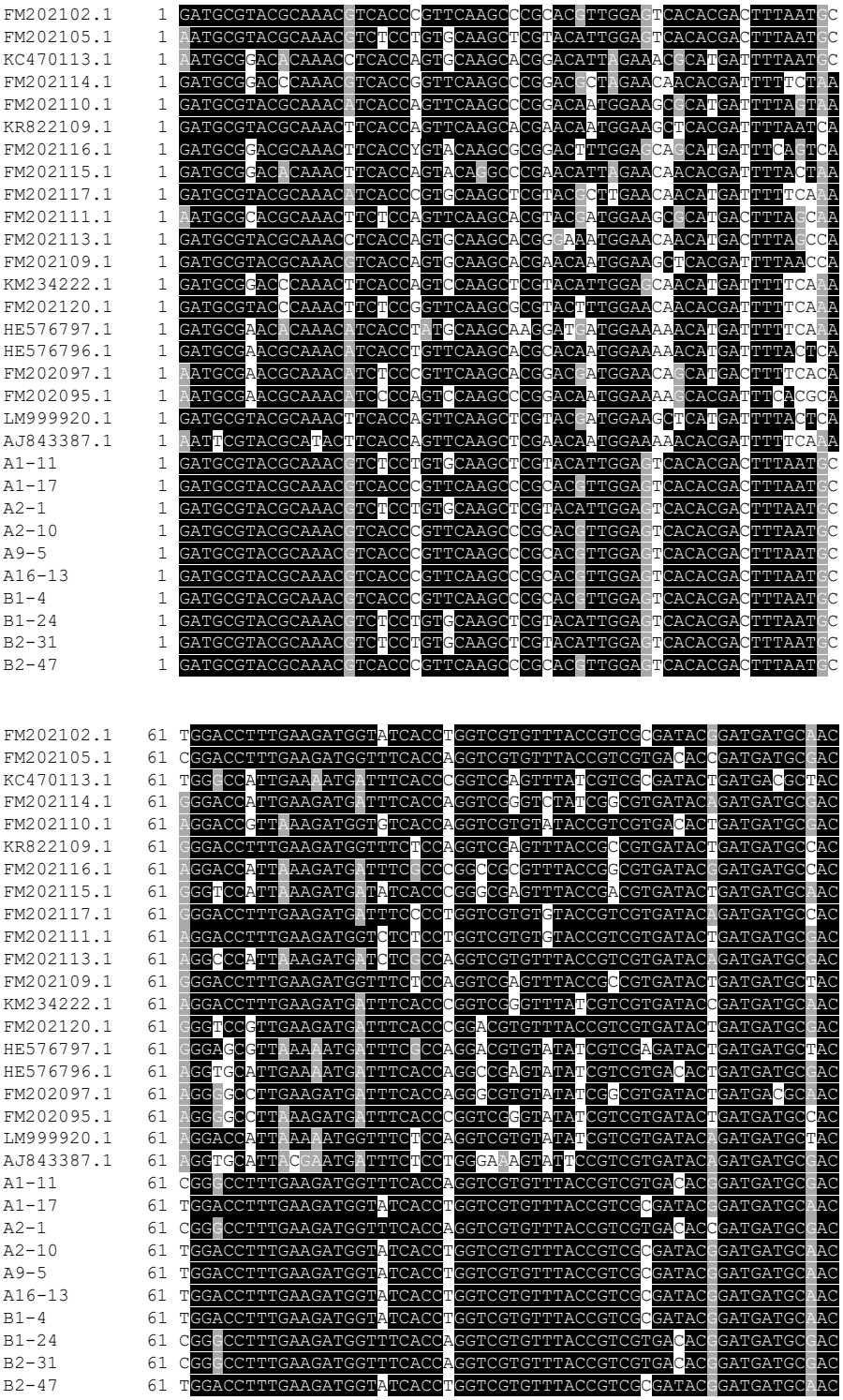
Fig. S2 Multiple sequence alignments of partial 16S rRNA genes of representative and reference *Weissella* strains used in this study. Sequences were aligned using the SILVA Incremental Aligner software version 1.2.11 [30] followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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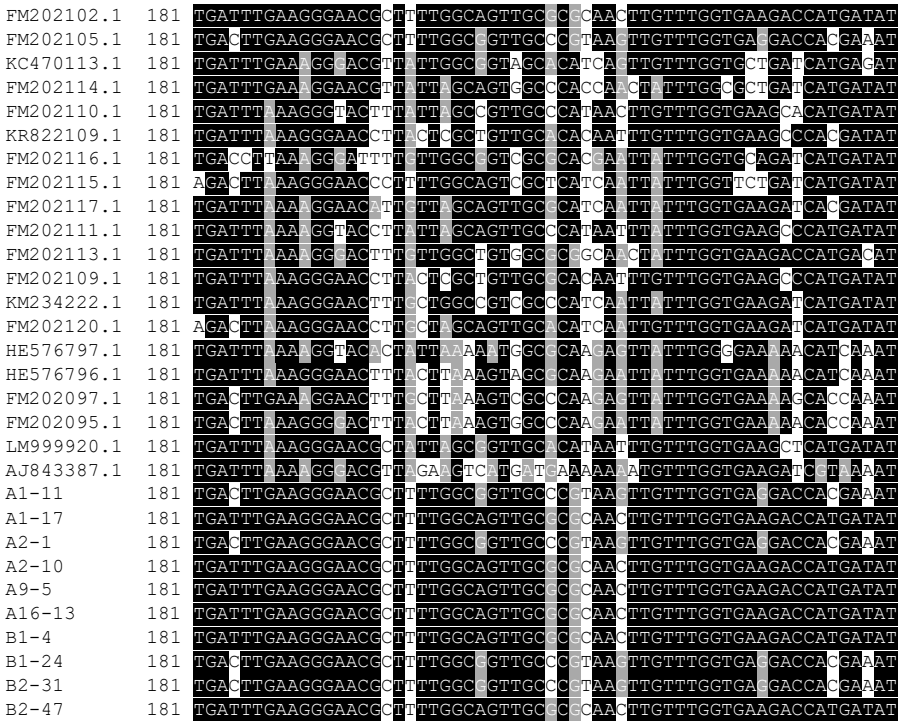

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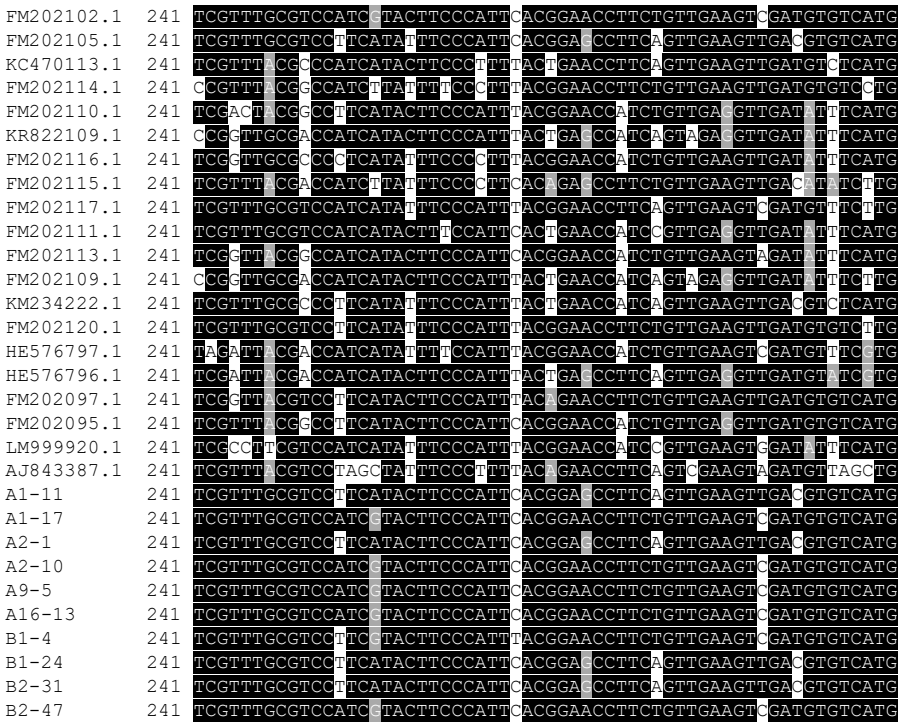

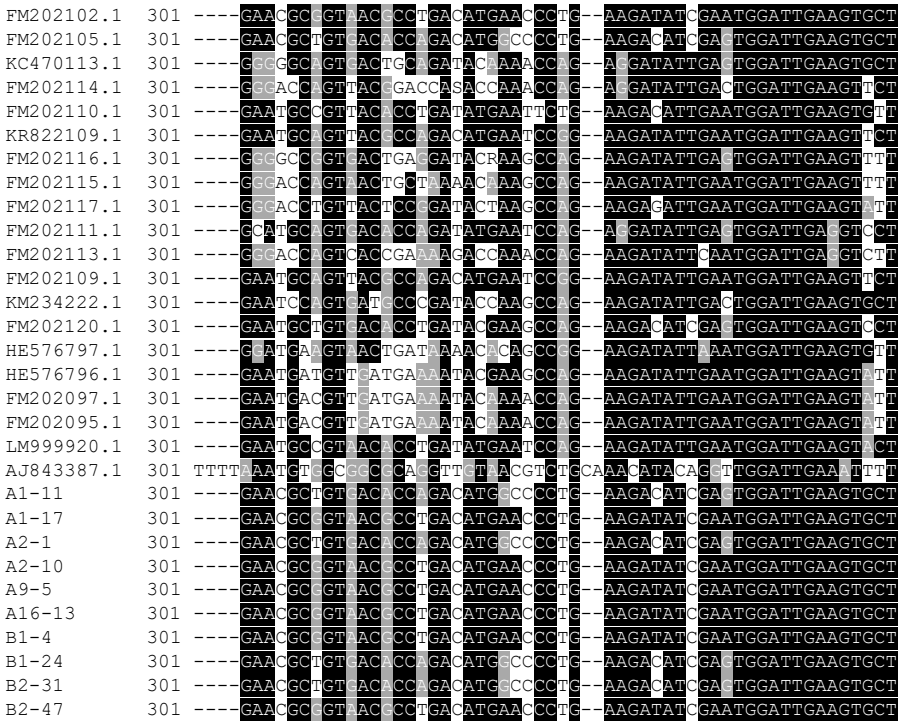

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LM999920.1  355 A
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A1-17       355 T
A2-1        355 T
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A16-13      355 T
B1-4        355 T
B1-24       355 T
B2-31       355 T
B2-47       355 T
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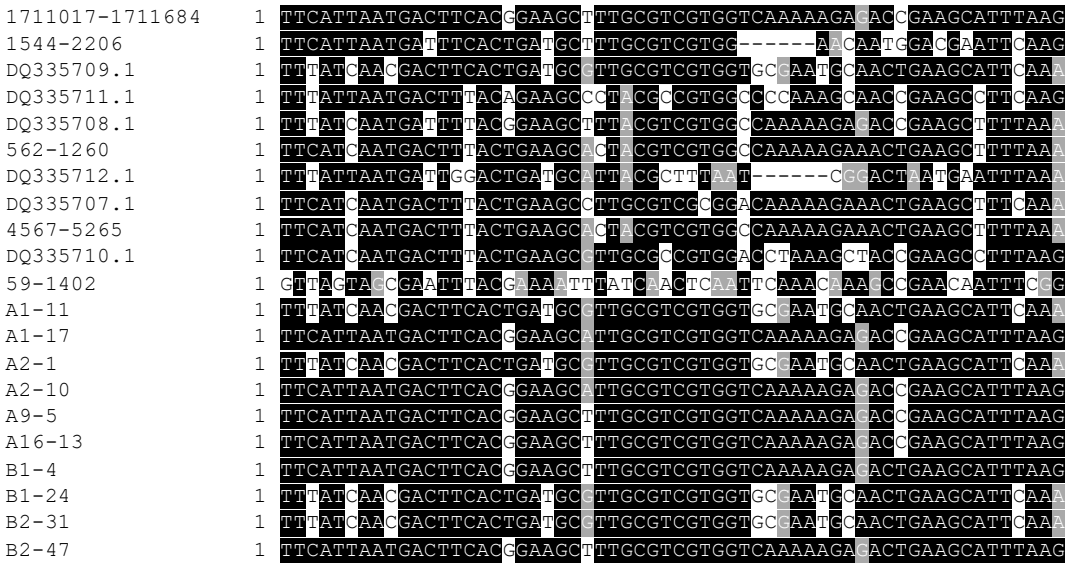
Fig. S3 Multiple DNA sequence alignments of partial *pheS* genes of representative and reference *Weissella* strains used in this study. Sequences were aligned with ClustalW [31] as implemented in the BioEdit Sequence Alignment Editor program [32] followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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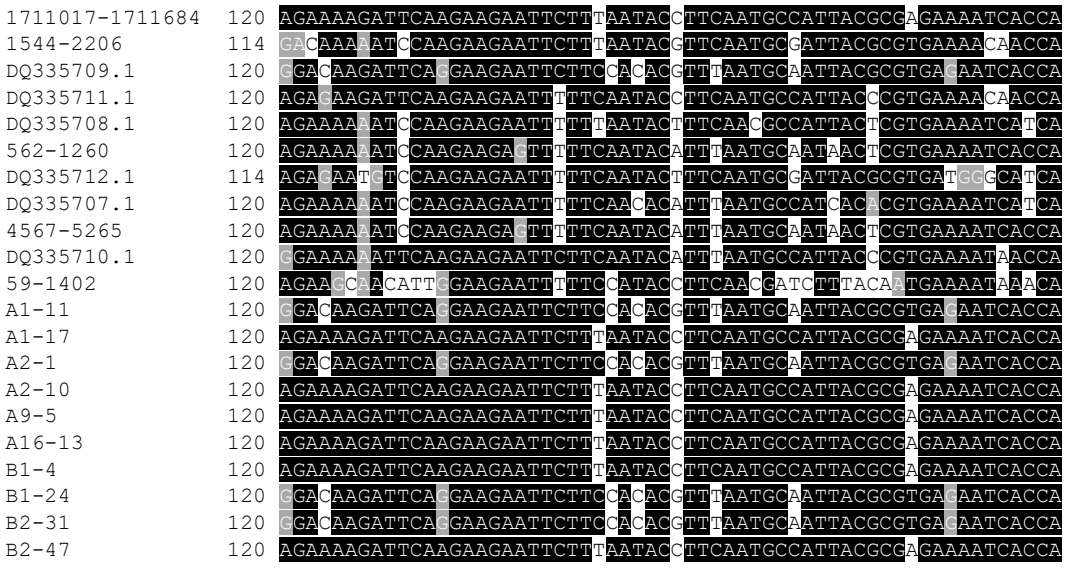

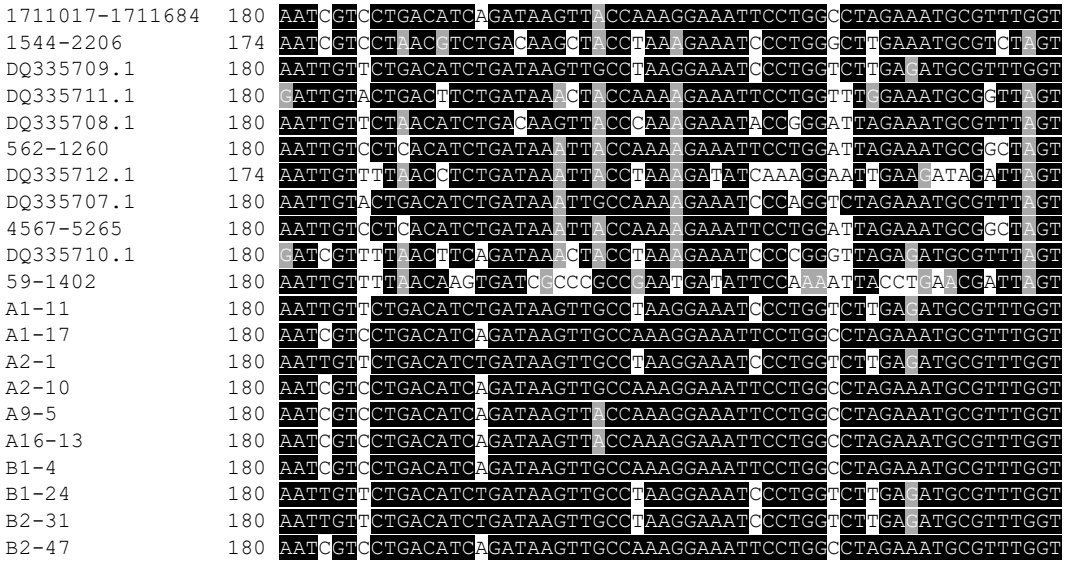

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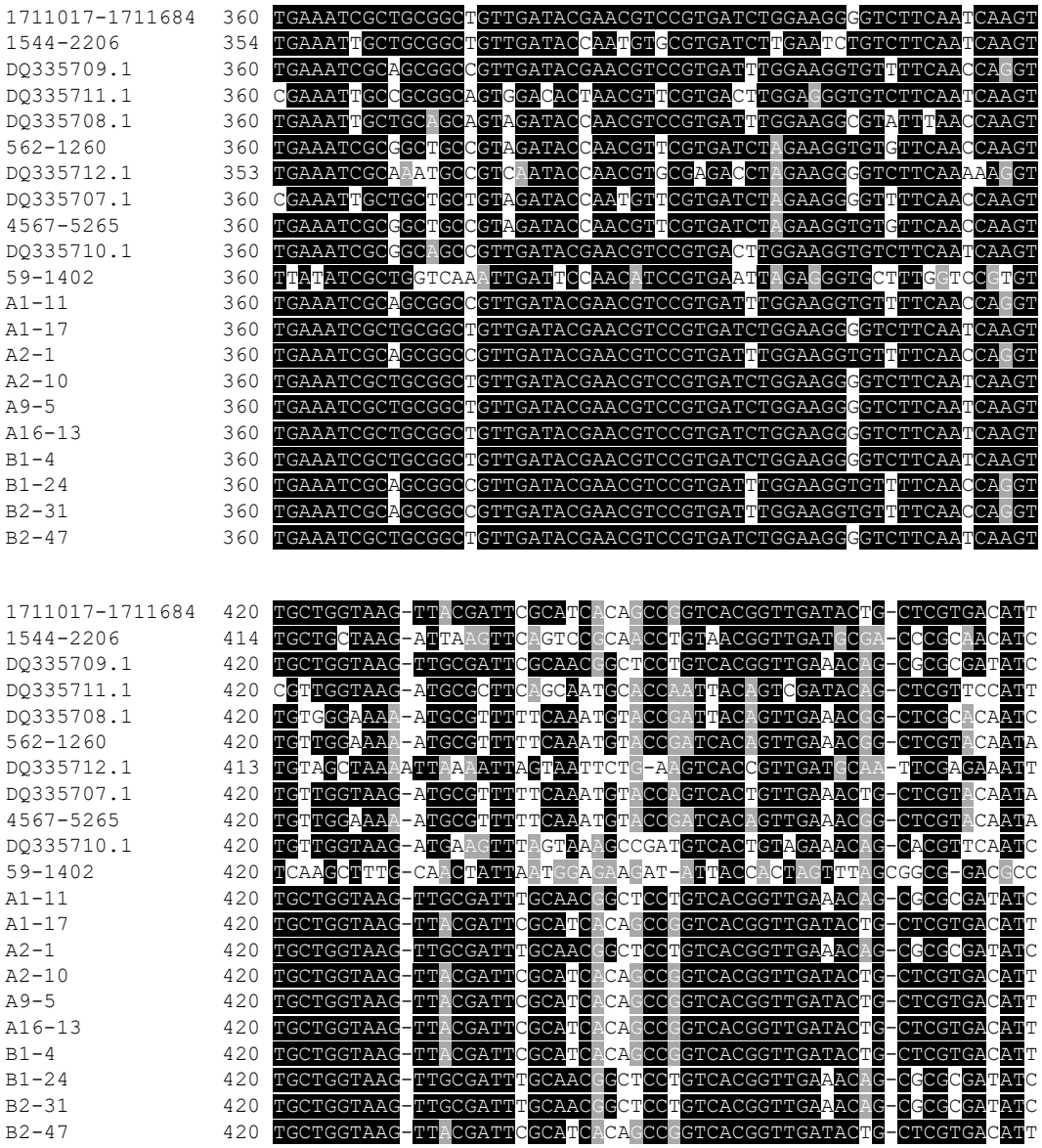

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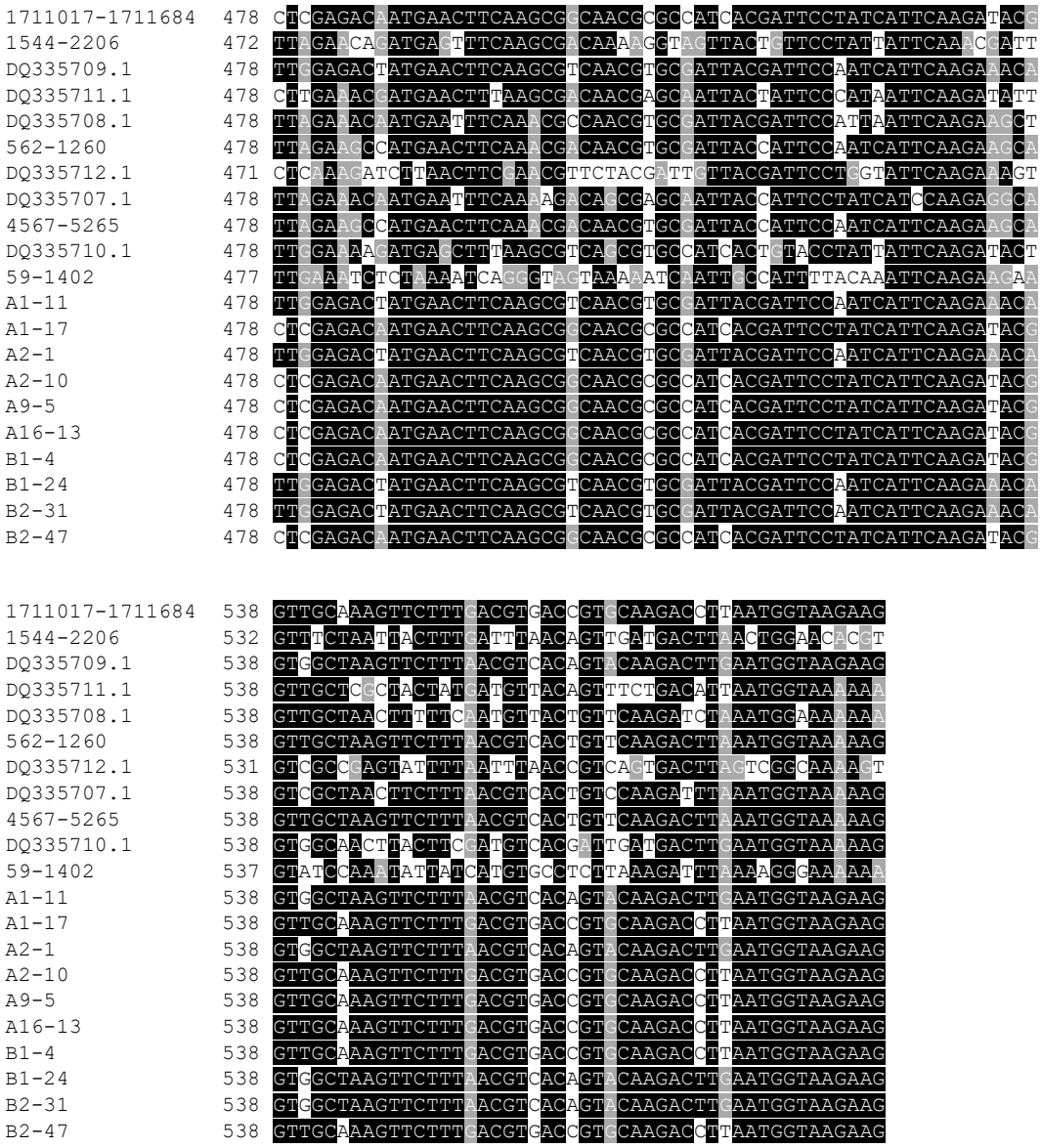
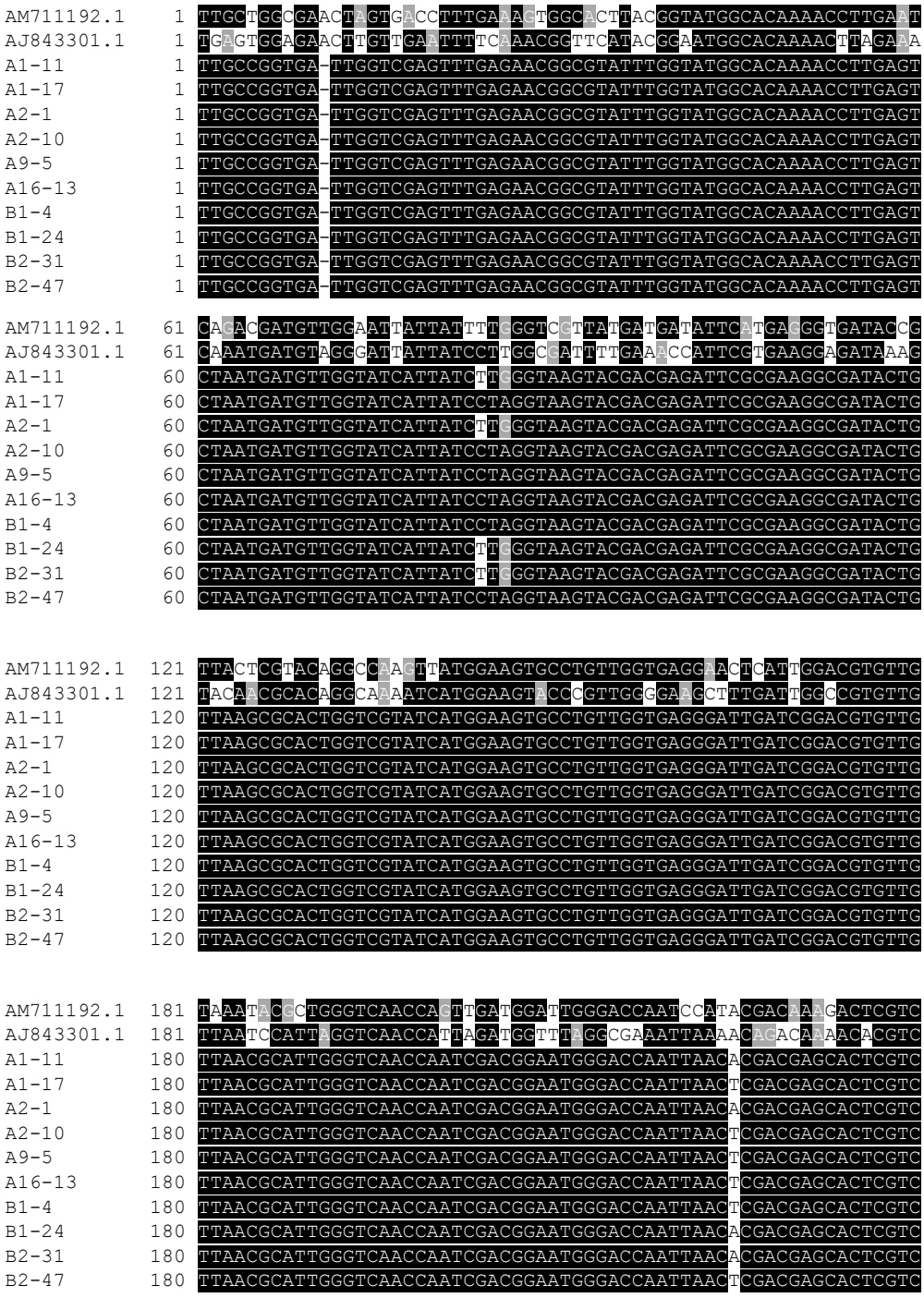


Fig. S4 Multiple DNA sequence alignments of partial *dnaA* genes of representative and reference *Weissella* strains used in this study. Sequences were aligned with ClustalW [31] as implemented in the BioEdit Sequence Alignment Editor program [32] followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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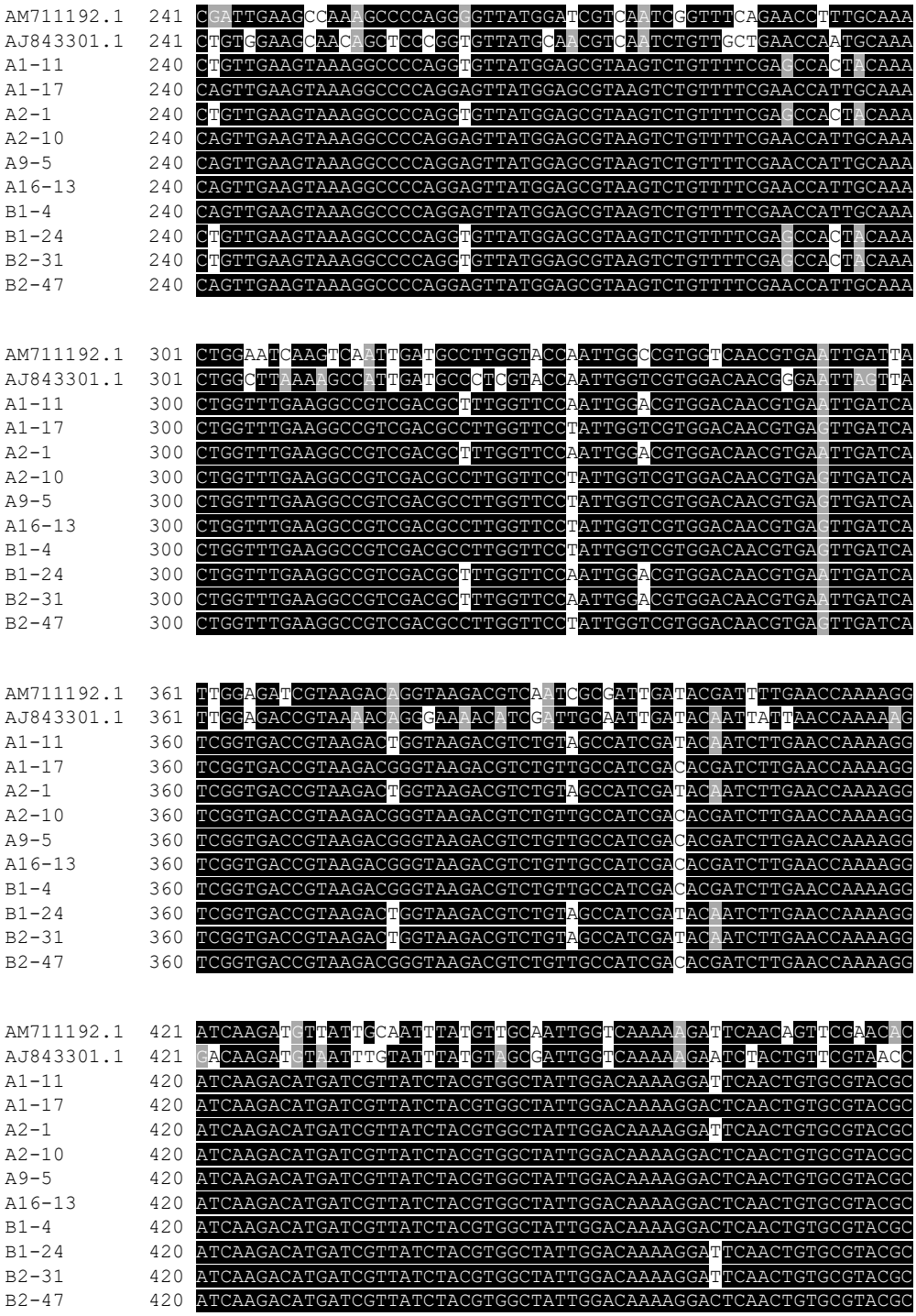

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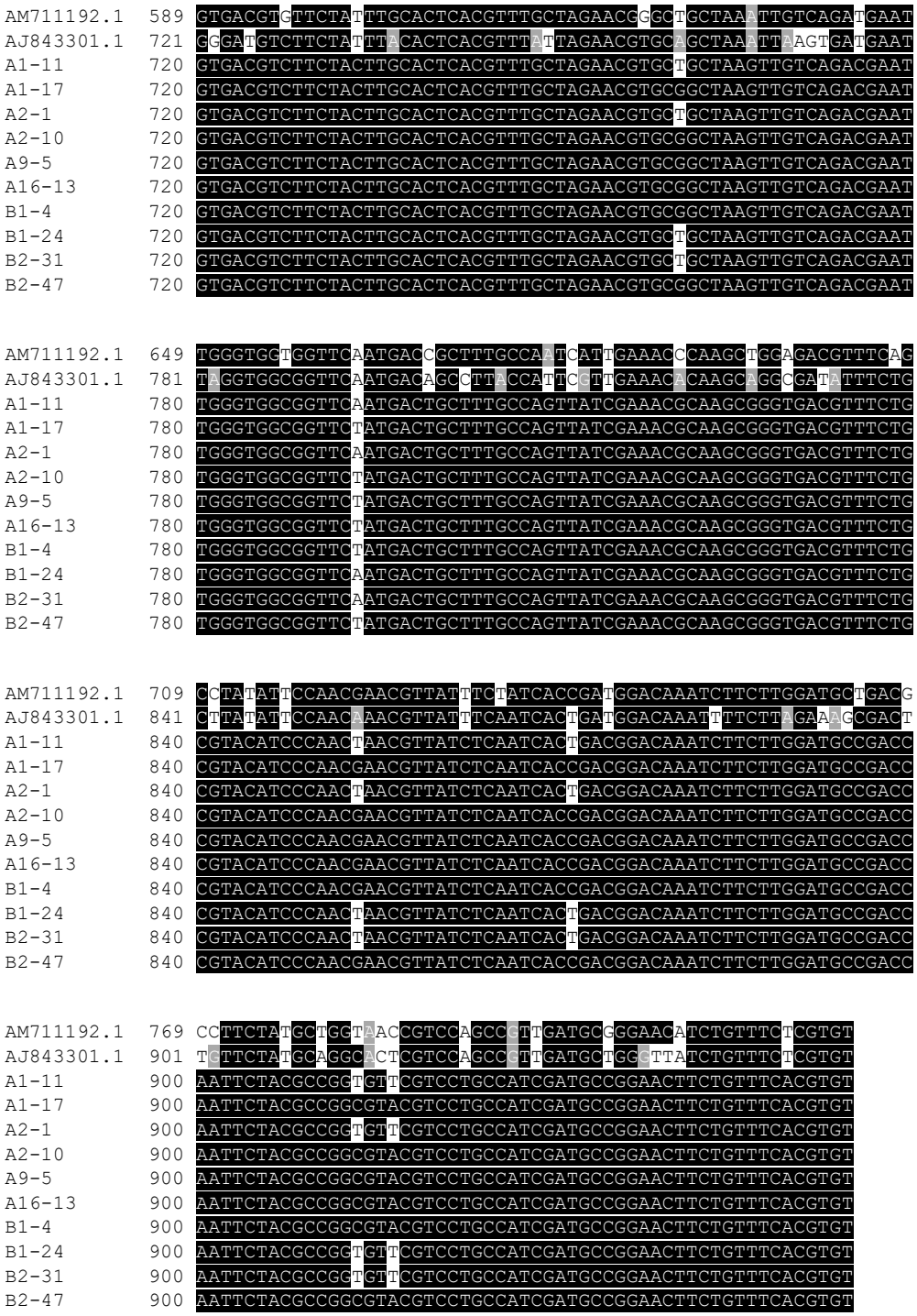

Fig. S5 Multiple DNA sequence alignments of partial *atpA* genes of representative and reference *Weissella* strains used in this study. Sequences were aligned with ClustalW [31] as implemented in the BioEdit Sequence Alignment Editor program [32] followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)

CHAPTER 6

Archives of Microbiology (2019) 201: 1453-1457

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

S Nel[1,2], SB Davis[1], A Endo[3] and LMT Dicks[2*]

[1]Sugar Milling Research Institute NPC, c/o University of KwaZulu-Natal, Durban 4041, South Africa

[2]Department of Microbiology, Stellenbosch University, Stellenbosch 7600, South Africa

[3]Department of Food, Aroma and Cosmetic Chemistry, Tokyo University of Agriculture, Hokkaido 099-2493, Japan

*Corresponding author: LMT Dicks (LMTD@sun.ac.za)

Keywords

Sugarcane processing, sucrose loss, phylogenetic analysis, ARDRA

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.

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 favourable 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 *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, *HhaI* 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). This 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 5x 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 visualised 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 x 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 were produced. The number of isolates that displayed identical banding patterns are 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. 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).

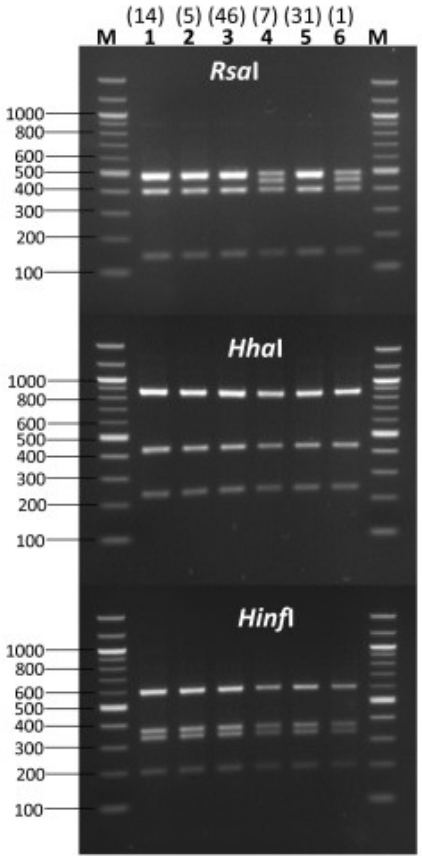

Fig. 1 ARDRA profiles recorded for 104 *Bacillus* isolates digested with *Rsa*I, *Hha*I and *Hinf*I, respectively. Each lane represents a specific sampling point and sampling time. The number of isolates with the same DNA profile are indicated in brackets. Lanes 1 and 2: DNA profiles of isolates sampled 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 Quick-Load® 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 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 with *rpoB* gene sequence analyses confirmed the separation of the representative isolates from *B. amyloliquefaciens,* as reported with ARDRA.

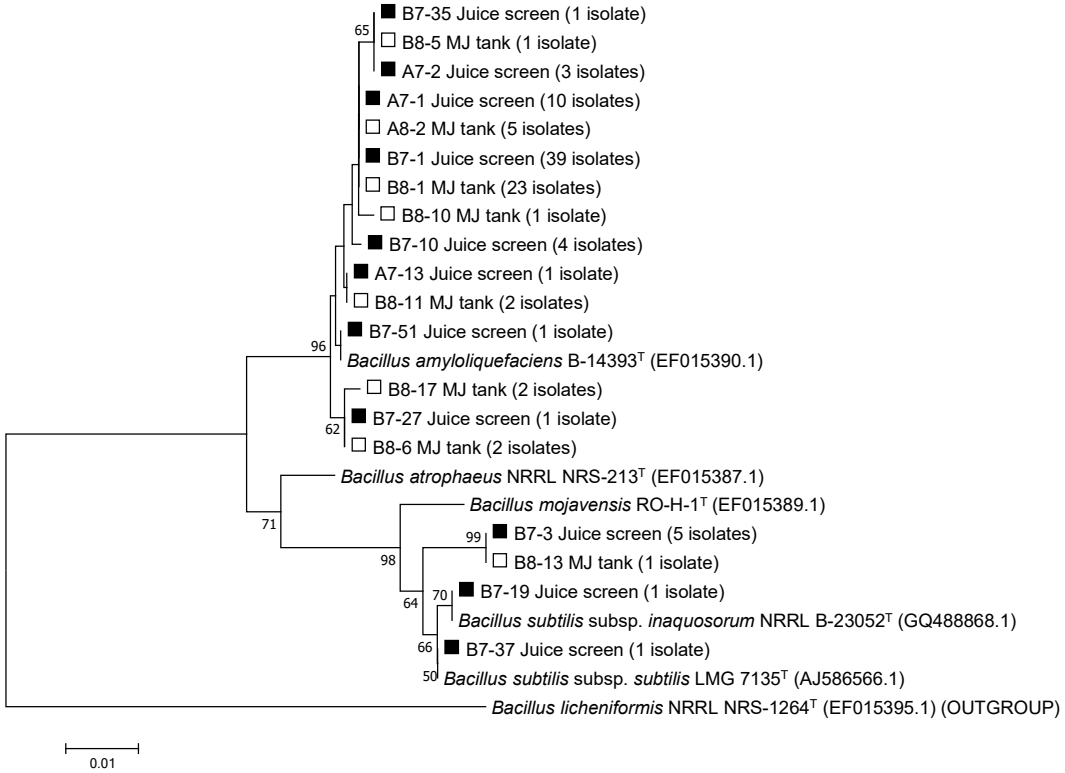

Fig. 2 Phylogenetic tree of the partial *rpoB* gene sequences amplified from *Bacillus* spp. isolated from the juice screen (black square) and mixed juice (MJ) tank (white square) of a South African sugarcane processing factory when low dextran concentrations (representative isolates prefixed by 'A') 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. *Bacillus lichenifiormis* NRRL NRS-1264^T was used as the outgroups used as the outgroup

The majority of *Bacillus* isolates (92%) isolated 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 to levan, and the build-up of exopolysaccharides may increase the viscosity of the sugarcane syrup and prevent the crystallisation 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.

Acknowledgements

DNA sequencing was performed by Dr Deborah Sweby (South African Sugarcane Research Institute, Mt Edgecombe, South Africa).

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Supplementary material

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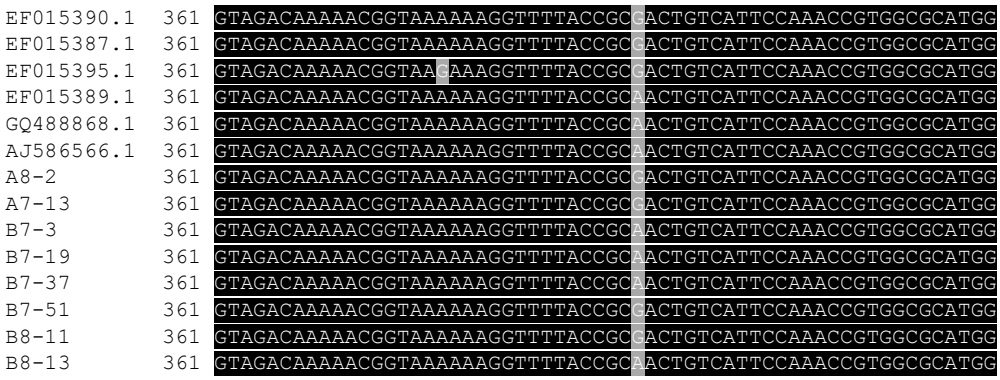


Fig. S1 Multiple DNA sequence alignments of partial *rpoB* genes of representative and reference *Bacillus* strains used in this study. Sequences were aligned with ClustalW (Thompson et al. 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)

CHAPTER 7

Submitted for publication in *MicrobiologyOpen*

Phylogenetic analysis of *Leuconostoc* and *Lactobacillus* species isolated from sugarcane processing streams

Sanet Nel[1,2], Stephen B. Davis[1], Akihito Endo[3] and Leon M. T. Dicks[2#]

[1]Sugar Milling Research Institute NPC, c/o University of KwaZulu-Natal, Durban 4041, South Africa

[2]Department of Microbiology, Stellenbosch University, Stellenbosch 7600, South Africa

[3]Department of Food, Aroma and Cosmetic Chemistry, Tokyo University of Agriculture, Hokkaido 099-2493, Japan

#Correspondence

LMT Dicks (LMTD@sun.ac.za), Tel.: +27-21-808 5849, Fax: +27-21-808 5846

Abstract

High levels of dextran, produced by *Leuconostoc* and *Lactobacillus* spp., have a severe impact on factory throughput and sugar quality. Previous studies that examined the microbial diversity in sugarcane processing factories were constrained by the absence of microbial identification methods with high discriminatory power. In this study we determined the phylogenetic relationships of 81 *Leuconostoc* isolates from five sugarcane processing streams based on the analysis of the housekeeping genes *rpoA* and *dnaA*. The phylogenetic inference for 43 *Lactobacillus* isolates from three factory streams was obtained by analysis of the *pheS* and *tuf* housekeeping genes. The *rpoA* gene proved highly discriminatory for the phylogenetic resolution of all *Leuconostoc* spp. isolated from a South African sugarcane processing factory. The *dnaA* housekeeping gene could be used to accurately infer the phylogenetic relatedness of isolates clustering with *Leuconostoc mesenteroides* and *Leuconostoc citreum.* Single locus analysis, as well as concatenation of the *pheS* and *tuf* housekeeping gene sequences, yielded identical phylogenies for 43 *Lactobacillus* isolates corresponding to *Lactobacillus fermentum.* The correct identification of spoilage bacteria at different stages of sugarcane processing is important for the development of effective sanitation management strategies.

KEYWORDS

Phylogenetic analysis, *Leuconostoc, Lactobacillus*, sugarcane, dextran

1. INTRODUCTION

Problems encountered with microbial degradation of harvested sugarcane, followed by further spoilage during processing, leads to a poor-quality product and severe economic losses. Dextran-producing strains of *Leuconostoc mesenteroides* have been implicated in the slowing down of factory throughput and quality of the final product (Eggleston et al., 2008). In the sugar industry dextran is also referred to as exopolysaccharides (EPS) or 'gums'. Some strains of *L. mesenteroides* produce as much as one-part dextran from 40 parts of sucrose after only 6 h (Cerutti de Guglielmone et al., 2000). Apart from an increase in viscosity, other metabolites produced during the degradation of sucrose reduce the purity of the cane juice and thus also sucrose recovery. Impure juice requires longer boiling times, leading to higher sucrose inversion losses. The impurities reduce evaporation rates and sugar crystals take longer to form (Godshall et al., 1996; Jimenez, 2005). Dextran shows high (20 %) transfer from juice to crystal, resulting in high carry-over from the factory to the refinery. Refiners and buyers of raw sugar prefer the product to have low levels of dextran (<100-150 mg/kg), even if the purchase contract does not specify dextran content (Ravnö, & Purchase, 2005). Economic losses due to microbial activities are, therefore, not limited to the direct loss of recoverable sucrose and indirect loss due to reduced factory throughput, but also finding financially attractive markets for high-dextran raw sugar (Moodley, & Khomo, 2018).

L. mesenteroides is not solely responsible for gum production in sugarcane processing. In a study on 430 gum-producing isolates from harvested sugarcane and sampled from a South African sugarcane processing factory (Nel et al., 2019a), *Leuconostoc pseudomesenteroides*, *Leuconostoc citreum*, *Leuconostoc lactis*, *Weissella confusa*, *Weissella cibaria*, *Lactobacillus fermentum*, *Bacillus amyloliquefaciens* and *Bacillus subtilis* have been identified in addition to *L. mesenteroides* subsp. *mesenteroides*, subsp. *dextranicum* and subsp. cremoris based on 16S rRNA gene sequence analysis. According to this study, 47% of EPS-producing bacteria belonged to the genus *Weissella*, followed by *Bacillus* (24%), *Leuconostoc* (19%) and *Lactobacillus* (10%). As pointed out in the study by Nel et al. (2019a), the unanticipated presence of *Weissella* and *Bacillus* spp. on cane and in a sugar processing factory reiterates the importance of accurately identifying spoilage bacteria.

Historically, sugar technologists had to rely on phenotypic identification methods which failed to accurately differentiate species within the genus *Leuconostoc* and between species of *Leuconostoc* and *Lactobacillus* (McNeil & Bond, 1980). The ambiguity of results obtained from earlier studies is evident from the report by McCleskey and co-workers (1947). The authors grouped 168 gum-producing bacteria, isolated from sugarcane juice, into four clusters

based on phenotypic and biochemical characteristics. Each group represented isolates with unique fermentation reactions, levels of gum production, acid and gas produced, and optimal growth temperature and growth pH. Despite these differences, all of the isolates were classified as *L. mesenteroides*. The difficulties with reliable differentiation between *Leuconostoc* spp. and heterofermentative *Lactobacillus* spp. by phenotypic identification methods are now well recognised (Collins et al. 1993). Although 16S rRNA gene sequences have been widely used as phylogenetic marker in bacterial taxonomy, the method has limitations and is not reliable in distinguishing species and subspecies with high sequence similarities (Jeon et al., 2017). *Leuconostoc mesenteroides* and *L. pseudomesenteroides* are good examples. The two species share almost identical 16S rRNA sequences, with differences in only 5 of the 1,483 nucleotides (Martinez-Murcia, & Collins, 1990). Comparisons amongst housekeeping gene sequences is commonly used to overcome the limitations of 16S rRNA sequencing (Chelo et al., 2007; De Bruyne et al., 2007; Naser et al., 2007; Yu et al., 2012). Single protein-coding genes do not reflect general phylogenetic relationships due to potential horizontal gene transfer (HGT) or lateral gene transfer (Gogarten et al., 2002; Macheras et al., 2011). Multiple gene-based phylogenies were introduced which have been used more frequently to overcome the bias caused by single gene sequence-based phylogenies (Glaeser and Kämpfer, 2015). Concatenation of several housekeeping genes may reduce the weight of HGT and it could accurately locate taxonomic positions for closely related species and strains (Glaeser and Kämpfer, 2015).

In this study, the phylogenetic relationships of *Leuconostoc* and *Lactobacillus* bacteria isolated from shredded (prepared) sugarcane, a diffuser sump, filtrate, mud trough and syrup tank in a sugarcane processing factory were determined by sequencing and analysis of housekeeping genes.

2. MATERIALS AND METHODS

2.1 Isolation of gum-producing bacteria

Samples of shredded (prepared) sugarcane, and samples from the diffuser sump, juice screen (Dutch State Mines; DSM screen), mixed juice tank (MJ tank), filtrate, mud trough and syrup tank in a South African sugarcane processing factory were collected and screened for the presence of gum-(polysaccharide) producing bacteria (Nel et al., 2019a). Once-off samples at each sampling location were taken during September 2013, when low dextran concentrations (<70 ppm) in the produced raw sugar were reported. This was repeated in November 2013, when high dextran concentrations (>500 ppm) in raw sugar were found.

Cane samples (10 g each) were added to 100 ml phosphate buffered saline (PBS; Green, & Sambrook, 2012) and incubated on a rotary shaker (30°C, 150 rpm) for 1 h. Liquid samples collected from each of the sampling points and PBS-cane suspensions were serially diluted in PBS. Serial dilutions were streaked onto modified dextransucrase-inducing agar with the following composition: sucrose 100 g/l, peptone 20 g/l, KH_2PO_4 20 g/l, agar 15 g/l and R-salts (4% $MgSO_4 \cdot 7H_2O$, 4% NaCl, 0.2% $FeSO_4 \cdot 7H_2O$ and 0.2% $MnSO_4 \cdot H_2O$) 5 ml (Tsuchiya et al., 1952). Plates were incubated at 30°C for 14 to 18 h. A total of 124 colonies with a glistening and slimy appearance were selected and streaked to purity on modified dextransucrase-inducing agar. From these plates, a single colony was inoculated into 5 ml MRS broth (Biolab, Merck South Africa) and the cultures incubated on a shaking incubator (150 rpm) for 14 to 18 h at 30°C. Cells were harvested by centrifugation (16 000 x g, 25°C, 2 min), re-suspended in sterile glycerol (200 µl; 50%, v/v) and stored at -70°C.

2.2 Genomic DNA extraction

An aliquot of ten microliters of stock culture was inoculated into 5 ml sterile MRS broth (Biolab, Merck, Modderfontein, South Africa) and incubated for 16 h at 30°C on a rotary shaker (150 rpm). Cells were harvested (16 000 x g, 25°C, 2 min) and genomic DNA extracted using the GeneJET Genomic DNA Purification kit (Thermo Scientific, Inqaba Biotechnical Industries, Hatfield Pretoria, South Africa) according to the manufacturer's instructions. Purified DNA was suspended in 50 µl elution buffer and used as template in amplification reactions.

2.3 Amplification of the 16S rDNA, and housekeeping genes *rpoA, dnaA*, *pheS*, and *tuf*

Genomic DNA was used as template to amplify sequences of the 16S rRNA genes of all species, *rpoA* and *dnaA* genes of *Leuconostoc* spp., and *pheS* and *tuf* genes of *Lactobacillus* spp. using the primers listed in Table 1. Reactions were carried out in 50 µl, containing 10 pmol of each primer, 200 µM of each deoxynucleoside triphosphate (Thermo Scientific), 10 µl of 5x One Taq Standard Reaction buffer, 1.25 U One Taq Hot Start DNA polymerase (Thermo Scientific) and 100 ng template genomic DNA. PCR reactions were 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 and elongation (see Table 1). Cycling was completed by a final elongation step (68°C, 10 min), followed by cooling to 4°C. The amplified fragments were purified using the DNA Clean and Concentrator™-25 kit (Zymo Research,

Inqaba Biotechnical Industries, Hatfield Pretoria, South Africa) according to the manufacturer's instructions.

2.4 Gene sequencing and phylogenetic analyses

Partial 16S rRNA, *rpoA, dnaA*, *pheS* and *tuf* gene sequencing was performed using BigDye Cycle Sequencing chemistry (Applied Biosystems, Johannesburg, South Africa), according to the manufacturer's instructions. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990). Reference 16S rRNA, *rpoA, dnaA*, *pheS* and *tuf* gene sequences from respective type strains with names in standing nomenclature were retrieved from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and included in the analyses. Reference strains and their species names are indicated in the respective figures. GenBank accession numbers for 16S rRNA, *rpoA, dnaA*, *pheS* and *tuf* gene sequences of representative strains for each sampling location, determined in this study, are listed in Table 2. For phylogenetic inference, seven separate alignments were created; five corresponding to the single locus alignment of 16S rRNA, *rpoA*, *dnaA*, *pheS* and *tuf* genes, and two alignments corresponding to the concatenation of the housekeeping genes *rpoA-dnaA* (for *Leuconostoc* spp.) and *pheS-tuf* (for *Lactobacillus* spp.). Sequences were aligned with ClustalW (Thompson et al., 1994), as implemented in the BioEdit Sequence Alignment Editor program (Hall, 1999). A data matrix for each alignment was created for the representative sequences of strains at each sampling location and sampling time. Phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 software (Kumar et al., 2016). Evolutionary histories were inferred using the Maximum Likelihood method with the Kimura 2-parameter model (Kimura, 1980) for 16S rRNA sequence analyses, *Lactobacillus tuf* sequence analyses and *Lactobacillus pheS-tuf* concatenated sequence analyses. The Tamura 3-parameter model (Tamura, 1992) was used for respective *Leuconostoc rpoA, dnaA* and *rpoA-dnaA* concatenated sequence analyses and *Lactobacillus pheS* sequence analyses. The strengths of the internal branches of the resultant trees were statistically evaluated by bootstrap analysis (Felsenstein, 1985) with 100 bootstrap replications.

Table 1 Primer sequences and PCR conditions for the amplification of the 16S rRNA gene and the housekeeping genes *rpoA, dnaA, pheS* and *tuf*

Gene	Primer name	Primer sequence (5'→3')	Annealing temp. (°C)	Elongation time (s)	Amplified fragment size (~bp)	Reference
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	50	90	1450	Lane (1991); Turner et al. (1999)
	1492R	GGTTACCTTGTTACGACTT				
rpoA	rpoA-21-F	ATGATYGARTTTGAAAAACC	46	60	800	De Bruyne et al. (2007)
	rpoA-23-R	ACHGTRTTRATDCCDGCRCG				
dnaA	dnaA445-F	GGTGGCGTTGGTCTAGGWAAAACMCAYYTRATG	55	60	800	Chelo et al. (2007)
	dnaA1253-R	TGCATCACAGTTGTATGATCYYKMCCRCCAAA				
	dnaA445-Fs*	GGTGGCGTTGGTCTAGG				
pheS	pheS-21-F	CAYCCNGCHCGYGAYATGC	56	30	400	De Bruyne et al. (2007)
	pheS-23-R	GGRTGRACCATVCCNGCHCC				
tuf	Tuf-for	ATGGCAGAAAAAGAACATTACG	52	90	1200	Sarmiento-Rubiano et al. (2010)
	Tuf-rev	AGTAACYTGACCRGCACCAAC				

* = sequencing primer only

Table 2 GenBank accession numbers of the sequences as determined in this study for representative *Leuconostoc* and *Lactobacillus* strains for each sampling location

Strain ID	16S rRNA	*rpoA*	*dnaA*	*pheS*	*tuf*
A2-5	MK673936	MK679630	-	-	-
A2-6	MK673937	MK679631	MK679647	-	-
A16-8	MK673938	MK679632	-	-	-
A16-9	MK673939	MK679633	MK679641	-	-
A19-15	MK673940	MK679634	MK679642	-	-
A19-37	MK673941	MK679635	-	-	-
B1-23	MK673942	MK679636	MK679643	-	-
B9-3	MK673943	MK679637	MK679644	-	-
B9-41	MK673944	MK679638	-	-	-
B16-2	MK673945	MK679639	MK679645	-	-
B19-1	MK673946	MK679610	MK679646	-	-
A2-7	MK673947	-	-	MK679648	MK679654
A9-3	MK673948	-	-	MK679649	MK679655
A19-103	MK673949	-	-	MK679650	MK679656
B2-4	MK673950	-	-	MK679651	MK679657
B9-17	MK673951	-	-	MK679652	MK679658
B19-10	MK673952	-	-	MK679653	MK679659

3. RESULTS AND DISCUSSION

One-hundred and twenty-four isolates of *Leuconostoc* and *Lactobacillus* spp. grouped into five distinct clusters based on 16S rRNA sequence analyses (Figure 1). Of the 124 isolates, 81 were classified as members of the genus *Leuconostoc*. Thirty-seven isolates formed a tight group with the type strains of *L. mesenteroides* subsp. *mesenteroides* (JCM 6124^T), *L. mesenteroides* subsp. *dextranicum* (NCFB 529^T) and *L. mesenteroides* subsp. *cremoris* (NCFB 543^T) in Cluster 1. Thirty-four of the 37 isolates in Cluster 1 were isolated from the mud trough and three were isolated from the syrup tank. Of the four isolates that grouped in Cluster 2 with the type strain of *L. pseudomesenteroides* (NRIC 1777^T), only one isolate was obtained in November (from the filtrate), when high dextran concentrations in the produced sugar were observed. Cluster 3 was the largest, with 38 isolates phylogenetically closely related (similarity values ranging 99.8-99.9%) to the type strain of *Leuconostoc lactis* (KCTC 3528^T). Two isolates grouped with the type strain of *Leuconostoc citreum* (ATCC 49370^T) in Cluster 4. All 43 isolates preliminary identified as members of the genus *Lactobacillus* grouped with the type strain of *Lactobacillus fermentum* (CIP 102980^T) in Cluster 5. Fifteen of these isolates were from the diffuser sump, 24 from the filtrate and four from the mud trough (Figure 1).

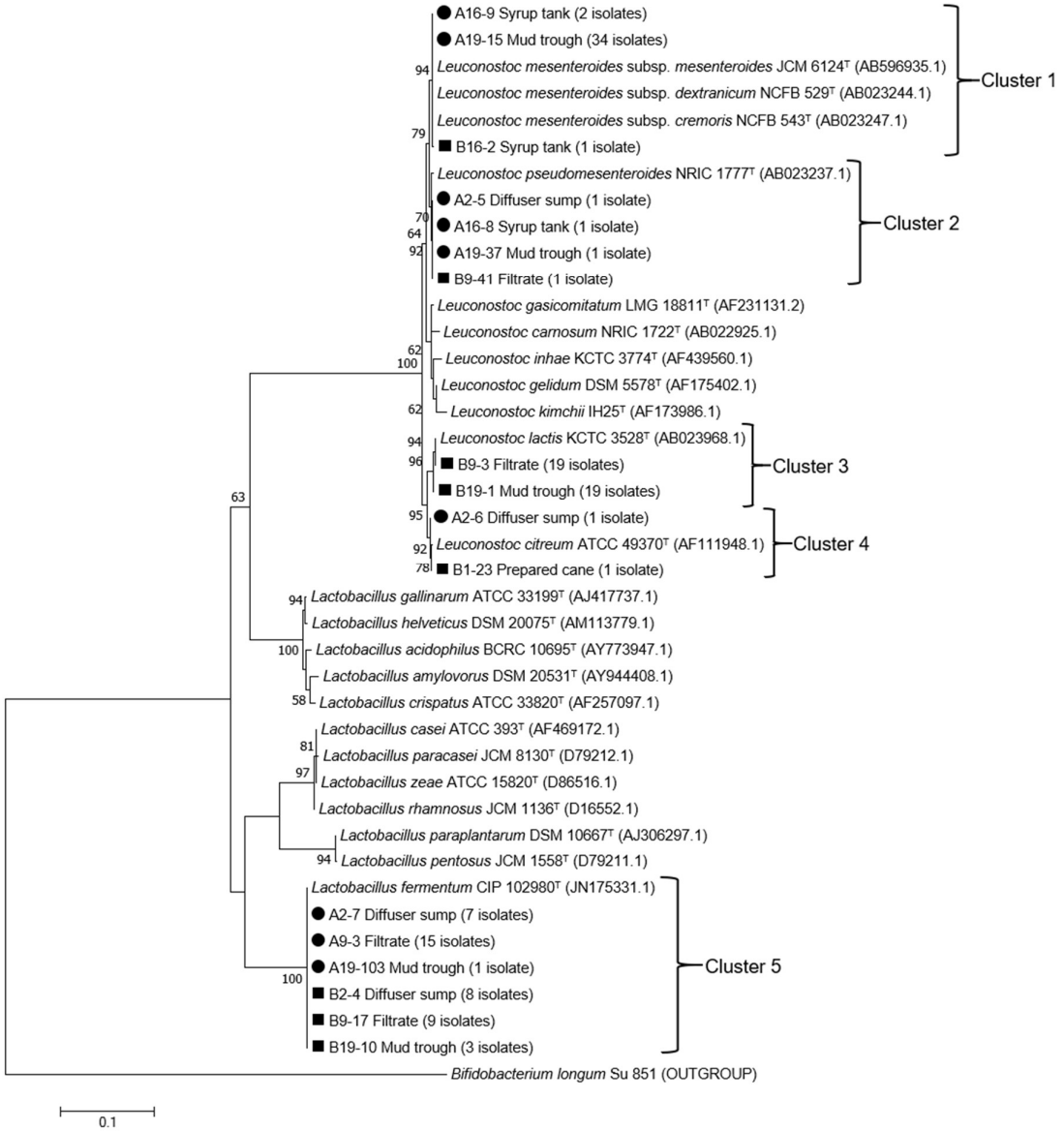


FIGURE 1 Phylogenetic tree based on partial 16S rRNA gene sequences of *Leuconostoc* and *Lactobacillus* species isolated from five sampling points in a South African sugarcane processing factory. Isolates from the sampling time when low dextran content was observed in raw sugar are labelled with a circle (●) and those when high dextran in raw sugar was reported with a square (■). The tree was constructed using the Maximum Likelihood method with MEGA 7.0 software and representative isolates from each sampling point is shown, with number of isolates indicated in brackets. Sequence data of reference strains were from GenBank. Genetic distances were computed by Kimura's 2-parameter model (Kimura 1980). The final dataset had a total of 897 positions. Bootstrap values over 50 % (based on 100 replications) are shown at each node. Bar, % estimated substitution per nucleotide position. *Bifidobacterium longum* Su 851 was used as the outgroup

Concluded from the 16S rRNA groupings, 67% of the leuconostocs, of which the majority clustered with the type strains of *L. mesenteroides* and *L. lactis*, were isolated from the mud trough. Filtration is a process used to recover sucrose form the mud, which is a mixture of juice and settled solids from the clarification process. The filtrate quality often does not receive the attention that it deserves, and severe sucrose losses may occur in filter stations through microbiological activity (Lionnet, 1996). The temperature of the mud in September, when low-dextran sugar was produced, was much lower (35°C) compared to the second sampling in November (64°C), possibly due to stoppages and longer retention times of the mud in the trough, resulting in cooling of the mud (Nel et al., 2019a). At this time, a considerable number of isolates which clustered with the type strains of *L. mesenteroides* subspp. *mesenteroides, dextranicum and cremoris* (42% of the total number of leuconostocs) were isolated from the mud. On the contrary, isolates which grouped with the type strain of *L. lactis* were present in the mud during the second sampling when temperature readings exceeded 64°C. It is likely that contamination occurs between the mud trough and the filtrate, which would account for the presence of high cell numbers of bacteria phylogenetically closely related to *L. lactis* in the filtrate sampled in November. *Leuconostoc lactis* has a higher heat resistance than *L. mesenteroides* (Logan, & de Vos, 2009), which may explain the absence of bacteria which clustered with *L. mesenteroides* in filtrate of 58 °C (sampled in September). High cell numbers of isolates related to *L. mesenteroides* were present in mud of 35 °C. The filtrate is recirculated to the mixed juice tank. However, none of the gum-producing bacteria isolated in the filtrate were detected in the juice sampled from the mixed juice tank (Nel et al., 2019a). This is presumably due to the high temperatures (67 °C and 73 °C, respectively) recorded for the juice samples from the mixed juice tank which allowed growth of endospore-forming *Bacillus* species, but not *Leuconostoc* and *Lactobacillus* spp. (Warth, 1978; Logan, & de Vos, 2009). Correct process control, especially of high-temperature factory streams, is critical to prevent microbial growth in a sugarcane processing factory.

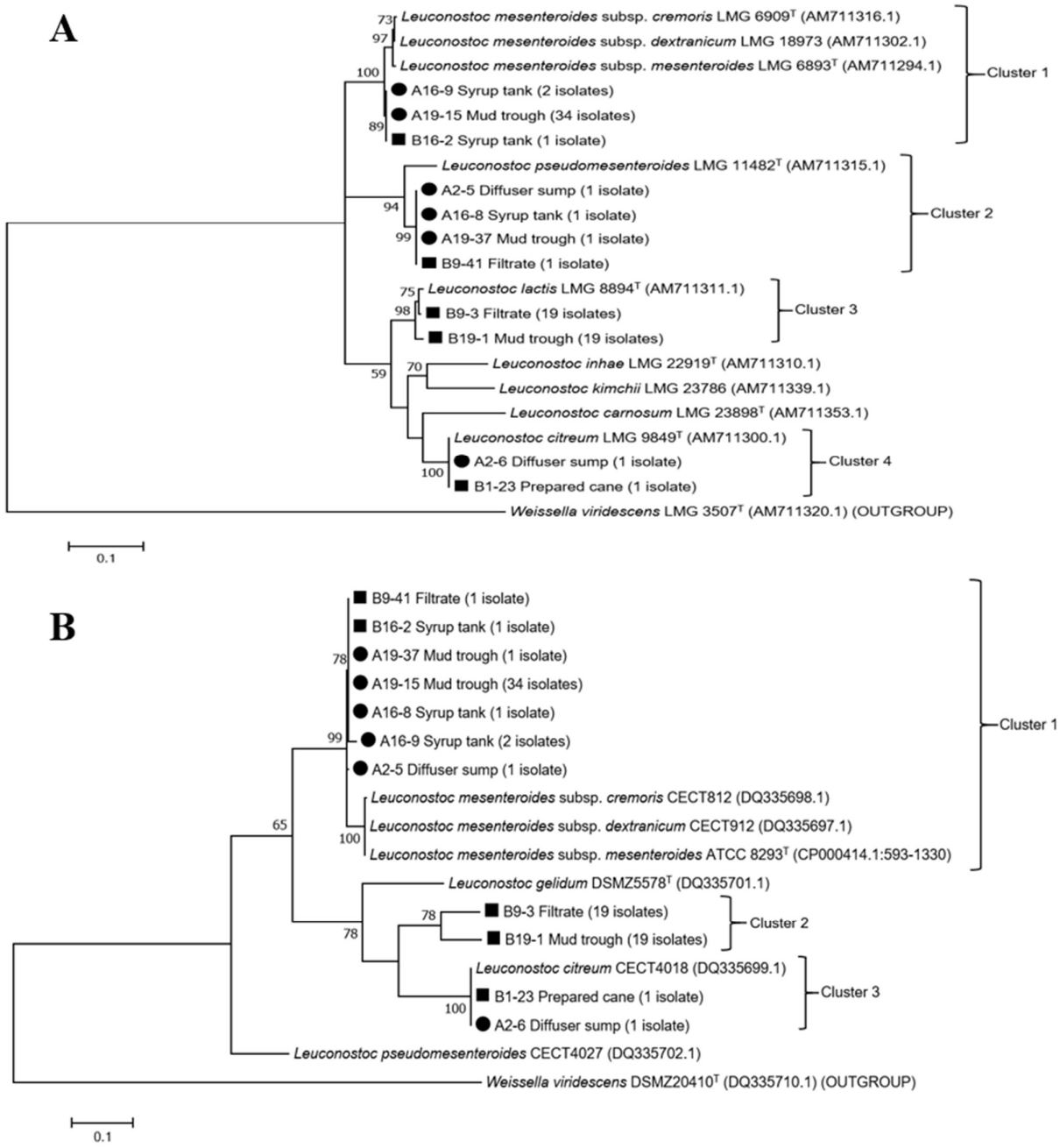
The presence of *Leuconostoc* and *Lactobacillus* spp. in sugarcane processing are unwanted due to the production of dextran and other metabolic products, including mannitol, lactic and acetic acids and ethanol (Daeschel et al., 1987; Eggleston et al., 2004). Although dextran is considered to be the most detrimental product to the factory because it is a high-viscosity polymer, *Leuconostoc* and *Lactobacillus* spp. are also capable of producing other polymers such as levan and alternan (Dutta et al., 2012; Kralj et al., 2004; Naessens et al., 2005) and their formation may be underestimated as contributors to impeding high viscosity problems in sugarcane processing. These bacterial metabolites may have a severe impact on the quality and quantity of produced sugar, and accurate identification of the spoilage bacteria in sugarcane processing is key for effective microbial control.

The *rpoA* housekeeping gene has previously been successfully applied for the phylogenetic resolution of *Leuoconostoc* spp. (De Bruyne et al., 2007; Rahkila et al., 2014). The *Leuconostoc* isolates (81 in total) grouped into four clusters based on partial *rpoA* gene sequence analysis (Figure 2A). In accordance with 16S rRNA sequence analyses, 37 isolates were phylogenetically related to *L. mesenteroides* subspp. *mesenteroides, dextranicum* and *cremoris*, with similarity values ranging from 98.3-98.5 % (Figure 2A). Four isolates were phylogenetically related to *L. pseudomesenteroides* (similarity value of 95.0 %), 38 to *L. lactis* (similarity values ranging 98.3-99.3 %) and two to *L. citreum* (100% similar) (Figure 2A).

The phylogenetic tree inferred from partial *dnaA* sequence analyses of *Leuconostoc* isolates is shown in Figure 2B. Eighty-one isolates grouped into three clusters. Cluster 1 contained 41 isolates related to *L. mesenteroides* subspp. *mesenteroides, dextranicum* and *cremoris*; Cluster 2 hosted 38 isolates which previously clustered with *L. lactis* 16S rRNA and *rpoA* gene sequences (Figures 1 and 2A) and Cluster 3 contained two isolates that grouped with *L. citreum*. A reference sequence for the *dnaA* gene from *L. lactis* was not available from GenBank. The phylogeny obtained for *dnaA* sequence analysis (Figure 2B) is in disagreement with the phylogeny of the trees inferred from 16S rRNA and *rpoA* sequence analyses (Figures 1 and 2A) for isolates A2-5, A16-8, A19-37 and B9-41, which previously clustered with *L. pseudomesenteroides*. Based on *dnaA* sequences, these four isolates are related to *L. mesenteroides* subspp. *mesenteroides, dextranicum* or *cremoris* (Figure 2B).

None of the loci examined (Figures 1, 2A and 2B), nor the concatenated *rpoA-dnaA* sequences (Figure 2C), allowed discrimination between subspecies within *L. mesenteroides*. Thirty-seven isolates were related to *L. mesenteroides* based on the phylogenetic analyses of 16S rRNA, *rpoA*, *dnaA* and the *rpoA-dnaA* concatenated sequences. Isolates A2-5, A16-8, A19-37 and B-41 formed a peculiar clade in the *rpoA-dnaA* concatenated tree phylogeny (Figure 2C, Cluster 2). The identities of these isolates could be confirmed by the phylogenetic analyses of additional housekeeping genes such as *atpA* (encoding alpha subunit of ATP synthase) or *pheS* (encoding phenylalanyl-tRNA synthase) (De Bruyne et al., 2007). The phylogenetic trees inferred from the partial *dnaA* gene sequences and the *rpoA-dnaA* concatenated sequences from the leuconostocs showed the clustering of 38 isolates (Figure 2B Cluster 2; Figure 2C Cluster 3) with high bootstrap support and an identical phylogeny of the isolates as obtained for the respective 16S rRNA and *rpoA* gene sequence analyses (Figure 1, Cluster 3 and Figure 2A, Cluster 3). It is suggested that, although no reference *dnaA* gene sequence for *L. lactis* is available, Cluster 2 (Figure 2B) represents *dnaA* gene sequences of bacteria phylogenetically related to *L. lactis*. Two isolates for which

identical phylogenies were observed in all four trees with high bootstrap support (Figure 1, Cluster 4; Figure 2A, Cluster 4; Figure 2B, Cluster 3 and Figure 2C, Cluster 4), were considered phylogenetically closely related to *L. citreum*.



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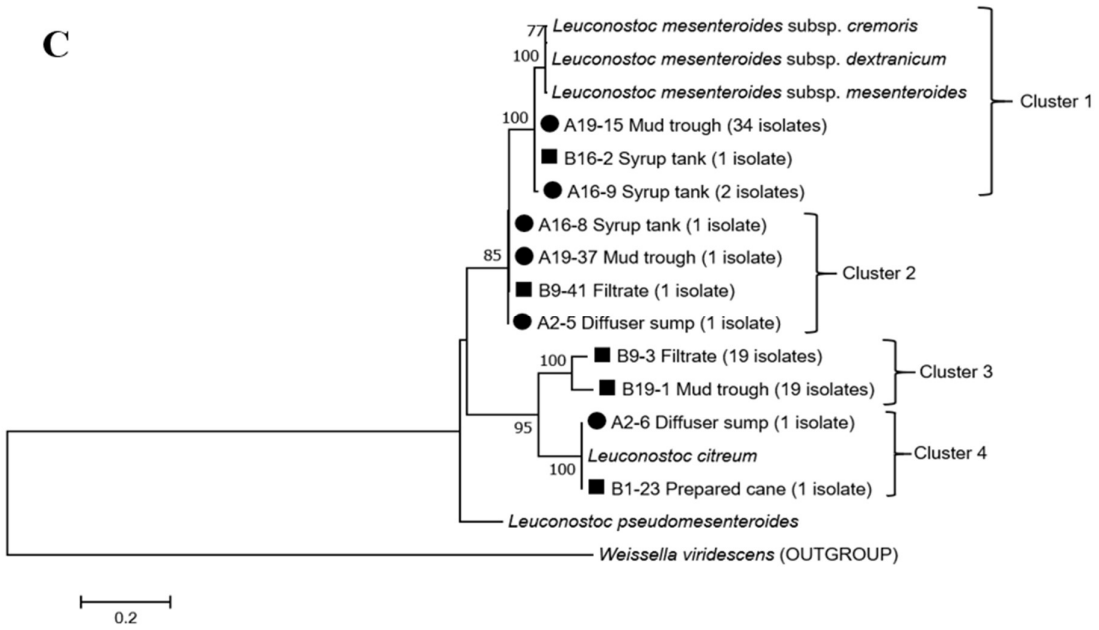
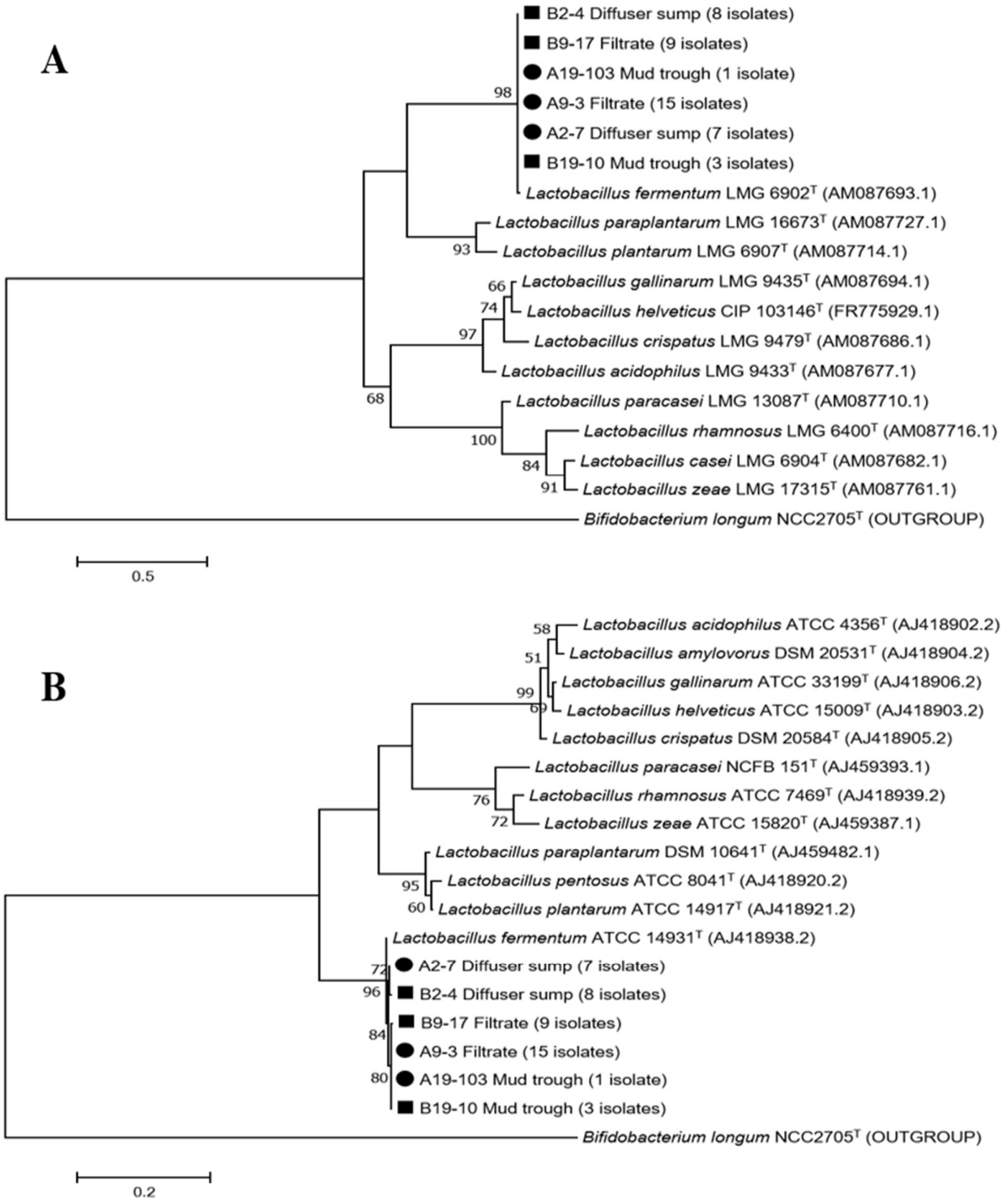


FIGURE 2 Phylogenetic trees based on partial *rpoA* (A), *dnaA* (B) and *rpoA-dnaA* concatenated (C) gene sequences of *Leuconostoc* species isolated from five sampling points in a South African sugarcane processing factory. Isolates from the sampling time when low dextran content was observed in raw sugar are labelled with a circle (●) and those when high dextran in raw sugar was reported with a square (■). The tree was constructed using the Maximum Likelihood method with MEGA 7.0 software and representative isolates from each sampling point is shown, with number of isolates indicated in brackets. Sequence data of reference strains were from GenBank. Bootstrap values over 50 % (based on 100 replications) are shown at each node. Bar, % estimated substitution per nucleotide position

The phylogenetic analyses of *pheS* (encoding phenylalanyl-tRNA synthase) and *tuf* (encoding elongation factor Tu) genes have proven to be a valuable tool for the taxonomic resolution of *Lactobacillus* spp. (Chavagnat et al., 2002; Naser et al., 2007; Sarmiento-Rubiano et al., 2010; Ventura et al., 2003; Yu et al., 2012). The 43 lactobacilli in this study clustered with *L. fermentum* in the phylogenetic trees inferred from partial *pheS* (Figure 3A), *tuf* (Figure 3B) and *pheS-tuf* concatenated (Figure 3C) sequences, with high bootstrap support in all three trees. This is in agreement with the clustering obtained from partial 16S rRNA gene sequence analyses (Figure 1).



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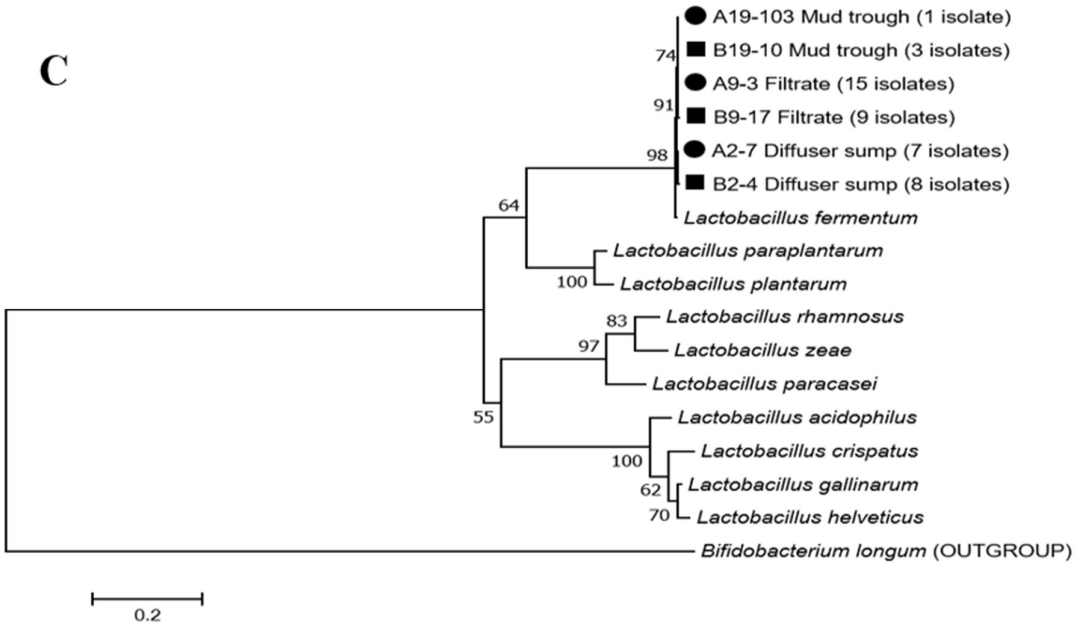

FIGURE 3 Phylogenetic trees based on partial *pheS* (A), *tuf* (B) and *pheS-tuf* concatenated (C) gene sequences of *Lactobacillus* species isolated from three sampling points in a South African sugarcane processing factory. Isolates from the sampling time when low dextran content was observed in raw sugar are labelled with a circle (●) and those when high dextran in raw sugar was reported with a square (■). The tree was constructed using the Maximum Likelihood method with MEGA 7.0 software and representative isolates from each sampling point is shown, with number of isolates indicated in brackets. Bootstrap values over 50 % (based on 100 replications) are shown at each node. Bar, % estimated substitution per nucleotide position

Contrary to previous reports (Egan, 1966; Solomon, 2000), *Leuconostoc* spp. were not the major gum-producing bacteria isolated from sugarcane (Nel et al., 2019a). This study showed that isolates closely related to *L. citreum* was the only *Leuconostoc* bacteria isolated from shredded (prepared) sugarcane, and it was shown previously (Nel et al., 2019b) that *W. confusa* and *W. cibaria* were the most prevalent gum-producing bacteria on the prepared cane. Isolates clustering closely with *Lactobacillus fermentum* were the dominant bacteria isolated from the diffuser sump, with isolates phylogenetically related to *L. lactis* and *L. fermentum* the most prevalent in the filtrate. The majority of bacteria isolated from the mud belonged to species clustering with *L. mesenteroides* and *L. lactis*, and isolates related to *L. mesenteroides* was the dominant bacteria isolated from the syrup tank.

4. CONCLUSIONS

Previous studies that examined the microbial diversity in sugarcane processing factories were constrained by the absence of microbial identification methods with high discriminatory power and could therefore not accurately differentiate species of *Leuconostoc* and *Lactobacillus.* We have demonstrated the potential of housekeeping gene sequence analysis for the phylogenetic resolution of *Leuconostoc* and *Lactobacillus* bacteria. The inclusion of additional housekeeping gene analysis, in combination with DNA-DNA hybridisation studies, would facilitate the assignment of species identities to the isolates. Ongoing efficiency improvements in the South African sugarcane processing industry is critical to remain globally competitive. Knowledge of the identities of spoilage bacteria at the various unit operations in sugarcane processing factories will influence correct process control, especially of high-temperature factory streams, and assist with sanitation management strategies, such as the evaluation of industrial biocide efficacy against specific bacterial species isolated from actual sugarcane processing streams.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Conceptualisation: Sanet Nel, Stephen B. Davis
Investigation, methodology and formal analysis: Sanet Nel
Supervision: Stephen B. Davis, Akihito Endo, Leon M. T. Dicks
Writing – original draft preparation: Sanet Nel
Writing – review and editing: Stephen B. Davis, Akihito Endo, Leon M. T. Dicks

ETHICS STATEMENT

None required.

DATA ACCESSIBILITY

All data are provided in full in the results section of this paper and the relevant DNA sequences were deposited in GenBank.

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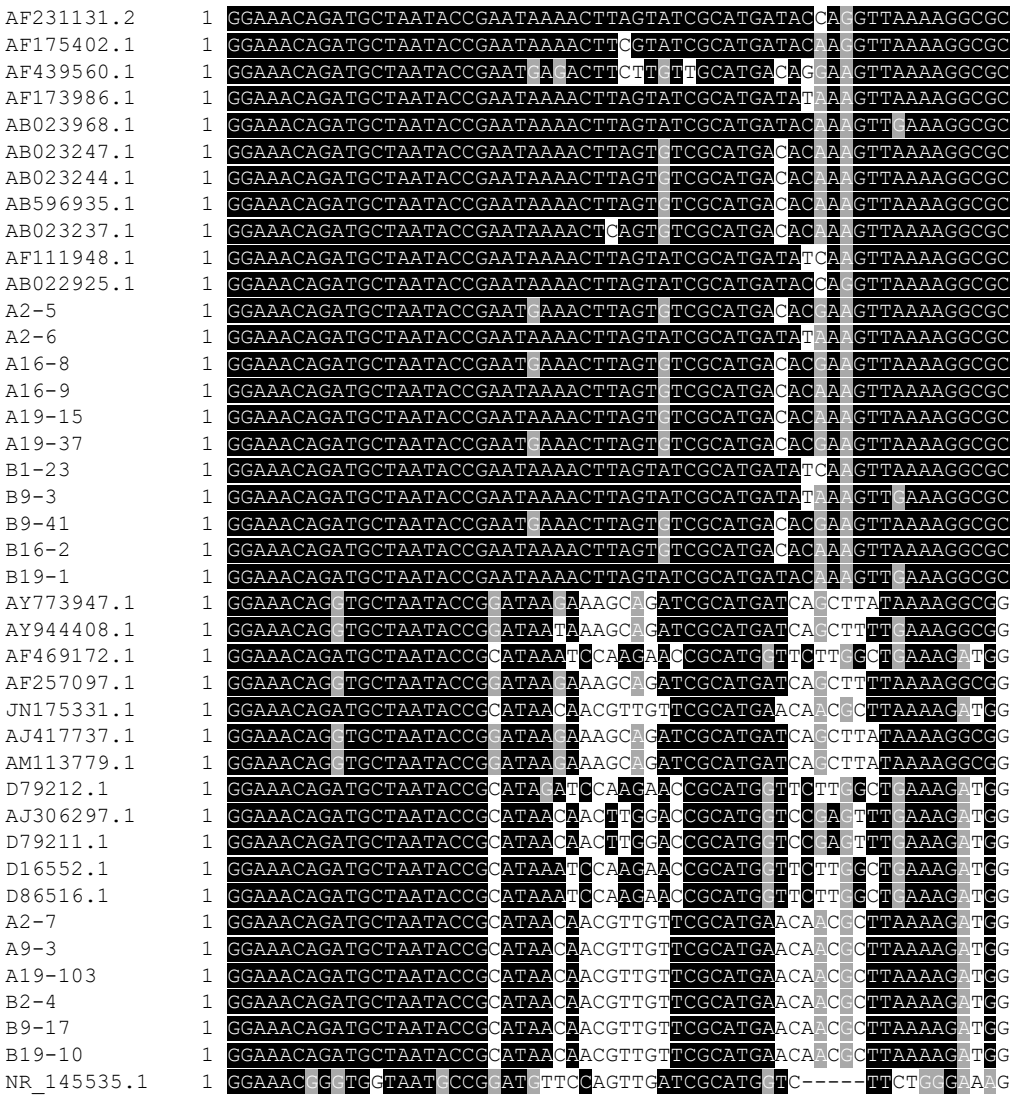
Supplementary material



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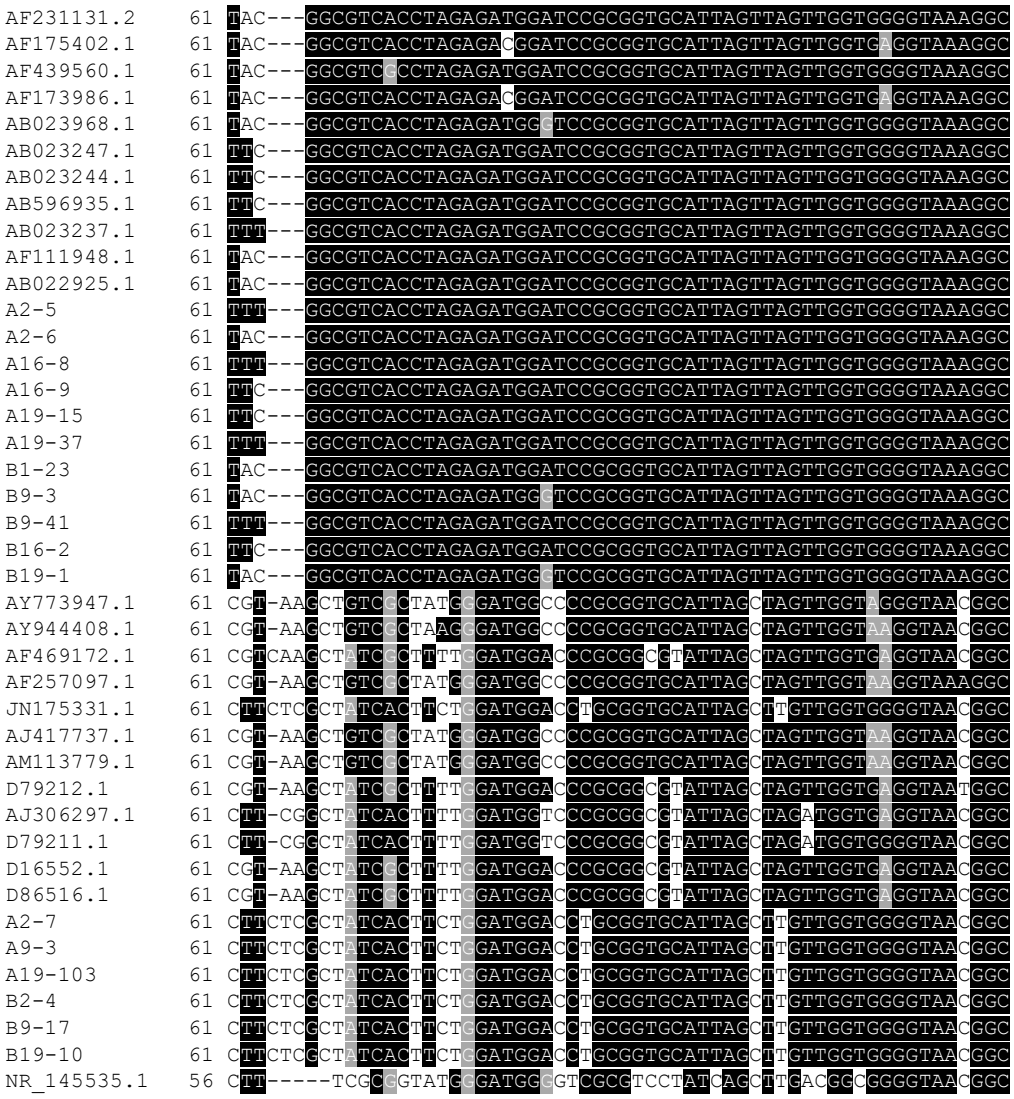

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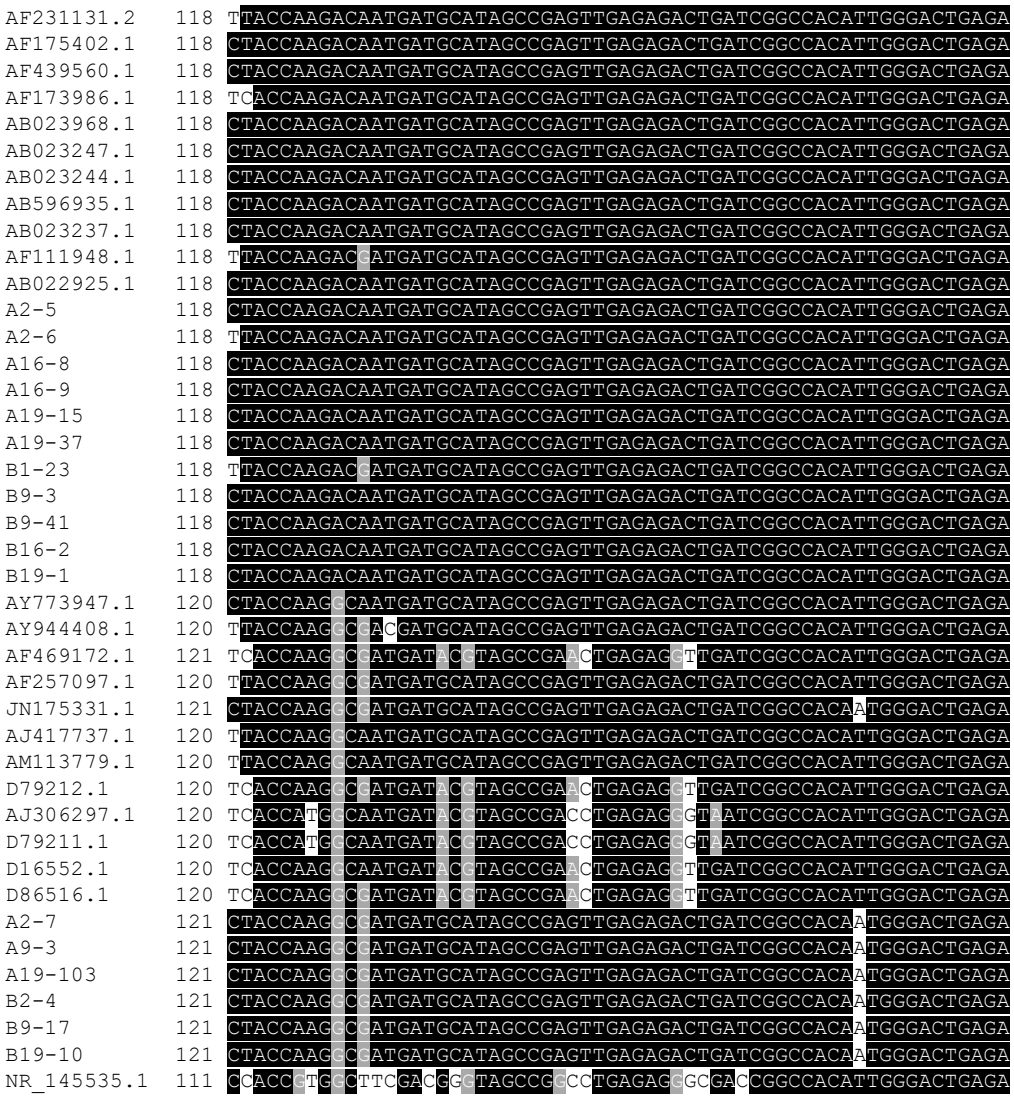

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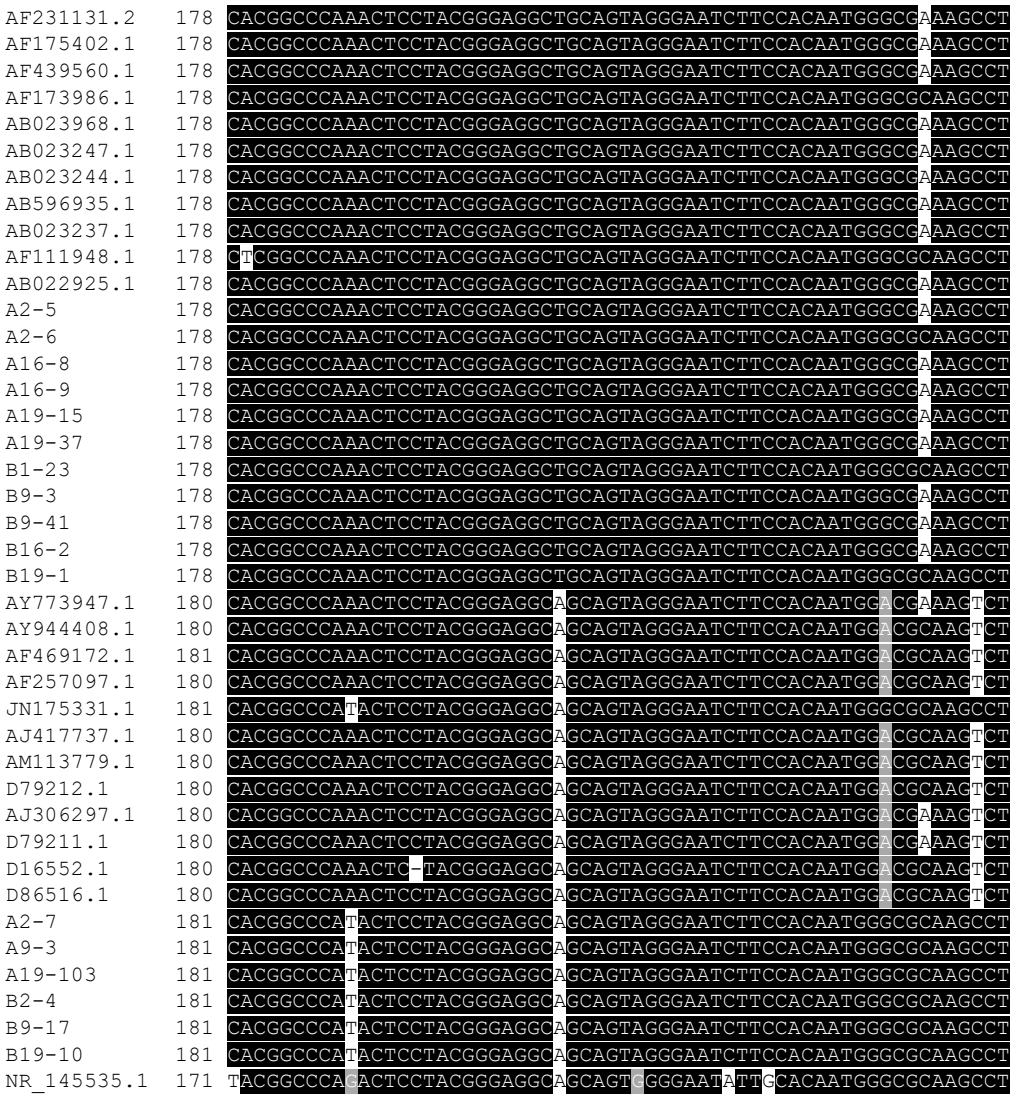

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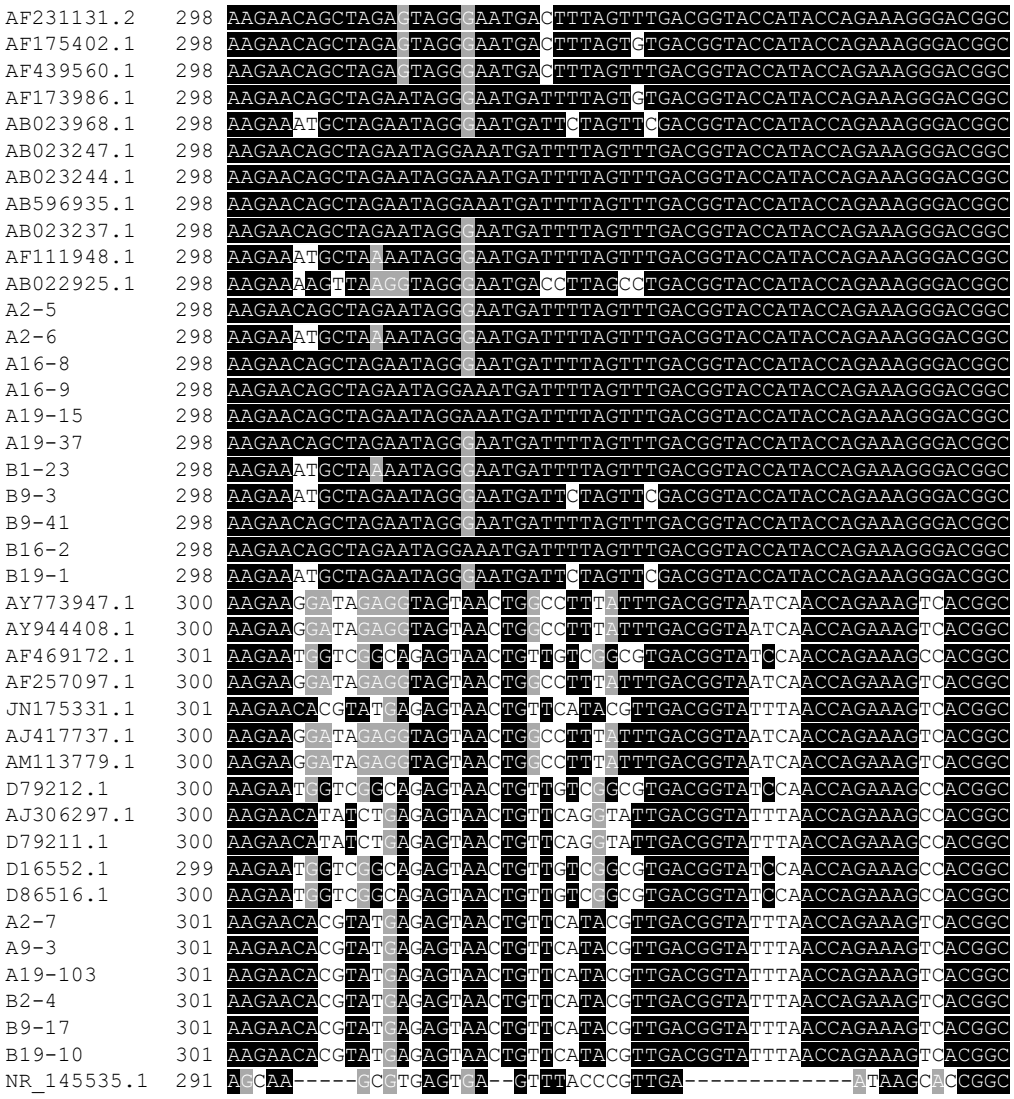

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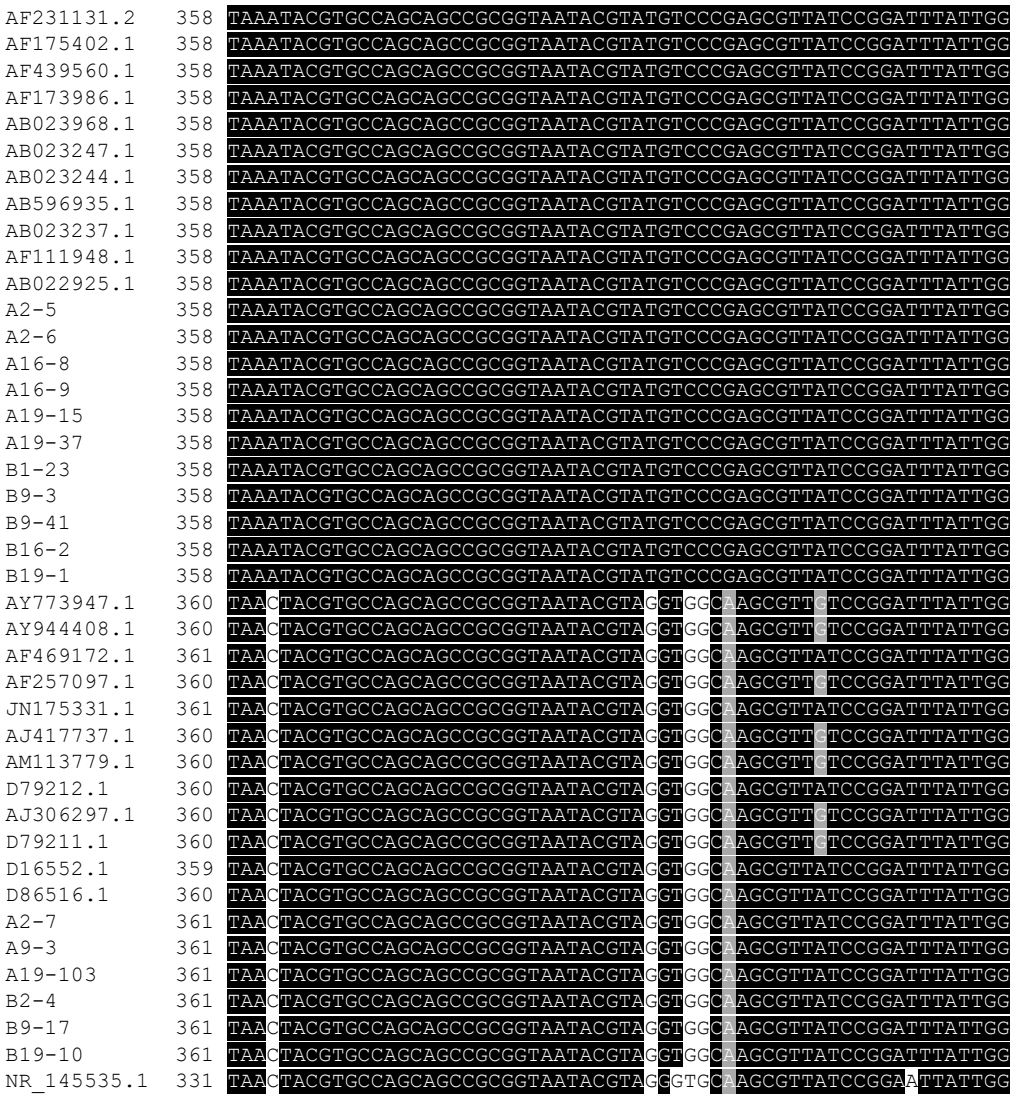

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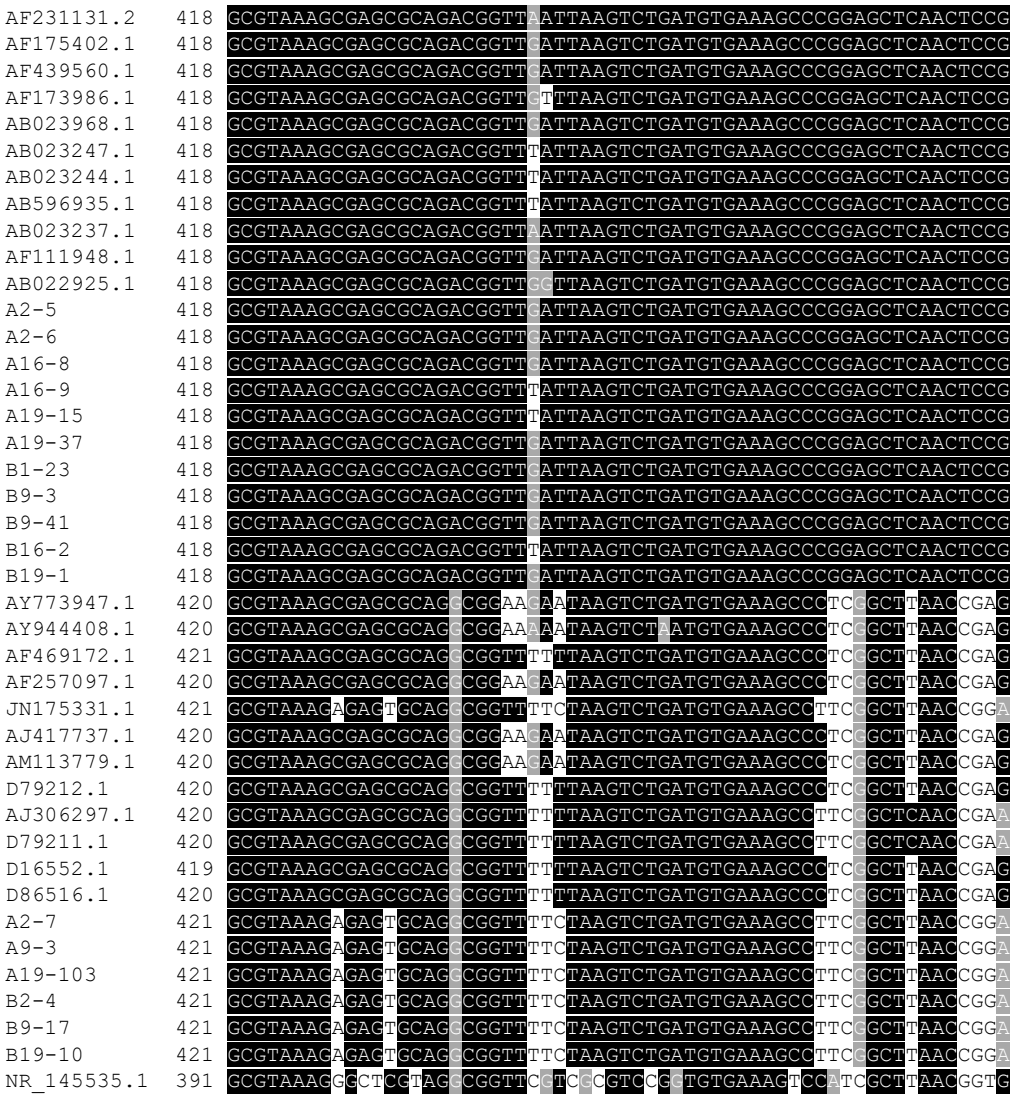

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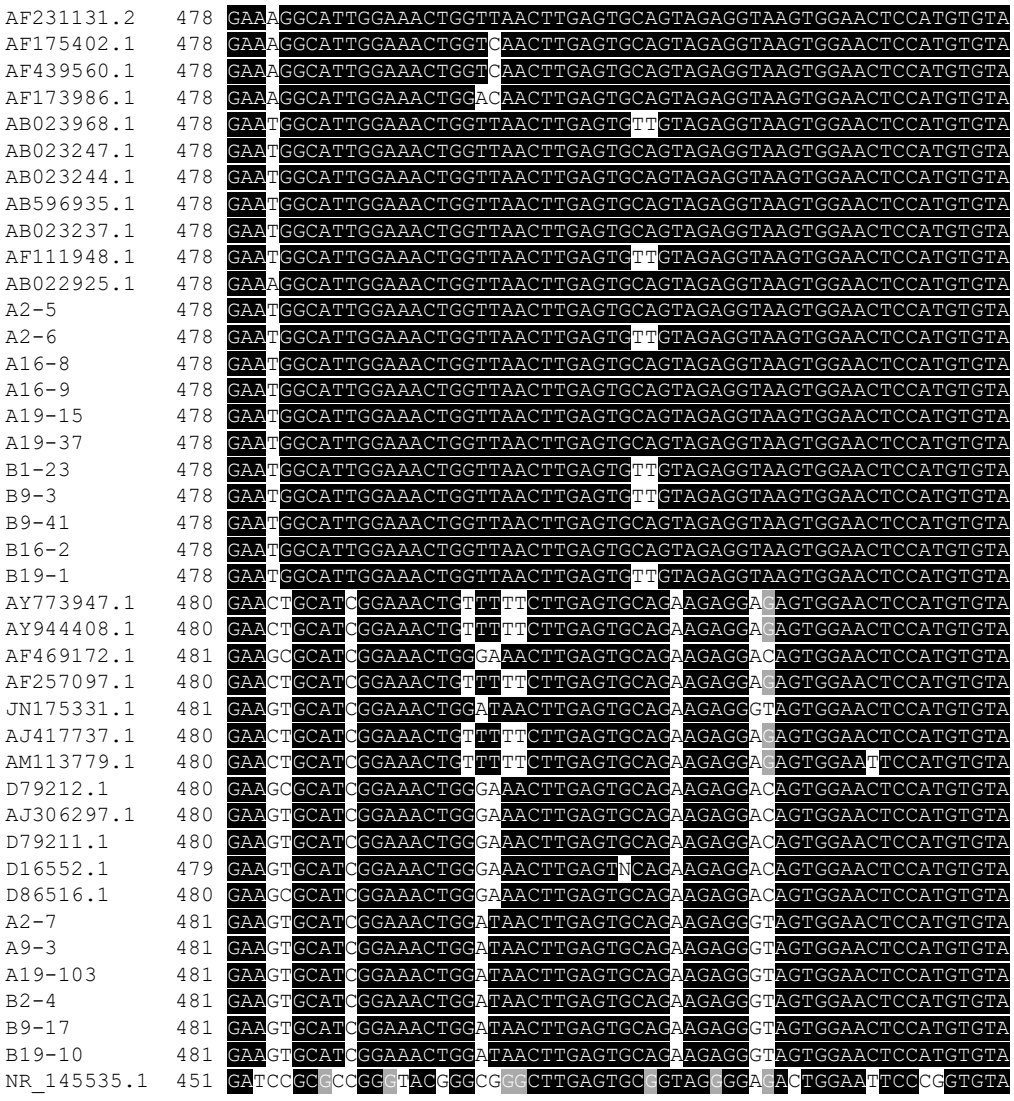

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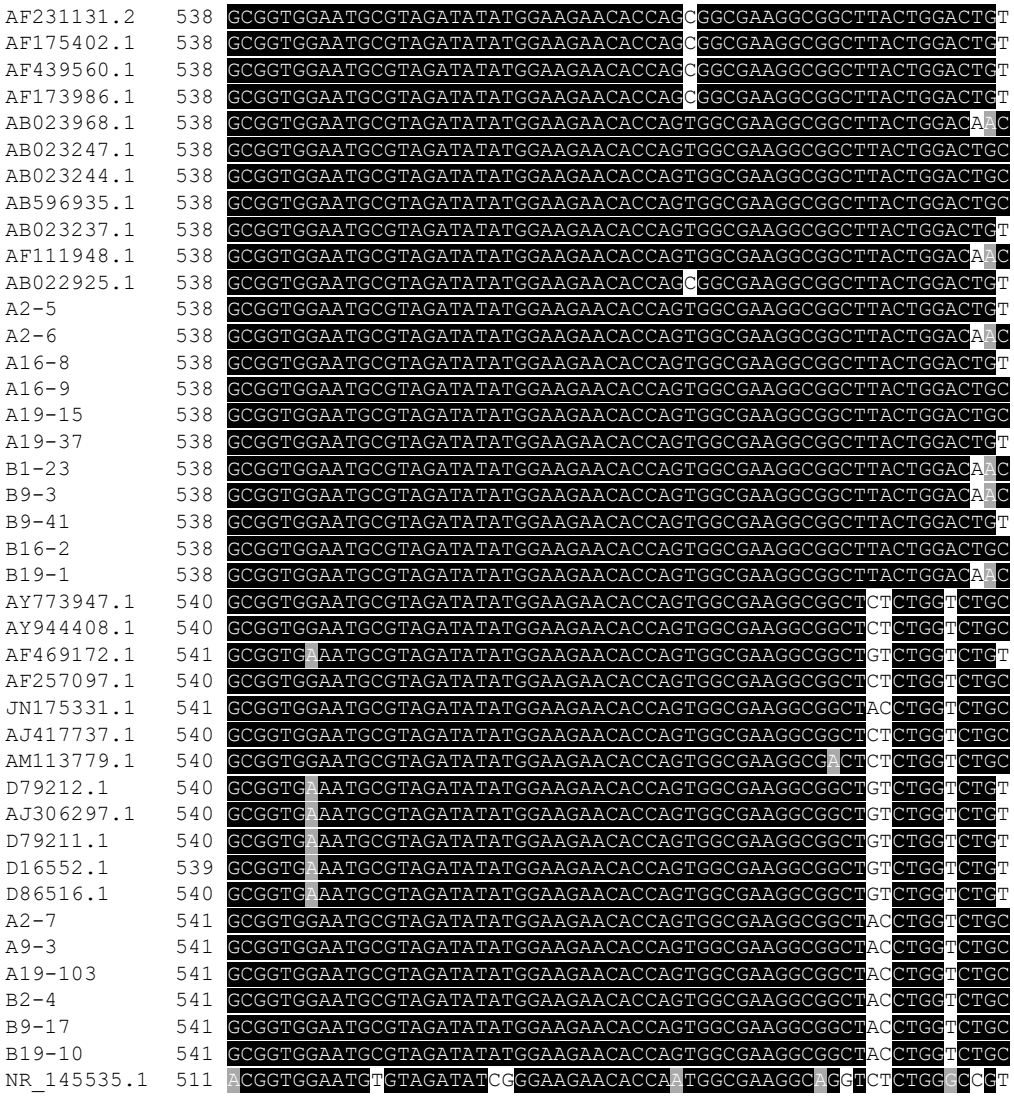

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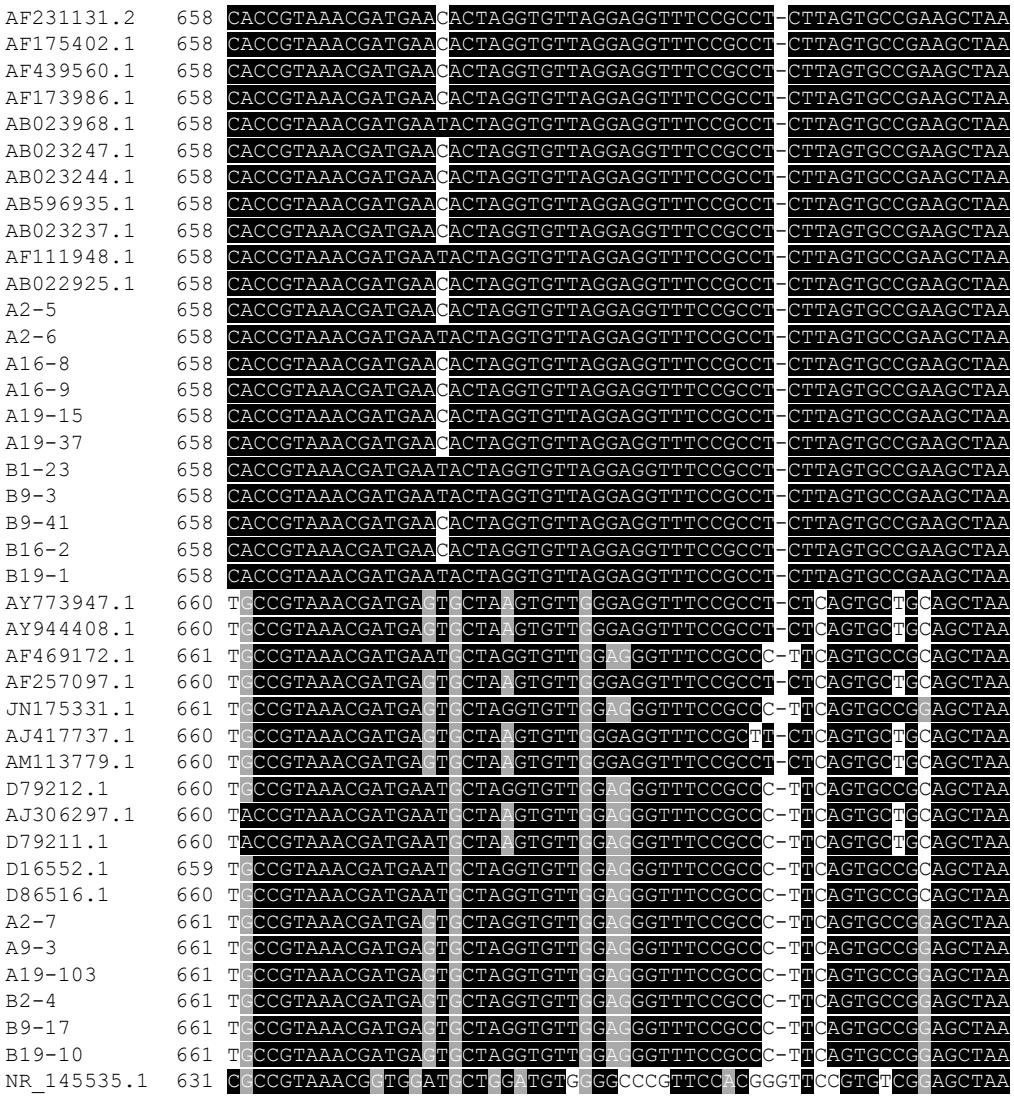

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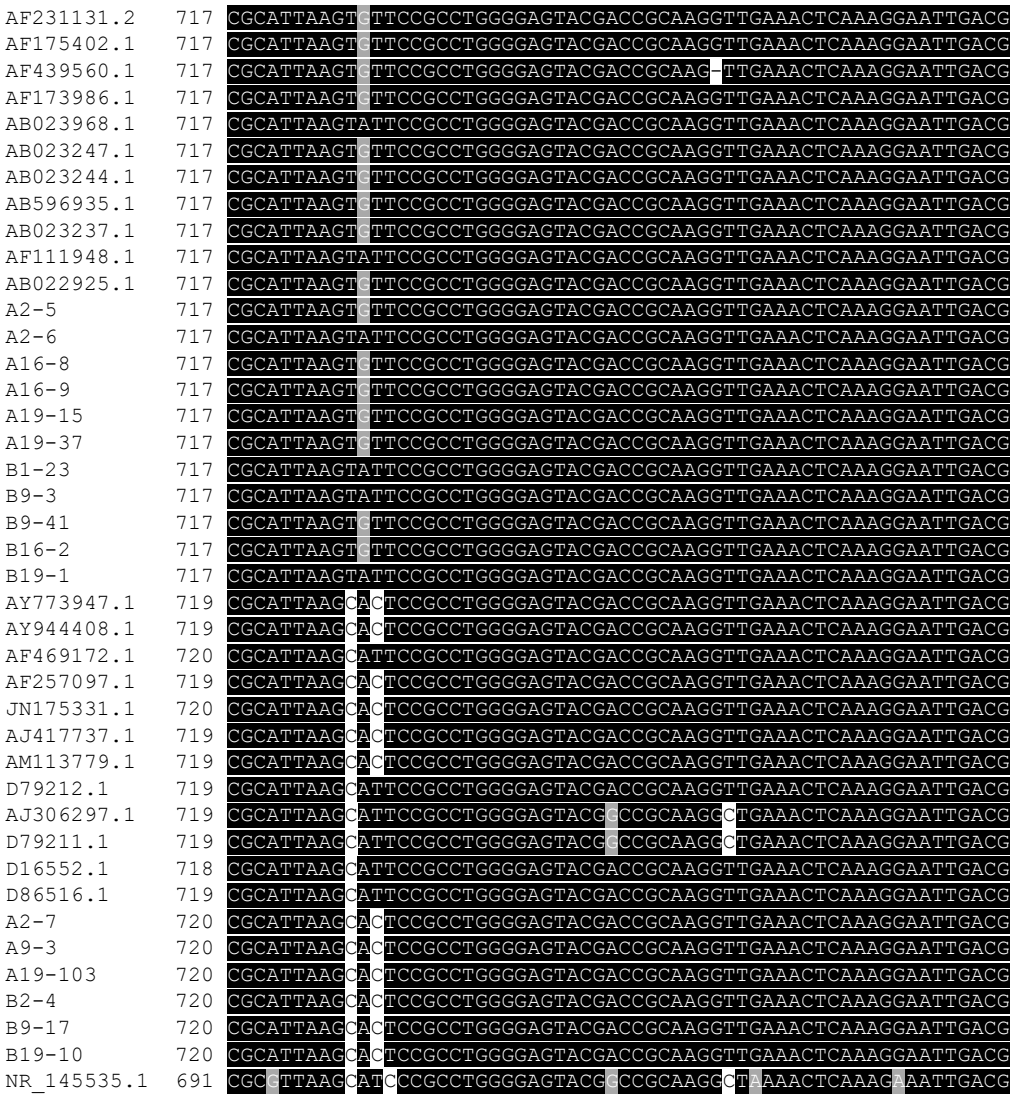

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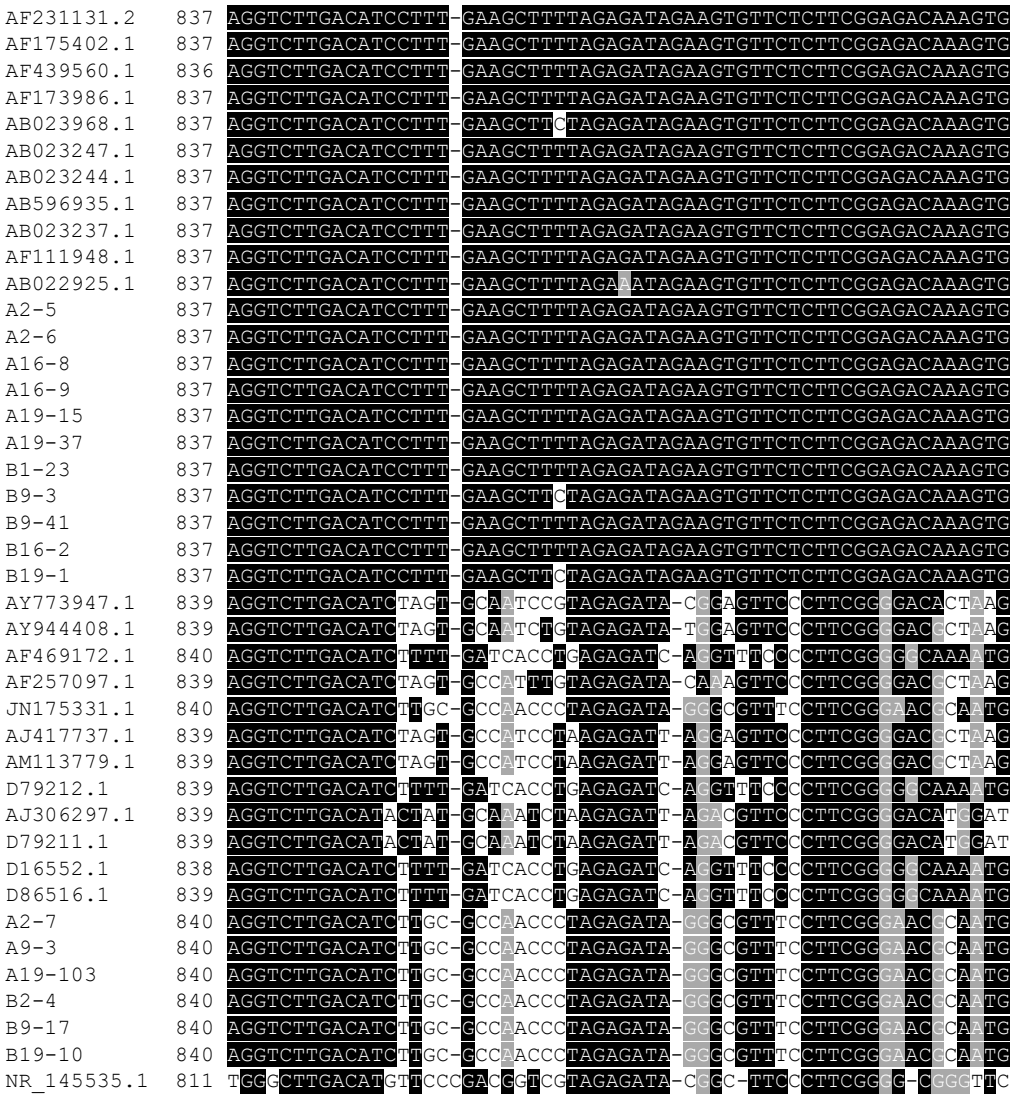

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JN175331.1   898 ACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTG
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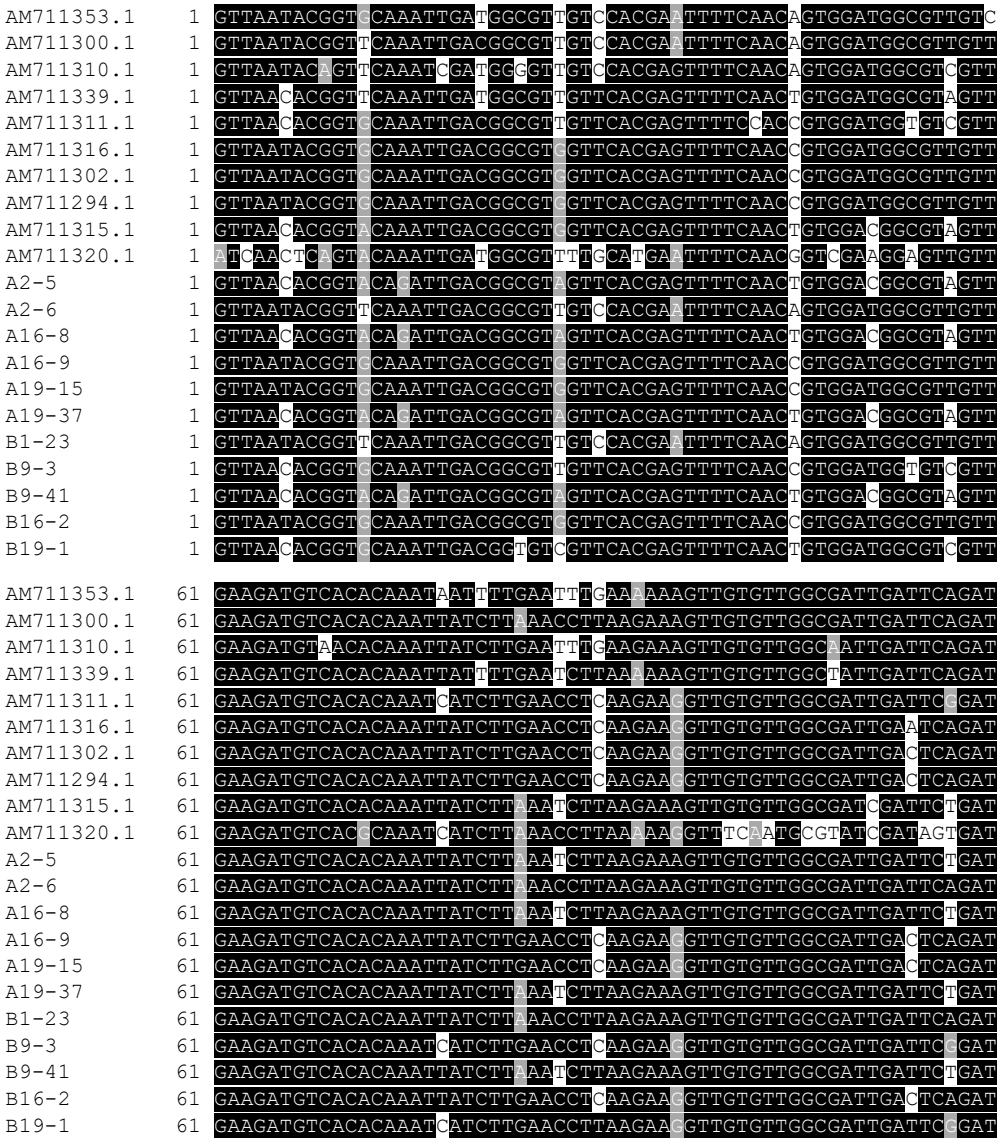
Fig. S1 Multiple DNA sequence alignments of partial 16S rRNA genes of representative and reference *Leuconostoc* and *Lactobacillus* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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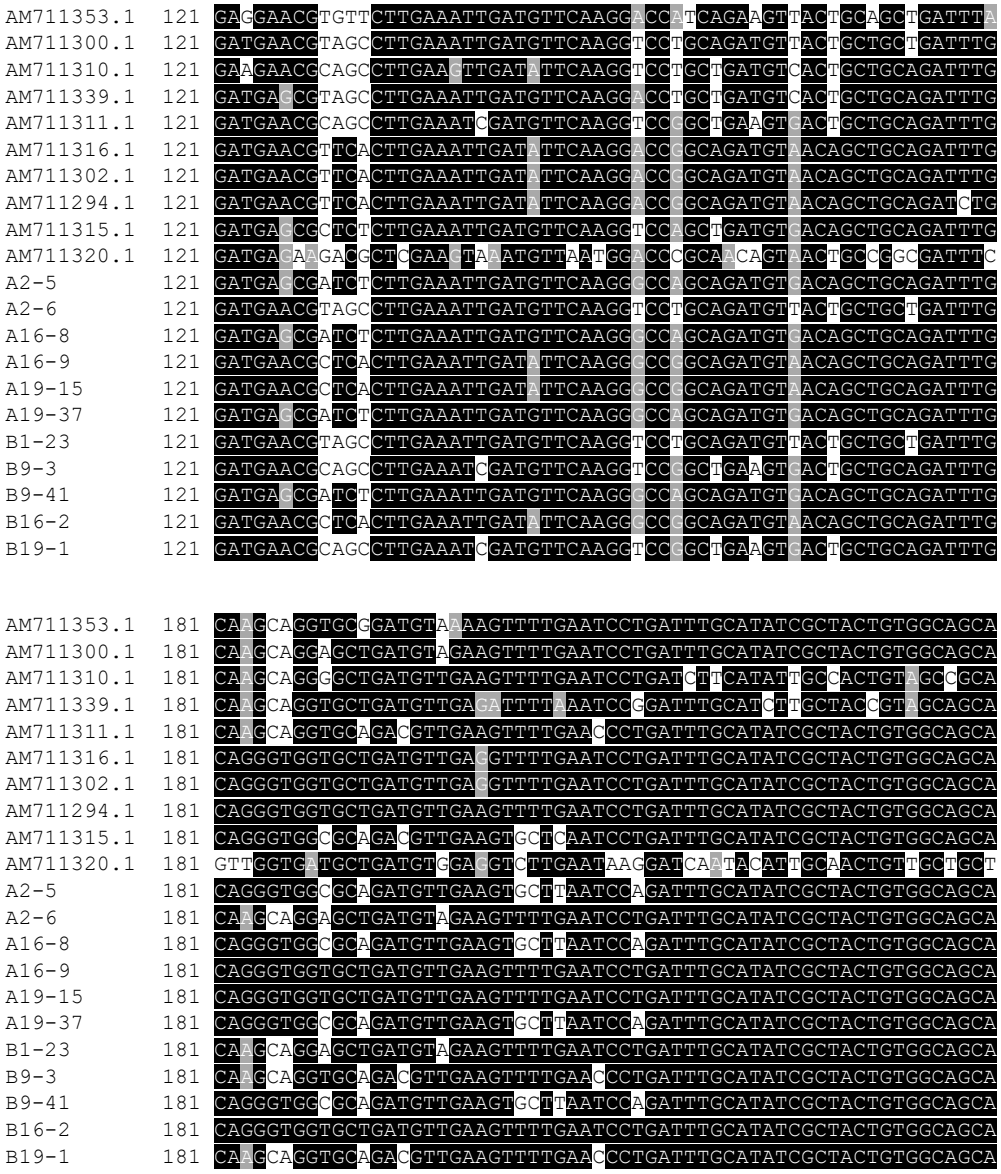

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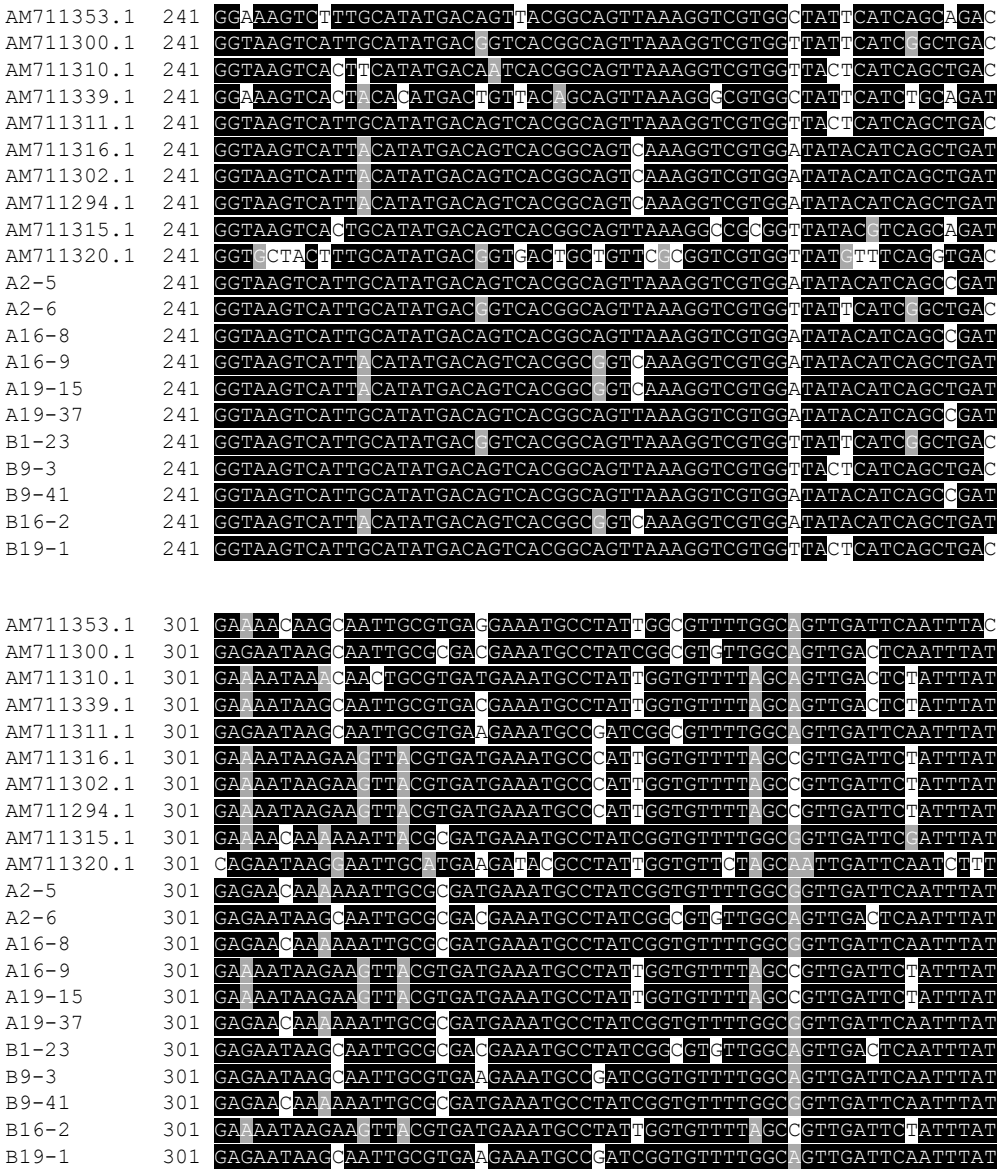

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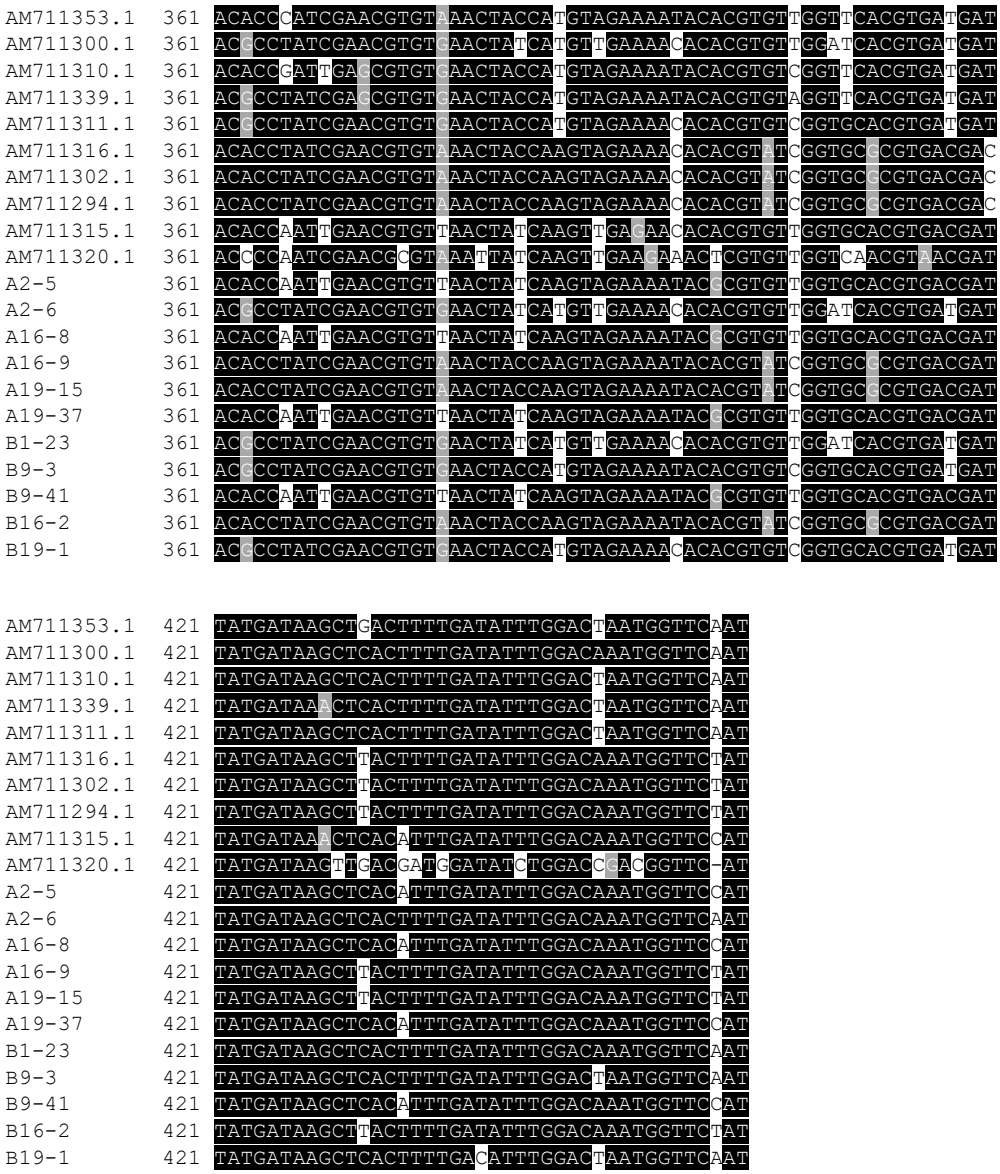
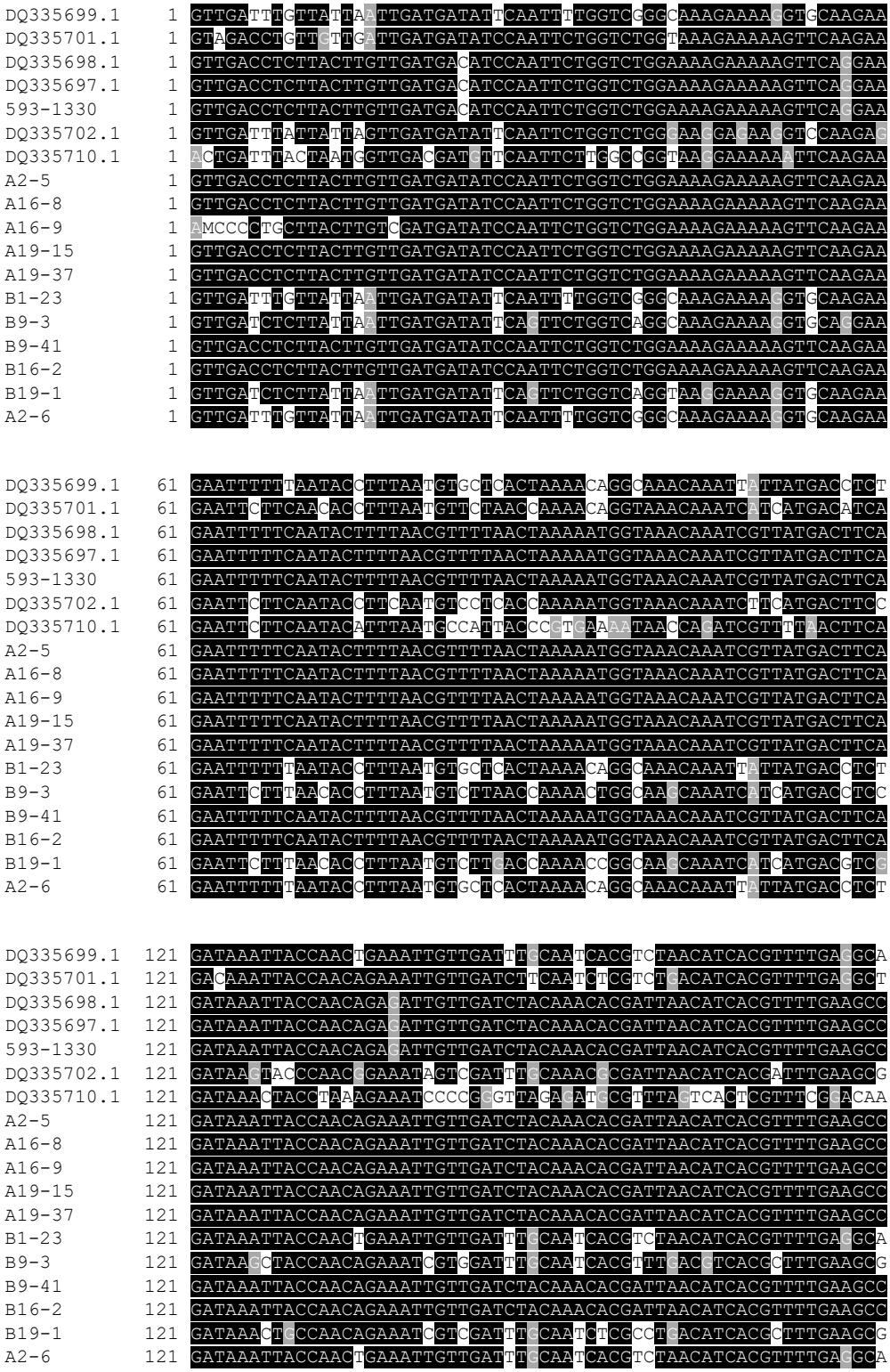


Fig. S2 Multiple DNA sequence alignments of partial *rpoA* genes of representative and reference *Leuconostoc* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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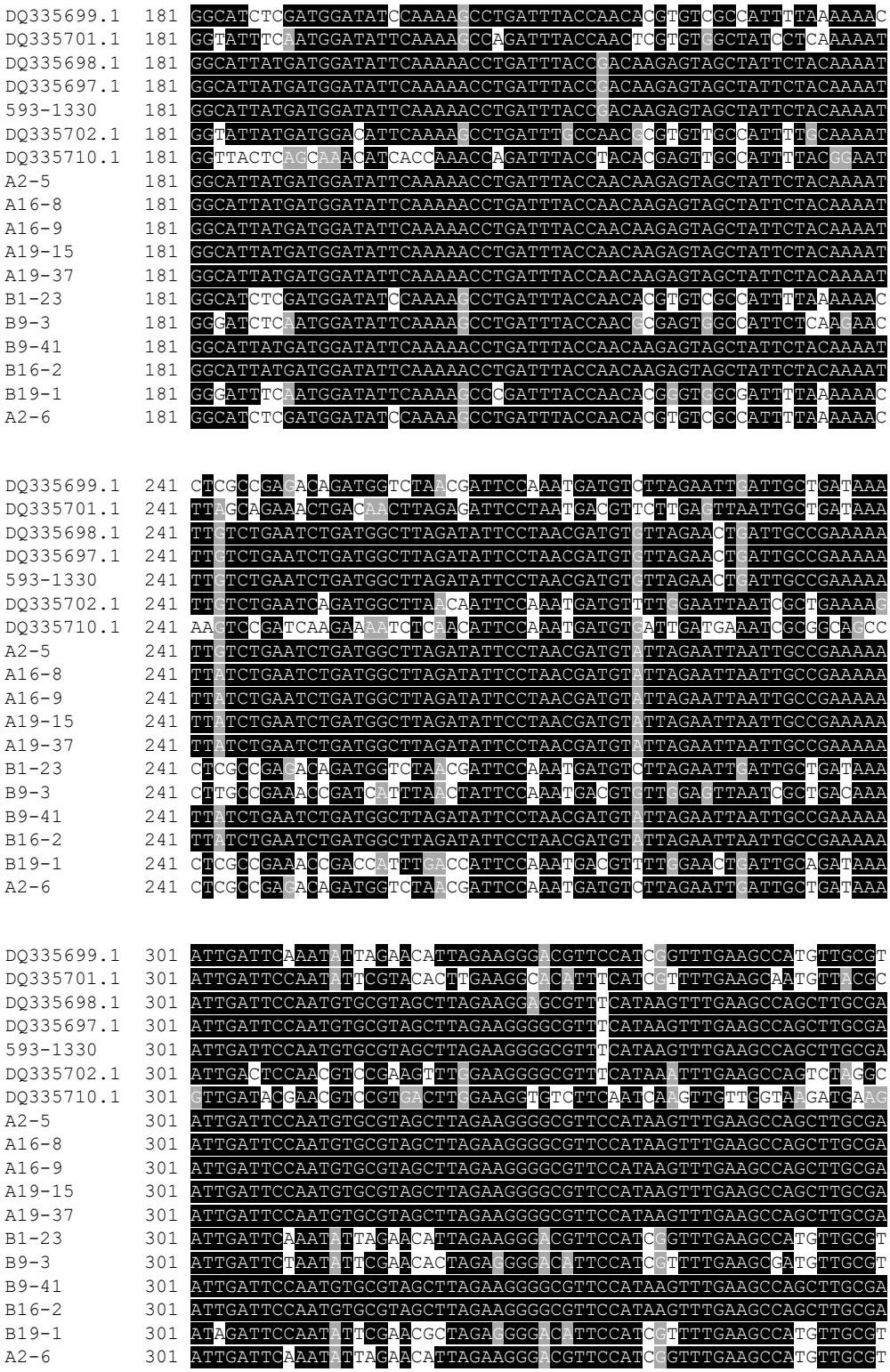

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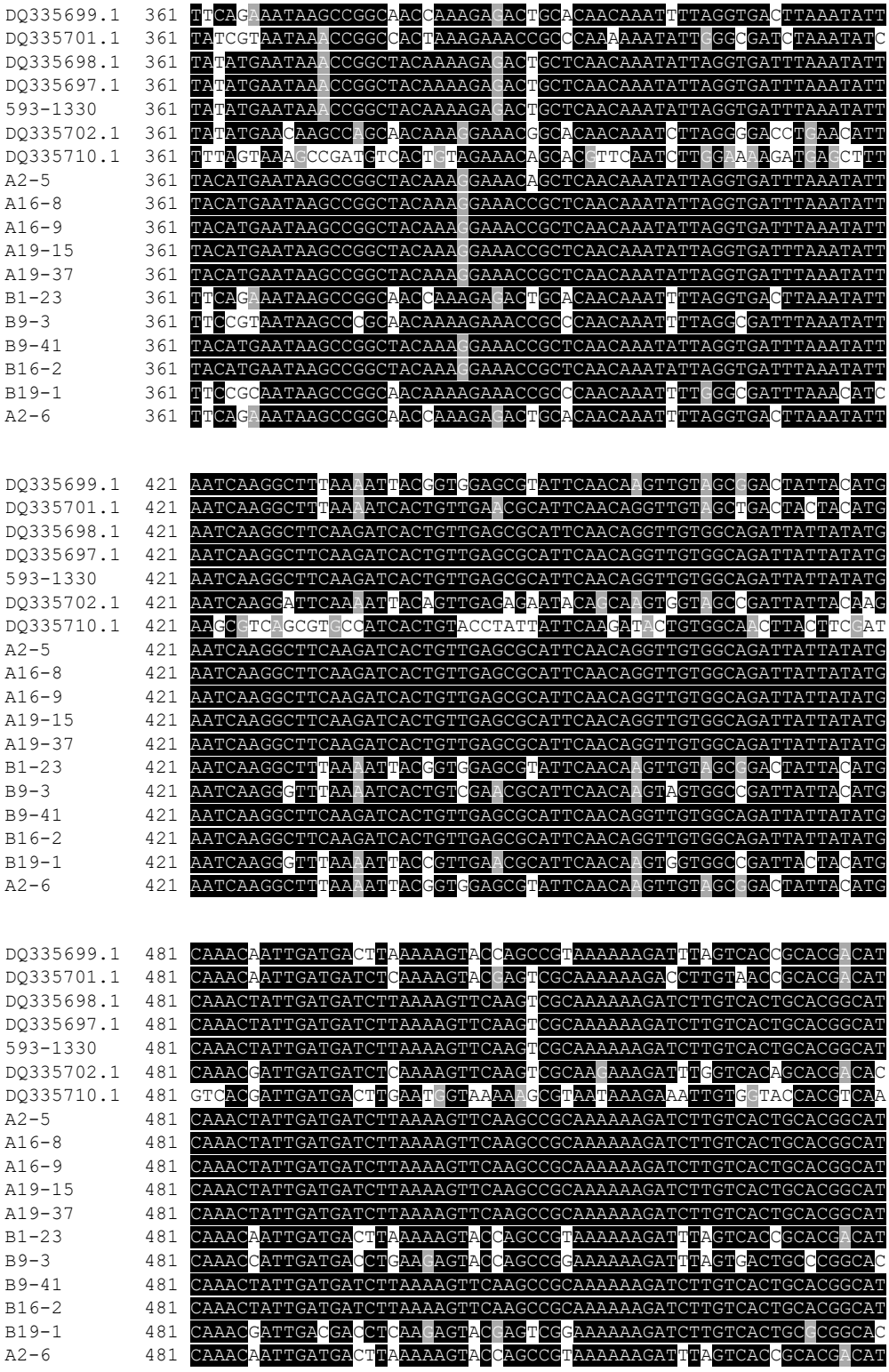

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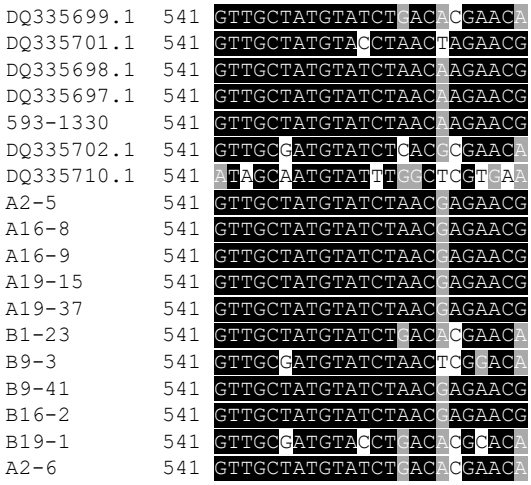

Fig. S3 Multiple DNA sequence alignments of partial *dnaA* genes of representative and reference *Leuconostoc* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)

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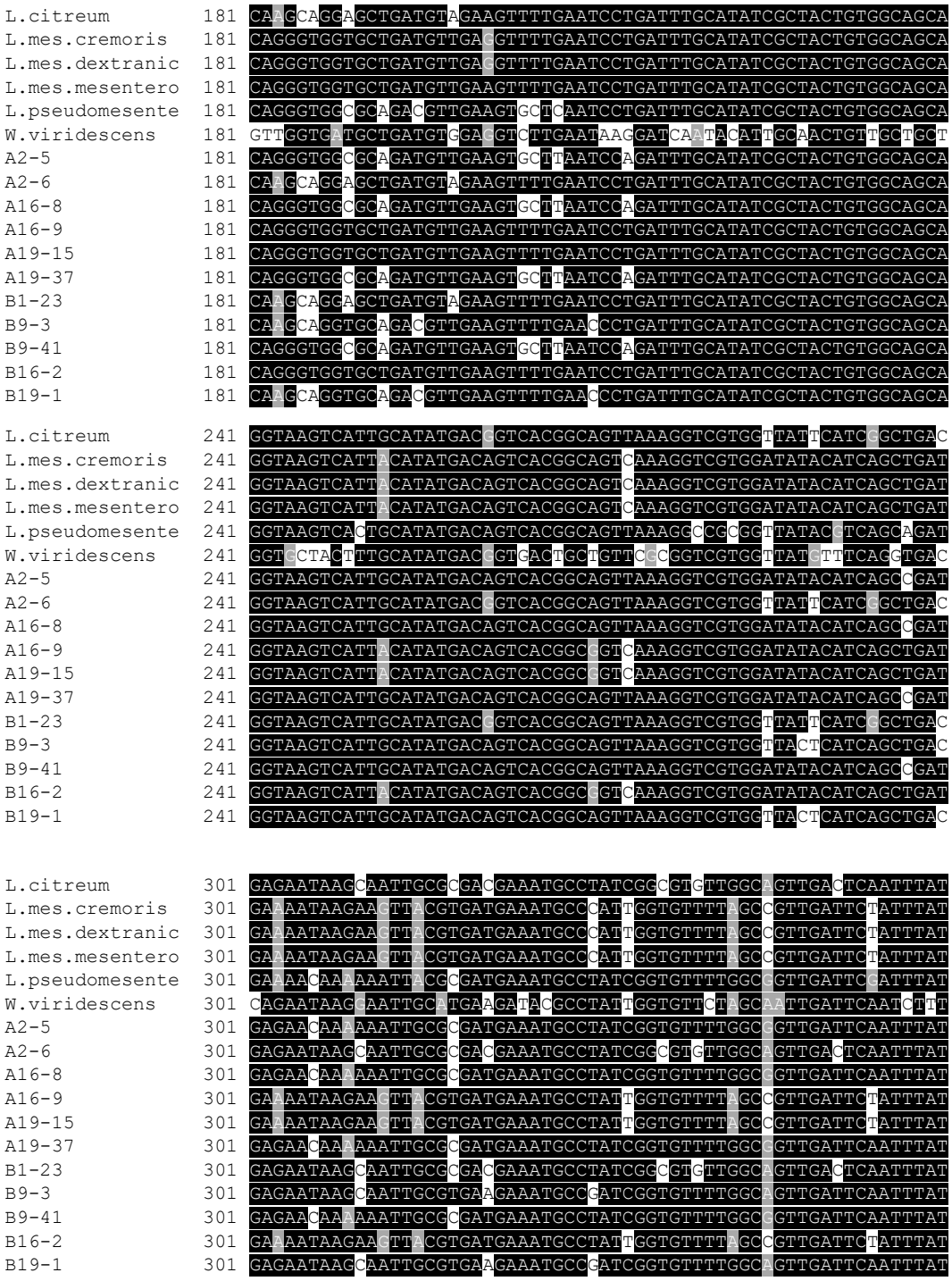

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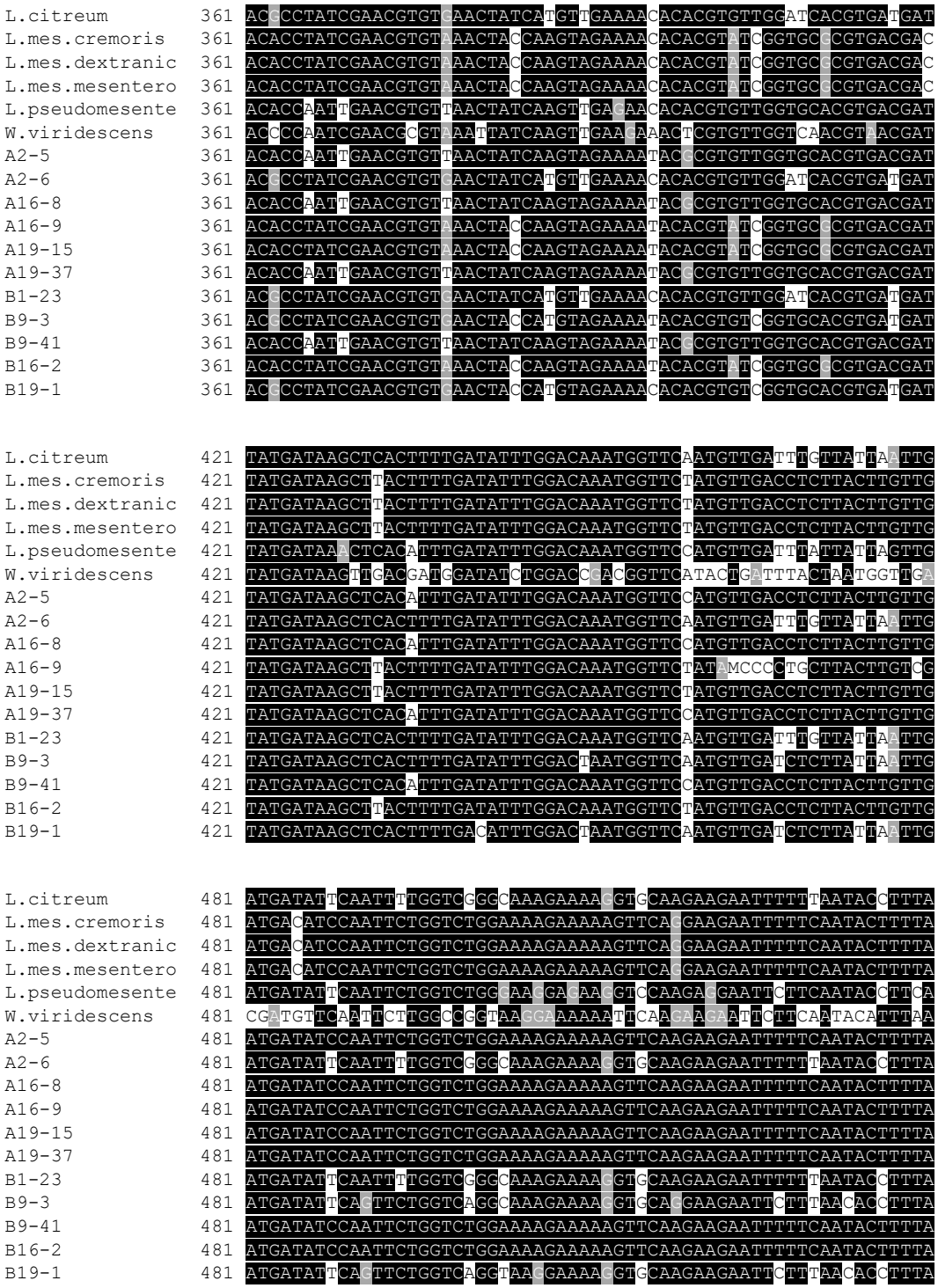

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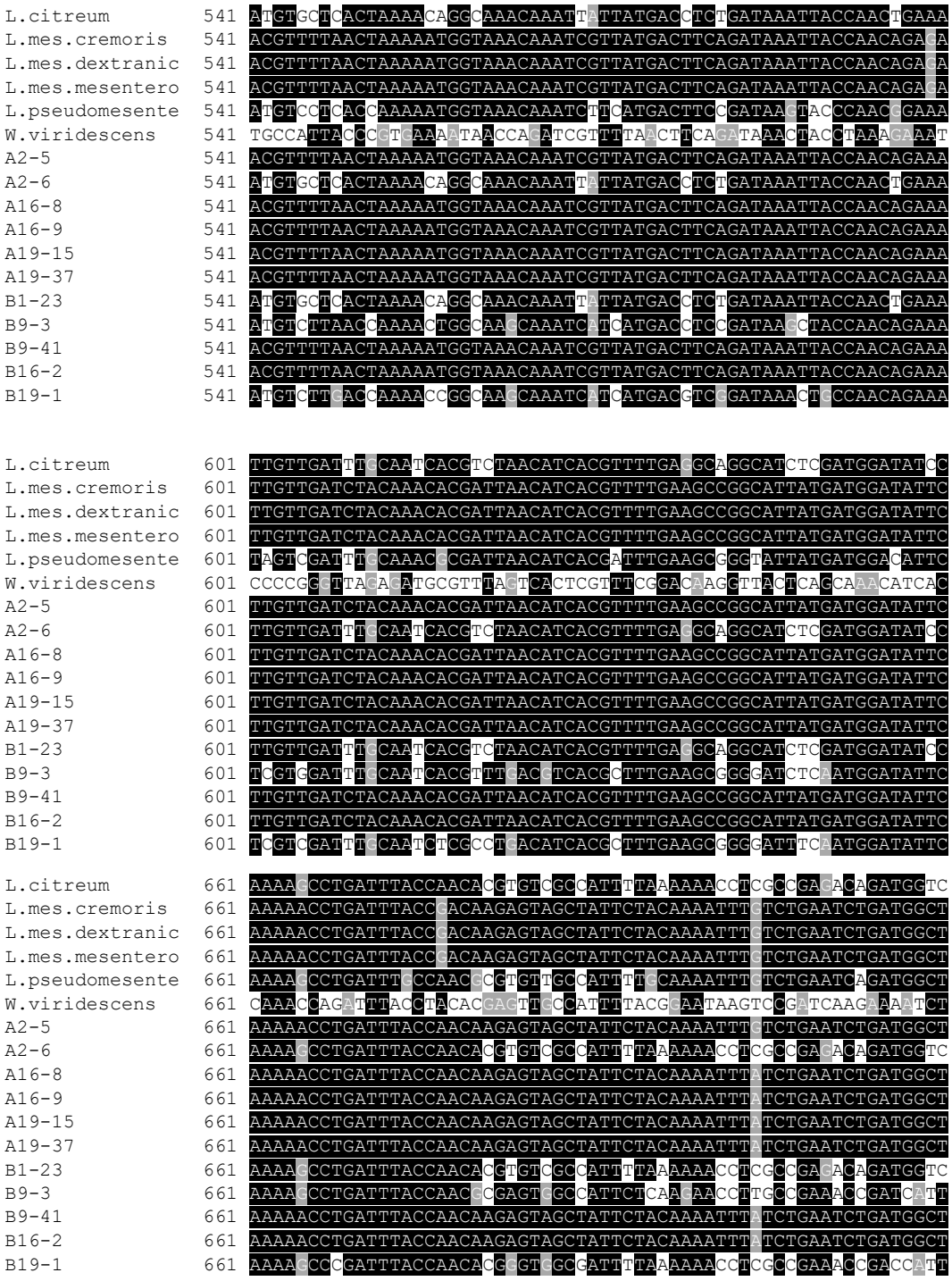

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L.mes.mesentero    781 GCTTAGAAGGGGCGTTTCATAAGTTTGAAGCCAGCTTGCGATATATGAATAAACCGGCTA
L.pseudomesente    781 GTTTGGAAGGGGCGTTTCATAAATTTGAAGCCAGTCTAGGCTATATGAACAAGCCAGCAA
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B16-2              781 GCTTAGAAGGGGCGTTCCATAAGTTTGAAGCCAGCTTGCGATACATGAATAAGCCGGCTA
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W.viridescens      841 TCTAGAAACAGCACGTTCAATCTTGGAAAAGATGAGCTTTAAGCGTCAGCGTGCCATCAC
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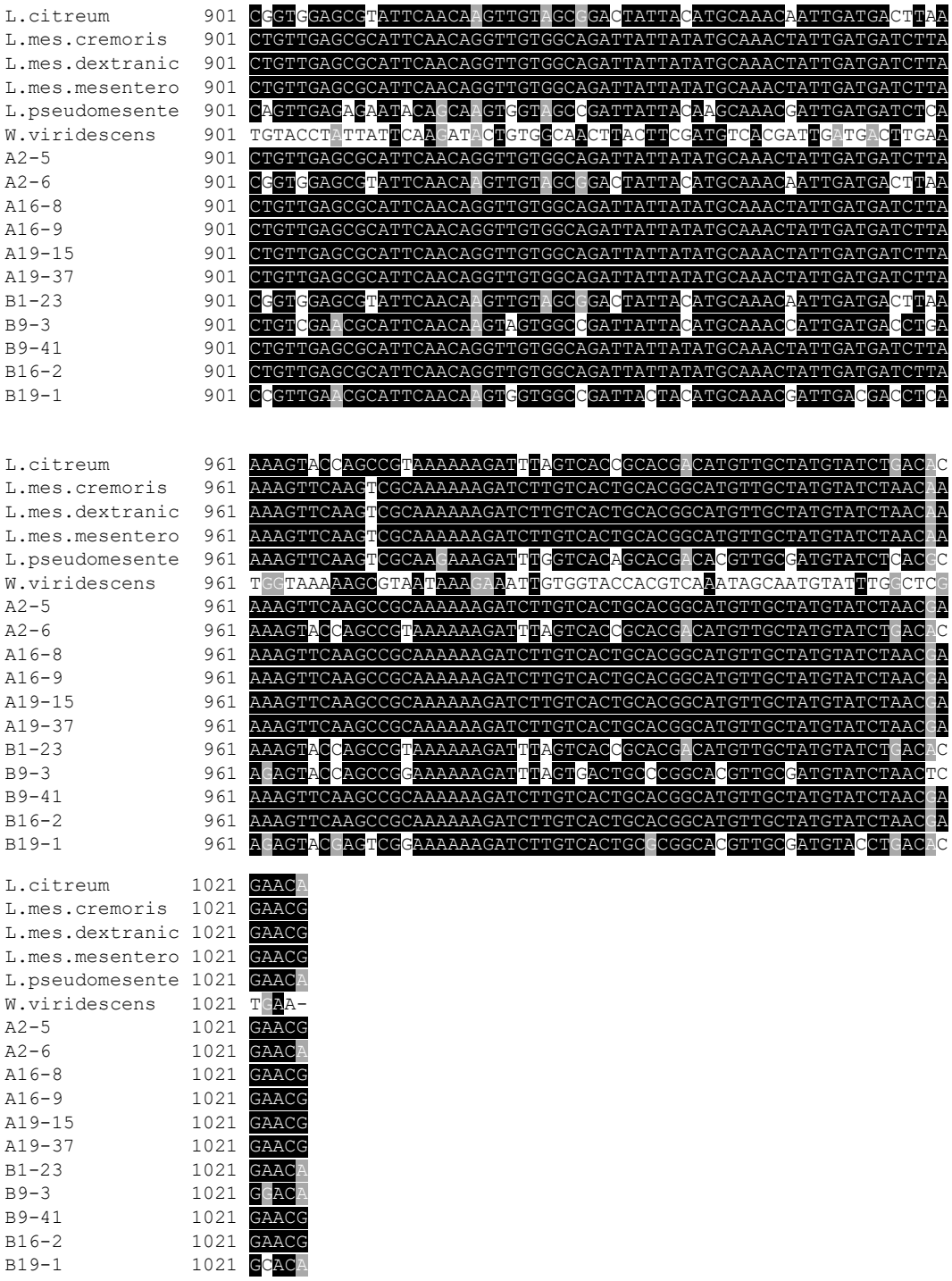
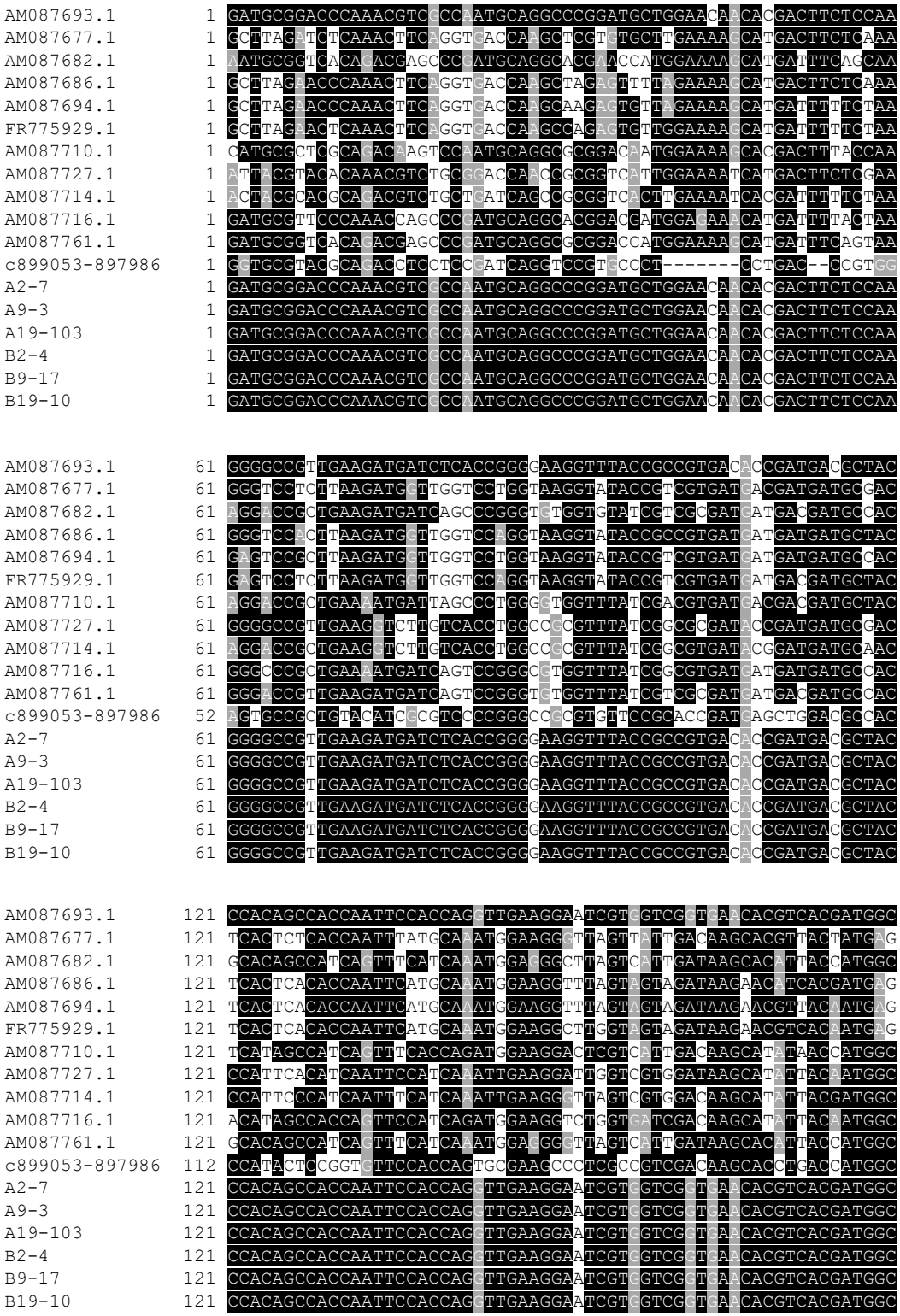


Fig. S4 Multiple DNA sequence alignments of partial *rpoA-dnaA* concatenated genes of representative and reference *Leuconostoc* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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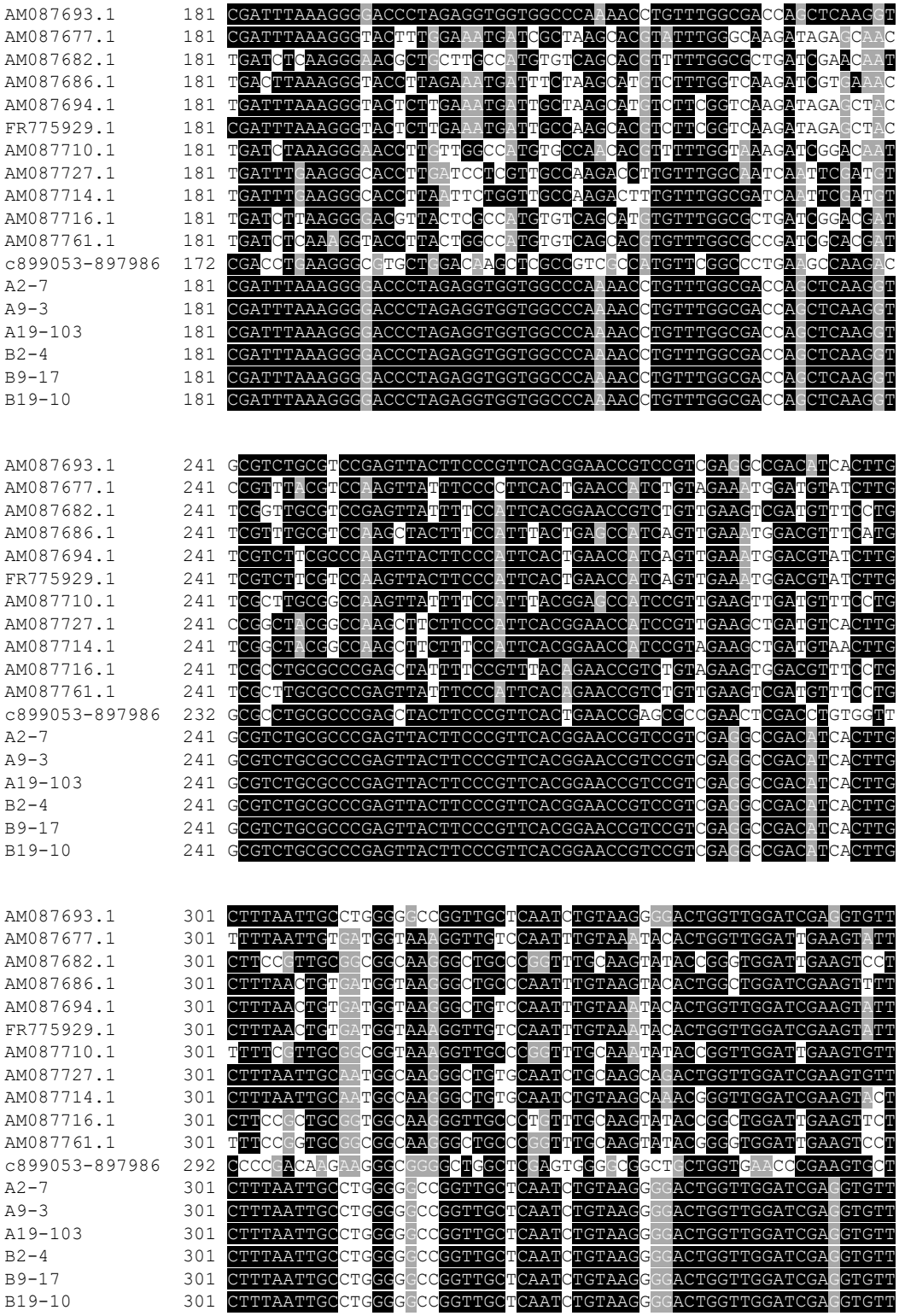

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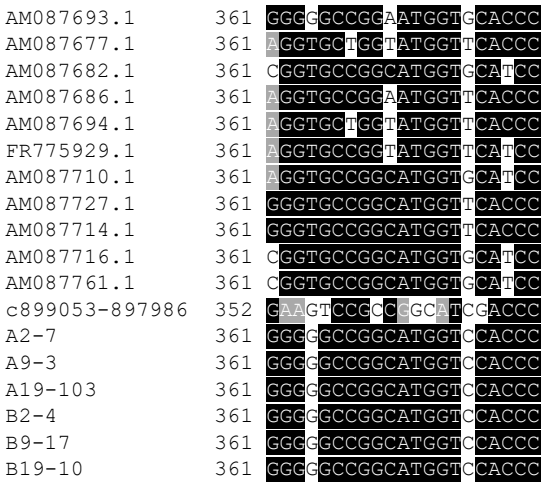
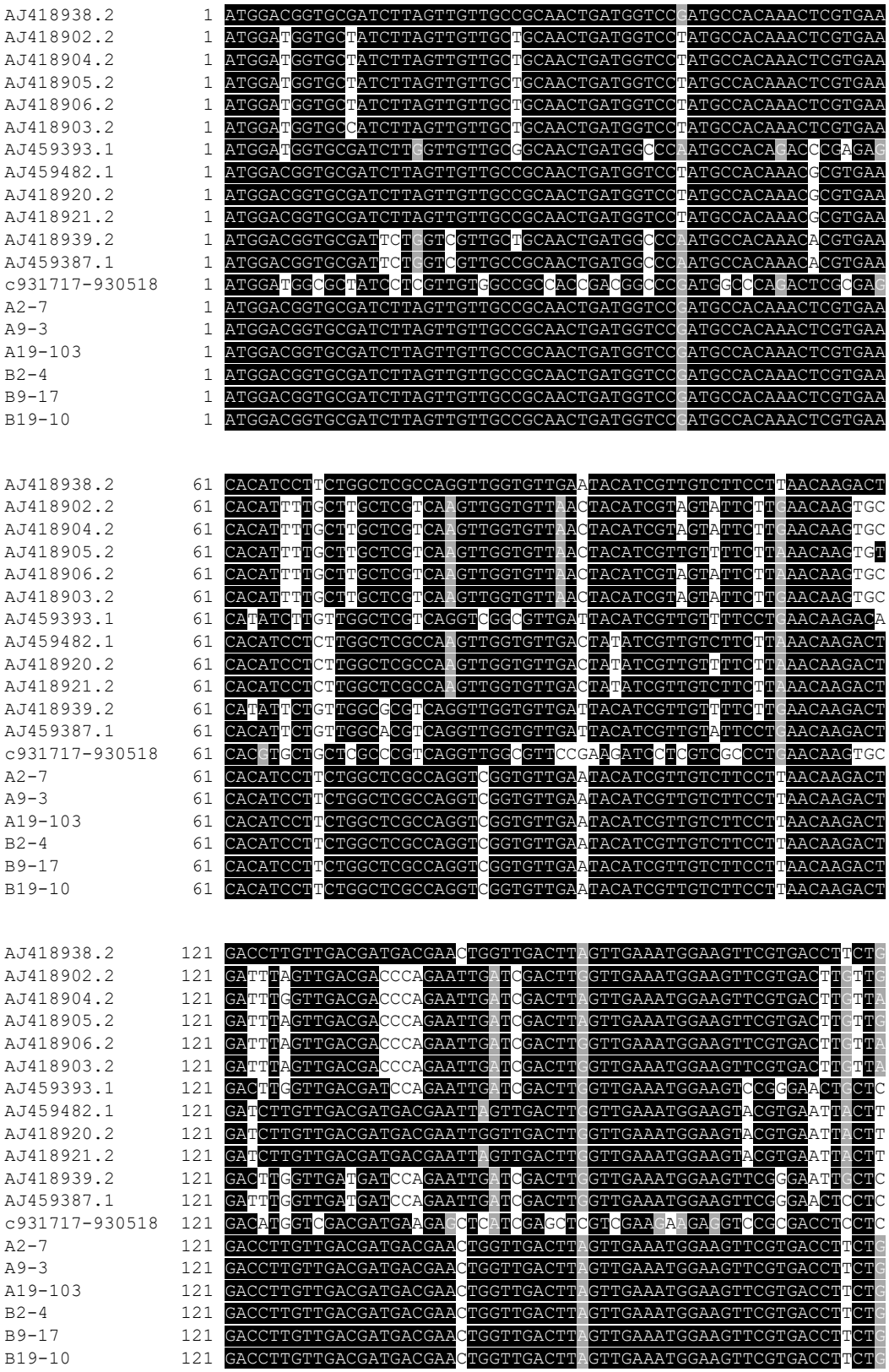


Fig. S5 Multiple DNA sequence alignments of partial *pheS* genes of representative and reference *Lactobcillus* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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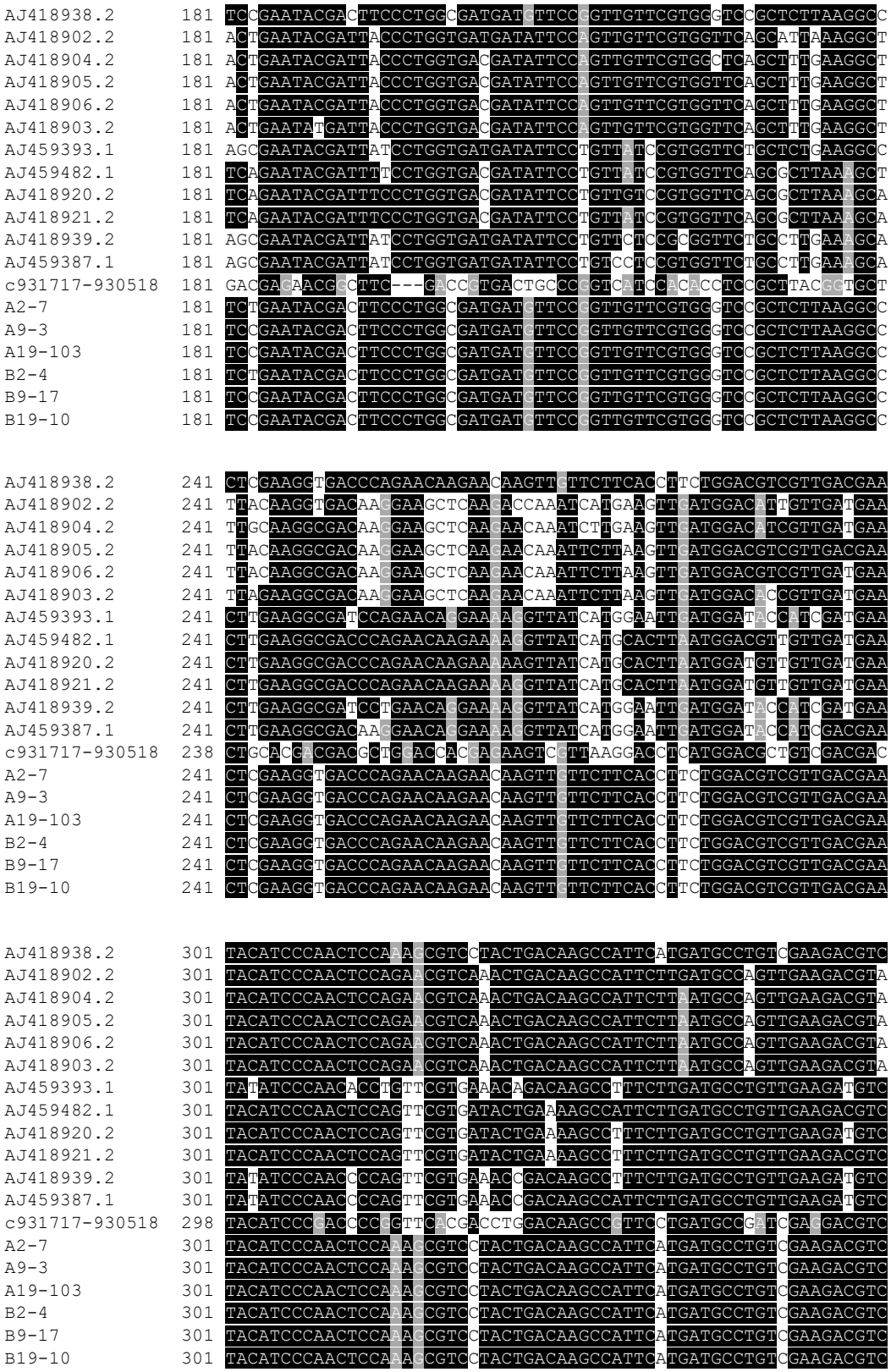

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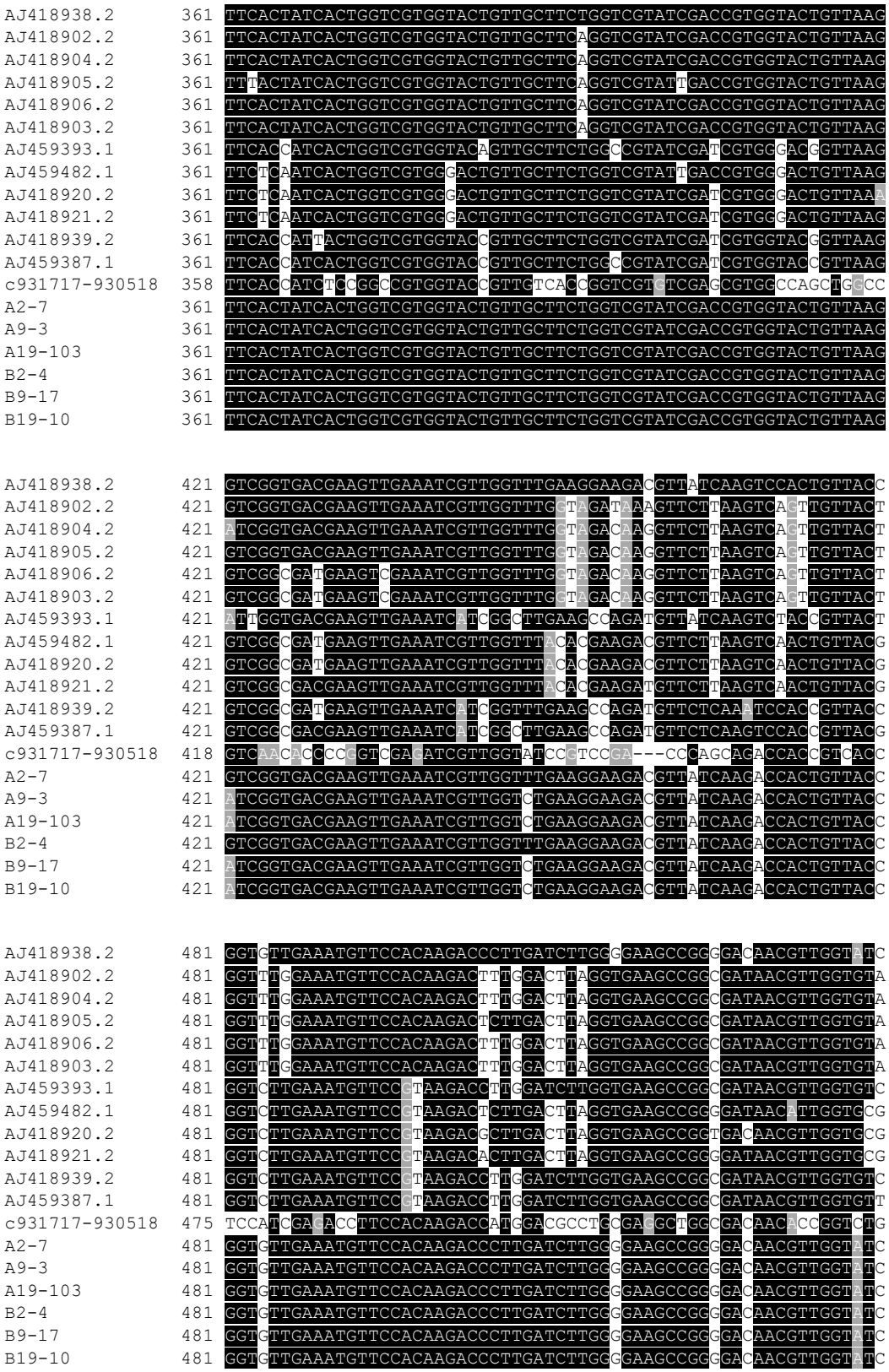

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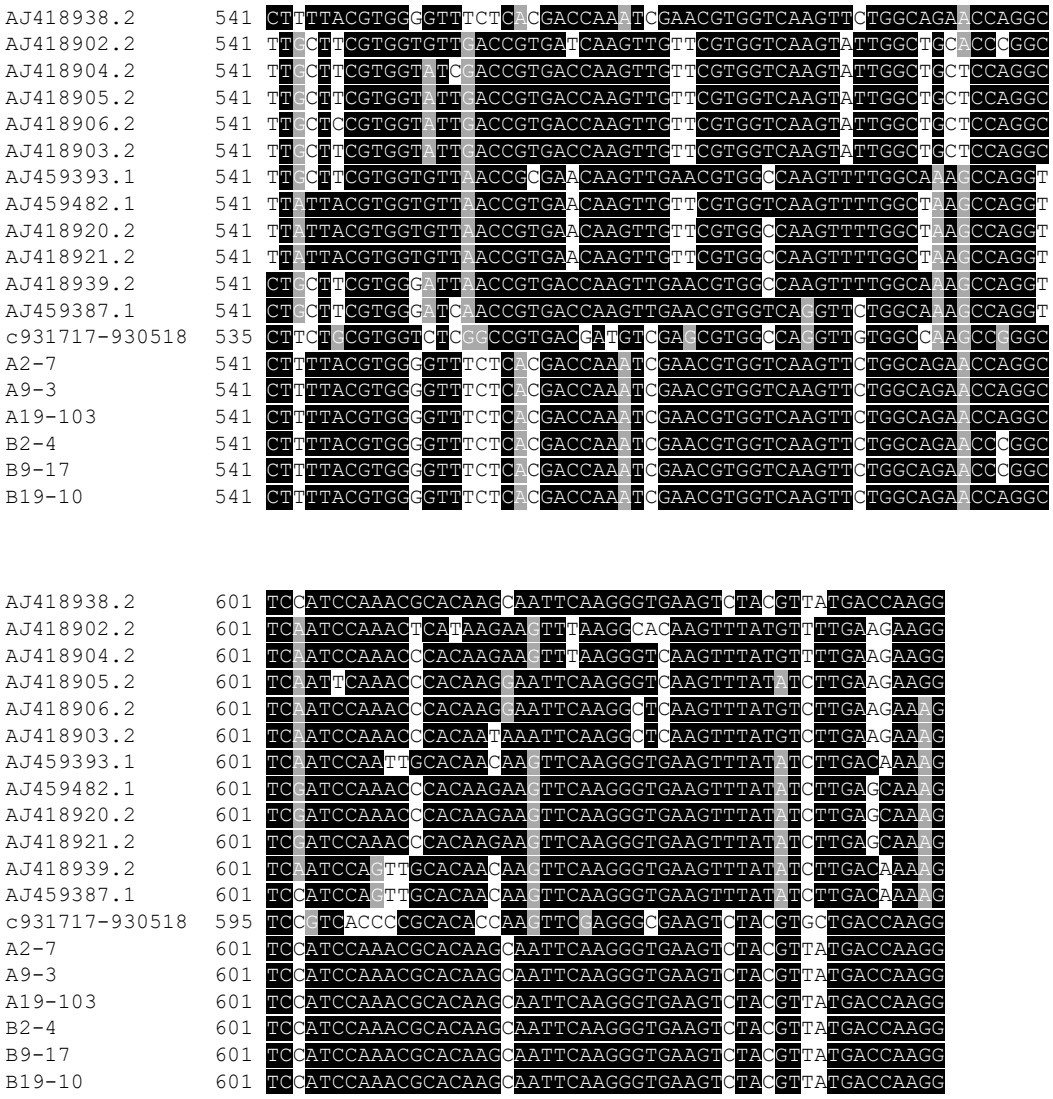
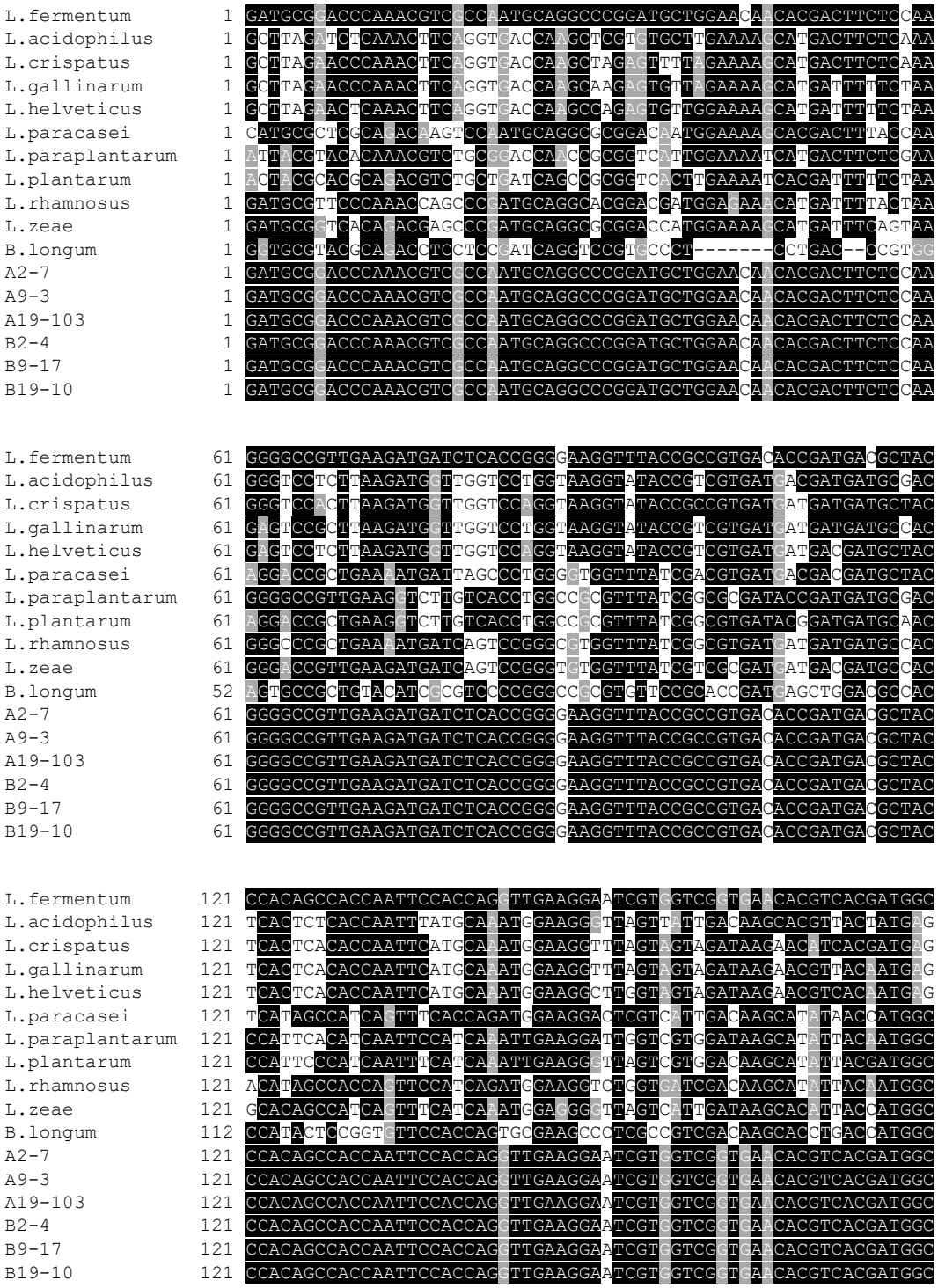


Fig. S6 Multiple DNA sequence alignments of partial *tuf* genes of representative and reference *Lactobcillus* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)



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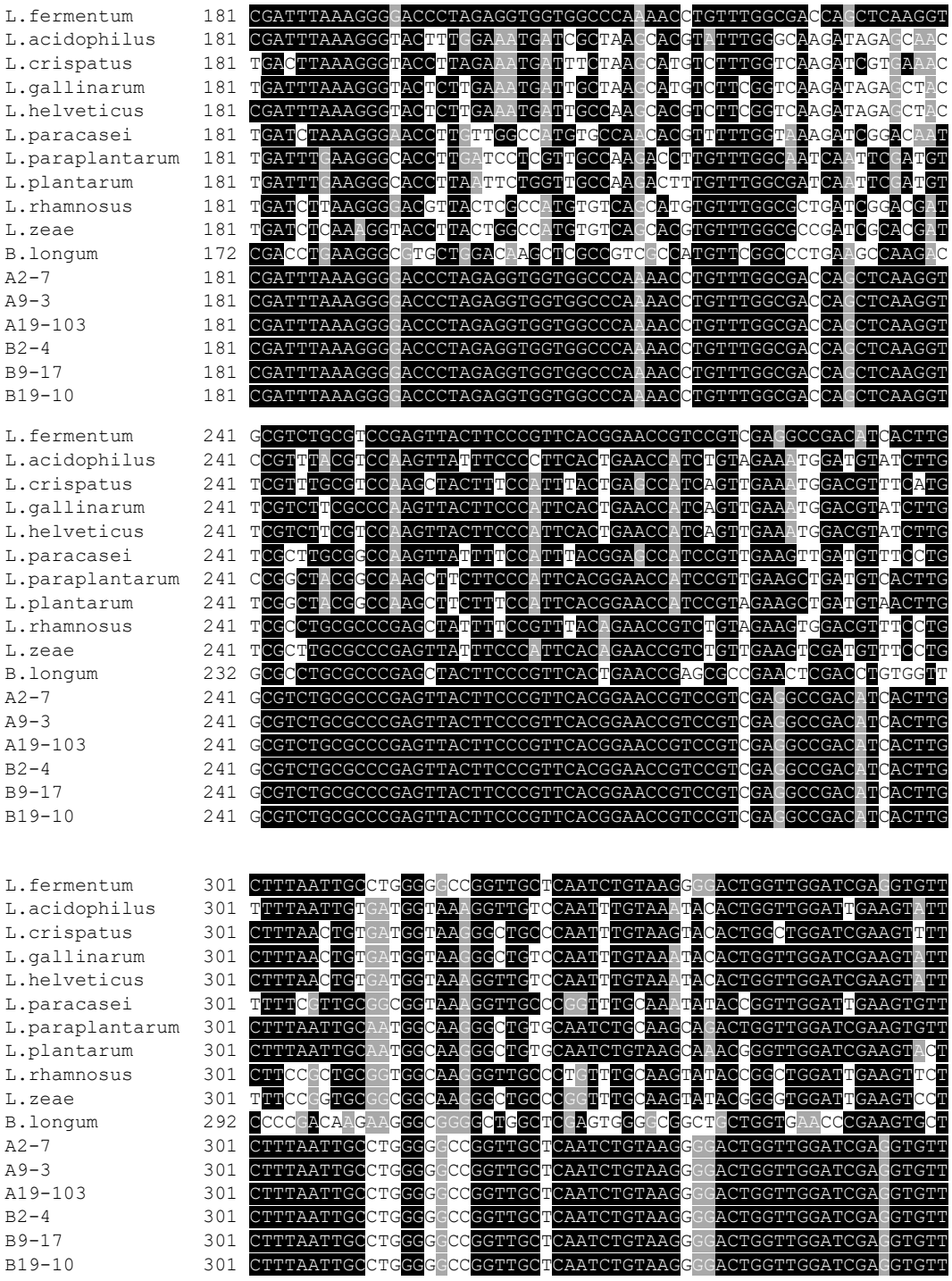

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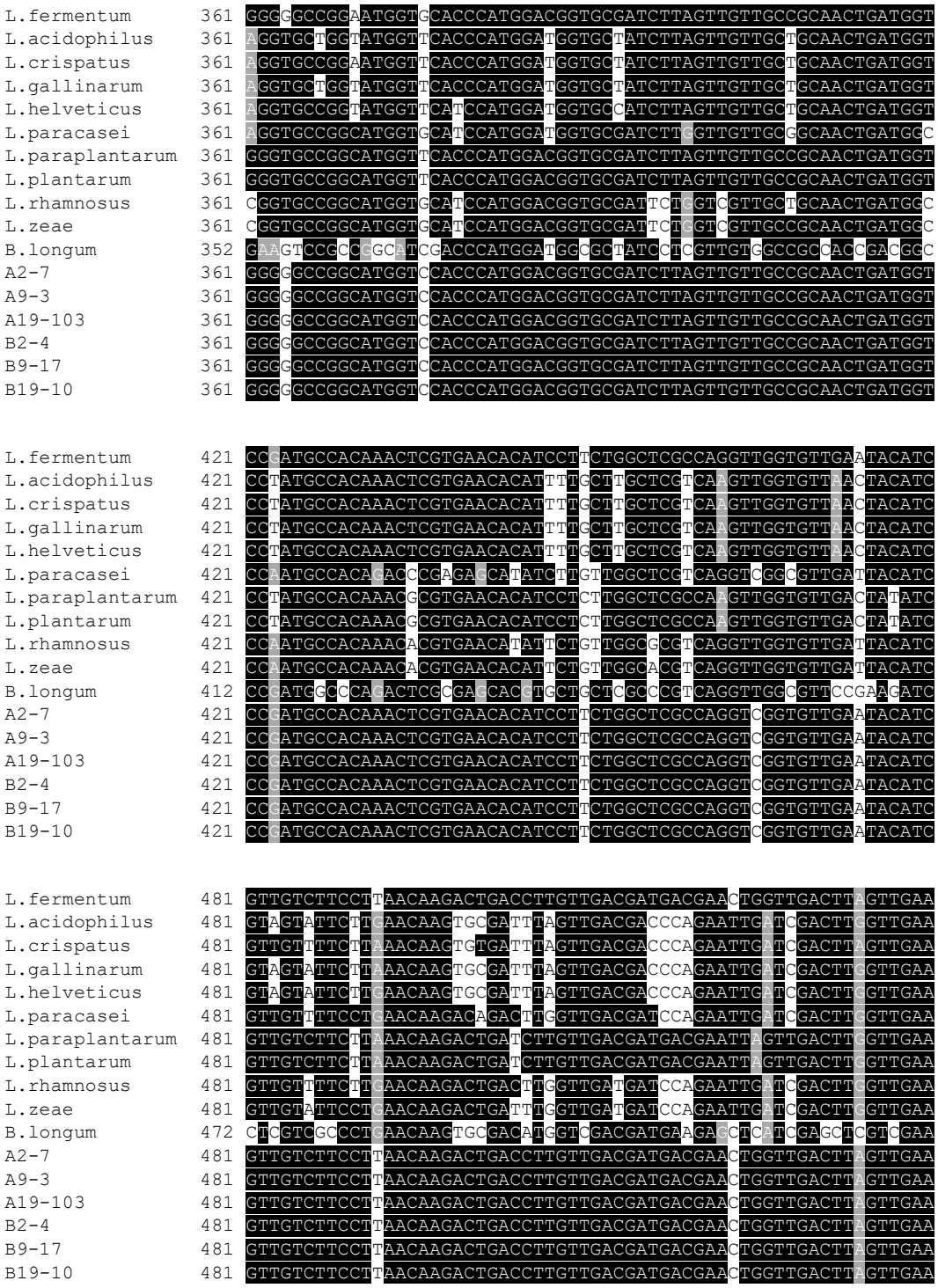

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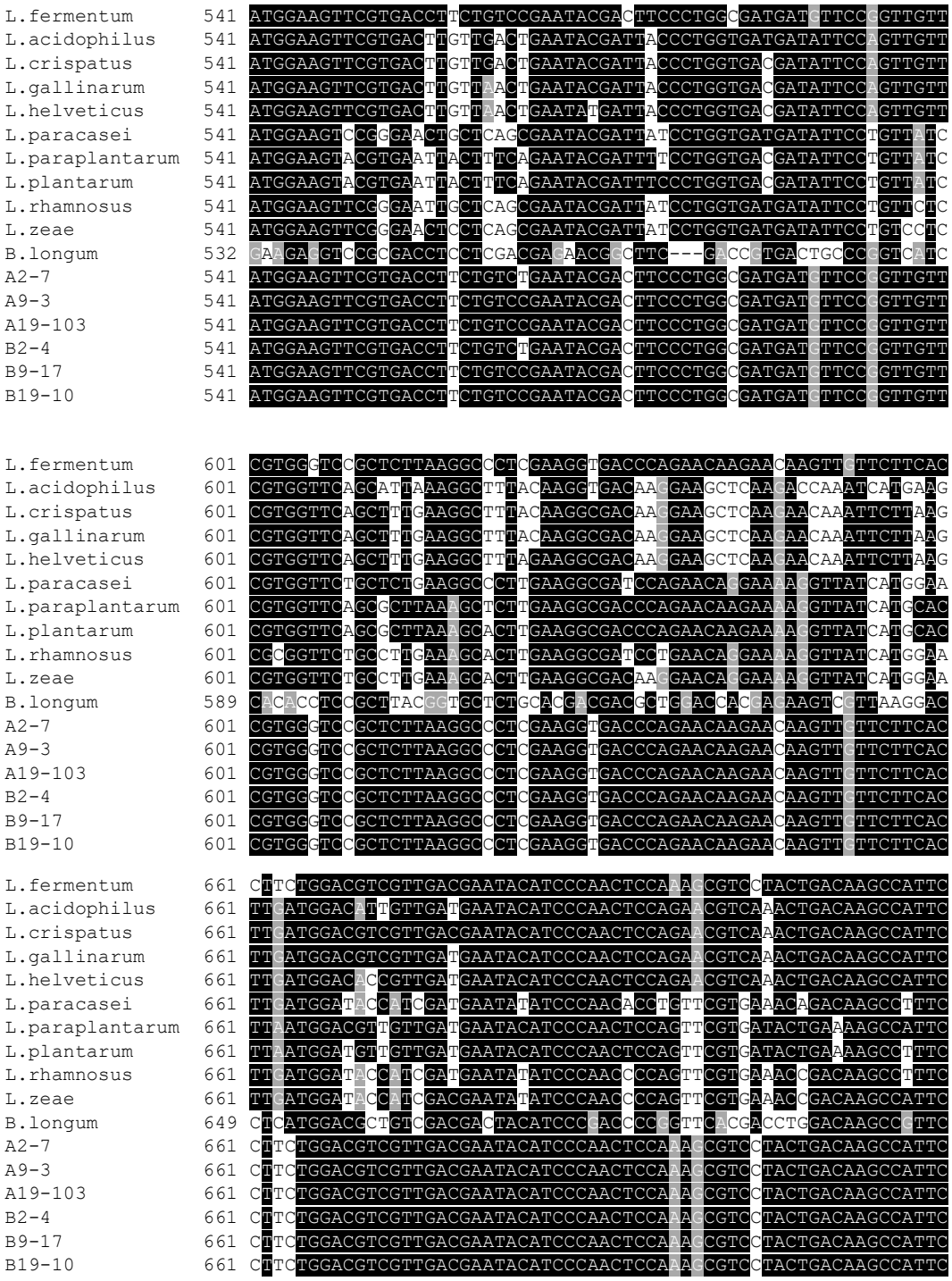

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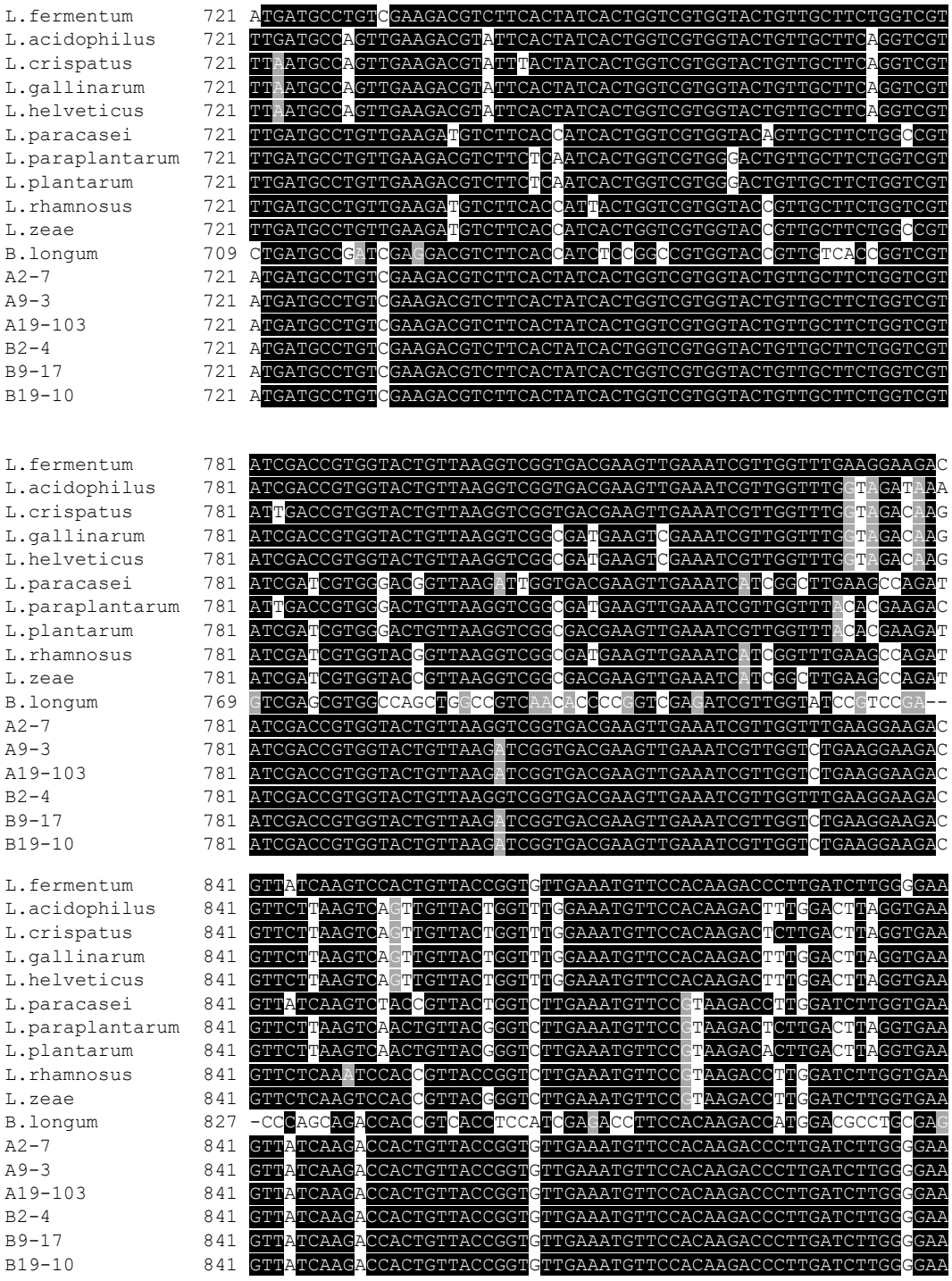

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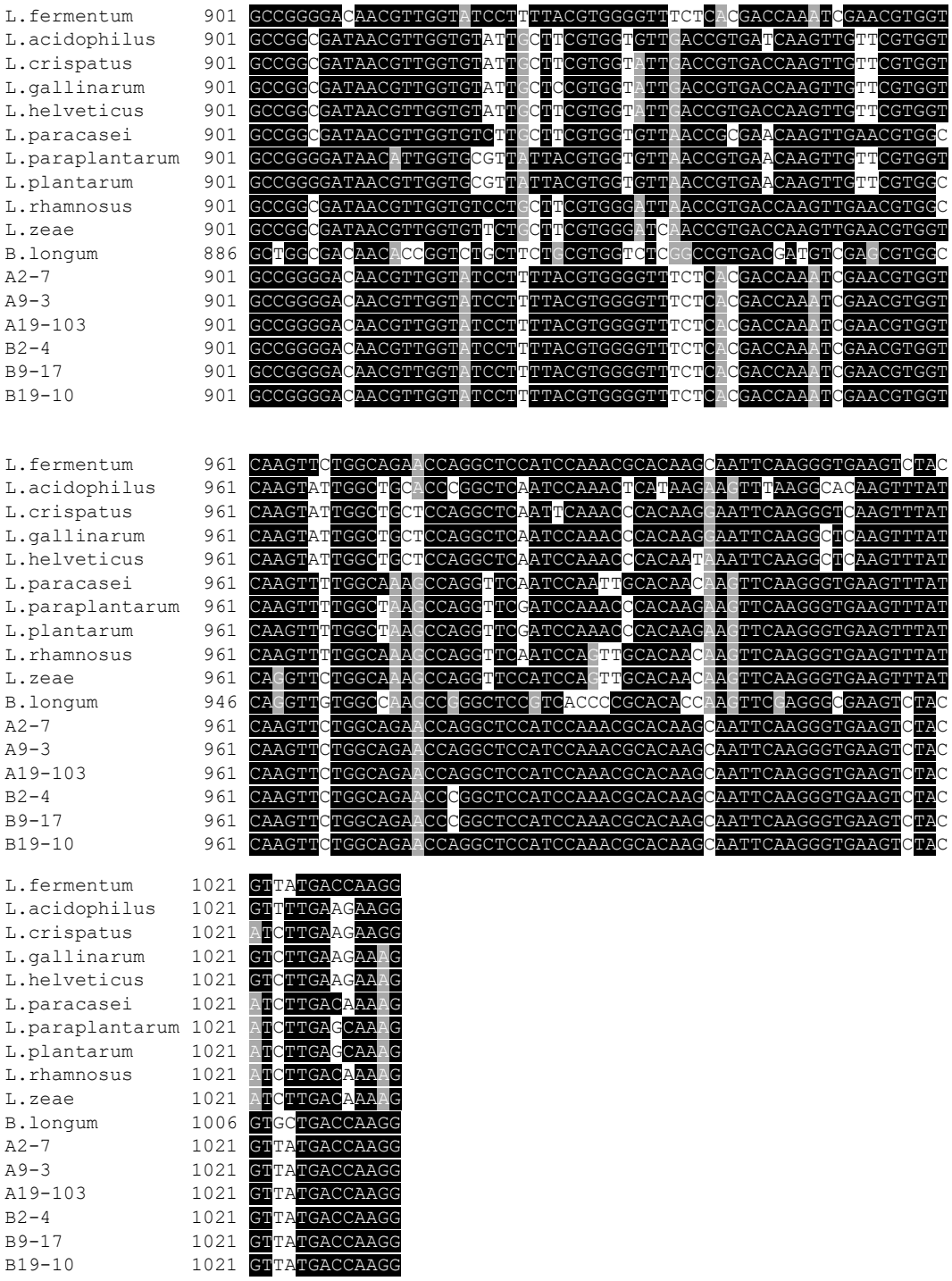

Fig. S7 Multiple DNA sequence alignments of partial *pheS-tuf* concatenated genes of representative and reference *Lactobcillus* strains used in this study. Sequences were aligned with ClustalW (Thompson et al., 1994) as implemented in the BioEdit Sequence Alignment Editor program (Hall 1999) followed by shading with Boxshade version 3.21 software (Hofmann and Baron, Institute Pasteur, France)

CHAPTER 8

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Effect of dithiocarbamate biocides on gum-producing bacteria isolated from a South African sugarcane processing factory

S Nel[1,2*], SB Davis[1], A Endo[3] and LMT Dicks[2]

[1]Sugar Milling Research Institute NPC, c/o University of KwaZulu-Natal, Durban 4041, South Africa

[2]Department of Microbiology, Stellenbosch University, Stellenbosch 7600, South Africa

[3]Department of Food, Aroma and Cosmetic Chemistry, Tokyo University of Agriculture, Hokkaido 099-2493, Japan

*Corresponding author: S Nel (snel@smri.org)

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Abstract

Exopolysaccharide (gum)-producing bacteria cause financial losses to the sugarcane processing industry. In previous studies, we have reported on the isolation of several strains of gum-producing lactic acid bacteria and *Bacillus* species from a South African sugarcane processing factory. Here we report on the antibacterial effect of Preventol®Z and Busan®1021, two dithiocarbamate biocides used in the sugar industry, on seven species of gum-producing bacteria. Preventol®Z, administered at 20 ppm, demonstrated a bactericidal (killing) effect against *Leuconostoc mesenteroides* A16-9*, Leuconostoc lactis* B9-3*, Bacillus subtilis* B7-19 and *Bacillus amyloliquefaciens* B7-51 after 6 h of contact, but had only a bacteriostatic (growth inhibiting) effect on *Weissella cibaria* A1-17*, Weissella confusa* B1-24 and *Lactobacillus fermentum* B19-18 when tested under the same conditions. Busan®1021 (20 ppm) had a bactericidal effect on all seven species. *Bacillus subtilis* B7-19 and *B. amyloliquefaciens* B7-51 were susceptible to both biocides, but only for the first 2 h of exposure, after which the killing effect remained constant. Based on results obtained in this study, the concentration of Preventol®Z and Busan®1021 may need to be increased, or dosage intervals altered, to kill all gum-producing bacteria.

Introduction

Microbial infection in harvested sugarcane and bacterial contamination in sugarcane processing factories lead to substantial losses in sugar production and reduces factory throughput (Cuddihy Jr et al., 2001; Eggleston and Grisham, 2003; Morel du Boil et al., 2005; Solomon, 2000). It is therefore critical that harvested cane reaches the factory in the shortest possible time and is processed as soon as possible to prevent microbial growth and spoilage. However, the average delay between burning, harvesting and processing of sugarcane is often between three and five days in South Africa (Harris, 2017). Poor factory sanitation and sump management may also contribute to microbial-related sucrose losses in the factory. Factory staff occasionally slug-dose biocides into the sumps to limit microbial growth and control contamination (Kalidass et al., 1996). Surfaces of juice screens need to be cleaned regularly as they have been implicated as sources of contamination, with reports of slime deposits on the juice screens as a consequence of bacterial activity (Lillehoj et al., 1984; Rein, 2007; Nel, 2019a). Foxon and Du Clou (2017) quantified gums in mixed juice and molasses from a South African sugarcane processing factory over an entire season and concluded that gums were produced in the factory during the last few weeks of the milling season. It may thus be more practical to add biocides at the end of a season, or when conditions are conducive to the proliferation of gum-producing bacteria.

Kulkarni (1999) remarked that there is a tremendous lack of understanding about factory sanitation, and biocides in particular, and that this is the result of a lack of knowledge regarding microorganisms and enzymes present in sugarcane processing which are responsible for major losses in recoverable sucrose. An extensive overview on the use of biocides in the sugar industry was published by Solomon (2000). The author noted that, although several disinfectants have been tested, their application is often restricted by availability, high cost and environmental concerns. Not all biocides that have been approved are effective. *Klebsiella pneumoniae, Enterobacter cloacae* and *Lactobacillus fermentum,* isolated from cane juice produced by a sugarcane processing factory in Thailand (Milintawisamai et al. 2009a), were resistant to the quaternary ammonium biocide dimethyl benzyl ammonium chloride (DBAC; Milintawisamai et al., 2009b). DBAC interacts with bacterial cell membranes, resulting in lysis and leakage of intracellular constituents (Ioannou et al., 2007). Binding of quaternary ammonium compound (QAC)-based biocides to divalent ions, such as Ca^{2+}, and proteins and bagasse in sugarcane leads to losses in antimicrobial activity. Sub-lethal doses of QACs may lead to the development of biocide-resistant strains (Gerba, 2015). As a result, higher doses of QAC-based biocides may be required to be effective (Solomon, 2000). A possible solution

to the problem would be to use broad-spectrum biocides that do not bind to Ca^{2+} and proteins, as suggested by Solomon (2000).

The use of dithiocarbamate (organosulfur) biocides in sugarcane processing factories has been approved by the Food and Drug Administration (FDA) of the United States of America (Solomon, 2000), and a variety of organosulfur-based formulations are commercially available. These biocides bind to molecules with SH-groups, e.g. invertase and dextransucrase (Solomon, 2000), and are thus ideally suited to control gum-producing bacteria in sugarcane processing factories. Interaction with these and other key enzymes in metabolic pathways leads to irreversible changes in a cell's redox potential and cell death (Rath et al., 2011).

Most studies evaluating biocides for sugarcane processing focus on changes in processing stream quality parameters and do not assess the killing ability of the biocide against specific spoilage bacteria. Historically, *Leuconostoc mesenteroides* had been implicated as the main causative agent of deteriorated sugarcane (Eggleston et al., 2009). However, earlier sugar technologists did not have access to microbiological identification methods of high discriminatory power which limited conclusive evidence that *L. mesenteroides* was indeed the main cause of cane deterioration and sucrose loss (Nel et al., 2017). Nel (2014) advocated the concept of microbial diversity profiling of spoilage bacteria in sugarcane processing for the development of a targeted approach for factory sanitation. In a comprehensive taxonomic study, Nel et al. (2019a) explored the comparative diversity of gum-producing bacteria in a South African sugarcane processing factory. Different bacterial populations were reported in sugarcane processing streams when low (<70 mg/kg) and high (>500 mg/kg) concentrations of dextran were present in raw sugar. Phylogenetic analyses of 16S rRNA gene sequences differentiated the gum-producing bacteria into four genera and nine species. The majority of these isolates belonged to the genus *Weissella* (47%), followed by members of *Bacillus* (24%), *Leuconostoc* (19%) and *Lactobacillus* (10%). Gum-producing bacteria isolated at various sampling points are listed in Table 1. Nel and co-workers (2019a) isolated *Weissella* spp. from prepared cane and *Bacillus* spp., in particular *Bacillus amyloliquefaciens*, from a juice screen and mixed juice tank.

Table 1 Gum-producing bacteria isolated from a South African sugarcane processing factory (Nel et al., 2019a)

Sampling point	**Species[a] isolated at times when:**	
	low dextran in raw sugar were reported	**high dextran in raw sugar were reported**
Cane Testing Service station (prepared cane)	*W. confusa* (14) *W. cibaria* (7)	*W. confusa* (25) *W. cibaria* (123) *Le. citreum* (1)
Diffuser sump	*W. confusa* (1) *W. cibaria* (1) *Le. pseudomesenteroides* (1) *Le. citreum* (1) *Lb. fermentum* (7)	*W. confusa* (15) *W. cibaria* (11) *Lb. fermentum* (8)
Juice screen	*B. amyloliquefaciens* (14)	*B. amyloliquefaciens* (46) *B. subtilis* (7)
Mixed juice tank	*B. amyloliquefaciens* (5)	*B. amyloliquefaciens* (31) *B. subtilis* (1)
Filtrate	*W. cibaria* (4) *Lb. fermentum* (15)	*Le. pseudomesenteroides* (1) *Le. lactis* (19) *Lb. fermentum* (9)
Mud trough	*Le. mesenteroides* (34) *Le. pseudomesenteroides* (1) *Lb. fermentum* (1)	*Le. lactis* (19) *Lb. fermentum* (3)
Syrup tank	*W. cibaria* (1) *Le. mesenteroides* (2) *Le. pseudomesenteroides* (1)	*Le. mesenteroides* (1)

[a]*W. = Weissella, Le. = Leuconostoc, B. = Bacillus, Lb. = Lactobacillus.* The number of strains obtained after a visual screening based on their slimy appearance on sucrose-based medium, is indicated in parenthesis

In the present study, we evaluated the antimicrobial effect of two commercial dithiocarbamate-based biocides on strains selected from the most prevalent gum-producing bacteria listed in Table 1. The antimicrobial effectiveness test was performed by spiking test bacteria individually into each biocide and determining the log reduction and percentage (%) kill of the bacteria at predetermined time intervals to quantitatively evaluate the effectiveness of the biocide to prevent microbial proliferation (bacteriostatic effect) and/or to kill the bacteria (bactericidal effect) (Moser and Meyer, 2011). This study did not evaluate the effectiveness of the biocides on biofilms, which are known to display increased resistance to biocide treatments (Bridier et al., 2011).

Materials and methods

Bacteria

Seven gum-producing bacteria, representing *W. confusa* B1-24, *W. cibaria* A1-17 (Nel et al., 2019b), *B. amyloliquefaciens* B7-51*, B. subtilis* B7-19 (Nel et al., 2019c), *Le. mesenteroides* A16-9*, Le. lactis* B9-3 and *Lb. fermentum* B19-18 (Nel et al., 2019d), were selected. Thawed cells of *Weissella, Leuconostoc* and *Lactobacillus* strains from a frozen glycerol stock culture were inoculated into 5 mL MRS broth (Biolab, Merck South Africa) and incubated at 30 °C for 18 to 24 h on a shaking incubator (IncuShake, Labotec South Africa), set at 150 rpm. The cultures were then streaked onto MRS Agar (Biolab) and incubated at 30°C. *Bacillus* strains from glycerol stock cultures were inoculated in 5 mL lysogenic broth (LB; Bertani, 1951), incubated at 30 °C on a shaking incubator (150 rpm) for 18 to 24 h, streaked onto LB agar and incubated at 30 °C. After 18 to 24 h, colonies were scraped from MRS and LB agar and suspended in sterile phosphate buffered saline (PBS; Green and Sambrook, 2012) to yield a final cell count of 10^6 colony forming units (CFU)/mL. These suspensions, which were standardised by spread plate counts of serial dilutions of the test cultures in sterile PBS against spectrophotometric measurements of the same dilutions at 600 nm (Lambda 25 UV/VIS spectrometer, Perkin Elmer, South Africa), were used as initial inocula for all tests.

Biocides

The dithiocarbamate-based biocides were Busan®1021 (Buckman Africa), a sodium dimethyl dithiocarbamate solution (SDDC; 25-50%) and Preventol®Z (Lanxess, Chemtrade South Africa), a SDDC solution (approximately 42%). The biocides were diluted with sterile PBS to a final concentration of 20 ppm as per manufacturers' dosage recommendations.

Antibacterial activity tests

Each test isolate was inoculated into three test tubes, one serving as a growth control containing sterile PBS (5 ml) only, and one test tube for each biocide (100 µl biocide in 4.9 mL sterile PBS), to a final concentration of approximately 10^6 CFU/ml. Immediately after addition of the biocides (time zero), 100 µl was removed from each test tube, serially diluted and plated onto MRS agar (for *Weissella, Leuconostoc* and *Lactobacillus* strains) and LB agar (for *Bacillus* strains). All plates were incubated at 30 °C for 24 h and the number of viable cells determined by colony counting. For the remaining part of the experiment the test tubes were incubated at 30 °C in a shaking water bath (ThermoFisher Scientific, South Africa), set at 160 rpm. After 2, 4 and 6 h, 100 µl was removed, serially diluted, plated onto respective growth media and incubated at 30 °C. Colonies were counted after 24 h of incubation. The

incubation time (up to 6 hours) and temperature (30 °C) of the antimicrobial activity assays reflect the possible conditions in factory sumps, which may contain a variety of microorganisms, including any one or a combination of the test bacteria. Three biological repeats were performed, and the number of surviving cells determined for each sampling time. The number of cells killed was expressed as a percentage value. Bactericidal activity is generally defined as a 3-log decrease in viable cell numbers, thus ≥99.9% killing (NCCLS, 1992). If a constant logarithmic rate of killing is assumed, a 90% kill at 6 h would be equivalent to 99.9% kill at 24 h (May et al., 1998). Bactericidal activity was defined as ≥1-log reduction (≥90% kill) and bacteriostatic activity as <1-log reduction (<90% kill) in viable cell numbers after 6 h of incubation. (Basri et al., 2014).

Results and discussion

The antibacterial effect (logarithm of the viable bacterial counts plotted against time exposed to the biocide) of Busan®1021 and Preventol®Z on gum-producing bacteria is shown in Figs 1 and 2, respectively. Changes in viable cell numbers, expressed as log reduction and percentage values, are listed in Table 2.

Leuconostoc mesenteroides A16-9 was killed by both of the biocides after 6 h of exposure (100% killing; 5-log reduction in viable counts). These results were not surprising, as *Le. mesenteroides* has historically been the major spoilage organism associated with deteriorated cane (Eggleston et al., 2009) and sugarcane processing factories (McNeil and Bond, 1980), and has therefore been the main target bacterium of sugar industry biocides. However, this association has not been based on comprehensive microbial diversity profiling studies using bacterial identification methods of high discriminatory power. A recent study on the isolation of gum-producing bacteria (Nel et al., 2019a) and identification of the bacteria using phylogenetic analyses of three housekeeping gene sequences (Nel et al, 2019b), showed that *W. cibaria* and *W. confusa* were the most prevalent gum-producing bacteria on prepared cane. Busan®1021 (20 ppm) had a bactericidal effect on *W. cibaria* A1-17 and *W. confusa* B1-24, with >90% cells killed (>1-log reduction) after 6 hours. Preventol®Z (20 ppm), on the other hand, had a bacteriostatic effect on *W. cibaria* A1-17 and *W. confusa* B1-24 when cells were exposed for 6 h (<90% killed; <1-log reduction). All cells of *Le. lactis* B9-3 were killed after 6 h exposure to Busan®1021 (20 ppm), and Preventol®Z (20 ppm) effected a bactericidal effect on *Le. lactis* B9-3 under the same conditions. Busan®1021 (20 ppm) was bactericidal to *Lb. fermentum* B19-18. However, Preventol®Z (20 ppm) had a bacteriostatic effect on *Lb. fermentum* B19-18. Busan®1021 and Preventol®Z (20 ppm each) killed >90% cells (>1-log reduction) of *B. subtilis* B7-19 and *B. amyloliquefaciens* B7-51 after

only 2 h of exposure. However, the antibacterial activity of the two biocides against *B. subtilis* B7-19 and *B. amyloliquefaciens* B7-51 remained relatively unchanged between 2 and 6 h of exposure, suggesting that the cells may have adapted to the biocides. Dosage levels may have to be increased, or contaminated areas may have to be treated more often. It may be necessary to use alternative biocides to kill *Bacillus* spp. The use of different combinations of biocides, or changing biocide combinations in consecutive treatments, may be another option. Further research has to be done to determine optimal dosage levels.

Slime deposits on juice screens have been attributed to the presence and gum-producing strains of *Le. mesenteroides* (Lillehoj et al., 1984; Rein, 2007). However, the presence of *B. subtilis* and *B. amyloliquefaciens* on juice screens in a South African sugarcane processing factory (Nel et al., 2019a) and the possible tolerance of these species to the two biocides tested, necessitates careful consideration for sanitation management of sugarcane processing factories. Biocide susceptibility of biofilms in sugarcane processing also requires additional investigation. Further studies on the microbial diversity in sugarcane processing factories need to be done to ensure that we select, or develop, the most effective biocides.

Conclusions

Busan®1021 (20 ppm) and Preventol®Z (20 ppm) proved effective in inhibiting the growth of seven gum-producing bacteria previously isolated from a South African sugarcane processing factory. Variations were recorded in the levels of sensitivity to these two biocides, and the possibility that some bacteria may develop resistance, suggest that different doses for different bacteria may be required to maintain a bactericidal effect. Studies pertaining to microbial diversity in sugarcane processing factories may be necessary to ensure effective sanitation strategies.

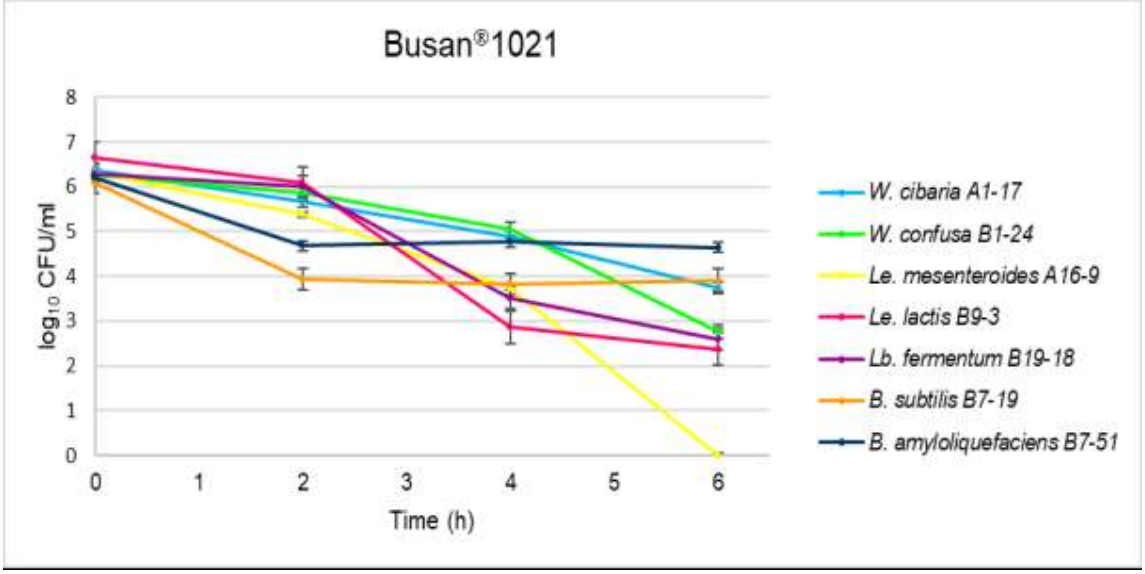

Fig 1 Antibacterial effect of 20 ppm Busan®1021 on seven gum-producing bacteria isolated from a South African sugarcane processing factory

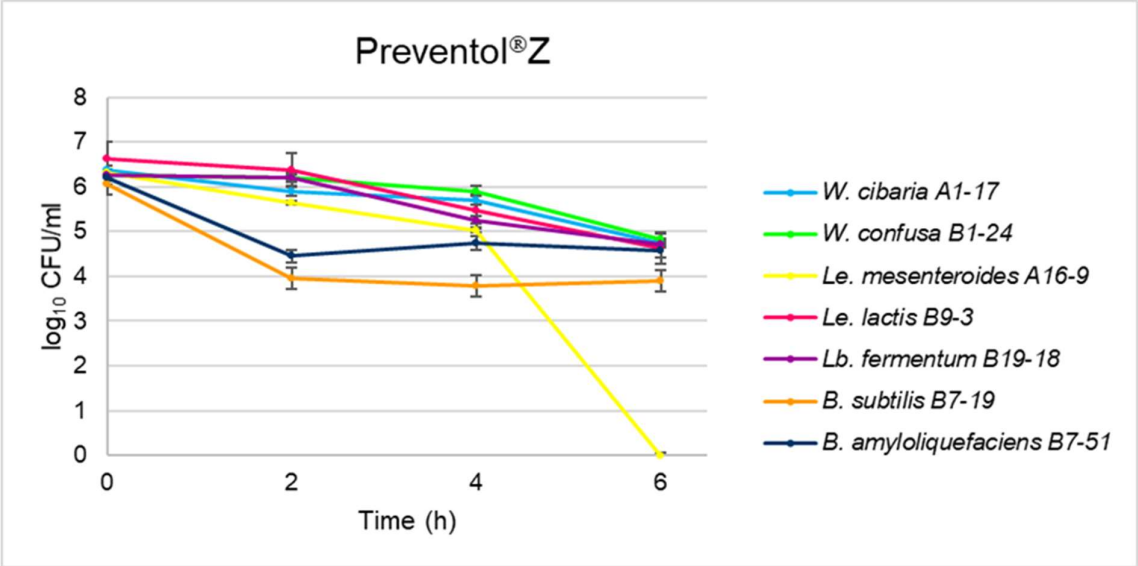

Fig 2 Antibacterial effect of 20 ppm Preventol®Z on seven gum-producing bacteria isolated from a South African sugarcane processing factory

Table 2 Changes in viable cell numbers of gum-producing bacteria after treatment with 20 ppm Busan®1021 and 20 ppm Preventol®Z

Test bacterium	% cells killed and log_{10} reduction	Time (h)		
		2	4	6
Busan®1021				
W. cibaria A-17	% kill	55.9	93.6	98.8
	log_{10} reduction	0.37	1.19	1.96
W. confusa B1-24	% kill	47.8	81.8	99.7
	log_{10} reduction	0.29	0.91	2.76
Le. mesenteroides A16-9	% kill	76.5	99.1	100
	log_{10} reduction	0.63	2.07	5.64
Le. lactis B9-3	% kill	70.6	100	100
	log_{10} reduction	0.53	3.75	4.04
Lb. fermentum B19-18	% kill	35.3	99.7	99.9
	log_{10} reduction	0.19	2.63	3.05
B. subtilis B7-19	% kill	96.0	94.0	95.0
	log_{10} reduction	1.69	1.31	1.31
B. amyloliquefaciens B7-51	% kill	91.4	91.7	89.4
	log_{10} reduction	1.07	1.09	0.99
Preventol®Z				
W. cibaria A-17	% kill	25.9	58.5	88.0
	log_{10} reduction	0.13	0.38	0.94
W. confusa B1-24	% kill	0	0	78.4
	log_{10} reduction	0	0	0.68
Le. mesenteroides A16-9	% kill	56.8	83.4	100
	log_{10} reduction	0.37	0.78	5.64
Le. lactis B9-3	% kill	40.6	87.6	97.7
	log_{10} reduction	0.23	1.15	1.78
Lb. fermentum B19-18	% kill	16.7	86.8	87.1
	log_{10} reduction	0	0.90	0.91
B. subtilis B7-19	% kill	97.2	93.7	95.0
	log_{10} reduction	1.65	1.36	1.34
B. amyloliquefaciens B7-51	% kill	94.6	92.0	91.0
	log_{10} reduction	1.30	1.11	1.05

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CHAPTER 9

General discussion and conclusions

General discussion and conclusions

Spoilage bacteria in sugarcane processing have historically been identified by phenotypic methods, which failed to accurately differentiate between the genera *Leuconostoc* and *Lactobacillus*, and between species within the genus *Leuconostoc* (McNeil and Bond, 1980). The last attempt at the profiling of spoilage bacteria in a sugarcane processing factory was more than 30 years ago. In that study, Lillehoj et al. (1984) identified *Leuconostoc mesenteroides* as the dominant species in the factory processing streams. The authors differentiated *Leuconostoc* from *Lactobacillus* based on the assumption that lactobacilli do not produce dextran from sucrose, despite reports to the contrary (Duncan and Seeley Jr., 1963, 1965; Pederson and Albury, 1955). Previous attempts to identify spoilage bacteria in sugarcane processing factories have all been hampered by the lack of available identification methods of high discriminatory power (as detailed in Chapter 3).

The use of phylogenetic analysis of 16S rRNA gene sequences in combination with housekeeping gene sequence analyses and amplified ribosomal DNA restriction analysis (ARDRA), is a novel approach for the identification of sugarcane processing spoilage bacteria. The advantage of this approach is the ability to accurately discriminate between closely related species (Hong and Farrance, 2015). In this study, the partial 16S rRNA gene sequences of the isolated bacteria were analysed as a first step in the identification process (detailed in Chapter 4). The generation of partial (*ca.* 500 bp) sequences is less expensive and faster than full-length gene sequencing, as it takes more sequencing reactions to generate the almost full-length 1 500 bp 16S rRNA sequence. The phylogenetic analysis of the partial 16S rRNA genes of 430 isolated gum-producing bacteria effectively grouped the isolates into four distinct clusters with high (100%) bootstrap values. The isolates belonged to the genus *Weissella* (47%), followed by members of *Bacillus* (24%), *Leuconostoc* (19%) and *Lactobacillus* (10%).

The second step in the identification process involved phylogenetic analyses of housekeeping genes and ARDRA. The isolates belonging to the genus *Weissella*, as determined by 16S rRNA gene sequence analysis, grouped with type strains of *Weissella confusa* and *Weissella cibaria*, respectively. *Weissella confusa* and *W. cibaria* are closely related, sharing 99.2% 16S rRNA sequence similarity (Bjorkroth et al., 2002). The additional phylogenetic analyses of the housekeeping genes *pheS* (encoding phenylalanyl t-RNA synthase alpha subunit), *dnaA* (encoding chromosomal replication initiation protein) and *atpA* (encoding alpha subunit of ATP synthase) were performed to differentiate between *W. confusa* and *W. cibaria*

(as detailed in Chapter 5). The single locus analysis of *pheS* and *dnaA* gene sequences provided high phylogenetic resolution of the two closely-related *Weissella* species. However, the *atpA* gene has not previously been used as phylogenetic marker for the identification of *Weissella* species, and the *atpA* gene sequence of only one *Weissella* type strain is currently available. Despite this limitation, phylogenetic analysis of the *atpA* gene showed the same grouping of representative *Weissella* isolates into two clusters as were observed with the phylogenetic analyses of *pheS* and *dnaA.* The *atpA* gene may therefore be a potential future phylogenetic marker to differentiate between strains of *W. confusa* and *W. cibaria.* The expansion of the number and type of currently available housekeeping gene sequences of *Weissella* type strains will enable the application of multilocus sequence analysis (*viz.* concatenation of several protein-coding gene sequences) to differentiate between closely related species of this genus.

Phylogenetic analysis of the partial 16S rRNA gene sequences showed that the isolated *Bacillus* strains clustered with the type strains of *Bacillus amyloliquefaciens* and *Bacillus subtilis*. However, *B. subtilis* and *B. amyloliquefaciens* share high 16S rRNA sequence similarity and cannot be differentiated by phenotypic characterisation methods (Wang et al., 2008). We used an ARDRA method (Jeyaram et al., 2011), in combination with phylogenetic analysis of *rpoB* (encoding the beta subunit of DNA-directed RNA polymerase) gene sequences (de Clerck et al., 2004), to distinguish between *B. amyloliquefaciens* and *B. subtilis* (as detailed in Chapter 6). The ARDRA method was faster and cheaper compared with the sequence-based identification methods, and the results obtained from ARDRA were confirmed by *rpoB* gene sequencing, showing that it was an effective method for the differentiation of the closely-related *B. amyloliquefaciens* and *B. subtilis.*

The isolated *Leuconostoc* and *Lactobacillus* species were identified by multilocus sequence analysis of housekeeping genes *rpoA* (encoding RNA polymerase alpha subunit), *dnaA* (encoding chromosomal replication initiation protein), *pheS* (encoding phenylalanyl t-RNA synthase alpha subunit) and *tuf* (encoding elongation factor Tu) (as detailed in Chapter 7). *Leuconostoc mesenteroides* could not be differentiated to subspecies level by single locus analysis of *rpoA* and *dnaA* gene sequences, or analysis of the concatenation of the two housekeeping genes. A high phylogenetic resolution was obtained for *rpoA* gene sequences to differentiate *Leuconostoc pseudomesenteroides, Leuconostoc lactis* and *Leuconostoc citreum*. However, the phylogeny obtained for *dnaA* sequence analysis was in disagreement with the phylogeny of the trees inferred from 16S rRNA and *rpoA* sequence analyses for four isolates which were previously identified as *Le. pseudomesenteroides*. In addition, a reference sequence for the *dnaA* gene from *Le. lactis* was not available from GenBank. The

rpoA housekeeping gene therefore proved more discriminatory for the identification of *Leuconostoc* species compared with the *dnaA* gene. Single locus analysis, as well as concatenation of the *pheS* and *tuf* housekeeping gene sequences, yielded identical phylogenies for the *Lactobacillus* isolates, corresponding to *Lactobacillus fermentum*.

The first objective of this study was to establish a profile consisting of the location and identity of gum-producing bacteria present in, or on, harvested sugarcane and factory processing streams during “good” (low concentrations of dextran in the produced sugar) and “bad” (high concentrations of dextran in the raw sugar) conditions (as reported by the factory). This objective was achieved and is detailed in Chapters 4 to 7. A significant finding of this study was the isolation of high numbers of *W. confusa* and *W. cibaria*, mostly present on the prepared (shredded) sugarcane, and in lower numbers in the diffuser sump. *Weissella* species have on only a few occasions been associated with deteriorated sugarcane; Hector et al. (2015) isolated *W. confusa* and *W. cibaria* from the cut-ends of three-day-old sugarcane stalks after milling, and they identified the bacteria by phylogenetic analysis of 16S rRNA gene sequences. Tilbury (1970) described the isolation of a new dextran-producing species, *Lactobacillus confusus*, from deteriorated cane, based on phenotypic identification methods. *Lactobacillus confusus* has since been reclassified as *W. confusa*. The difficulties experienced by early sugar technologists in differentiating species of *Leuconostoc* and *Lactobacillus* (Lillehoj et al., 1984; McNeil and Bond, 1980) have been established. It is most probable that *Weissella* spp. have been present on deteriorated cane and in factories at times when earlier researchers attempted to identify sugarcane processing spoilage bacteria. The identification methods available at the time were just not powerful enough to differentiate these related taxa. In addition, the genus *Weissella* was only established in 1993, after in-depth study based on molecular taxonomical techniques (Collins et al., 1993). The outcomes from study by Collins et al. (1993) resulted in the reclassification of *Leuconostoc paramesenteroides* as *Weissella paramesenteroides*. The atypical lactobacilli *Lb. confusus, Lactobacillus minor, Lactobacillus kandleri, Lactobacillus halotolerans* and *Lactobacillus viridescens* were reclassified as *W. confusa, Weissella minor, Weissella kandleri, Weissella halotolerans* and *Weissella viridescens*, respectively (as discussed in Chapter 2). This study showed that the application of bacterial identification methods of high discriminatory power can accurately identify species of *Weissella, Leuconostoc, Lactobacillus* and *Bacillus* which are present in sugarcane processing factories.

The presence of *W. cibaria* and *W. confusa* in sugarcane processing is significant due to their ability to produce copious amounts of dextran (Fusco et al., 2015). In 2017, 77% of raw sugar which was sent to the South African Sugar Terminals (SAST) contained dextran

concentrations above the maximum dextran specification of 150 mg/kg. These high dextran concentrations prevented the sale of this raw sugar on financially attractive markets (Moodley and Khomo, 2018). Currently dextran formation in the sugar industry cannot be determined by any method which is specific, quantitative and rapid, and a modified alcohol haze method (Anon, 2015) is used. This method quantifies gums against commercial dextran standards. Gums are defined as polysaccharides of high molecular weight precipitated from aqueous solutions by acidified ethanol (Imrie and Tilbury, 1972). Commercial dextran standards are produced by *Le. mesenteroides* NRRL B-512 and consist of 95 % α-(1,6)-glycosidic linkages in the main linear backbone and 5 % α-(1,3)-glycosidic linked branches (Khalikova et al., 2005). Dextrans produced by *W. confusa* and *W. cibaria* have a similar structure, with 97 % α-(1,6)- and 3 % α-(1,3)-linkages (Fusco et al., 2015). It is therefore expected that dextrans produced by *Weissella* spp. would be quantifiable by the modified haze dextran method, if they are of high molecular weight.

The chemical structure of dextrans in sugarcane processing is an important consideration for potential dextranase treatment of high dextran processing streams to reduce the dextran concentration. Commercial dextranases catalyse the hydrolysis of the α-(1,6)-linkages in the dextran chain (Khalikova et al., 2005), and therefore a dextran with high percentage of α-(1,6)-linkages would be more susceptible to commercial dextranase enzymes (under optimal conditions) compared to a highly branched dextran with a low percentage of α-(1,6)-linkages. The application of dextranase enzymes in South African sugarcane processing factories is currently considered uneconomical due to the high cost of the enzymes. However, the Tongaat Hulett Sugar Refinery (Hulref), which receives a significant amount of raw sugar from SAST to process, considered the application of dextranase enzymes to reduce high dextran concentrations in raw sugar during 2017. High dextran raw sugar caused a significant decrease in the refinery throughput, which had a major negative financial impact on the refinery (Moodley and Khomo, 2018). Based on the reported structure of dextrans from *W. confusa* and *W. cibaria* (Fusco et al., 2015), it is expected that commercial dextranase enzymes, under optimum conditions, would be able to hydrolyse these dextrans if present in sugarcane processing streams and in raw sugar.

In this study, the isolation of *B. amyloliquefaciens* from a sugarcane processing factory is reported for the first time. The presence of *B. amyloliquefaciens* and *B. subtilis* is significant due to the ability of these bacteria to produce levan, a fructose-based polysaccharide (Marvasi et al., 2010; Tian et al., 2011) from sucrose. Malang et al. (2015) has shown that *W. confusa* can also produce levan from sucrose. As previously discussed, the method used to quantify

dextran in the sugar industry is non-specific and involves the precipitation of all high molecular weight polysaccharides with acidified alcohol. Should high concentrations of levan be present in the factory streams or raw sugar, it would be erroneously quantified as dextran. Levan would also not be susceptible to dextranase hydrolysis. The impact of dextran on factory throughput and sugar quality has been well described and is widely acknowledged. However, the possible contributions of other bacterial polysaccharides, such as levan, on factory performance and sugar quality have not received any attention. This is because sugar technologists are largely unaware of the presence of bacteria other than *Leuconostoc* in sugarcane processing, and the potential impact of bacteria such as *Bacillus* and *Weissella* on processing and the quality of the final product. The information obtained in this study should broaden the knowledge and insight of sugar technologists on spoilage bacteria and their actions in sugarcane processing. Future research should include the quantification of levan in sugarcane processing streams and raw sugar to get an indication of the magnitude of levan as a portion of the total gums present in sugarcane processing.

The second objective of this study was to evaluate the efficacy of commercially available microbicides used by the sugar industry to control bacterial growth. The diversity profile of gum-producing bacteria established in this study not only provided the identities of the spoilage bacteria, but also indicated where in the process, and under which conditions, they were isolated. Correct process control, especially of high temperature streams, is critical to prevent microbial growth in the sugarcane processing factory. In this study, filtrate temperatures of 58 °C and 29 °C were recorded when sampled at times of low and high dextran concentrations in raw sugar, respectively. Filtrate temperatures are usually around 60 °C. Factory staff acknowledged that the low filtrate temperature recorded during the second sampling was due to a processing error. At this time, strains of *Le. lactis* and *Lb. fermentum* were isolated. *Lactobacillus fermentum* dominated the filtrate sample taken at the first sampling. The temperature of the mud in the mud trough at this time (35 °C) was much lower compared to the second sampling (64 °C), possibly due to stoppages and longer retention times of the mud in the trough, resulting in cooling of the mud. A considerable number of *Le. mesenteroides* strains (31% of the total number of strains isolated during the first sampling) were from mud at 35 °C. On the contrary, *Le. lactis* was the major gum-producer in the mud during the second sampling when the temperature was higher (64°C). *Le. lactis* has a higher heat resistance than *Le. mesenteroides* (Logan and De Vos, 2009). Although the filtrate is recirculated to the mixed juice tank, none of the gum-producing bacteria isolated in the filtrate were detected in the juice sampled from the mixed juice tank. This is presumably due to the high temperatures (67 °C and 73 °C, respectively) recorded for juice

samples, which allowed growth of endospore-forming *Bacillus* species, but not *Leuconostoc* and *Lactobacillus* spp. (Berendsen et al., 2016; Logan and De Vos, 2009; Warth, 1978).

The two dithiocarbamate biocides evaluated in this study (detailed in Chapter 8) showed promise to prevent the growth of the lactic acid bacteria which were tested. However, the effect of biocides on biofilm formation on juice screens was not evaluated. Future research on biocide efficacy on biofilms is important because bacterial biofilms are often more resistant to biocides compared to their planktonic cells (Bridier et al., 2011), and this study showed the presence of *B. amyloliquefaciens* and *B. subtilis* on the juice screens. These bacteria are known to form biofilms when present on surfaces (Dogsa et al., 2013; Odeniyi and Amoo, 2015). Although factories have no control over cane deterioration in the field, poor sanitation in the factory and incorrect process control can also contribute to sucrose loss due to microbial activities and subsequent gum formation. Factories should be mindful of correctly controlling high-temperature processes and reduce the recirculation of sump contents that are not treated with biocides.

The final outcome of this study was to gain knowledge on the identities of spoilage bacteria in sugarcane processing factories, to provide a foundation for the development of processes and/or recommendations on how to reduce post-burning/post-harvest deterioration of sugarcane and subsequent processing streams, and the associated revenue losses. This study was exploratory in nature, executed at one factory and during one season, with two sampling periods. The cost and time associated with the bacterial identification methods prevented the extension of the study to include more factories over more seasons, or to include various consignments of deteriorated cane, for example. This study showed that *Le. mesenteroides* is not the only/major gum-producing bacterium in sugarcane processing, and that dextran may therefore not be the only 'gum' responsible for problems in factory throughput and sugar quality. Other bacteria with the abilities to convert sucrose to a variety of metabolic products, which would be detrimental to sugarcane processing if present in high concentrations, were identified in this study. The majority of gum-producing bacteria, isolated at a time when high dextran raw sugar was produced, were found on the incoming sugarcane. Future research efforts should be directed at ensuring that good quality sugarcane enters the factory. Although it is widely recognised that a reduction in the burn/harvest-to-crush delays to an absolute minimum (preferably to 24 hours) is the most practical approach to minimising losses (Harris, 2017), the lack of the inclusion of a deterioration parameter in the cane payment formula does not incentivise stakeholders to improve operations. In-field control of spoilage bacteria, aimed at specific species identified as gum-producers, at the time of harvesting or when deposited at loading zones or cane yards, may prove an effective strategy

for reducing sucrose loss and gum-production, before the deteriorated cane with high concentration of gums reaches the factory.

In this study data were collected to identify gum-producing bacteria in a sugarcane processing factory. We have contextualised the data to provide information to sugar technologists on the specific isolated bacteria and what possible effects their presence in sugarcane processing may have, and how to mitigate these effects. It is anticipated that the knowledge gained by this study will influence sugarcane processing operations to improve sugar quality and production efficiency to ensure that maximum value can be recovered from the sugarcane crop.

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