STRAIN-SPECIFIC MOLECULAR DIAGNOSTICS FOR

***Pediococcus acidilactici*: FUNCTIONAL GENOMICS IN FOOD SAFETY**

A Thesis

by

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ABSTRACT

Commercial strains of *Pediococcus acidilactici* are incorporated into food safety cultures that are used to inhibit the proliferation of foodborne pathogens. We employed comparative genomics and molecular biology to characterize *Pediococcus acidilactici* D3, an industrial food safety strain provided by the company Guardian Food Technologies. The objective of this research was to develop strain-specific molecular probes at unique genomic targets to uniquely identify *P. acidilactici* D3. The antimicrobial activity of *P. acidilactici* D3 was examined in this study. In addition, a carbohydrate utilization profile was prepared for this strain, from which it was observed that sucrose was readily metabolized by *P. acidilactici* D3. In order to identify the genes responsible for the observed antimicrobial activity and sucrose utilization, a draft sequence of the *P. acidilactici* D3 genome was generated. The genes putatively responsible for the expression of an antimicrobial peptide (pediocin) and the sucrose utilization loci were annotated. Using a combination of the two operons as genomic targets, strain-specific probes were successfully developed and validated with quantitative-PCR.

DEDICATION

This is dedicated to my Pop, Rajendran Thangadurai.

Thank you for your inspiration to strive for excellence.

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CHAPTER I

INTRODUCTION

INTRODUCTION

Biopreservation

The most widely used traditional preservatives are the chemical preservatives propionates, sorbates, and benzoates (21, 45). Alternatively, there is a growing trend of consumer demand for natural preservatives. In 2010, 39% of consumers cited chemicals in foods as the most important food safety issue (42). Food developers are investing effort into improving the quality and shelf life of foods with natural preservatives.

Biopreservation or biologically based preservation technologies is the use of lactic acid bacteria (LAB) and their metabolic products to improve the safety and quality of foods (28). *In situ* acidification by the production of lactic acid, the production of diacetyls, hydrogen peroxide, and bacteriocins by LAB are all widely recognized methods for biopreservation. Bacteriocins in particular have been studied to be highly beneficial in inhibiting food spoilage pathogenic bacteria that are otherwise less susceptible to traditional inhibitory/food preservation techniques.

Food Safety Cultures

Last year, it was estimated that 31 major pathogens cause 9.4 million cases of food-borne illnesses in the United States alone (38). Such astonishing statistics appeals for the improvement of food safety technologies.

The microbial quality of a product plays a valuable role in the modification of several characteristics such as nutritional value, taste, texture, etc., that can influence the shelf life of foods. In addition, certain antimicrobial compound producer strains of LAB can prolong the shelf life of food products by reducing the load of food-borne pathogenic and/or spoilage bacteria (28).

Lactic acid bacteria have been used previously to control pathogens such as *E. coli* O157:H7, *Salmonella* and *Listeria*. The effectiveness of a *Lactobacillus*-based intervention strategies was studied by using a combination of 4 strains of lactic acid bacteria (Lactiguard, Guardian Food Technologies, Kansas City, MO) for the reduction of *Salmonella* in turkey products (9). Both bacteriostatic and bacteriocidal activity was observed when the competitive inhibition of *L. monocytogenes* at refrigeration (5°C) temperatures by lactic acid bacteria, isolated from commercially available ready-to-eat meat products, was studied (2).

Bacteriocins

Bacteriocins are antimicrobial proteins produced by bacteria to inhibit the growth of other species of bacteria that are competing for the same nutrients. This heterologous group of proteins varies in its size, mode of action and effectual concentration.

The genetic organization of the bacteriocin operon generally appears as a contiguous set of open reading frames, responsible for bacteriocin synthesis and transport. Genes transcribing accessory proteins for modifying or cleaving a pre-bacteriocin, and those coding for immunity proteins, which are usually membrane

proteins that prevent bacteriocins from causing self-lysis, are also a part of the bacteriocin operon.

Since proteins responsible for the modification, export and regulation of bacteriocin production are often encoded in the same operon as the bacteriocins. Bacteriocin production related genes are among those that are often transferred horizontally. Horizontal gene transfer is very common in LAB due to the presence of mobile genetic elements, insertion elements, and conjugative and mobilizable plasmids.

Pediococcus acidilactici

Pediococcus acidilactici is a gram positive homofermentive lactic acid bacteria that belongs to the family *Lactobacillus*. This species has a long history of safe use as a starter culture in mean and vegetable fermentation. A few strains of *P. acidilactici* produce bacteriocins; and these bacteriocins produced by *Pediococcus* are called pediocins (18).

Genomics

In simple terms, genomics is the study of genes and their functions. Functional genomics is the characterization of these genes and their interaction with the environment or other genes. Genome sequencing and functional genomics has provided a molecular basis for important traits in LAB such as sugar metabolism, flavor formation, stress response, etc. Bioinformatics has been an essential tool in handling and analyzing the huge volume of data generated by genomic sequencing (40).

PROJECT OBJECTIVES

We employed comparative genomics and molecular biology to characterize *Pediococcus acidilactici* D3, an industrial food safety strain provided by the company Guardian Food Technologies. The three central deliverables of the project include: (i) the generation of a partial draft genome sequence, (ii) the characterization of the phenotypic profile, and (iii) the development strain-specific molecular diagnostics to detect and quantify *P. acidilactici D3*, a component of LactiGuard.

Figure 1 describes the project outline and the approach used to attain the deliverables. The phenotypic study was used to identify functional traits: bacteriocin production, carbohydrate utilization, and exopolysaccharide production, that would later serve as gene targets for probe development: Each phenotypic study was done in comparison with related strains, including the 'Type' strain for each species of *Pediococcus*. Once these characteristics were confirmed, and determined to be exclusive targets, they were selected as genomic targets for strain-specific probe design. This included a detailed study of the operons putatively involved in the expression of these phenotypes. The *P. acidilactici* D3-encoded genes/operons were examined for single nucleotide polymorphisms (SNPs) and other larger-order genetic changes in comparison to other closely related strains. Diagnostic regions on the genome were used as targets for the design of primers/probes, which were then tested for strain-specific amplification compared to other strains. Following probe design, quantitative-PCR was developed to screen for specificity and quantification.

THESIS STRUCTURE

Chapter II (**Expression of antimicrobials by *Pediococcus acidilactici*: Functional genomics in food safety**) includes two main sections of the research: (i) strain verification and genome sequencing, and (ii) the study of antimicrobials produced by *P. acidilactici* D3. The initial verification tests were conducted to confirm the speciation of our target strain. These tests included light microscopy to observe shape and clustering, gram staining to attest that the strain was gram positive, 16S rRNA for a molecular level confirmation and. scanning electron microscopy to observe the size and shape of *P. acidilactici* D3 in closer detail. Whole genome sequencing of the strain was carried out with the Ion Torrent Personal Genome Machine. The sequence data obtained was used to explain the genomic basis for pediocin production, which was phenotypically observed by bacteriolytic zymograms. Annotation of the pediocin operon of *P. acidilactici* D3 was accomplished in this chapter.

Carbohydrate utilization studies using the OMNILOG (BIOLOG phenotypic microarray plates) and Analytical Profile Index (API) strips were conducted to characterize *P. acidilactici* D3 phenotypically in Chapter III (**Strain-specific molecular diagnostics for *Pediococcus acidilactici***). Sucrose metabolism was an interesting phenotype observed because previous work has cited plasmid-linked sucrose utilization in *P. pentosaceus* (17). Similar to Chapter II, the sucrose operon, consisting of six open reading frames, was annotated. The sucrose operon contained several single nucleotide polymorphisms which provided ideal strain-specific targets for probe design, which

concluded the final deliverable of the project – strain specific diagnostics to detect *P. acidilactici* D3. In this chapter, the method for primer/probe design is outlined. Validation of the probes by quantitative PCR (qPCR) and successive band detection on agarose gels is described. A combination of probe pairs to be used for detection of *P. acidilactici* D3 in a strain-specific manner is listed, thereby accomplishing the objectives of this research.

Chapter IV (**Summary)** briefly describes the result of each aspect of the project and summarizes the overall project conclusion.

CHAPTER II

EXPRESSION OF ANTIMICROBIALS BY *Pediococcus acidilactici*: FUNCTIONAL GENOMICS IN FOOD SAFETY

INTRODUCTION

Strains of *Pediococcus acidilactici* have long been incorporated into starter cultures designed to drive the commercial fermentation of plant materials (*e.g.*, cucumbers, olives) and meats (*e.g.*, fermented sausages, fresh and marinated fish). When used in this capacity, a few strains of *Pediococcus acidilactici* act preserve the product and to inhibit spoilage microorganisms such as gram-negative bacteria: *Escherichia* spp. (5) and *Salmonella* spp. (9, 36), gram-positive spore-formers: *Bacillus* spp. and *Clostridium* spp. (30), nonstarter lactic acid bacteria, and foodborne and feedborne pathogens: *Listeria* spp. (2, 31). Much of the preservative and protective activities are due to the expression of diffusible, low-molecular weight factors including byproducts of metabolism and antimicrobial peptides.

Ribosomally-encoded bacteriocins are antimicrobial peptides that are typically effectual against a narrow range of target microorganisms (10). A universal system for bacteriocin taxonomy, based on the foundations of previously proposed schemes (6, 23), categorizes bacteriocins into four main classes (19). Class I bacteriocins are 'lantibiotics', post-translationally modified peptides with atypical amino acids like lanthionine residues; Class II bacteriocins are 'unmodified peptides', and are subdivided into IIa: 'pediocin-like', IIb: 'miscellaneous', and IIc: 'multicomponent'. Bacteriolytic

and non-lytic 'large proteins' are Class III bacteriocins, and Class IV antimicrobials are 'cyclic peptides'. Regardless of their classification, antimicrobial factors are typically more effective in combination (synergism) (13), which parallels the hurdle concept for food safety and stability (24). Bacteriocins produced by bacteria of the genus *Pediococcus* are termed pediocins (Class IIa) and are small membrane-permeabilizing peptides (22) (**Figure 2**) that are effectual bacteriolytics at low concentrations (32) and are the subject of this study.

The pediocin-encoding operons are located on a 9.6 kb plasmid (18) and an 11.4 kb plasmid (34) in *Pediococcus acidilactici* PAC1.0 and *Pediococcus acidilactici* H, respectively. In these two strains, the four genes responsible for pediocin production and transport are: *pedA* (bacteriocin, *Uniprot: P29430*), *pedB* (immunity protein, *Uniprot: P36496*), *pedC* (protein biosynthesis, *Uniprot: P37249*), and *pedD* (transport and ATP-binding, *Uniprot: P36497*) (**Figure 3**). Although the genes for bacteriocin production are among some features found in *Pediococci* in a strain-specific fashion, these genes could serve as biomarkers for the identification of food safety strains. The utility of food safety strains, especially bacteriocin-producing strains, is highly significant and could confer an extended shelf-life and an improved spoilage-prevention strategy in consumer foods.

We studied the functional genomics of pediocin production in *P. acidilactici* D3 by studying the strain's capability to produce antimicrobial peptides after identifying the presence of pediocin producing genes in the *P. acidilactici* D3's genome. Further analysis of these genes led to the annotation of the pediocin operon.

MATERIALS AND METHODS

Bacterial Strains and Cultivation Conditions

All of the strains (12, 15, 29, 41, 43) used in this study are listed in **Table 1**. *P. acidilactici* D3 was provided by Guardian Food Technologies (Overland Park, KS). Additional *P. acidilactici* strains, and strains of related species, were also acquired from various sources to enable phenotypic, genetic, and genomic comparisons to the strain provided by Guardian Food Technologies.

All lactic acid bacteria were streak purified three times on de Man-Rogosa-Sharpe (MRS) agar (Difco Laboratories, Detroit, MI, USA) and cultivated at 37°C. Stocked cultures were maintained at —80°C in MRS broth supplemented with 20% (v/v) glycerol. Strains were passed 2X before being used.

Colony and Cell Morphology

Because a butyrous consistency might suggest the expression of complex extracellular polysaccharides (*e.g.*, exopolysaccharides like homopolysaccharides), *P. acidilactici* D3 colonies were observed for shape, size, color, margin type, and consistency. Strains were also observed under a light microscope in order to identify the shape of the cells, and determine if the cells were found singly, in pairs or tetrads.

The gram-stain was used to assay the physiochemical properties of *P. acidilactici* D3's cellular envelope, especially the abundance of peptidoglycan. The strain

Escherichia coli K-12 BW25113 was used a gram-negative control and the strain *L. amylovorus* ATCC 33620^{T} served as the gram-positive control.

Scanning Electron Microscopy (SEM)

An overnight broth culture of *P. acidilactici* D3 was observed at up to 50,000X using the TOPCON Aquila Scanning Electron Microscope (Microscopy & Imaging Center, Texas A&M University). Preparation of the broth culture for observation was carried out over four days with four main steps: sample fixing, sample dehydration, sample coating, and final imaging. Fixing the sample consisted of mixing and equal volume of the fixative (2% gluteraldehyde, 2% paraformaldehyde and 1X of minimal media buffer containing sodium acetate, potassium dihydrogen-phosphate, manganese sulphate, magnesium sulphate, ammonium citrate, and dipotassium phosphate) to the broth culture. This was followed by pelleting the cells and storing them in 1% (w/v) osmium tetroxide (OsO_4) in hydroxyethyl piperazineethanesulfonic acid (HEPES), pH 7.4. The sample was then stored at 4°C overnight. On the second day, samples were dehydrated by microwave processing with an ascending grade of methanol (10 a 100% in 5% increments), 1 or 6 minutes in each step, and finally washed with hexamethyl disitizane (HMDS) for three cycles of 30 minutes each on the rotator, and finally left overnight. The samples were then splutter coated with ruthenium vapor and finally observed under scanning electron microscope.

16S rRNA Gene Sequencing and Taxonomy

Universal primers/ oligonucleotides (Invitrogen) (**Table 2**) were selected for PCR amplification of the 16S rRNA region of the *P. acidilactici* D3 genome using a gradient cycler (Eppendorf Gradient Cycler). Each 25µL reaction contained 1.5ng/µL of template bacterial DNA, 1X (1.5mM $MgCl_2$) of Optimized DyNAzyme EXT Buffer, 200µM of each dNTP, 0.5µM of each primer and 0.5U of DyNAzyme EXT DNA Polymerase. With the primers 27F and 1522R, the PCR conditions were an initial denaturation cycle for 2 min at 94°C, 30 cycles of denaturation for 0.5 min at 94°C, annealing for 0.75 min at 56°C, and extension for 2 min at 72°C. This was followed by a final extension for 7 min at 72°C and cooling to 4°C, at which temperature the products were maintained. The PCR products were loaded on a 0.8% agarose mini gel and run at 80V. 3µL of each sample was taken, mixed with 2µL of 50% (v/v) 6X gel loading dye (New England BioLabs) in glycerol, and 1.43X of Sybergold DNA stain. The gel was visualized under a light cabinet (Alpha Innotech Multi Image Light Cabinet) under transilluminance UV.

A pure genomic DNA sample of *P. acidilactici* D3 was sent to Eton Bio for 16S rRNA sequencing to confirm taxonomy of the strain. The Basic Local Alignment Search Tool (BLAST) was used to conduct 16S rRNA sequence comparison to verify the nomenclature of *P. acidilactici* D3 as *Pediococcus acidilactici*. A homology search of the 16S rRNA sequence was performed using BlastN to determine the closest known relatives of the *Pediococcus acidilactici* D3 strain. In addition, the genomic DNA

sample was sent to Accugenix for identification of the strain based on 16S rRNA analysis with the vendor's database and primer set.

A phylogenetic tree based on 16S rRNA sequence from Accugenix for *P. acidilactici* D3 against related type strains (**Table 3**) for the species in the *Pediococcus* cluster (11) by using BLAST pairwise alignments and by applying the neighbor joining algorithm with a bootstrap analysis using 100 replicates was completed.

Whole Genome Sequencing and Bioinformatics

In order to achieve a total DNA yield of 10-20µg of high quality DNA (OD A260/280 purity ratio ~ 1.8) for sequencing, DNA extraction, precipitation and purification was optimized using a combination of the Masterpure Gram Positive DNA Purification Kit (Epicenter Biotechnologies), phenol chloroform precipitation, and the DNeasy Blood Purification Kit (Qiagen) spin protocol.

In brief, to extract genomic DNA from the gram-positive *Pediococcus* cells, 5mL of bacterial culture, grown overnight (OD ~ 2), was pelleted in a swing bucket centrifuge (Eppendorf centrifuge 5810 R). The pellet was resuspended in 750µL of TE buffer (EpiCentre Biotechnologies) and lysozyme (Ambresco) was added to give a final concentration of 30mg/mL. The solution was incubated for 3 hours at 37°C. 5µL of Proteinase K (50µg/µL) was diluted into 750 µL of lysis buffer (EpiCenter Biotechnologies) to yield a 140µg/mL enzyme concentration. This was followed by incubation for 15 mins at 67°C with intermittent vortexing every 5 mins. The samples were then cooled to 37°C and placed on ice for 5 mins. A half volume of MPC Protein

Precipitation Reagent (EpiCenter Biotechnologies) was added and vortexed for 10 secs. The proteinaceous debris was pelleted by centrifugation at 4,000 rpm for 15 mins at 4°C. An RNase blend of 5µL RNase A (EpiCenter Biotechnologies), 5 µL of Riboshredder (Epicenter Biotechnologies) and 5 µL of Purelink RNase A (Life Technologies) was added to the supernatant and incubated at 37°C for 30 mins. A 1:1 Phenol chloroform precipitation and subsequent 24:1 chloroform isoamyl alcohol wash was conducted to remove any contaminants. An equal volume of isopropanol was added to the supernatant obtained from the above steps to precipitate the DNA by inversion of the sample tubes 30-40 times. At this point, filamentous DNA was pelleted by centrifugation at 4,000 rpm for 10 mins at 4°C and the isopropanol was removed. Once the pellet was rinsed with 70% ethanol, the DNA was resuspended in 150µL of sterile deionized water. The resuspended DNA was then divided into two spin columns (DNeasy Spin Kit for Blood, Qiagen) such that each column contained 75µL of sample or ~48-57ng of DNA, and the DNeasy spin protocol was followed.

The extracted DNA was primed for sequencing by carrying out the Ion Xpress Template kit preparation protocol. Whole genome sequencing of *Pediococcus acidilactici* D3 was conducted partially using the Ion Torrent Personal Genome Machine and the Illumina GAII sequencer.

Sequencing conducted on the Ion Torrent Personal Genome Machine was performed by real-time measurement of hydrogen ions produced during DNA replication on the surface of ion spheres distributed on the 316D Ion chip. The sequencing,

including base calling, in conjunction with measurement, was carried out on the Torrent Server.

The CLC Genomics Workbench 4.7.2 software was used to assemble the partial genome sequence of *P. acidilactici* D3 by *de novo* assembly. Two runs of Ion Torrent and two runs of Illumina GXII high-throughput sequencing were carried out sequentially to increase coverage of the genome. The results of all four runs were combined and mapped against the reference genome sequence of *Pediococcus acidilactici* DSM 20284^{T} contigs: *NZ_AEEG01000001.1, NZ_AEEG01000002.1, NZ_AEEG01000003.1, NZ_AEEG01000004.1, NZ_AEEG01000005.1, NZ_AEEG01000006.1, NZ_AEEG01000007.1, NZ_AEEG01000008.1, NZ_AEEG01000009.1, NZ_AEEG010000010.1, NZ_AEEG010000011.1 and NZ_AEEG010000012.1*.

Spot-on-agar Antimicrobial Activity Assay

Samples for antimicrobial screening were prepared as described in **Figure 4**. Briefly, the supernatant of an overnight culture of *Pediococcus acidilactici* D3 was ultrafiltered in the Amicon 3K centrifugal filter unit (Millipore). The ultrafiltration cartridge separated the filtrate, containing low molecular weight compounds (<3000 Da, *e.g.* lactic acid) from the retentate, containing peptides >3000 Da (e.g. bacteriocins). Different fractions of this culture (the cell pellet, supernatant, retentate and the filtrate) were then spotted onto MRS agar plates overlaid with 9mL of MRS soft agar seeded with 500µL of an overnight culture of the pediocin-sensitive indicator strain, *Pediococcus acidilactici* DSM 20284^{T}. In order to control for the antimicrobial

properties of lactic acid (to account for acid inhibition), uninoculated control fractions (supernatant, filtrate and retentate acidified with lactic acid, made comparable to the final pH of the inoculated filtrate and retentate, respectively) was also prepared. Fractionated control suspensions were similarly assayed for antimicrobial activity. Spotted plates were incubated at 37°C overnight and then observed for zones of inhibition. If present, zones of inhibition suggest the presence of antimicrobial compound(s) that may have caused the lysis of the indicator strain.

Bacteriolytic-zymogram

In order to estimate the size of the antimicrobial(s) expressed by the strain, the retentate obtained by ultrafiltration was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Since the expected size of the bacteriocin is of the range 4,000 Da (4), the NuPage Bis Tris gel (Life Technologies) was used with the NuPage MES SDS Running Buffer (Life Technologies).

The amount of protein in the retentate was determined by the Quant-IT Protein Assay Kit (Invitrogen), methodology followed according to the given protocol. The retentate was then diluted with the uninoculated control retentate for comparable analysis with the 10ng/µL of commercial pediocin (Sigma-Aldrich).

Following electrophoresis, half the SDS gel (containing the marker, a commercial pediocin-positive control, and the retentate of *P. acidilactici* D3) was washed three times for 1 hour each and was overlaid on MRS soft agar (0.75% w/v agarose) seeded with the indicator strain, *P. acidilactici* DSM 20284^{T}. For band-size

comparison, the other half of the gel (containing the marker and the retentate of strain D3) was stained with SimplyBlue SafeStain (Invitrogen) and was then washed thoroughly with distilled water. The size of the band corresponding to the antimicrobial activity (a zone of inhibition) was used to estimate the size of the bacteriocin.

Mapping of the Pediocin Operon

The preliminary genome sequence data for *P. acidilactici* D3, generated from the Ion Torrent sequencing run, was assembled by *de novo* assembly using the CLC Bio Genomics Workbench. Sequence data was mapped using the *Pediococcus acidilactici* H plasmid pSMB74 (*GenBank: U02482.2*) as a scaffold, which is known to encode a pediocin operon. For the pediocin D3, a draft sequence of the operon was generated. Using multiple sequence alignment and comparative genomics, primers were designed in the conserved regions to amplify various regions of the *pedABCD* operon. These PCR products were then sequenced by Sanger Sequencing in order to fill gaps in the pediocin operon sequence (**Figure 5**).

It is well known that these genes encoding pediocin production share highly conserved regions within the pediocin operon. Based on sequence similarity to *P. acidilactici* MTCC 5101 plasmid pCP289 ped operon (*GenBank: GQ214404.1*), the presence of the pediocin operon was once again validated.

Genomic sequence data from Illumina GAIIx sequencing was mapped against *Pediococcus acidilactici* strain K10 pediocin operon (*GenBank: AY705375.1*). The mapped reads were extracted, reassembled by *de novo* and then once again aligned with

the *P. acidilactici* strain K10 pediocin operon. Identification of the pediocin operon in *P. acidilactici* D3 plays an important role in not only identification of the strain *P. acidilactici* D3, but also utilization of the strain in food safety cultures.

Pediocin Operon

The pediocin operon was annotated by identifying the start codons (ATG, GTG, TTG, ATT, CTG), the stop codons (TAG, TAA, TGA), the ribosome binding site (GGAG) upstream of each gene, and the -10 regulatory signal with consensus TATAA. The nucleotide sequence of the pediocin-encoding *P. acidilactici* PAC 1.0 plasmid pSRQ11 fragment was used as a reference for annotation (27).

RESULTS AND DISCUSSION

Cell and Colony Morphology

P. acidilactici D3, the *P. acidilactici* type strain DSM 20284^T and the other pediococci, including *P. pentosaceus* type strain DSM 20336^T were cocci and generally observed singly or in pairs, although a few tetrads and short chains were also perceived (20). Light microscopy captures are illustrated in **Figure 6**.

P. acidilactici D3 retained the purple-iodine complex in the gram reaction. Thus, they are gram-positive. The gram-negative assay control strain (*Escherichia coli* K-12 BW25113) stained pink/red in color (3), whereas the gram-positive assay control strain (*L. amylovorus* ATCC 33620^T) stained dark purple/blue.

When grown on an agar plate, colonies were opaque and circular in form with entire, slightly translucent margins. The colony surfaces were typically smooth and slightly convex in elevation. Interestingly, *Pediococcus acidilactici* D3 had a butyrous and viscid consistency, which might suggest the expression of complex extracellular polysaccharides (e.g., exopolysaccharides, capsular polysaccharides) (47).

Scanning Electron Microscopy

Scanning Electron Microscopy revealed that the size of each *P. acidilactici* D3 cell was approximately 700nm in diameter. In different fields, *P. acidilactici* D3 was predominantly found in singles, pairs, or clustered. Representative micrographs of *P. acidilactici* D3 are shown in **Figure 7**.

16S rRNA Identification

Taxonomic classification schemes have evolved over time. Currently, a variety of genotype-based methods are routinely used to classify microorganisms into different taxonomic groups. The 16S rRNA gene is well conserved within bacteria, including species of the same genus. Therefore, the evaluation of this gene has become a standard method for species determination.

Nomenclature for *Pediococcus* has often been revised. *P. acidilactici* DSM 20284 was proposed as the neotype or 'Type' strain for *P. acidilactici* by rejecting the previous neotype strain *P. acidilactici* ATCC 33314 (IFO 3884=DSM 20333=NCDO

1859). *P. acidilactici* ATCC 33314 was replaced by DSM 20284^T due to *P. acidilactici* ATCC 33314's high DNA homology with the strain *P. pentosaceus* DSM 20336^T (16)

The 16S rRNA sequence for *P. acidilactici* D3 is illustrated in **Figure 8**. The identification reports from Accugenix based on similarity search against their private database reported *P. acidilactici* D3 to be *P. lolli.* However, a BlastN of the Accugenix-generated 16S rRNA sequence against available databases resulted in a 99% identity and 99% coverage with *P. acidilactici* (*GenBank: AJ305320.1*) and a 98% identity with 96% coverage with *P. lolli* (*GenBank: AB362985.1*). In 2009, *P. lolli* was proposed as a new species of *Pediococcus* based on similarity of 16S rRNA gene sequencing results to *P. acidilactici* DSM 20284^T (98.2%), *P. pentosaceus* DSM 20336^T (96.9%), and *P. stilesii* LMG 23082^T (96.3%) and DNA-DNA relatedness (8). DNA-DNA relatedness between the strain *P. lolii* NGRI 0510Q^T and *P. acidilactici* DSM 20284^T and *P. pentosaceus* DSM 20336^T was found to be lower than the recommended DNA-DNA relatedness threshold for a species (70%) (49) and hence was recommended as a new species for *Pediococcus*.

However, based on previous nomenclature (7, 33) and by BlastN of the 16S rRNA sequence against the available public database, we have determined our strain to be *P. acidilactici.* The phylogentic tree depicting 16S rRNA relatedness of the Pediococci is shown in **Figure 9**.

Whole Genome Sequencing

A distribution of read lengths from the preliminary Ion Torrent run, after mapping against the reference is shown in **Figure 10**. The average read length for reads that mapped against the reference and reads that did not map against the reference was approximately 100 base pairs long. A larger volume of data, 20,979,013 reads, each of length 106 base pairs, was generated in the first run with the Illumina GAIIx sequencer. The reads were mapped against the previously assembled contigs of the type strain *P. acidilactici* DSM 20284^{T}. The assembled reference sequence length was 1,926,844 base pairs long, to which 16,546,761 Illumina reads mapped to.

Antimicrobial Activity Assay

In contrast to the uninoculated controls, the cell pellet, supernatant, and retentate after ultrafiltration, of *P. acidilactici* D3 created zones of inhibition (**Figure 11**) on a lawn of the type strain, *P. acidilactici* DSM 20284^{T}. These results suggest that one or more antimicrobial substances were produced.

As shown in **Figure 12**, the antimicrobial spot assay of *P. acidilactici* D3 on soft agar seeded with a sensitive indicator strain *P. acidilactici* DSM 20284^{T} demonstrated the efficiency of *P. acidilactici* D3 in causing cell lysis (illustrated by the zones of inhibition) over the commercial pediocin (Sigma-Aldrich).

Bacteriolytic Zymogram

The bacteriolytic zymogram demonstrated that at least one of the antimicrobials was a protein, approximately 4,000 Da in size (**Figure 13**). The approximate length of the commercial pediocin was 30-55 amino acids, or approximately 4,000 – 5,000 Da in size.

Pediocin Operon

Besides the above phenotypic studies, we have identified the potential for *Pediococcus acidilactici* D3 to produce pediocins based on sequence homology to pediocin operons on other bacteriocin producing strains.

The four genes of the pediocin operon: *pedA*, *pedB*, *pedC*, and *pedD* are shown in **Figure 14**.

We have demonstrated that *P. acidilactici* D3 causes antimicrobial activity and this is due to the production of proteins (bacteriocins called pediocins) of approximately 4Da in size. It is also likely that the pediocin operon is linked to this antimicrobial activity. Pediocin production in *Pedioccous* is unique to few strains and is an essential trait in its ability to act as a food safety culture. In assessment of our ultimate goal in producing strain-specific probes, a molecular probe within the pediocin operon region could serve the purpose of eliminating non-pediocin producing related strains and hence more easily distinguish *P. acidilactici* D3 from a mixed culture.

CHAPTER III

STRAIN-SPECIFIC MOLECULAR DIAGNOSTICS FOR *Pediococcus acidilactici*

INTRODUCTION

Previous studies explain that certain strains of *Pediococcus damnosus* cause ropiness in wine due to exopolysaccharide (EPS) production (26, 48). Integral EPS can help to bind the cell to the surface, while sloughed EPS and substrate-bound cells can be used to disrupt biofilms (37) and/or inhibit the adhesion of microorganisms involved in spoilage, pathogenesis, or both (25, 46). In addition, EPS production has been known to augment the ability of producer strains to co-aggregate with harmful microorganisms, thus presenting a barrier that could inhibit the colonization of pathogenic bacteria (35).

Many substrates are known to regulate EPS production in bacteria. For instance, sucrose is the major substrate for glycosyltransferases (39) that are necessary for the synthesis of homopolysaccharides (HoPS) (44), a desirable attribute in food-grade starter cultures. Thus, sucrose metabolism could also be an important trait linked to the efficacy of food safety cultures (14).

There are six genes, expressed as an operon, required for sucrose transport and utilization: *scrK* (fructokinase), *agaS* (α-galactosidase), *scrA* (PTS EII transport protein), *scrB* (sucrose 6-phosphate hydrolase), *scrR* (sucrose regulator), and *agl* (α-glucosidase) (**Figure 15**). The proteins encoded by these genes are responsible for the regulation of

uptake and metabolism of sucrose into the cell. The genes associated with sucrose utilization are plasmid encoded on *P. acidilactici* PAC1.0 (18) and *P. pentosaceus* (17).

Our objective in this study was to identify interesting phenotypes demonstrated by *P. acidilactici* D3 (such as sucrose utilization) and use these phenotypes to identify genes that are responsible for these phenotypes. These genes could then be used as targets for probes for strain-specific diagnosis.

MATERIALS AND METHODS

Carbohydrate Utilization

Phenotypic data from API CHL 50 strips (BioMeriux) and Biolog Phenotype Microarray (1) plates framed a platform for probing for genes involved in biosynthetic and catabolic pathways.

Phenotype Microarray Analysis

Phenotype microarrays were used in order to determine the uptake and utilization of selected sugar substrates, including sucrose. Briefly, *P. acidilactici* D3 was streak purified on MRS agar plates incubated at 37°C overnight. A sterile cotton swab was used to inoculate an individual colony into the inoculating fluid, containing minimal media, at a cell density of T=65%. 100µL of this inoculating fluid was dispensed into a 96 well PM plate, each well containing a single substrate. Utilization of the substrate was determined by a redox reaction that caused a color change in the medium, which was detected and recorded by the OMNILOG PM during incubation at 37°C for 48 hours.

Analytical Profile Index (API)

For comparison, carbohydrate utilization profiles will also be verified based on the API 50 CHL test kits (bioMérieux, France). The API CHL medium was used for the identification of lactobacilli. The procedure was followed in accordance to the manufacturer's instructions. A positive reaction was scored when a color change from blue (lack of fermentation, pH ~ 6.2) or green (pH ~ 5.2) to yellow (below approximately pH 4) at 37ºC after 48 hours of incubation was observed. A colorimetric reference (**Figure 16**) based on the pH dependent colour change of bromocresol purple (0.017% w/v), which is used in the API CHL media, was developed to standardize the scoring of API strips.

Sucrose Operon

The *P. acidilactici* D3 genomic sequences were queried for the sucrose operon genes by mapping all generated sequences (Ion Torrent and Illumina data) to the raffinose and sucrose operon of *Pediococcus pentosaceus* (*GenBank: L32093.1*). The mapped sequence reads were then extracted and reassembled by *de novo* assembly. BlastN of each contig generated with the *P. pentosaceus* reference determined the location of each contig on the operon.

The sucrose operon was annotated by identifying the start codons (ATG, GTG, TTG, ATT, CTG), the stop codons (TAG, TAA, TGA), the ribosome binding site (GGAG) upstream of each gene, and the -10 regulatory signal with consensus TATAA

using the CLC Genomics Workbench. The nucleotide sequence of the *P. pentosaseus* raffinose and sucrose operon was once again used as a reference for annotation.

Probe Development

To conclude our research, the final deliverable of strain-specific diagnostics was initiated. Previously, we identified pediocin production and sucrose utilization as key phenotypes of interest. These two features are distinguishing to *P. acidilactici* as typically, *P. acidilactici* does not ferment sucrose and only a few strains produce pediocins.

Once the sequence data was assembled, each operon of interest (pediocin operon and the sucrose utilization operon, in particular) was identified by mapping the *P. acidilactici* D3 sequence reads to similar operons/genes/sequences submitted into the NCBI (National Center for Biotechnology Information) database up to that time. The genomic DNA sequence reads of *P. acidilactici* D3 were extracted after mapping against a reference. These sequences were then re-assembled by *de novo* assembly into contigs, which were mapped again to the reference. The putative regulatory signals (-35, -10 promoter regions), ribosome binding sites, start codons, stop codons, and restriction sites in the operons were predicted. After annotation of the operons, regions with unique nucleotide changes were used to design forward and reverse primers (approximately 18-22 bases in length); PCR conditions for these primers were optimized and tested on *P. acidilactici* D3 and closely related strains. Since the goal of the primers/probes was to facilitate strain-specific identification, the assembled sequences were examined for

single-nucleotide polymorphisms and larger-order differences in sequence. Thereafter, a variety of primer pairs were used to preferentially detect *P. acidilactici* D3 and thereby constrain the PCR amplification.

SNP Detection

SNPs or single nucleotide polymorphisms are single nucleotide base differences from reference sequences. Once the sucrose operon for *P. acidilactici* D3 was assembled and annotated, the operon was aligned with the raffinose and sucrose operon of *P. pentosaceus* (*GenBank: L32093.1*). Conflicting bases were identified by means of the SNP detection tool in the CLC Genomics Workbench software. The SNP detection table, containing information about the position of the SNP in relation to the reference, the frequency of occurance of the SNP across all the generated reads, the number of reads containing the SNP, the base pair change (and any allele variations, if present) and coverage (the number of reads at the particular SNP region) was generated. SNPs with frequencies ≥ 95% (counts of reads with the SNP by the total coverage) were chosen as regions for probe design (**Figure 17**).

Primer Design

For the unique identification of *Pediococcus acidilactici* D3 by the isolation of DNA (from a product supplemented with the strain, for example) and subsequent qPCR detection with molecular probes, the probes were designed at two operons, the pediocin operon and the sucrose operon, which either provided constraints to enable unique

detection, or contained strain-specific changes in the genome, which together, identified *P. acidilactici* D3 when compared to other related strains.

Regions containing SNPs were identified and used to design primers. Forward and reverse primers (with an annealing temperature between 58.5°C to 60°C) were designed to amplify an approximate 500 base pair region, with at least one SNP in either the forward or reverse primer. In addition, primer probes were redesigned to ensure specific binding by choosing primers with SNPs on the 3' end of the primer. In **Figure 18**, screenshots of probe design on the CLC Genomics Workbench is illustrated. The highlighted regions on the consensus show SNPs in comparison to the *P. pentosaceus* raffinose and sucrose operon.

Quantitative-Polymerase Chain Reaction and Gel Electrophoresis

Quantitative-Polymerase Chain Reaction (qPCR) was optimized for the selected probes with the final conditions as shown in **Figure 19**. With each probe pair, the qPCR run was repeated at least twice, with triplicate samples in each run, for each of the previously selected related strains of *P. acidilactici* D3 (**Figure 20**). Detection was executed on the Bio-Rad iQ5 Real-Time PCR Detection System with the Fast SYBER Green Master Mix (Applied Biosystems). Each 20µL reaction tube of the 96-well PCR contained 10µL of the Fast SYBER Green Master Mix (2X), 1ng of template, 0.75-1µL of 10pmol forward and reverse primer working solution, and make up to 20µL with nuclease-free water. The plate was centrifuged briefly before each run. Agarose gel

electrophoresis of the samples was carried out after each run to confirm amplification of the targeted 500bp region.

RESULTS AND DISCUSSION

Carbohydrate Utilization

The phenotypic study of the effect of various substrates and conditions on the growth of *P. acidilactici* D3 is shown in **Figure 21**. The array shown in the figure is a collection of growth signals using the BIOLOG protocol A and C1, the BIOLOG phenotype microarray plates P1 and P2, and the API strip trials 1 and 2.

API Strip Analysis

P. acidilactici D3 metabolized a wide range of carbohydrates using the API strip analysis. Pentoses (such as L-arabinose, ribose and D-xylose), hexoses (such as galactose, glucose, fructose, and mannose), modified hexoses (such as *N*-acetyl glucosamine) and disaccharides (like cellobiose, saccharose, trehalose and gentibiose) all showed a positive signal. As per the API CH 50 identification table, *P. acidilactici* does not ferment sucrose. However, *P. acidilactici* D3 did metabolize sucrose (D-saccharose) within 24 hours. This positive result was likely due to the presence of the sucrose operon in the genome of *P. acidilactici* D3.

Differences in fermentation pattern were also observed as a function of time (24h vs. 48h). Of the 49 sugars tested for their ability to be fermented by *P. acidilactici* D3 and *P. acidilactici* DSM 20284^{T} strain, 14 sugars (L-arabinose, D-ribose, D-xylose, D-

galactose, D-glucose, D-fructose, D-mannose, *N*-acetyl glucosamine, arbutine, esculine ferric citrate, salicine, D-cellobiose, D-saccharose, gentibiose) showed a positive fermentation profile for both strains after a 48 hour incubation. The sugars D-trehalose and D-tagatose exhibited an intermediate color standard (green) when fermented by *P. acidilactici* D3. In contrast, *P. acidilactici* DSM 20284^{T} fermented both sugars within 24 hours.

BIOLOG Phenotype Microarray Analysis

Comparable to the results of the API analysis, several carbohydrates that showed a positive signal using the API strips also exhibited a positive signal with the BIOLOG PM analysis. The well containing sucrose had a high growth signal, once again suggesting the expression of the sucrose operon. In addition, *P. acidilactici* D3 displayed strong (signal >100) metabolism of compounds such as dihydroxy acetone, tween 40, and pectin.

Sucrose Operon

Five contigs (**Figure 22, panels A-E**) were generated from de novo assembly of the extracted reads (initially mapped to the *P. pentosaceus* sucrose and raffinose operons). In contrast to the *P. pentosaceus* sucrose and raffinose operons that are contiguous with each other, *P. acidilactici* D3 contains only the sucrose operon, as illustrated in **Figure 23**. Contig 5 is represented in the figure because it was the longest contig and contained genes *scrK* to the initial sequence of the *agl* gene. The green

(forward) and red (reverse) lines show millions of reads that were generated from Illumina and Ion Torrent sequencing data.

The sucrose operon for *P. acidilactici* D3 was annotated (**Figure 24**). It consists of six contiguous genes, of with the three upstream genes are reverse. The genes *scrK*, *agaS*, *scrA* are reverse on the plus strand. The genes *scrB*, *scrR*, and *agl* are forward on the minus strand. **Figure 25** represents a consolidated sucrose operon of *P. acidilactici* D3.

Verification by Amplification

When PCR was conducted for *P. acidilactici* D3 and related strains for amplification of the *ped*A-B region on the pediocin operon, it was found that several strains were eliminated from the screening process. These pediocin probes could therefore be used to specifically target *P. acidilactici* D3. However, the strains *P. acidilactici* PS and *P. pentosaceus* DSM 20336^{T} also showed amplification at this region. Since the pediocin operon is highly conserved among strains, the sucrose operon, containing SNPs was chosen as a second target to constrict the molecular diagnostic to *P. acidilactici* D3.

Quantitative - PCR amplification of *P. acidilactici* D3 with probe pair 'pedjmsF' and 'pedjmsR' is shown in **Figure 26**. The experiment was done in technical triplicates. 1 ng of genomic DNA was amplified for all three replicates of *P. acidilactici* D3. This concluded that the primer pair could be used as a probe for our strain. At cycle 30, the non-template control (light green) started showing non-specific amplification which

could be due to the production of primer-dimers. Similarily, **Figure 27** depicts the results of qPCR for *P. acidilactici* D3 using the probes pair '1SUCD3F' and '1SUCD3R'. Using this probe pair, all replicates of our strain showed amplification, signifying optimal qPCR conditions.

We expected the related strains to behave similar to the non-template control to validate strain-specificity of the probe. When qPCR was conducted for the related strains, only the three replicates for reactions containing the *P. acidilactici* D3 template showed amplification up until cycle 30 (**Figure 28)**; therefore verifying strain-specificity.

The products of amplification were observed on agarose gel electrophoresis as distinct band of size 500bp. The combination of probes was determined to specifically identify *P. acidilactici* D3 from related strains. **Figure 29**, Panel A, describes the PCR products generated using primers 'pedjmsF' and 'pedjmsR' for *P. acidilactici* D3 and related strains. When the pediocin operon was used as a target, amplification was observed in a few related strains due to high sequence conservation of the pediocin operon. In Panel B**,** PCR products generated using primers '1SUCD3F_N' and '1SUCD3R' for *P. acidilactici* D3 and related strains is shown. *P. acidilactici* D3 showed amplification when both the pediocin and sucrose probe targets were used. In contrast, the related strains showed specific amplification, represented by a bright band on the gel, for either the pediocin target, sucrose target, or neither target.

Based on the above data, the diagnostic probes chosen for strain-specific diagnosis of *P. acidilactici* D3 is shown in **Figure 30**.

CHAPTER IV

SUMMARY

Biopreservatives not only prevent spoilage and extend the shelf life of a product, but they are also perceived as a natural and healthier option to chemical preservatives. The food safety bacterial culture *P. acidilactici* D3, provided by Guardian Food Technologies, was determined to produce antimicrobial peptides called pediocins, demonstrated by both phenotypic and genomic studies. These pediocins are effective antimicrobials, previously identified to inhibit the growth of food spoilage bacteria.

In order to develop molecular probes to specifically identify *P. acidilactici* D3, whole genome sequencing was carried out. The combination of Ion Torrent Next Generation Sequencing and Illumina Sequencing provided sequence data to probe for unique genes that related to characteristic phenotypes such as bacteriocin production and sucrose utilization. Sucrose utilization was identified by carbohydrate utilization studies, including phenotype microarrays, a high-throughput method to identify possible phenotypes of interest.

Single nucleotide polymorphisms on the genome of *P. acidilactici* D3 were used to design probes in unique regions of the genome (verified by comparative genomic analysis). Amplification of target genes were confirmed by qPCR and PCR and compared to strains related to *Pediococcus acidilactici*. The methodology followed in this project resulted in the successful identification of strain-specific probes, unique to the strain *P. acidilactici* D3.

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damnosus and Oenococcus oeni strains isolated from wine and cider. International Journal of Food Microbiology **98:**53-62.

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Species	Strain	Relevant Information	Source
Pediococcus acidilactici	ATCC 12697	-	ATCC[a], Duong Culture Collection
	ATCC 8042	-	ATCC[a]
	ATCC 8081	-	ATCC[a]
	D3	-	Guardian Food Technologies
	DSM 20284^T	Type strain	DSMZ[b]
	PS	-	Sturino Culture Collection
Pediococcus pentosaceus	ATCC 25744	-	ATCC[a]
	ATCC 33314	-	ATCC[a]
	DSM 20333	-	DSMZ[b]
	DSM 20336^T	Type strain	DSMZ[b]
Pediococcus stilesii	DSM 18001	Type strain	DSMZ[b]
Lactobacillus amylovorus	ATCC 33620^T	Type strain	ATCC[a]
Escherichia coli	K -12 BW25113	-	Sturino Culture Collection

Table 1. List of strains used in this study. [a]ATCC: American Type Culture Collection, [b]DSMZ: German Collections of Microorganisms and cell cultures.

Primer Name	Primer Sequence (5´-3´)	T_m (°C)	
		Basic	Base-Stacking
27F	AGAGTTTGATCCTGGCTCAG	52	54
R518 (R)	ATTACCGCGGCTGCTGG	52	58
LPW290	ATTACCGCGGCTGCTGG	53	57
SP4	CTCGTTGCGGGACTTAAC	50	53
M920F	AAACTCAAAGGAAATTGACGG	49	52
1522R	AAGGAGGTGATCCAACCGCA	54	58

Table 2. List of primers used for 16S rRNA region amplification

Species	Strain	GenBank Accession
Pediococcus acidilactici	D3	-
	DSM 20284^T	AJ305320.1
Pediococcus pentosaceus	DSM 20336^T	AJ305321.1
Pediococcus stilesii	LMG 23082^T	AJ973157.1
Pediococcus claussenii	DSM 14800^T	AJ621555.1
Pediococcus inopinatus	DSM 20285^T	AJ271383.1
Pediococcus damnosus	DSM 20331^T	AJ318414.1
Pediococcus parvulus	-	D88528.1
Pediococcus cellicola	Z-8	AY956788.1
Pediococcus etanolidurans	Z-9	AY956789.1

Table 3. List of Pediococcus cluster type strains used for neighbor joining tree analysis and their GenBank Accession numbers

APPENDIX II

FIGURES

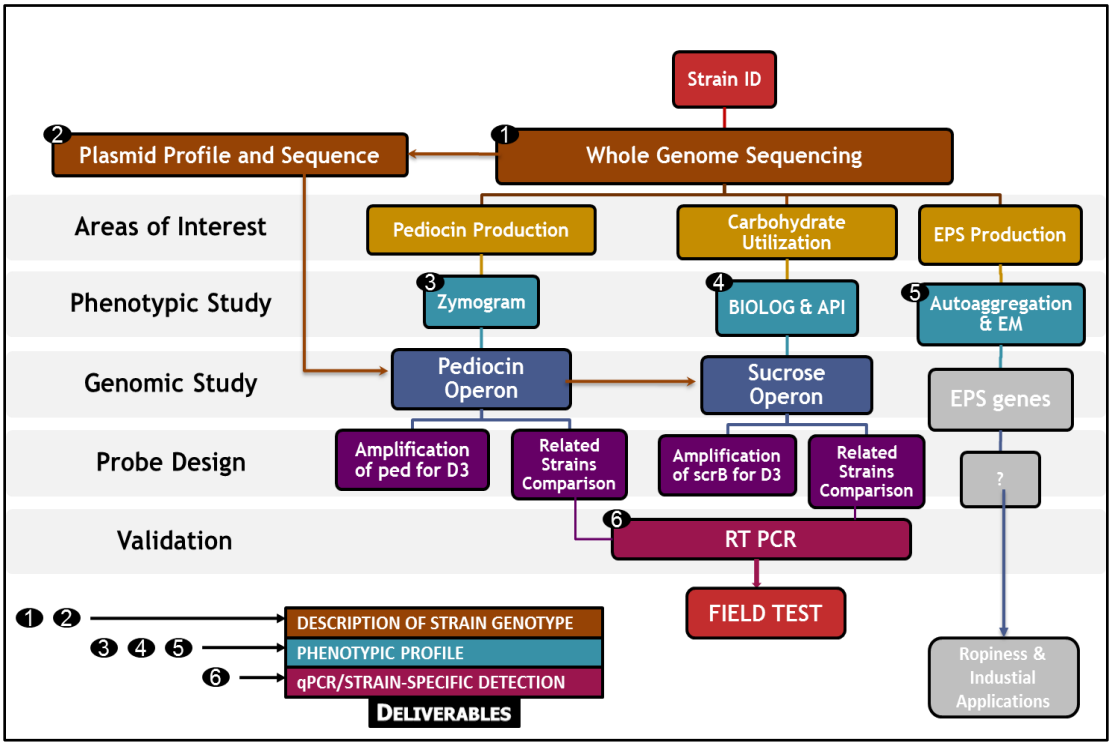


Figure 1. Project outline and description of deliverables.

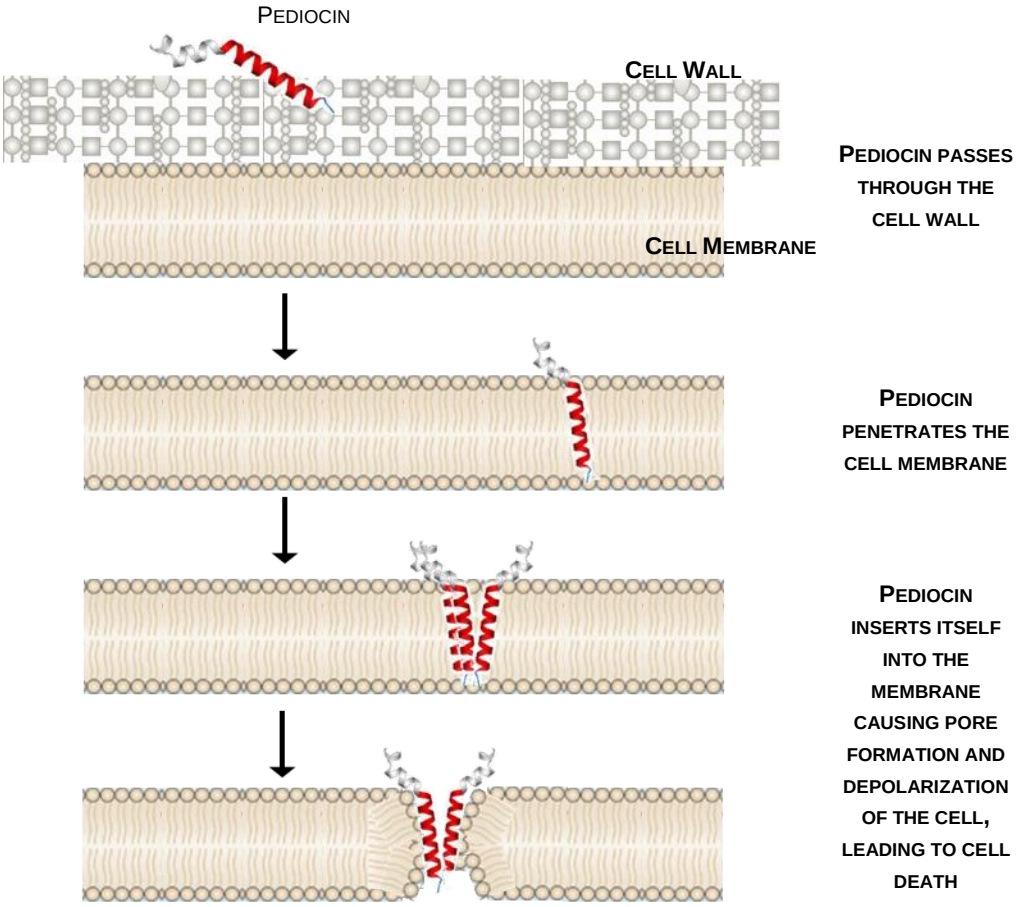


Figure 2. Schematic of the mode of action of pediocin. In the figure, the pediocin peptide is shown as the helix, the N-terminus in gray and the C-terminus in red.

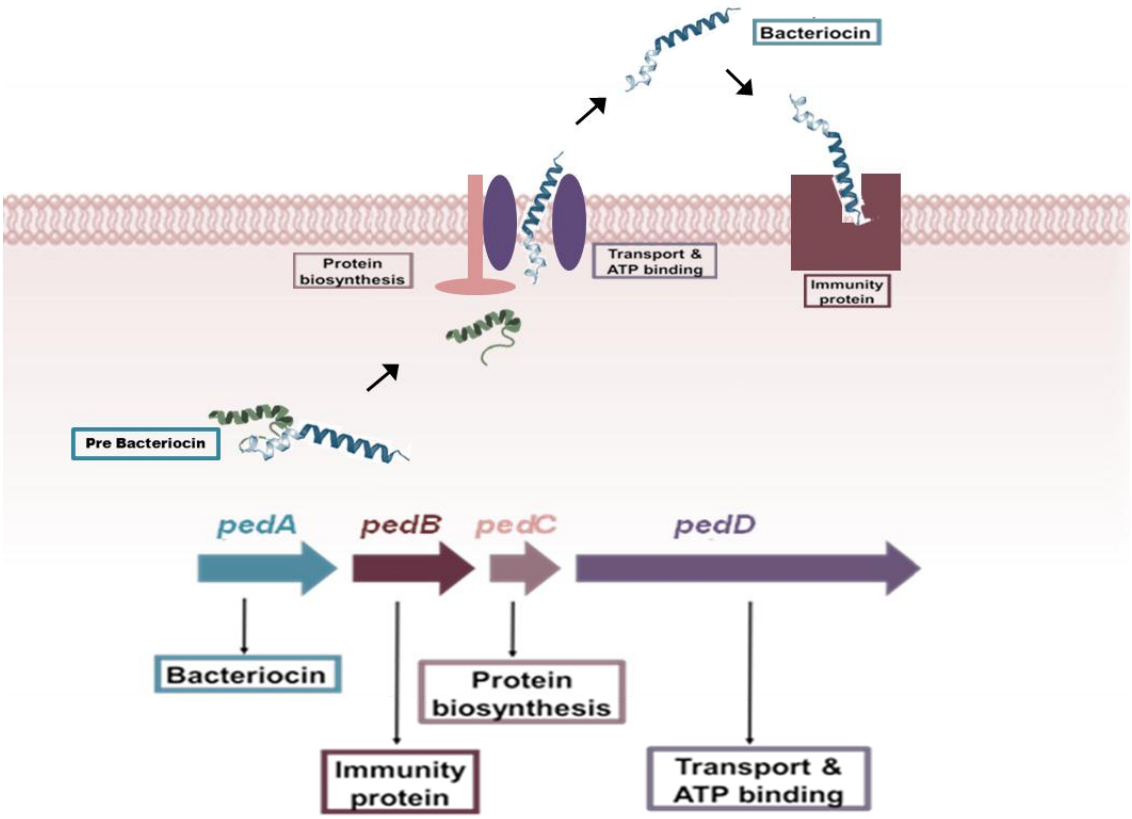


Figure 3. The pediocin operon. It consists of four open reading frames coding for four genes involved in pediocin biosynthesis, transport and antimicrobial immunity.

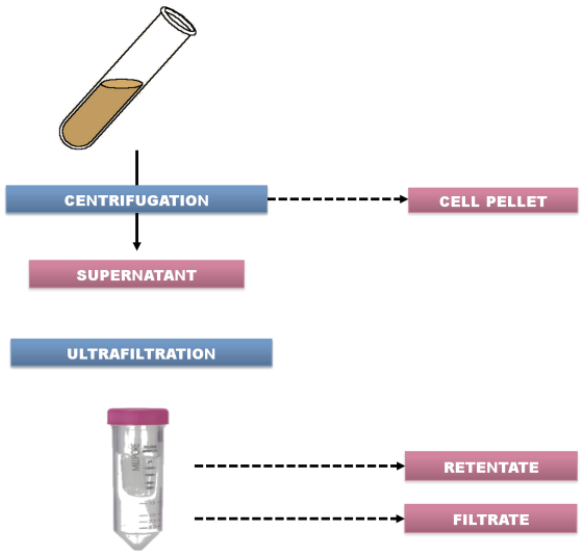


Figure 4. Schematic depicting the preparation of samples for the spot-on-agar antimicrobial activity assay and bacteriolytic zymograms.

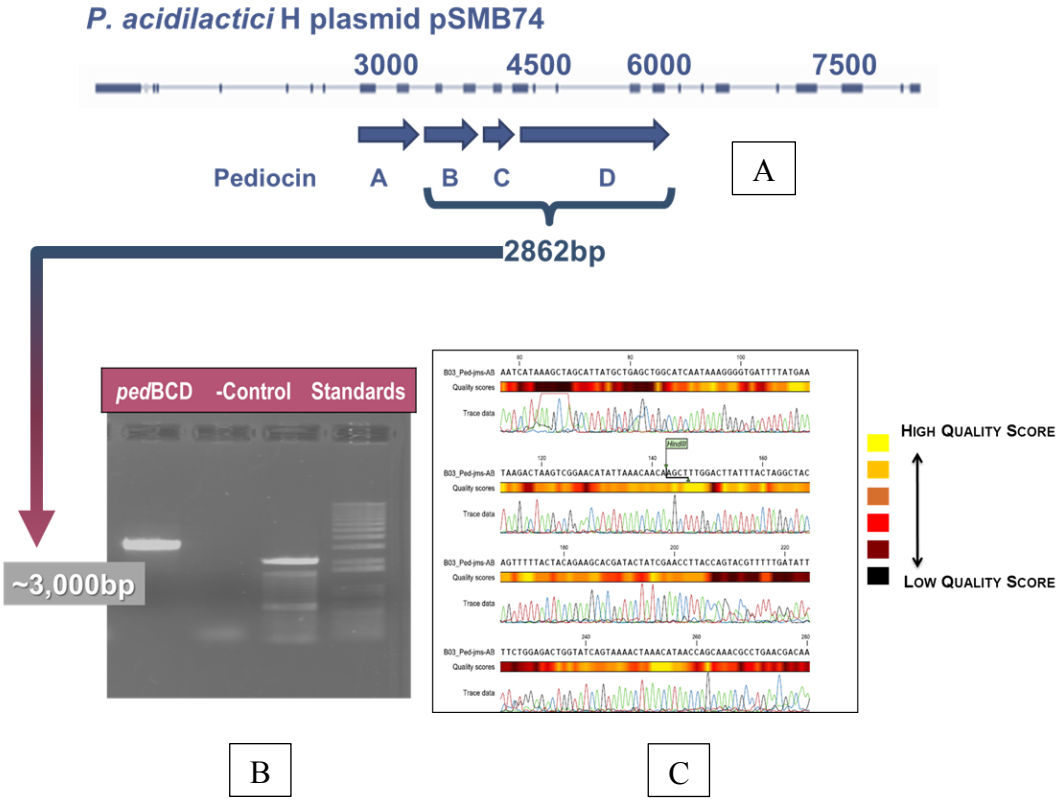


Figure 5. Sequence alignment and gene confirmation. Panel A. A sequence alignment of Ion Torrent reads with the *P. acidilactici* H plasmid pSMB74 revealed the presence of the pediocin operon. **Panel B.** In this example, the region *ped*BCD, which had an expected size of 2,862 bp was amplified. The product was run on an agarose gel and the band size of approximately 3,000bp was observed. **Panel C.** The amplified region was then sequenced by Sanger sequencing.

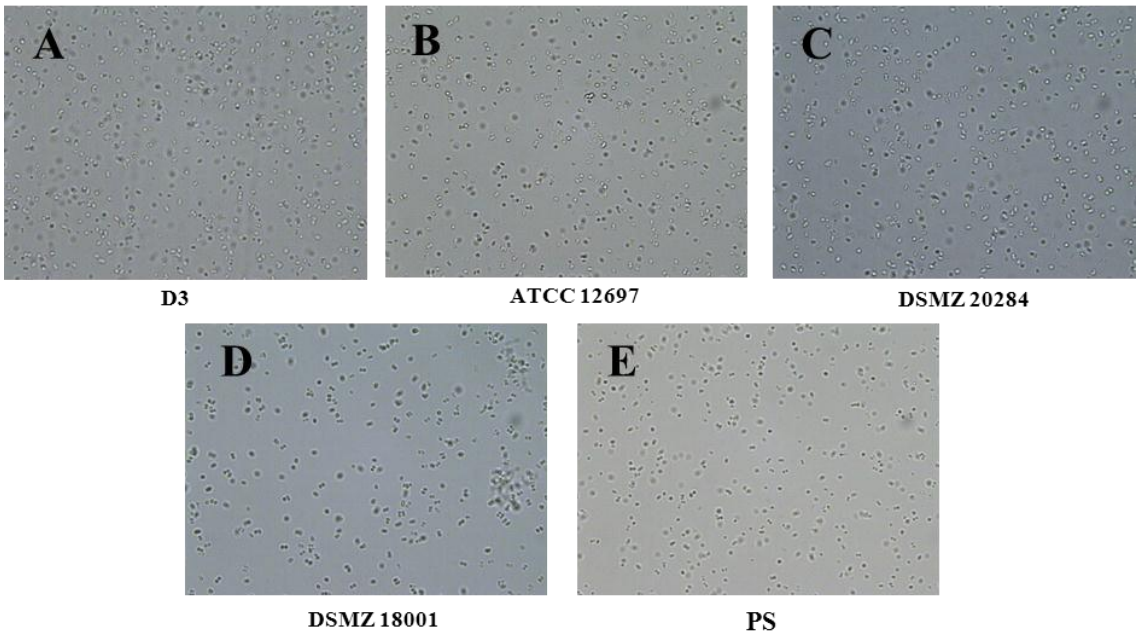


Figure 6. Light microscope captures of few Pediococcus strains
Panels A, B, C and E are *P. acidilactici*. Panel E is *P. stilesii*. Samples were unstained and fields were at 100X magnification

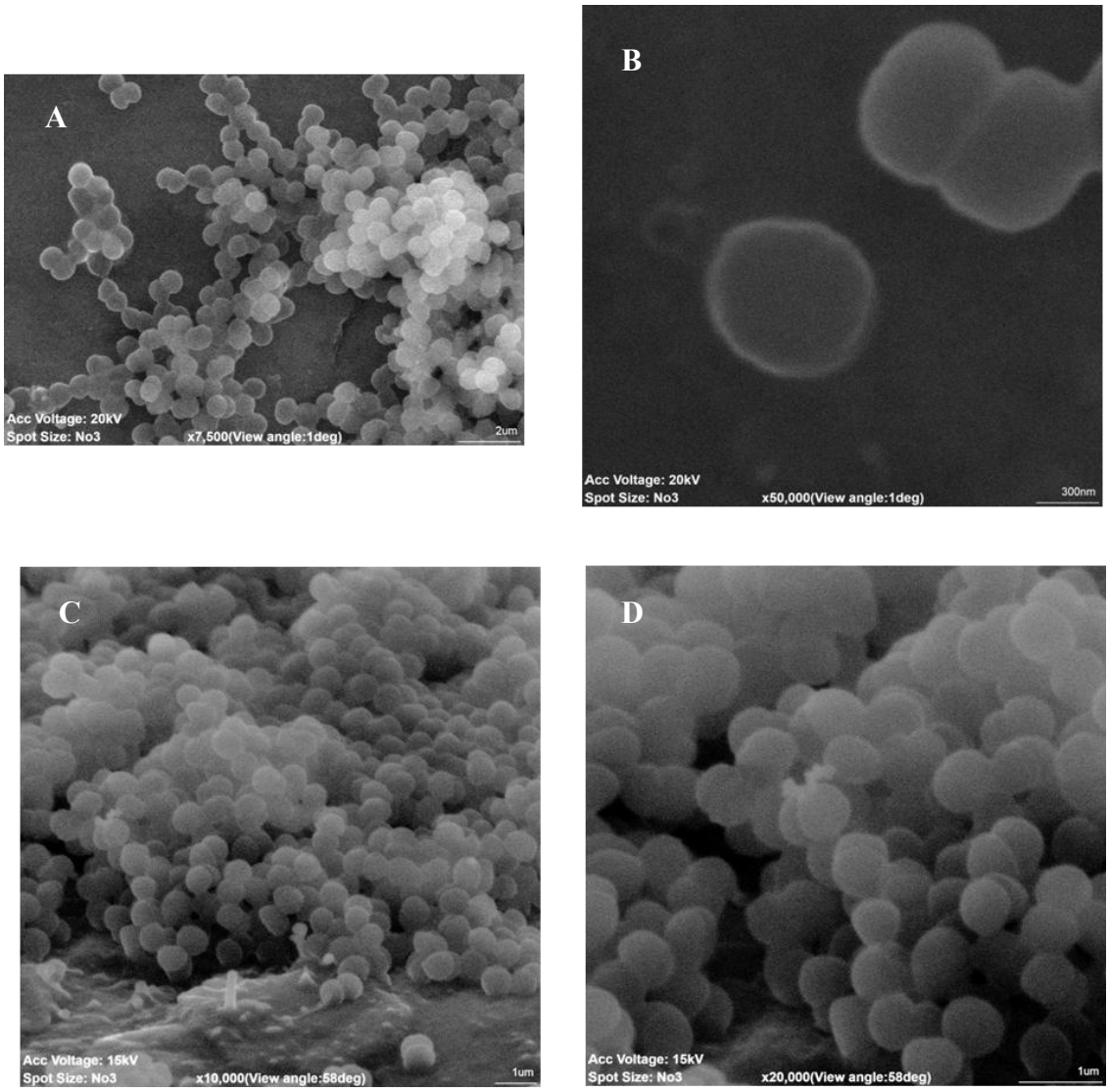


Figure 7. Scanning Electron Microscopy. Panels A-D, representative fields of *Pediococcus acidilactici* D3.

Figure 8. 16S rRNA Sequence for *Pediococcus acidilactici* D3

Red highlighted sequence indicates 5F primer sequence and green highlighted sequence indicates 1540R primer sequence.

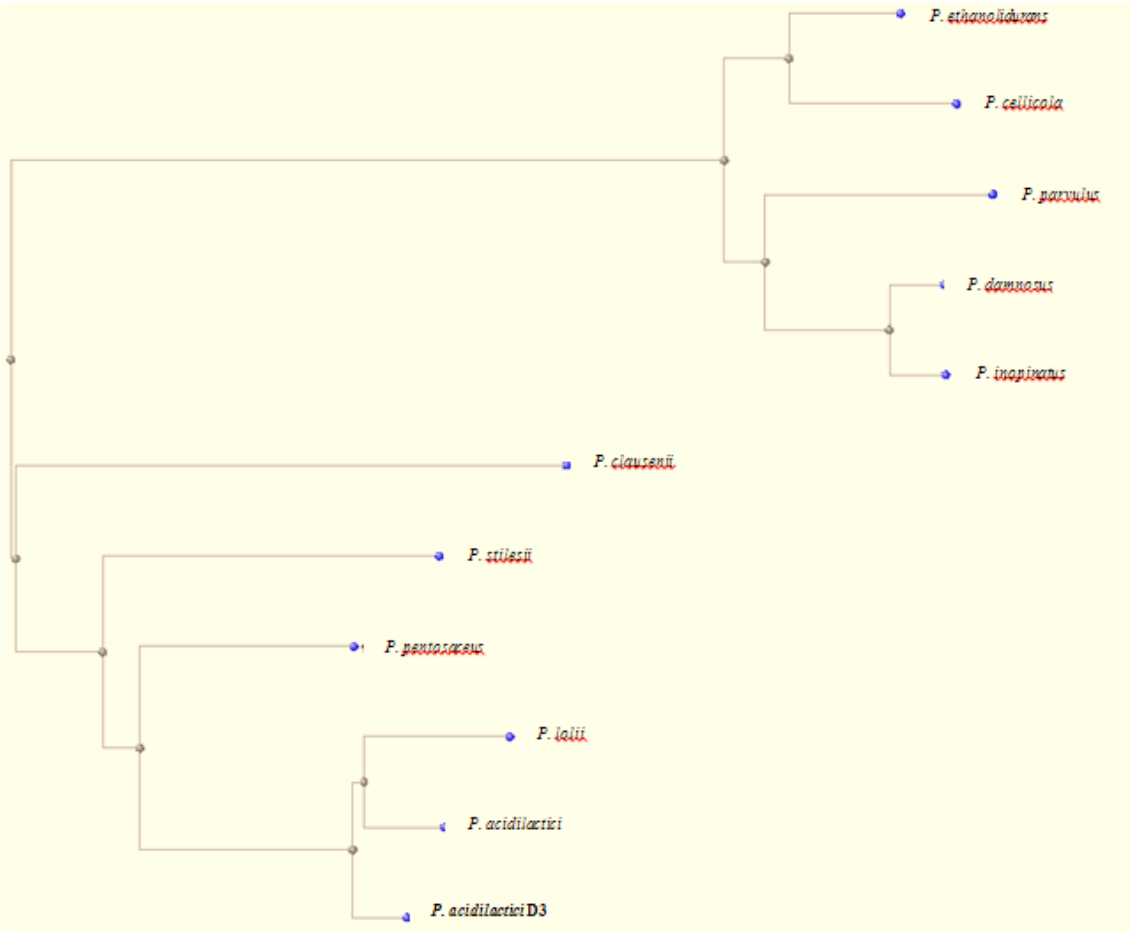


Figure 9. Tree view by neighbor joining method for Pediococcus cluster type strains and *P. acidilactici* D3.

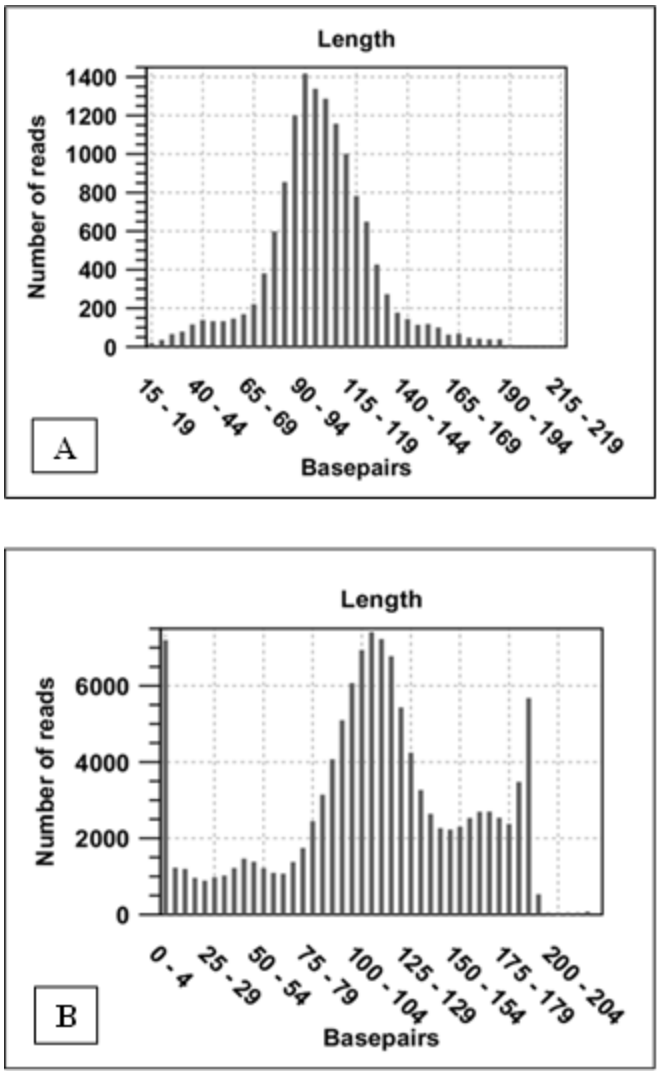


Figure 10. Distribution of read lengths

Panel A, distribution of read lengths that matched the reference and Panel B, distribution of read lengths that did not match the reference.

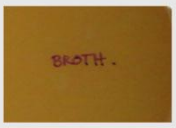
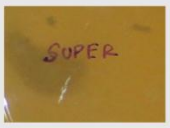
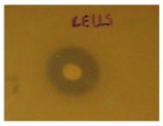
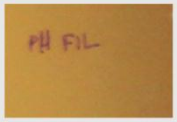
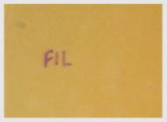
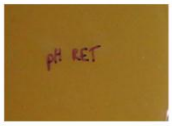
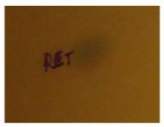
Fraction Tested	MRS (uninoculated)	*P. acidilactici*
SUPERNATANT	– 	+ 
CELL PELLET		+ 
FILTRATE	– 	– 
RETENTATE	– 	+ 

Figure 11. Antimicrobial spot assay with various ultrafiltration fractions. Spot assays demonstrate no antimicrobial activity in the uninoculated MRS media and *P. acidilactici* D3 filtrate. In the inoculated fraction (supernatant, cell pellet and retentate) a zone of inhibition (antimicrobial activity) is observed.

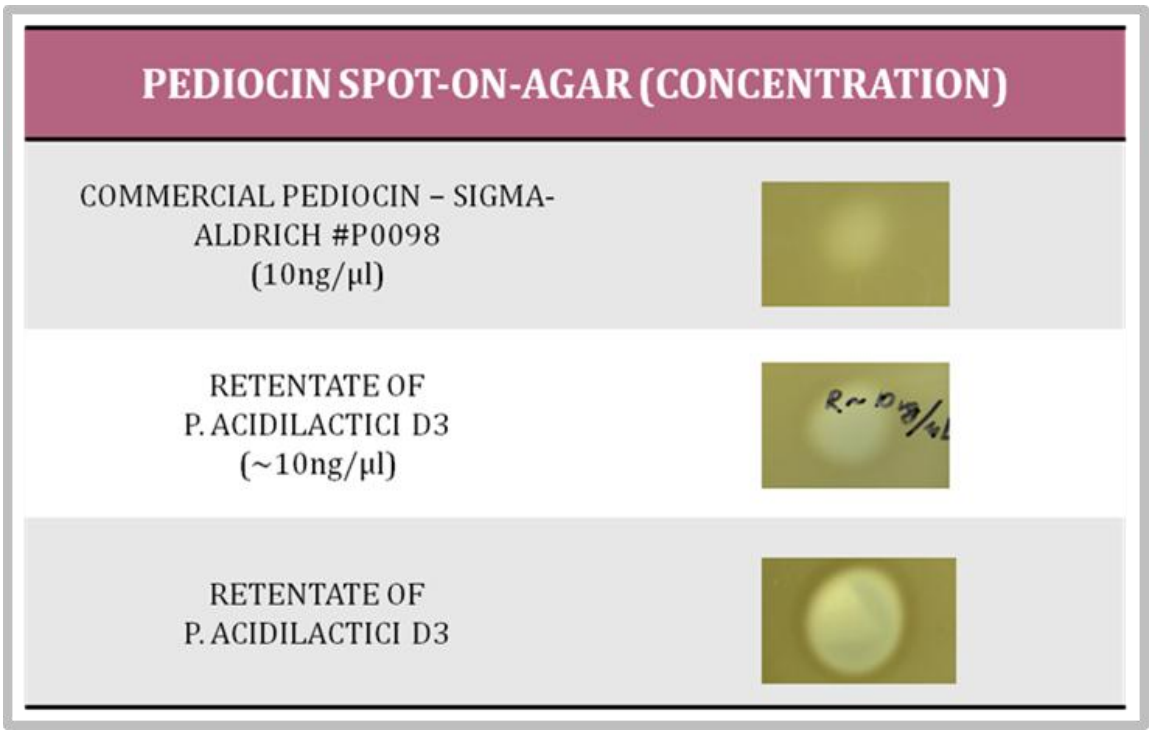


Figure 12. Bacteriocin spot-on-agar assay demonstrating relative bacteriocin activity by *Pediococcus acidilactici* D3. Bacteriocin spot assay of *P. acidilactici* D3 on soft agar seeded with a sensitive indicator strain *Pediococcus acidilactici* DSM 20284^{T} demonstrated the efficiency of *P. acidilactici* D3 in causing cell lysis (illustrated by zones of inhibition) over the commercial pediocin (Sigma-Aldrich).

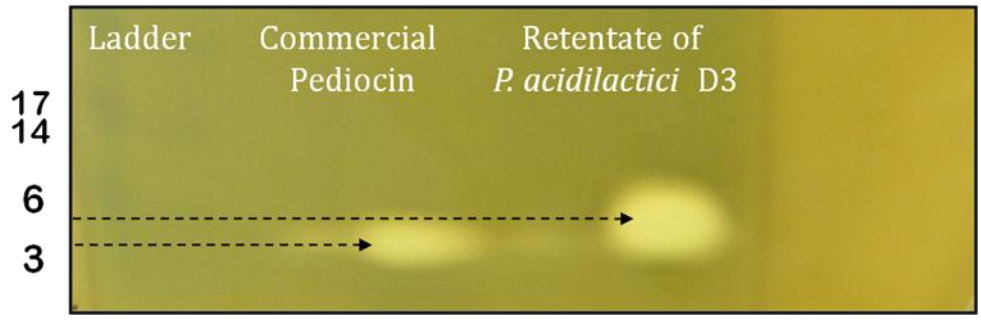


Figure 13. SDS-PAGE and bacteriolytic zymogram demonstrating relative size of bacteriocin produced by *Pediococcus acidilactici* D3. SDS-PAGE was used to resolve the proteins (> 3,000 Da) present in the *Pediococcus acidilactici* D3 culture retentate into separate bands. The size of the band corresponding to antimicrobial activity on a bacteriocin-sensitive indicator was determined to be ~4,000 Da.

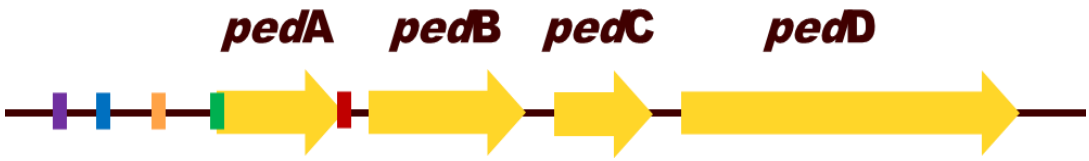
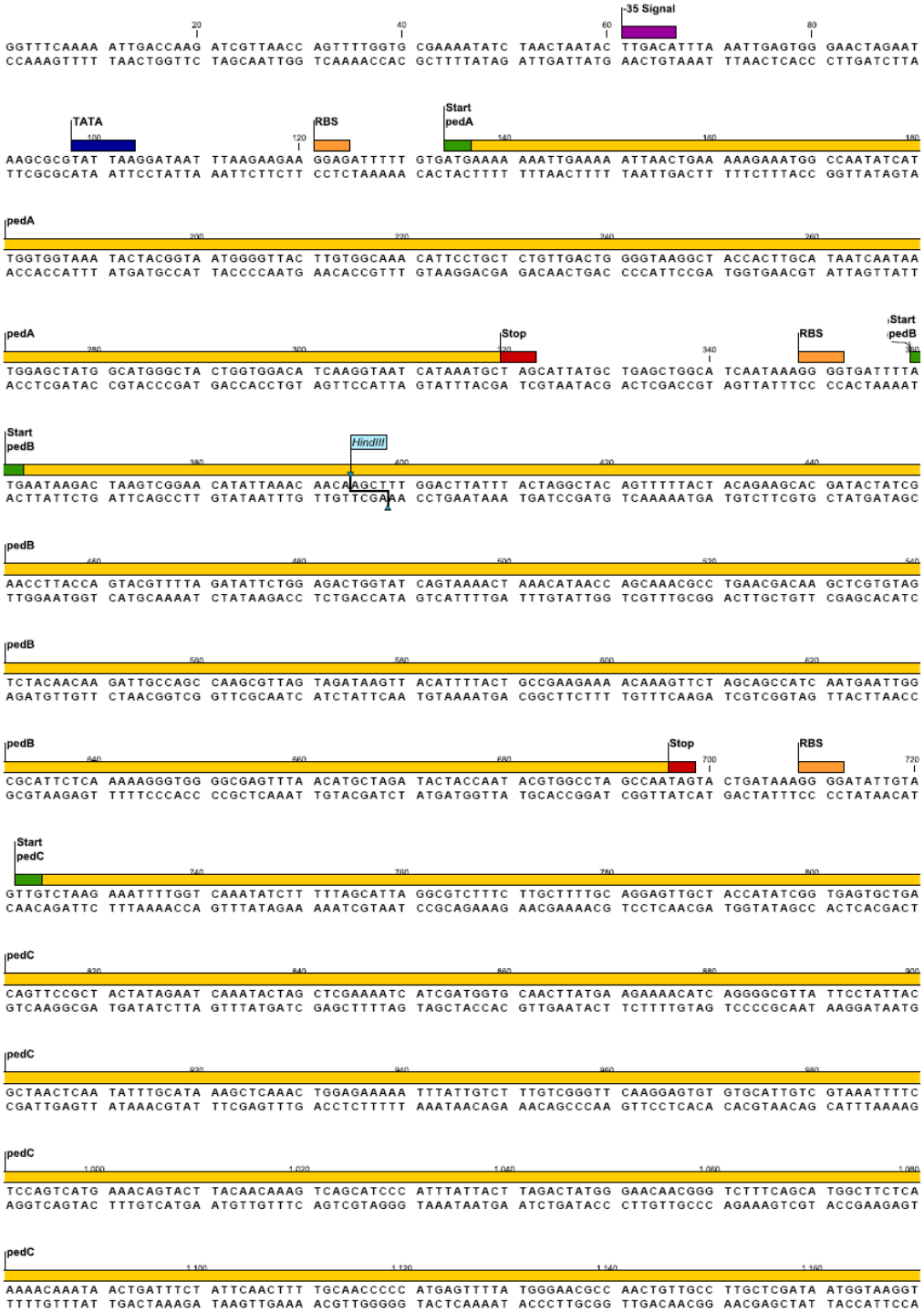
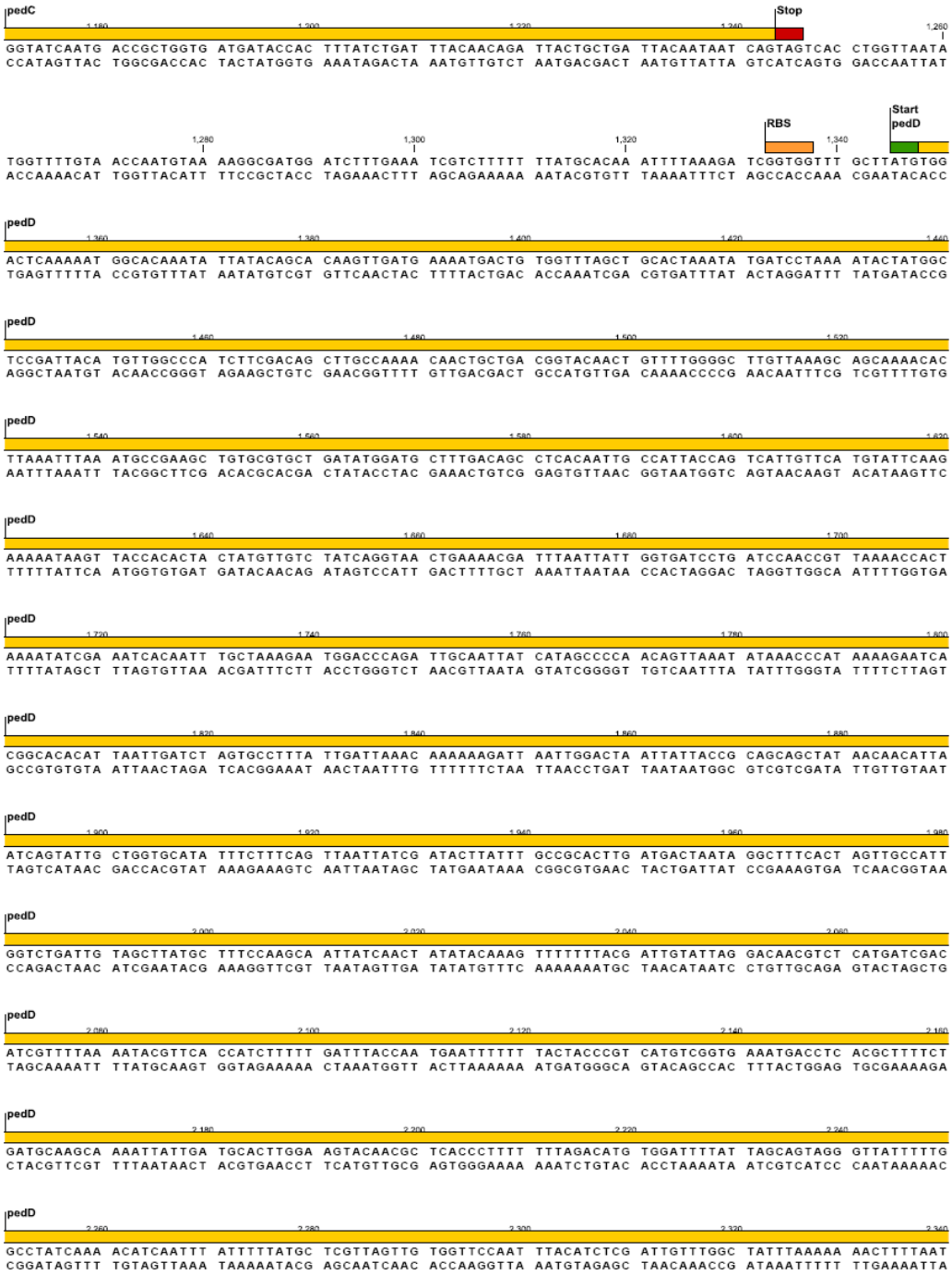


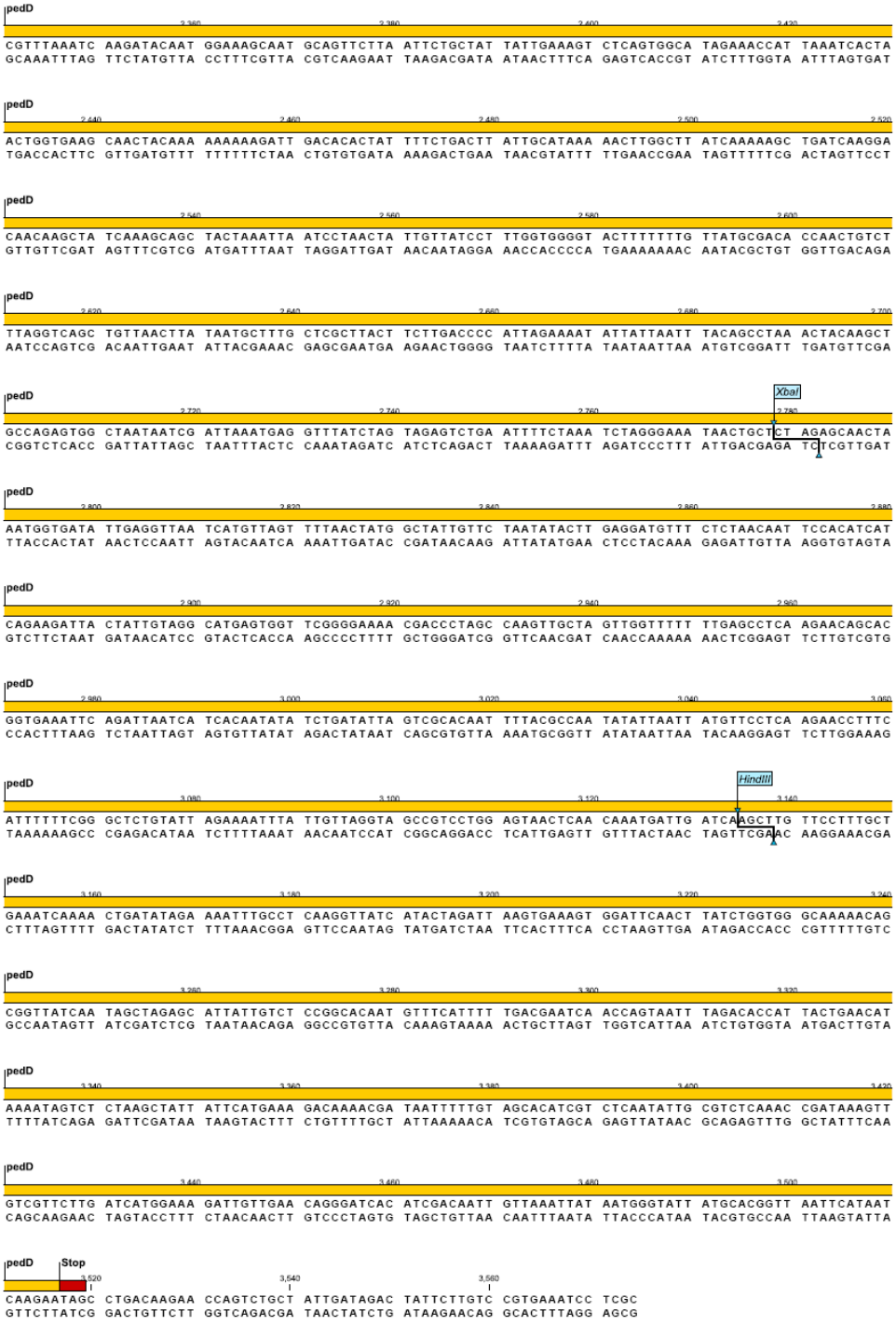
Figure 14. The pediocin operon of *Pediococcus acidilactici* D3. It consists of four open reading frames coding for four genes involved in pediocin biosynthesis, transport and antimicrobial immunity. The upstream -35 signal (purple), the -10 (blue), the ribosome binding site (RBS), the start codon (green), and the stop codon (29) are shown for the gene *ped*A.



Continued: Figure 14. The pediocin operon of *Pediococcus acidilactici* D3.



Continued: Figure 14. The pediocin operon of *Pediococcus acidilactici* D3.



Continued: Figure 14. The pediocin operon of *Pediococcus acidilactici* D3.

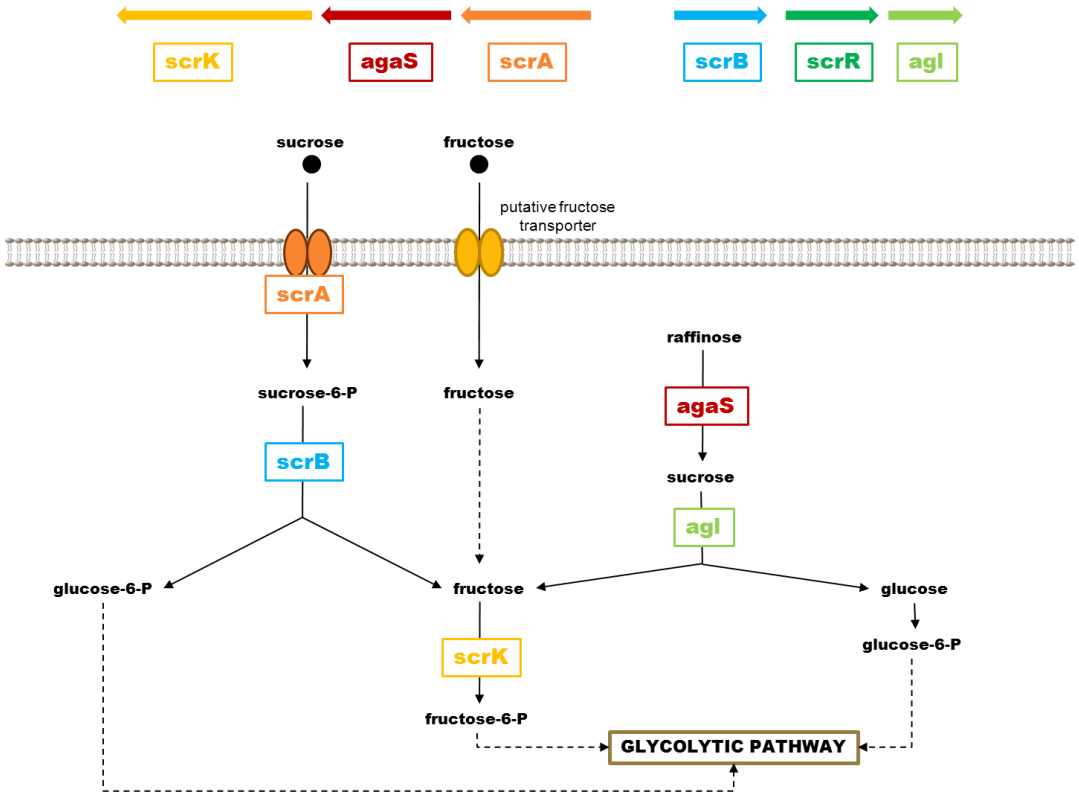


Figure 15. Sucrose operon and metabolic pathway. There are six open reading frames in the sucrose operon and each open reading frame codes for a gene that acts in the metabolic pathway for the uptake and degradation of sucrose, ending in the glycolytic pathway.

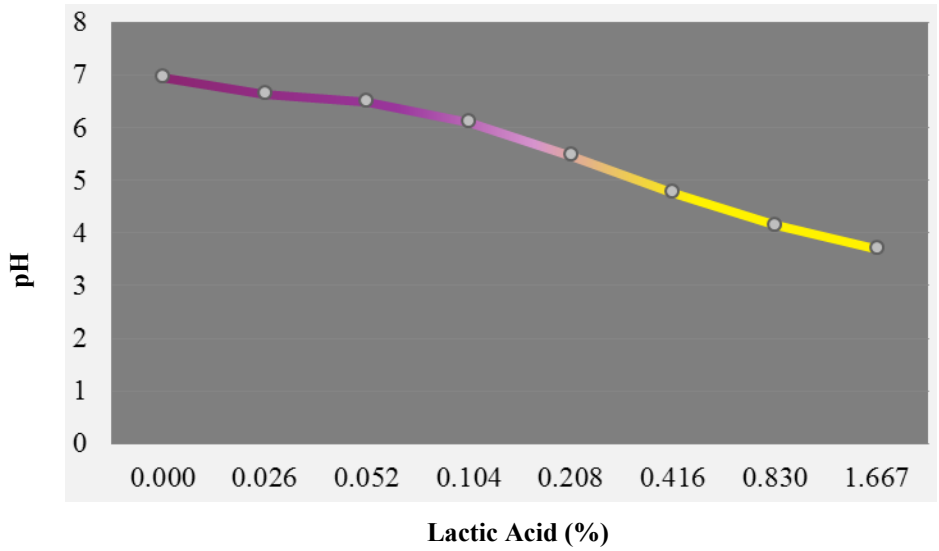


Figure 16. Colorimetric reference standard for API 50 research strip. Graph of pH change based on lactic acid percentage. Lactic acid dissolved was in aqueous bromocresol purple (0.017% w/v) (BCP) at different concentrations (from left to right: 0.026%, 0.052%, 0.104%, 0.208%, 0.416%, 0.830%, to 1.667%). The pH (*y*-axis) of the colorimetric reference as a function of lactic acid concentration (%) (*x*-axis), including deionized water (0.00% lactic acid). Each reading was taken thrice.

SNPs on Sucrose

Reference Position	Consensus Position	Variation Type	Length	Reference	Allele Variations	Frequencies	Counts	Coverage
10165	923	SNP	1	T	T/C	56.0/44.0	2465/1935	4401
10217	975	SNP	1	G	G/C	55.8/44.2	2343/1856	4201
10226	984	SNP	1	A	A/C	56.0/44.0	2326/1827	4153
10367	1125	SNP	1	G	A/G	51.1/48.8	2150/2054	4205
10643	1402	SNP	1	G	T	100.0	2000	2000
10650	1409	SNP	1	A	C	99.9	1987	1988
10975	1734	SNP	1	T	C	99.7	1874	1879
11149	1908	SNP	1	G	A	100.0	1841	1841
11395	2154	SNP	1	G	C	99.9	1721	1723
11842	2601	SNP	1	C	G	99.7	1951	1957
11843	2602	SNP	1	G	C	99.8	1943	1946
11900	2659	SNP	1	T	G	100.0	2176	2177
11901	2660	SNP	1	C	T	100.0	2184	2184
11985	2744	SNP	1	T	C	100.0	2153	2154
12027	2786	SNP	1	C	T	100.0	2093	2094
12321	3080	SNP	1	G	A	99.9	1732	1734
12350	3109	SNP	1	G	A	99.9	1782	1783
12369	3128	SNP	1	C	T	99.9	1767	1768
12405	3164	SNP	1	G	A	100.0	1842	1842
13536	4295	SNP	1	T	G	99.9	2022	2025
13888	4647	SNP	1	A	T	100.0	1992	1992
13907	4666	SNP	1	T	G	99.9	2043	2046
14226	4985	SNP	1	A	C	99.9	1658	1660
14317	5076	SNP	1	C	T	100.0	1880	1880
15022	5781	SNP	1	G	A	99.9	1881	1882

Figure 17. SNP detection table. The SNP detection table was generated after alignment of the sucrose operon of *P. acidilactici* D3 to the raffinose operon genes of *P. pentosaceus* (*GenBank: L32093.1*). SNPs with frequencies ≥ 95 were chosen for probe design.

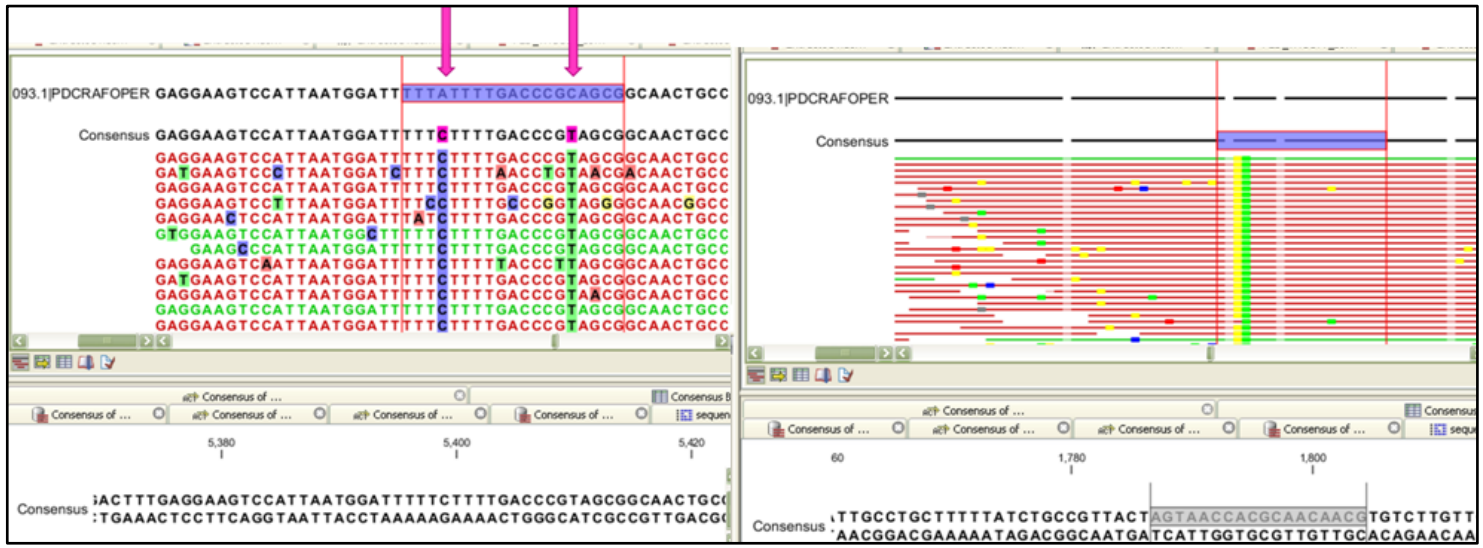


Figure 18. Screenshots of probe design using CLC Genomics Workbench.

Regions containing SNPs were identified and used to design primers. In the figure, the highlighted regions on the consensus show SNPs in comparison to the *P. pentosaceus* raffinose operon

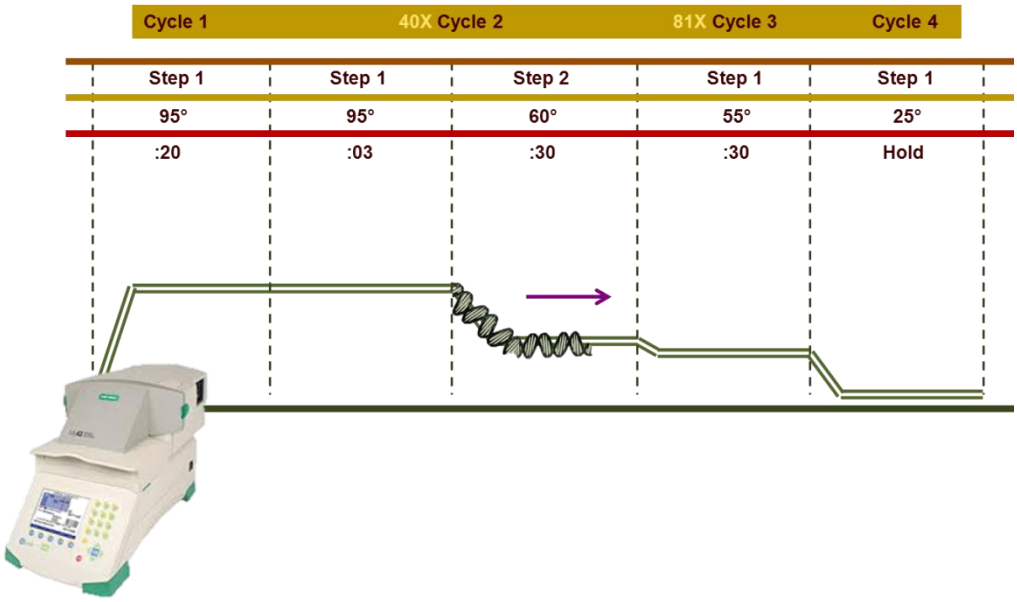


Figure 19. Optimized qPCR conditions for amplification of *P. acidilactici* D3.

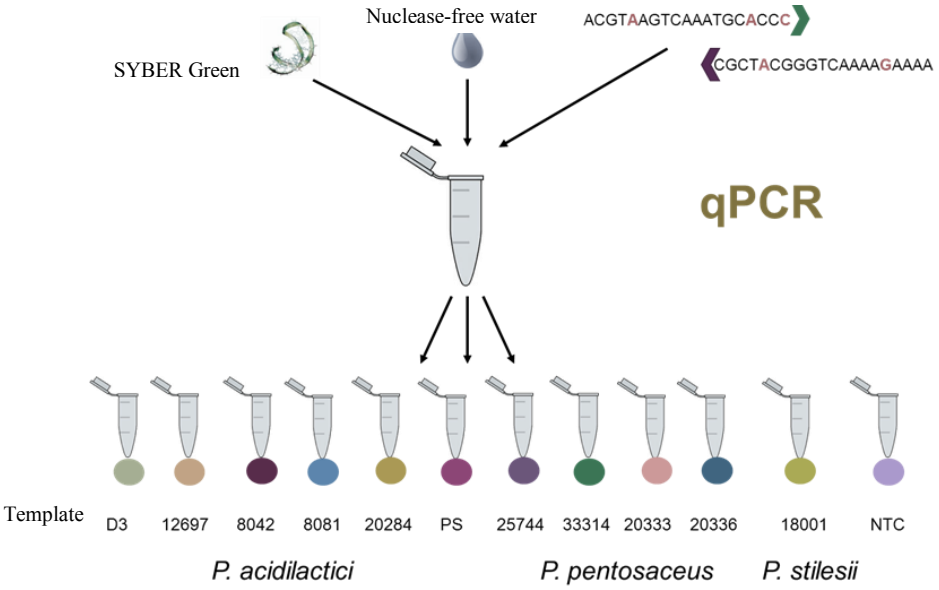


Figure 20. Methodology for qPCR to quantify *P. acidilactici* D3 in comparison to related strains. For each strain, 1ng of DNA was tested for amplification; Fast SYBER Green MasterMix (Applied Biosystems) was used for detection; NTC (non-template control).

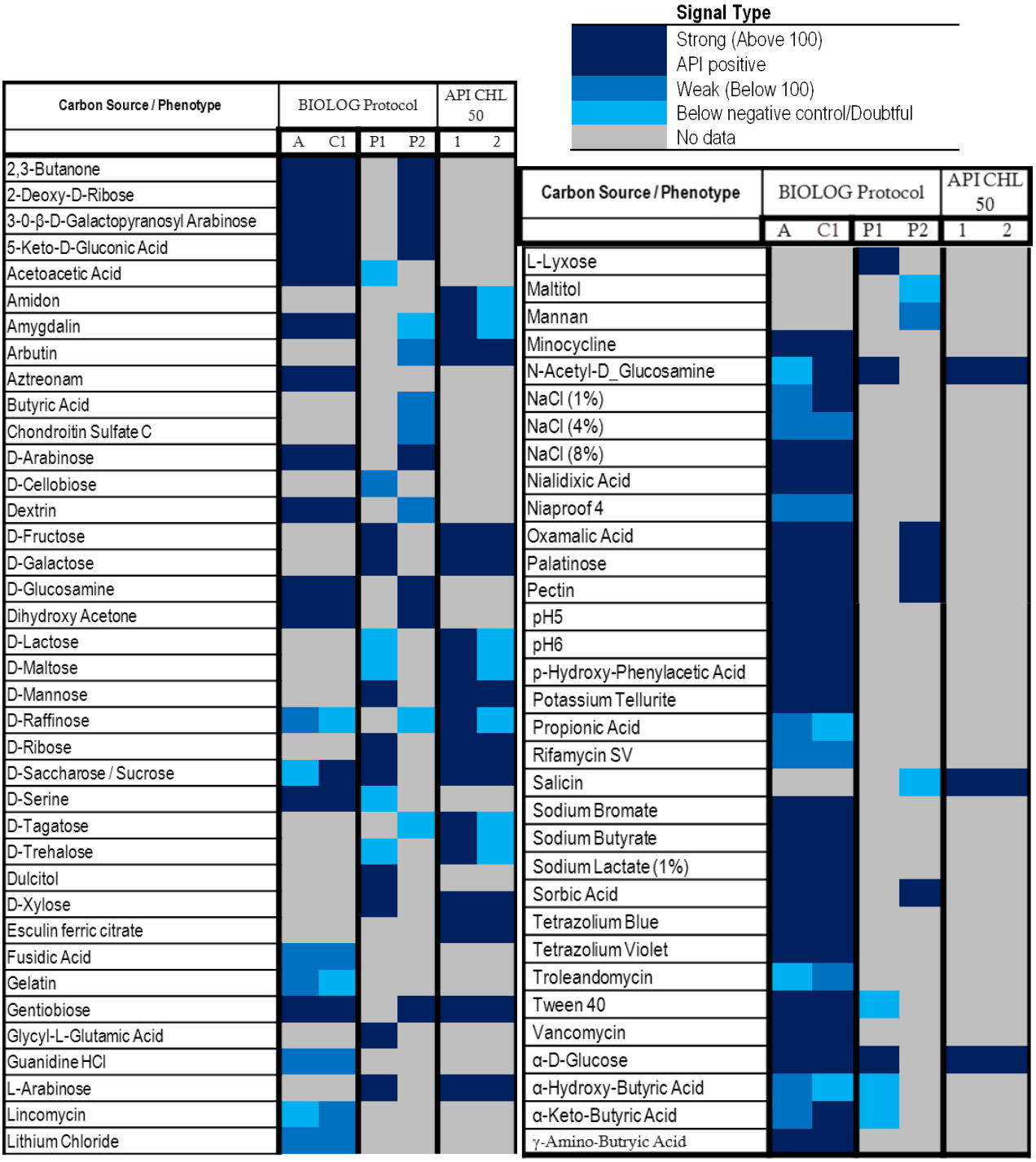


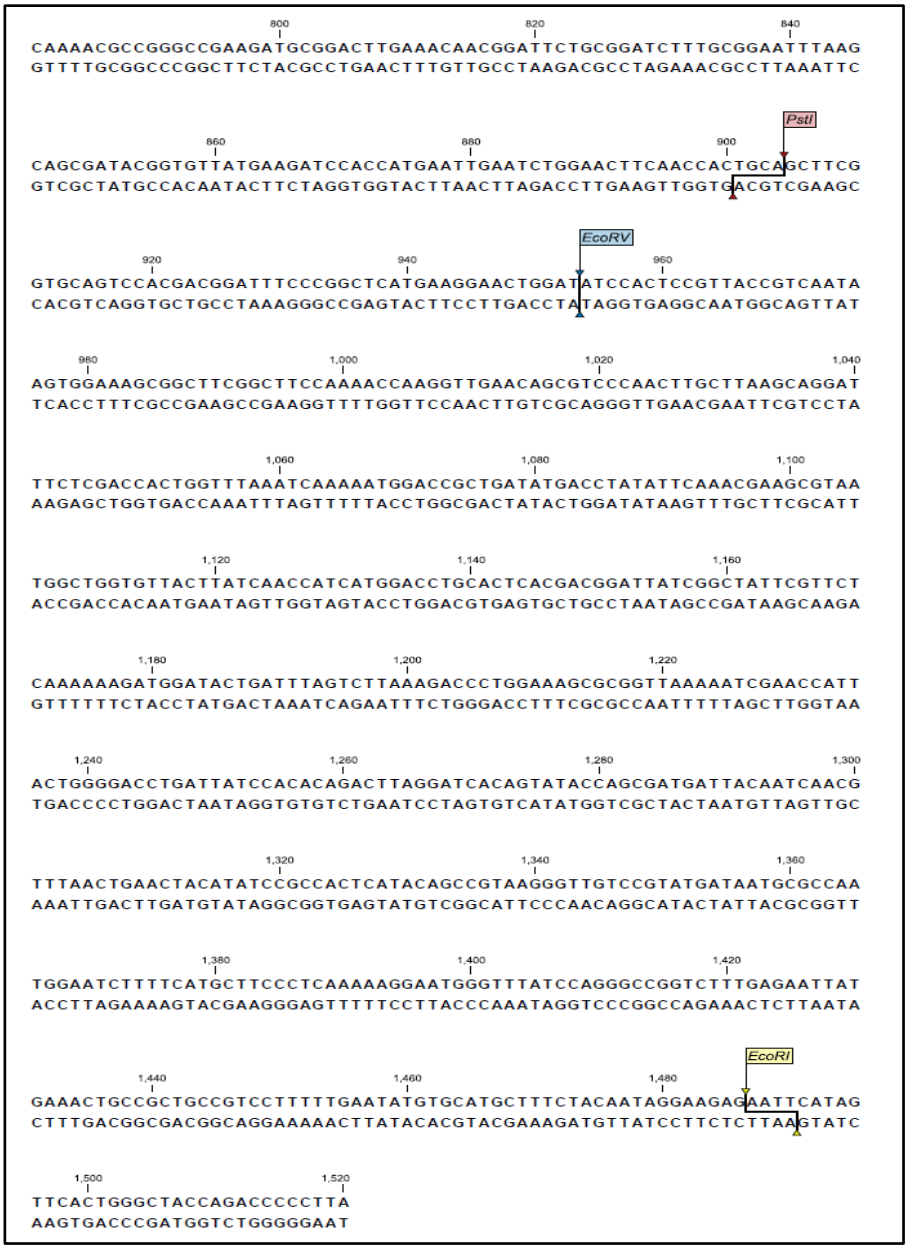
Figure 21. BIOLOG and Analytical Profile Index data.

A

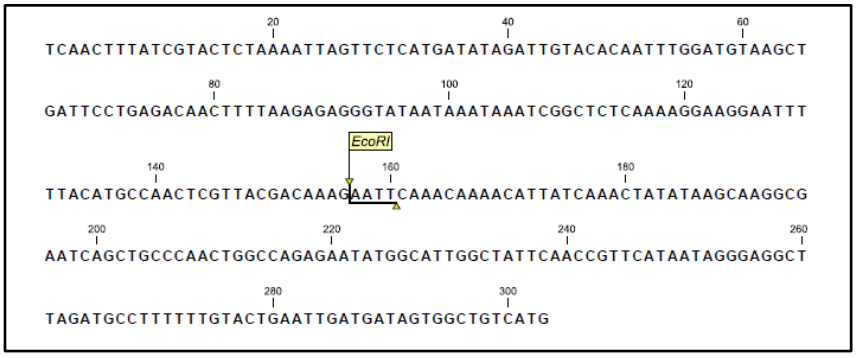
Figure 22. Contigs of the sucrose operon. Panel A: Contig 1; Panel B: Contig 2; Panel C: Contig 3; Panel D: Contig 4; Panel E: Contig 5

B

Continued: Figure 22. Contigs of the sucrose operon.



Continued: Figure 22. Contigs of the sucrose operon.



C

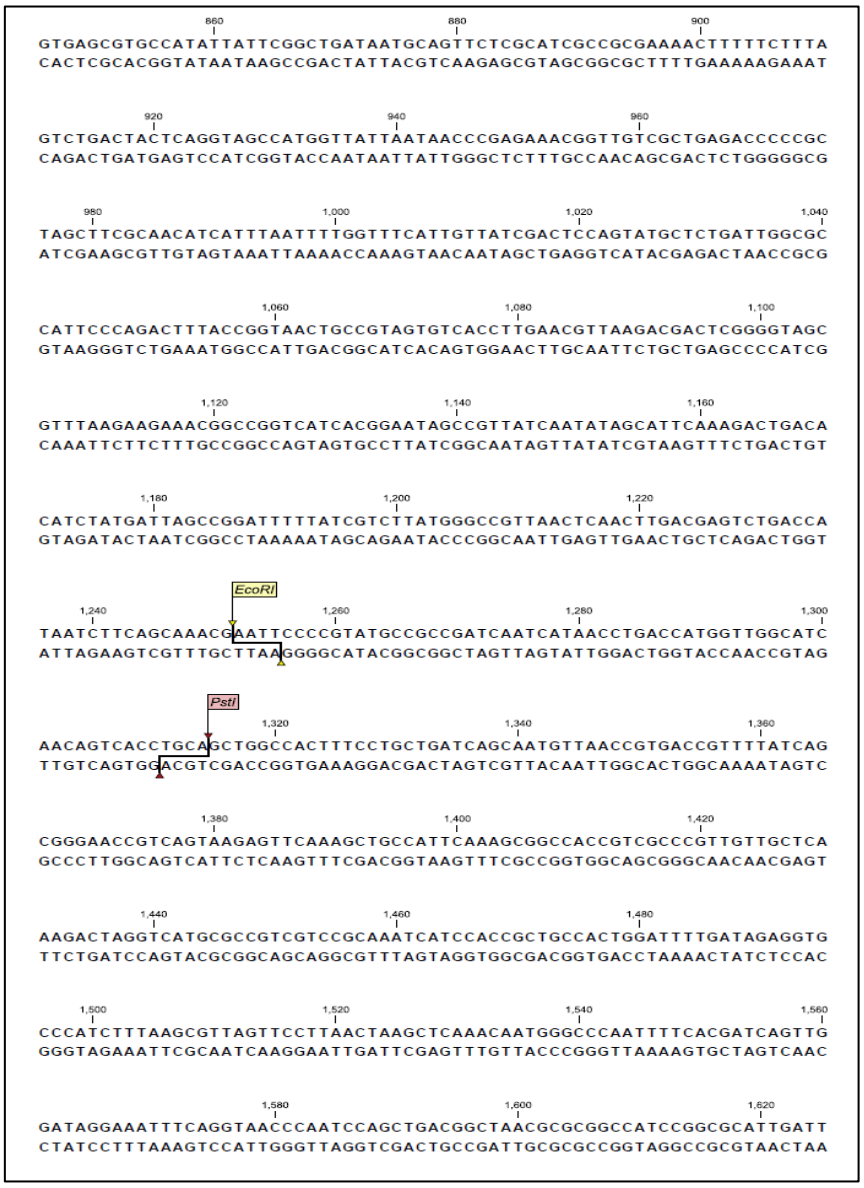
Continued: Figure 22. Contigs of the sucrose operon.

D

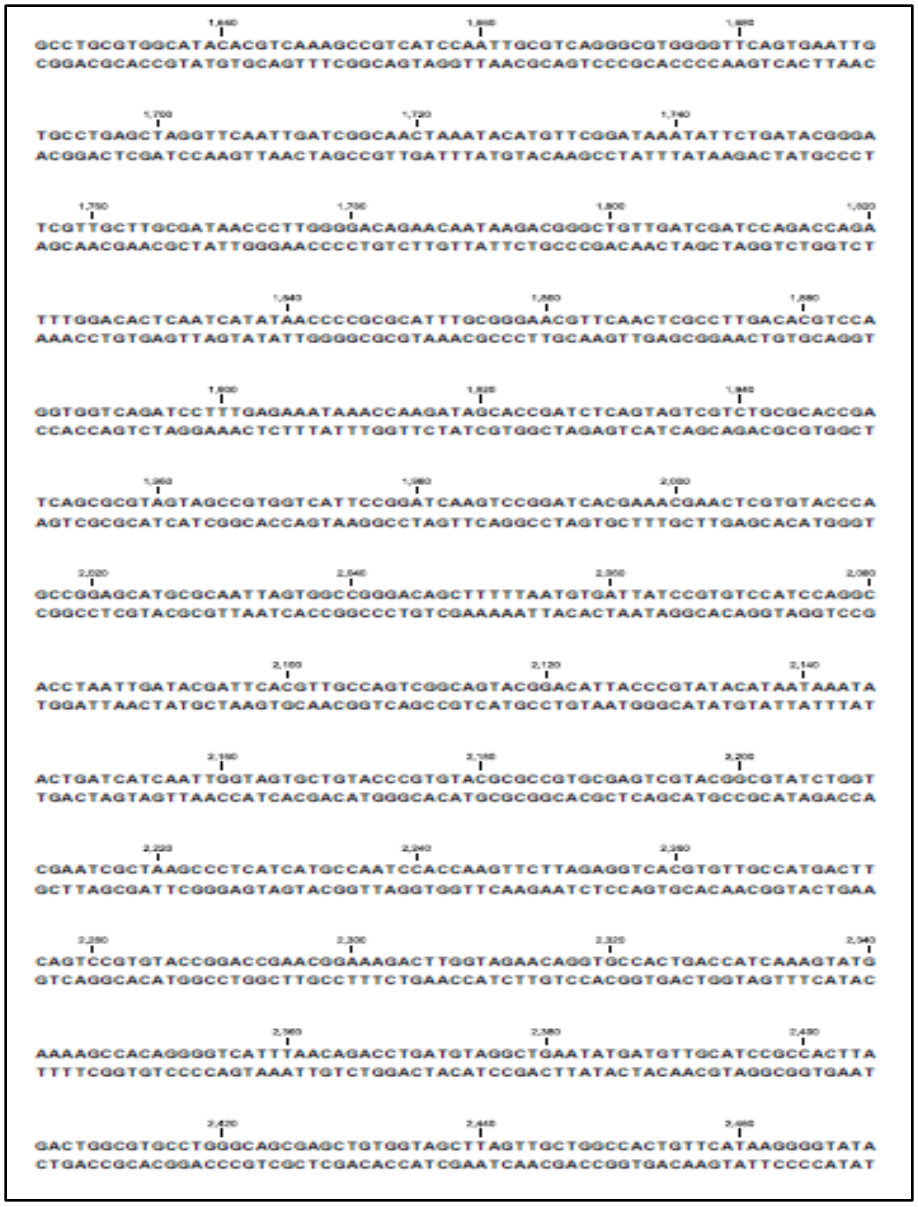
Continued: Figure 22. Contigs of the sucrose operon.

E

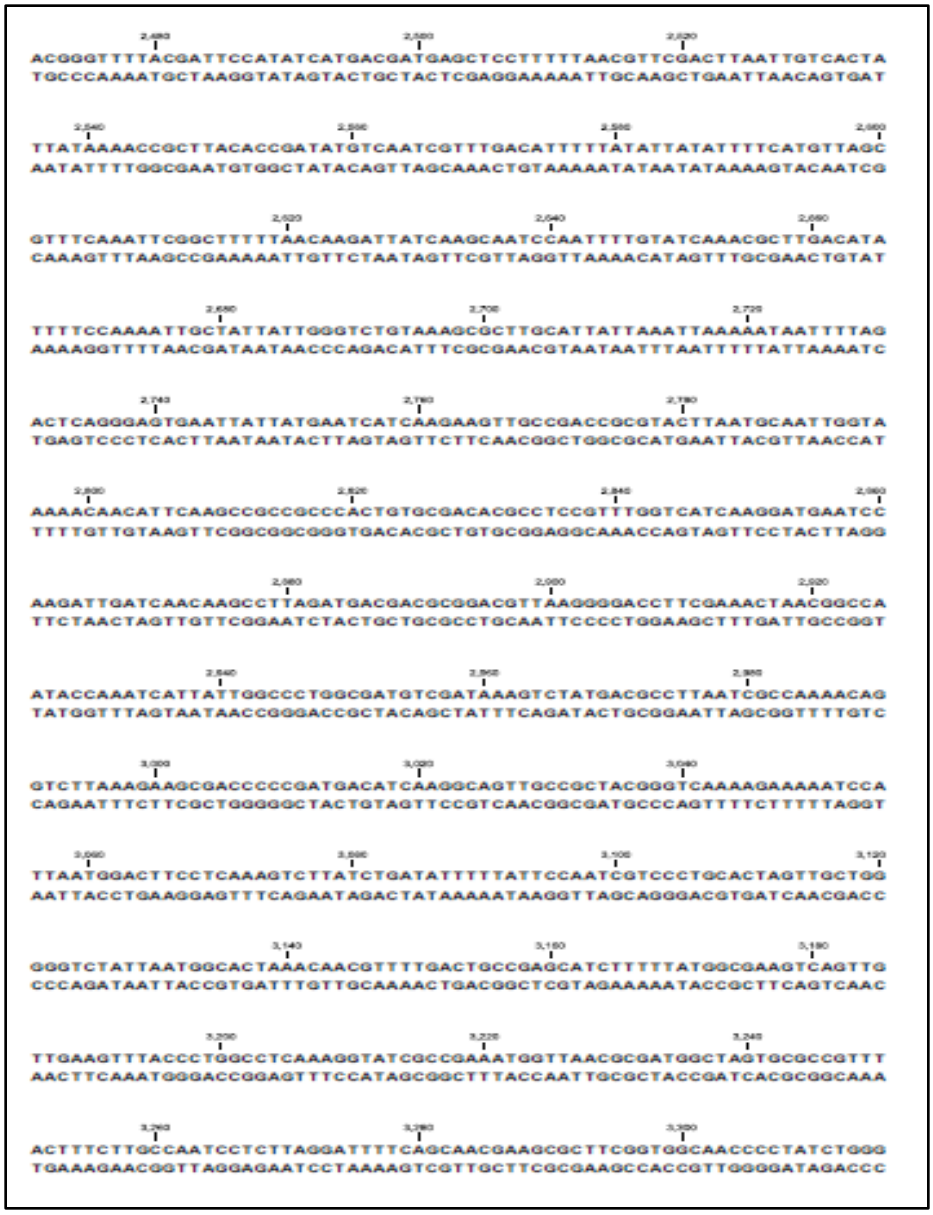
Continued: Figure 22. Contigs of the sucrose operon.



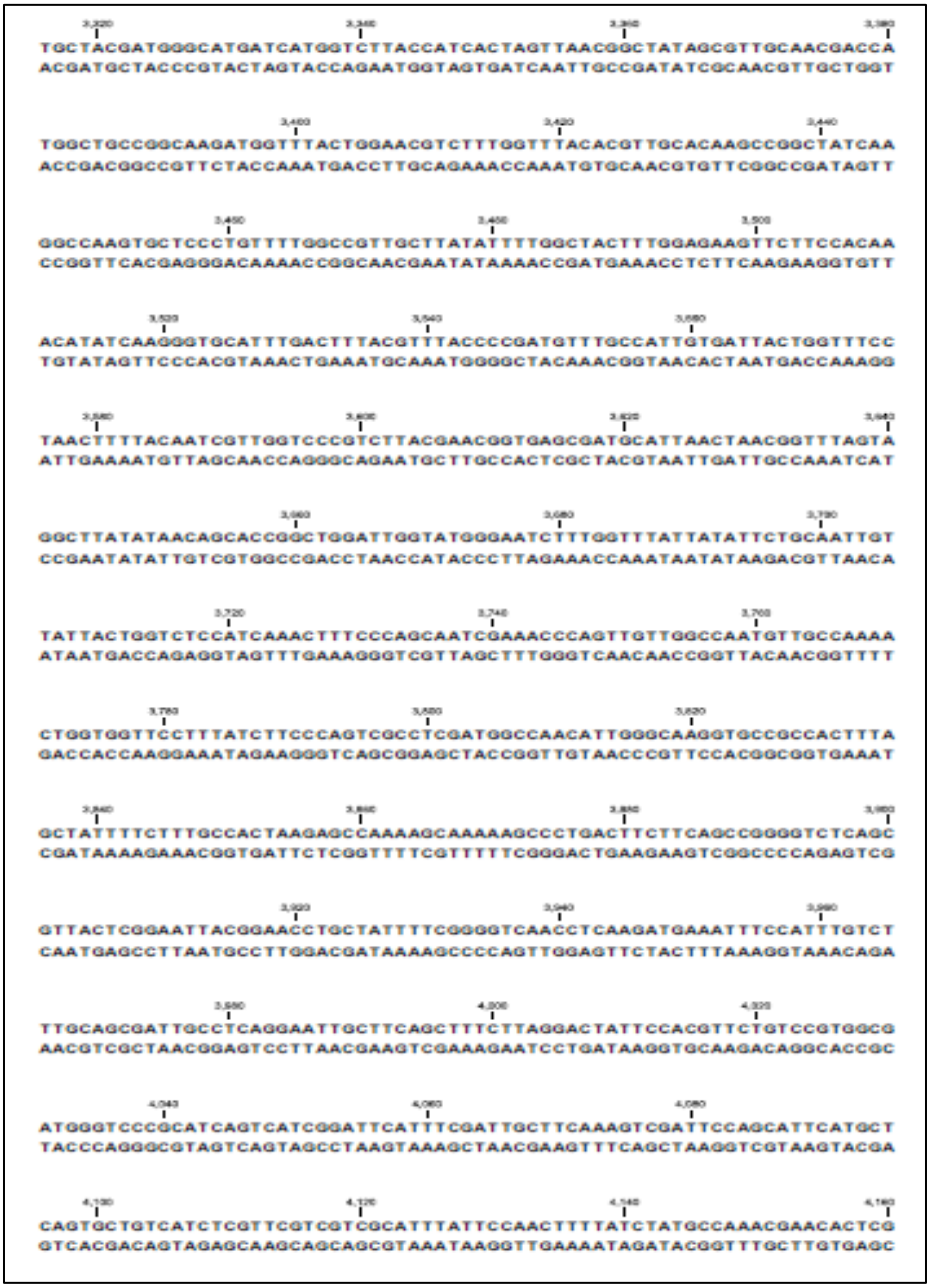
Continued: Figure 22. Contigs of the sucrose operon.



Continued: Figure 22. Contigs of the sucrose operon.



Continued: Figure 22. Contigs of the sucrose operon.



Continued: Figure 22. Contigs of the sucrose operon.

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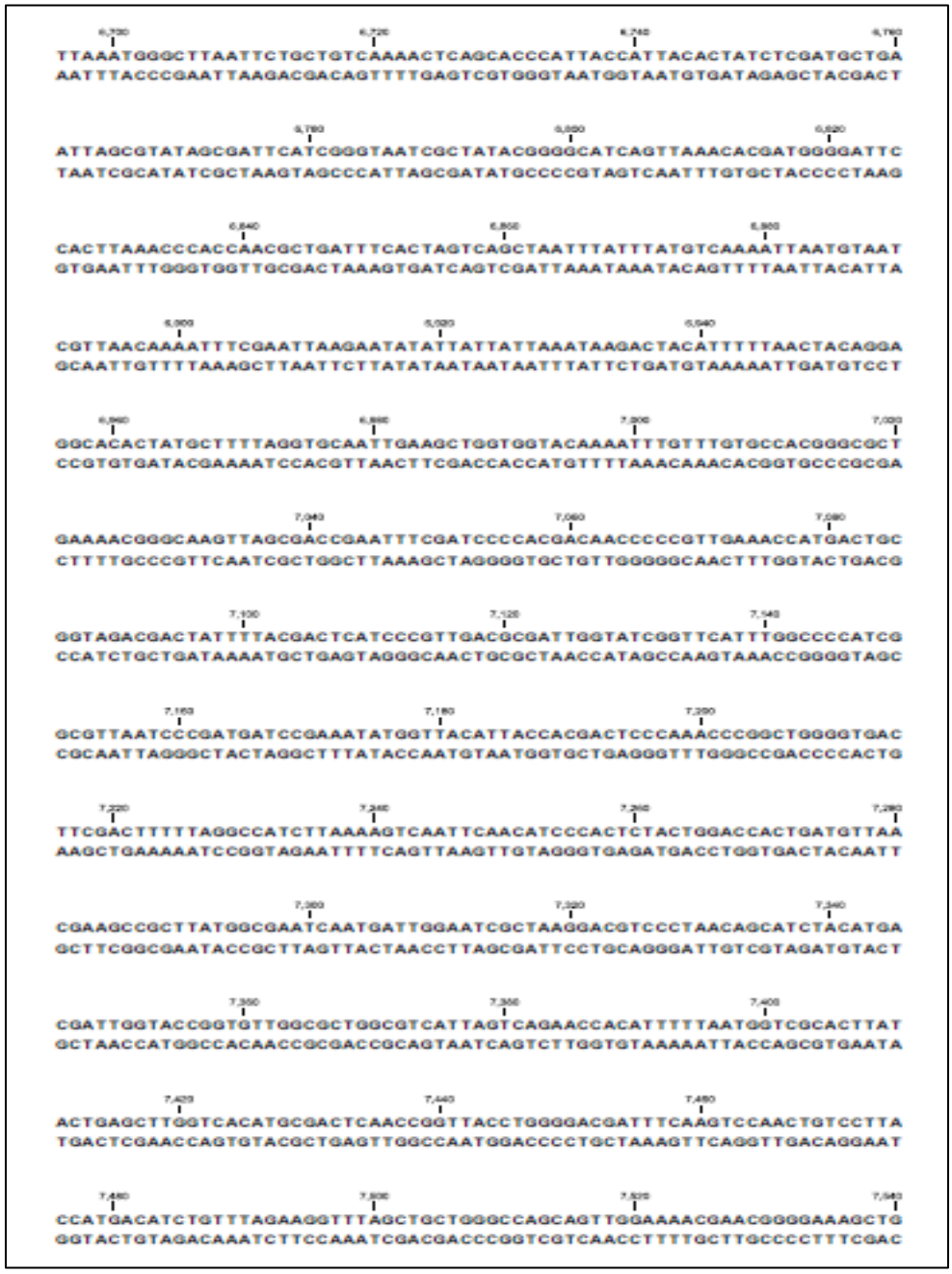
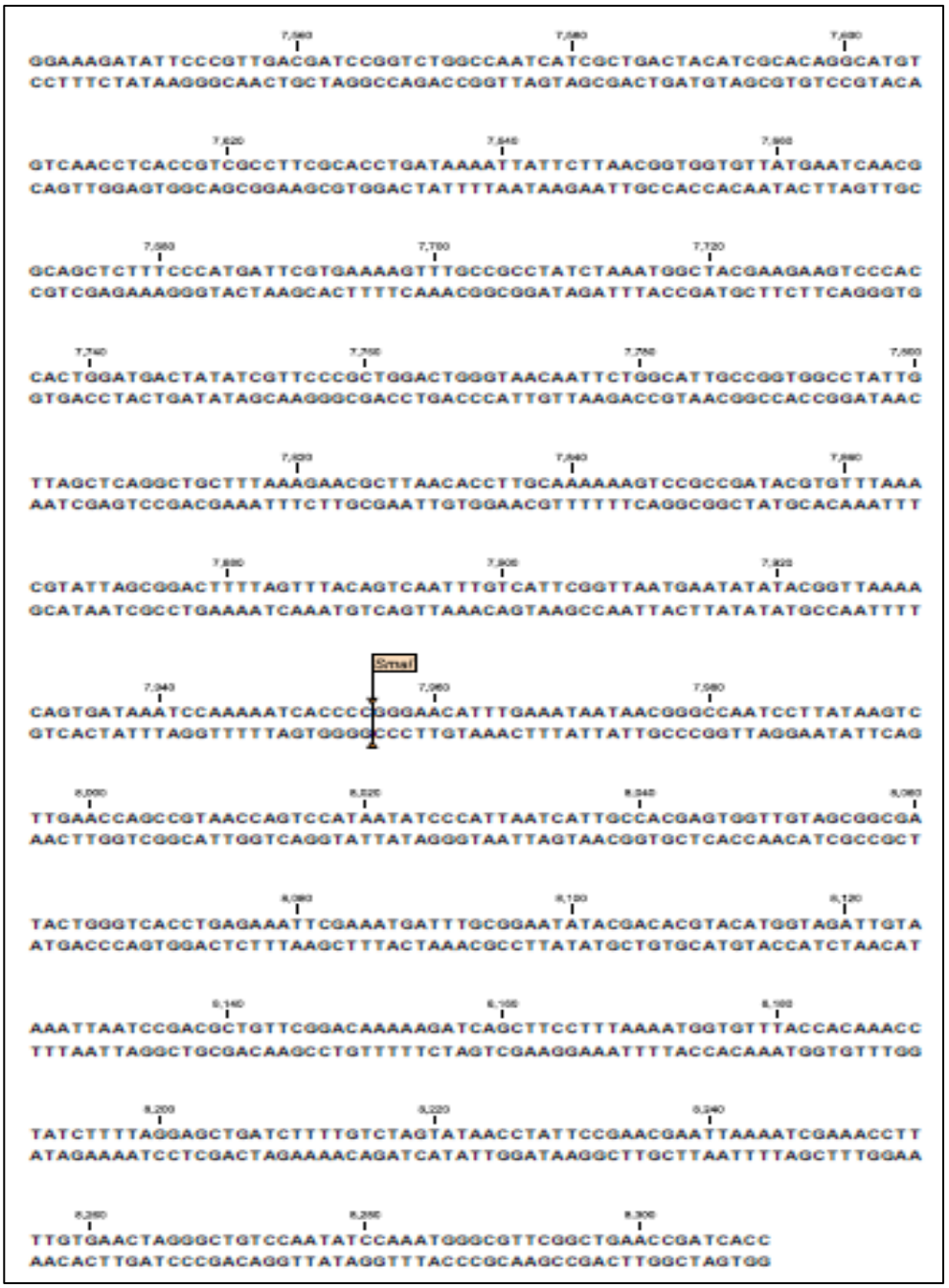


Continued: Figure 22. Contigs of the sucrose operon.



Continued: Figure 22. Contigs of the sucrose operon.

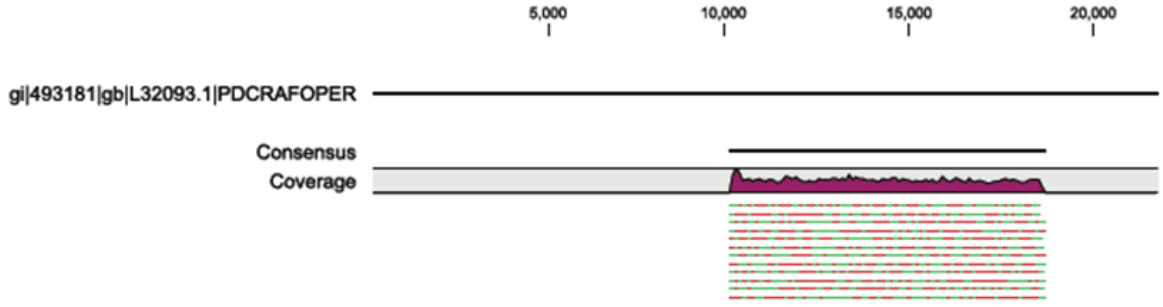


Figure 23. Contig 5 of the *Pediococcus acidilactici* D3 sucrose operon mapped against the raffinose and sucrose operon of *Pediococcus pentosaceus* (*GenBank: L32093.1*).

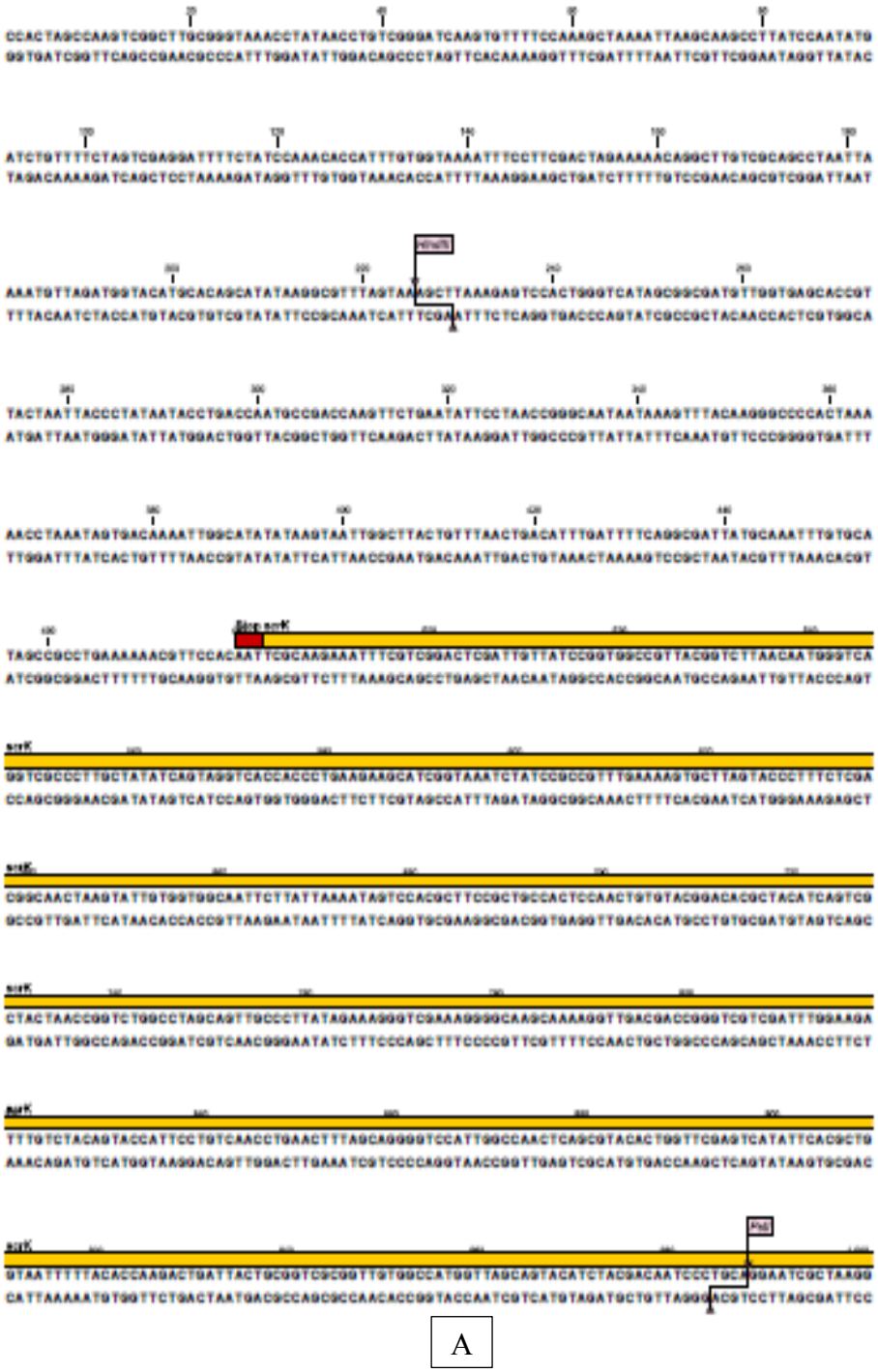
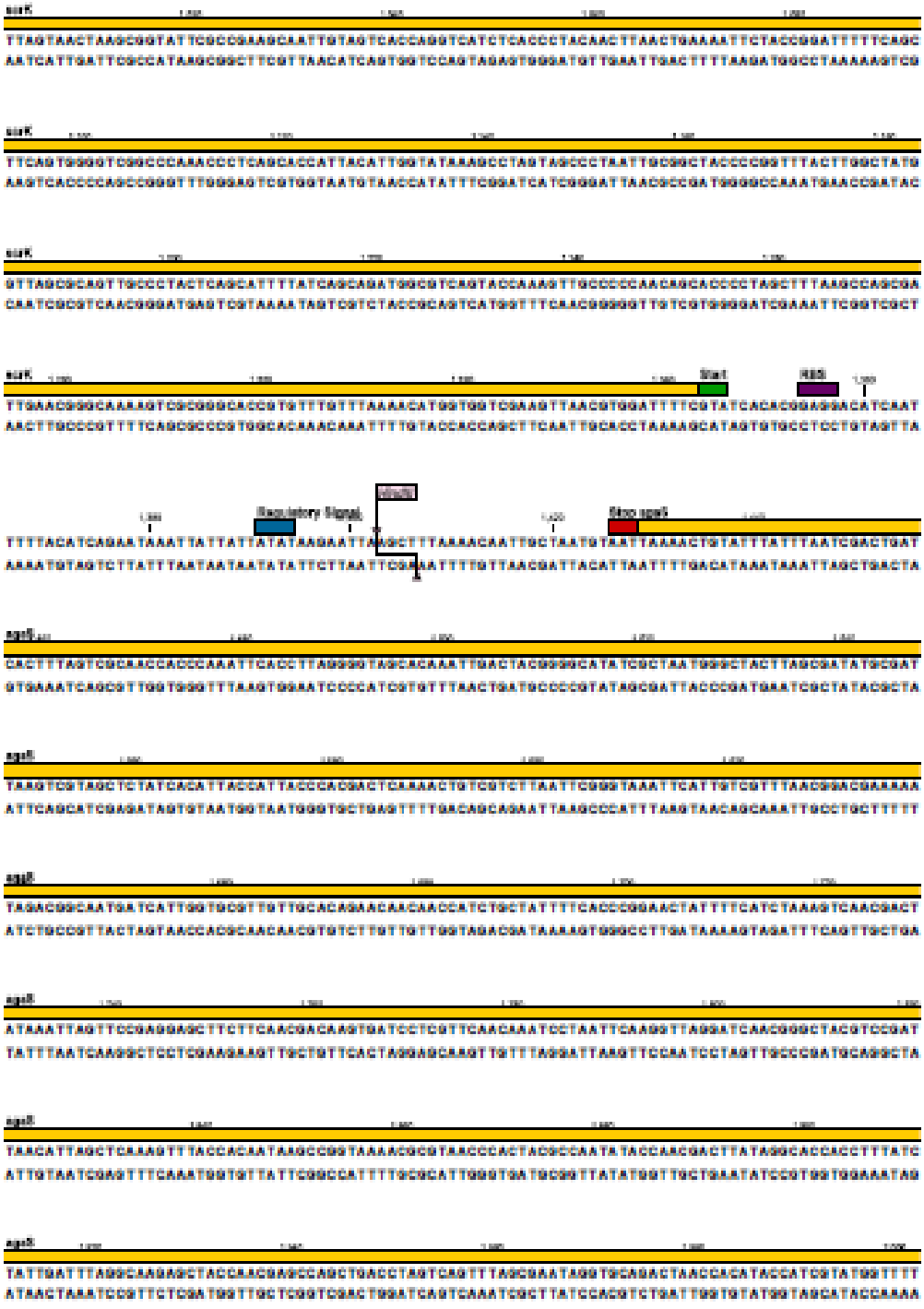
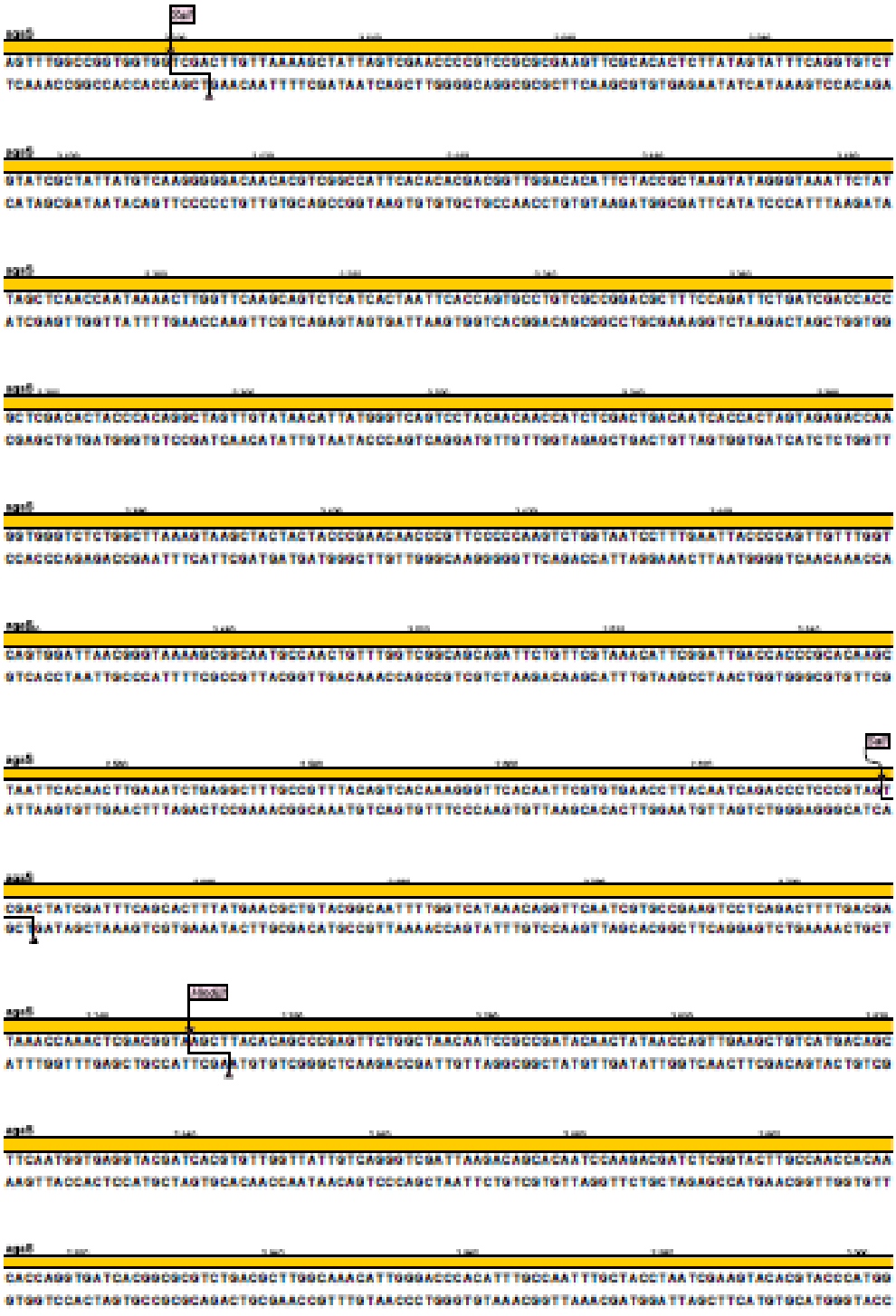


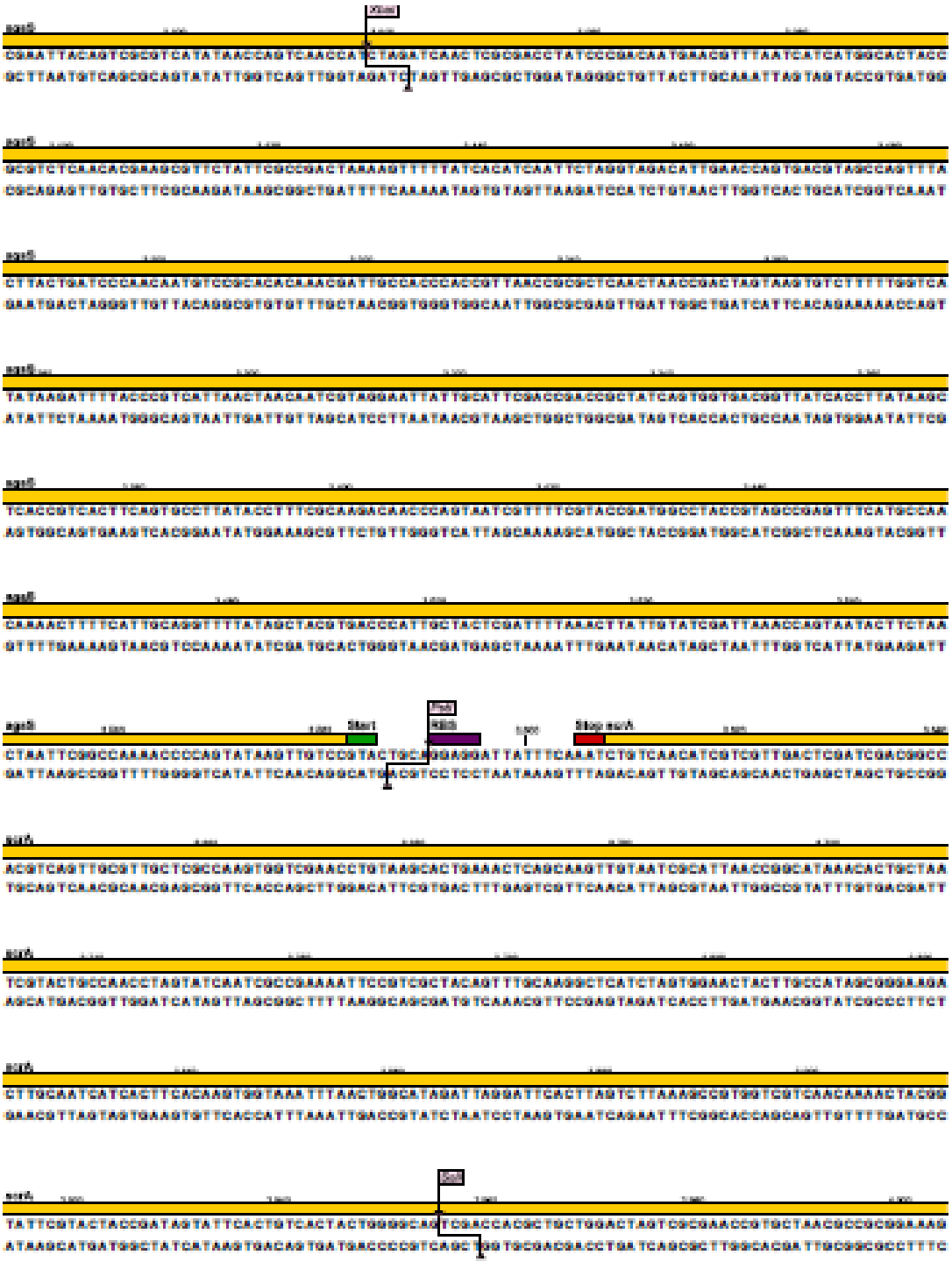
Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3. Panel A is the annotated sucrose operon with genes *scrK* to the introduction of *agl*. Panel B contains the minus strand of the *agl* gene after a 66bp gap from the other contiguous genes.



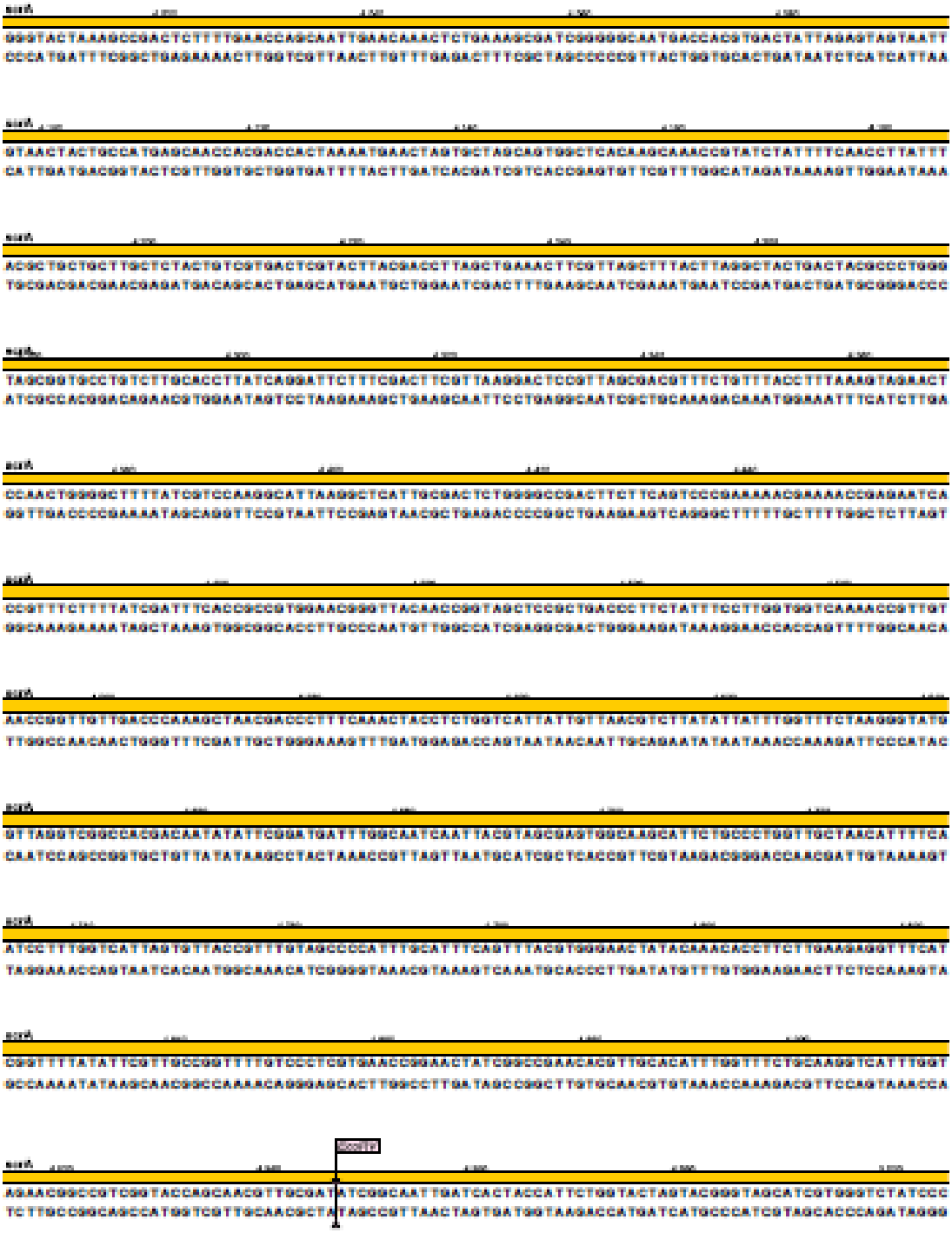
Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



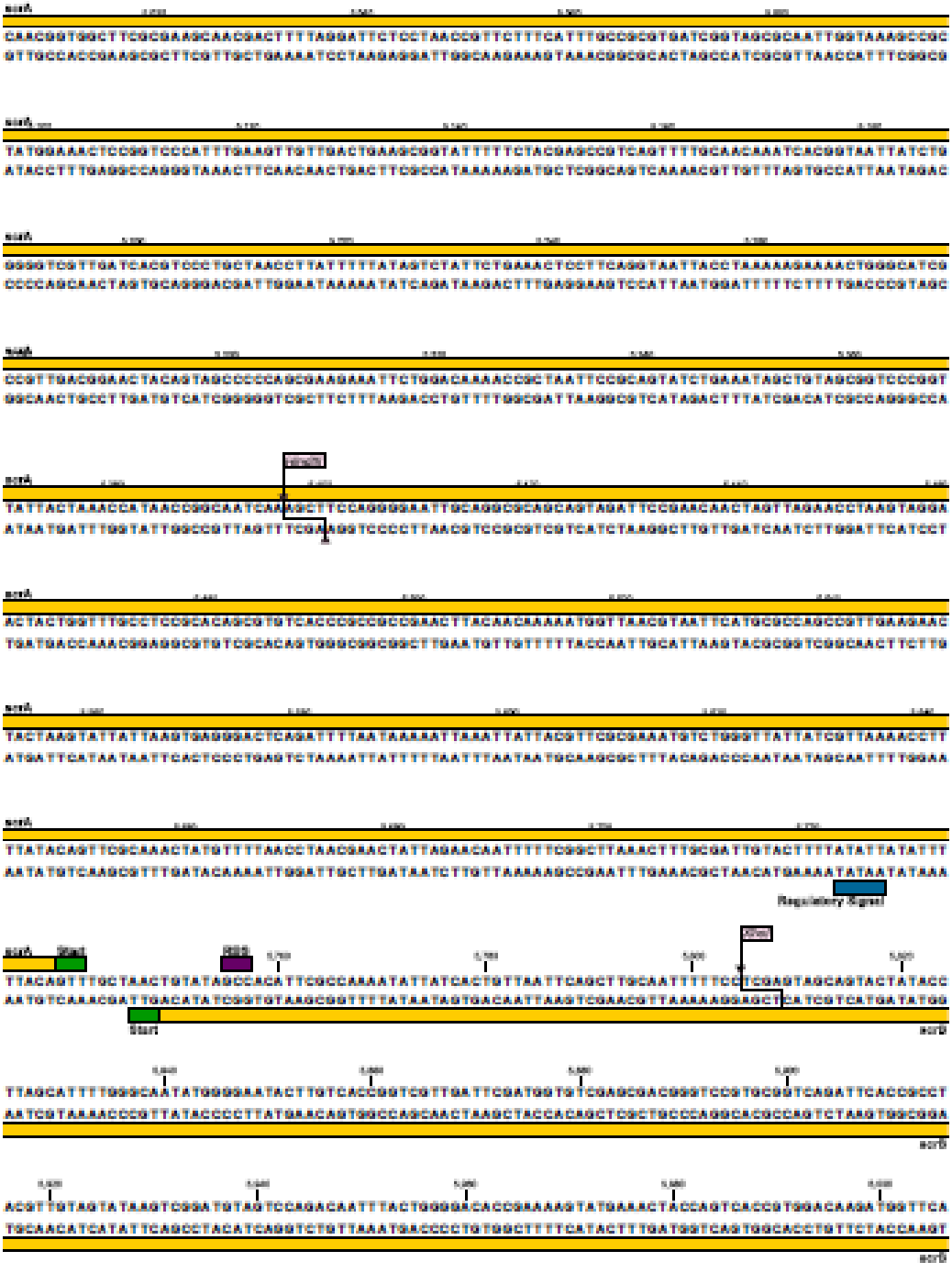
Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



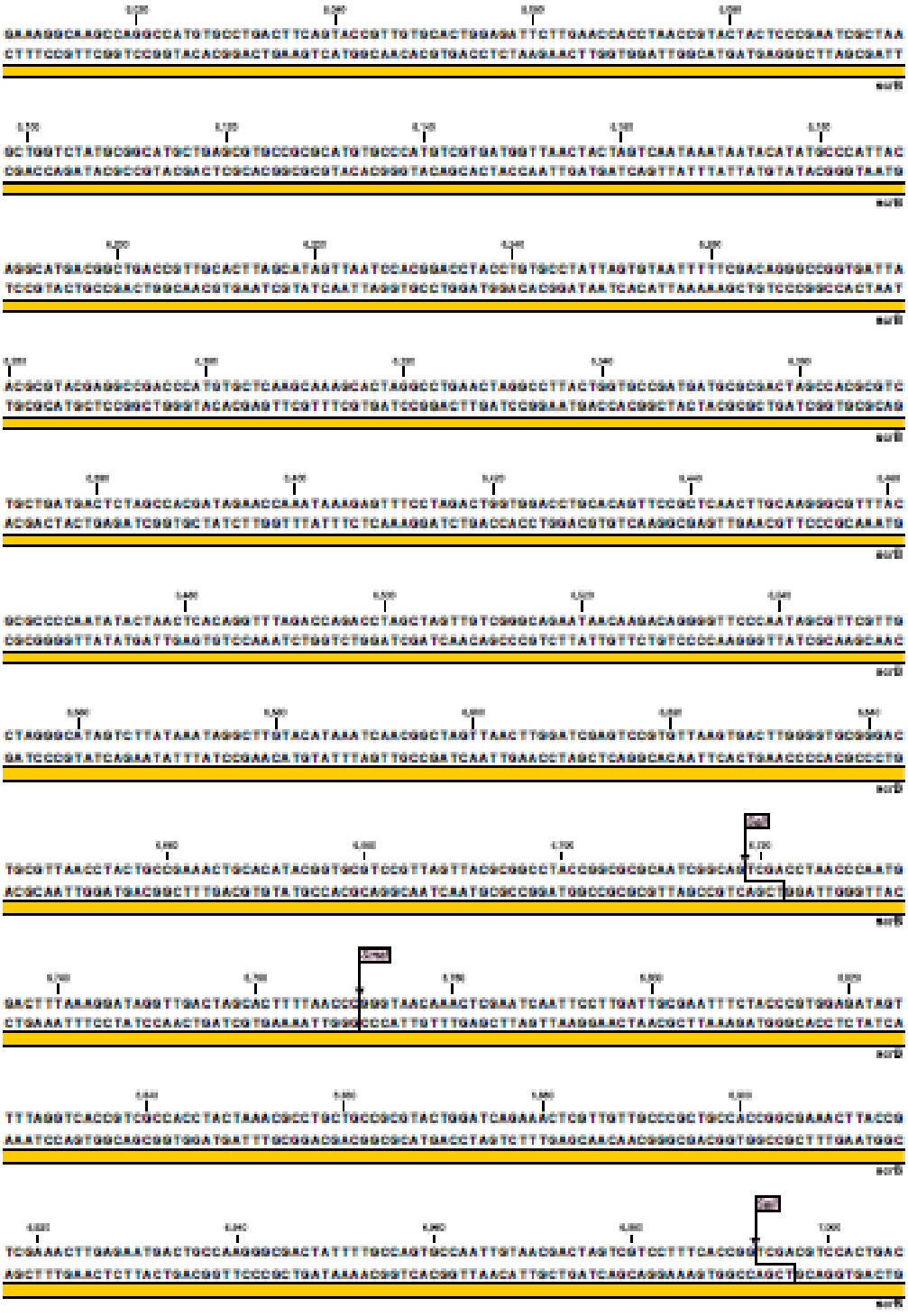
Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



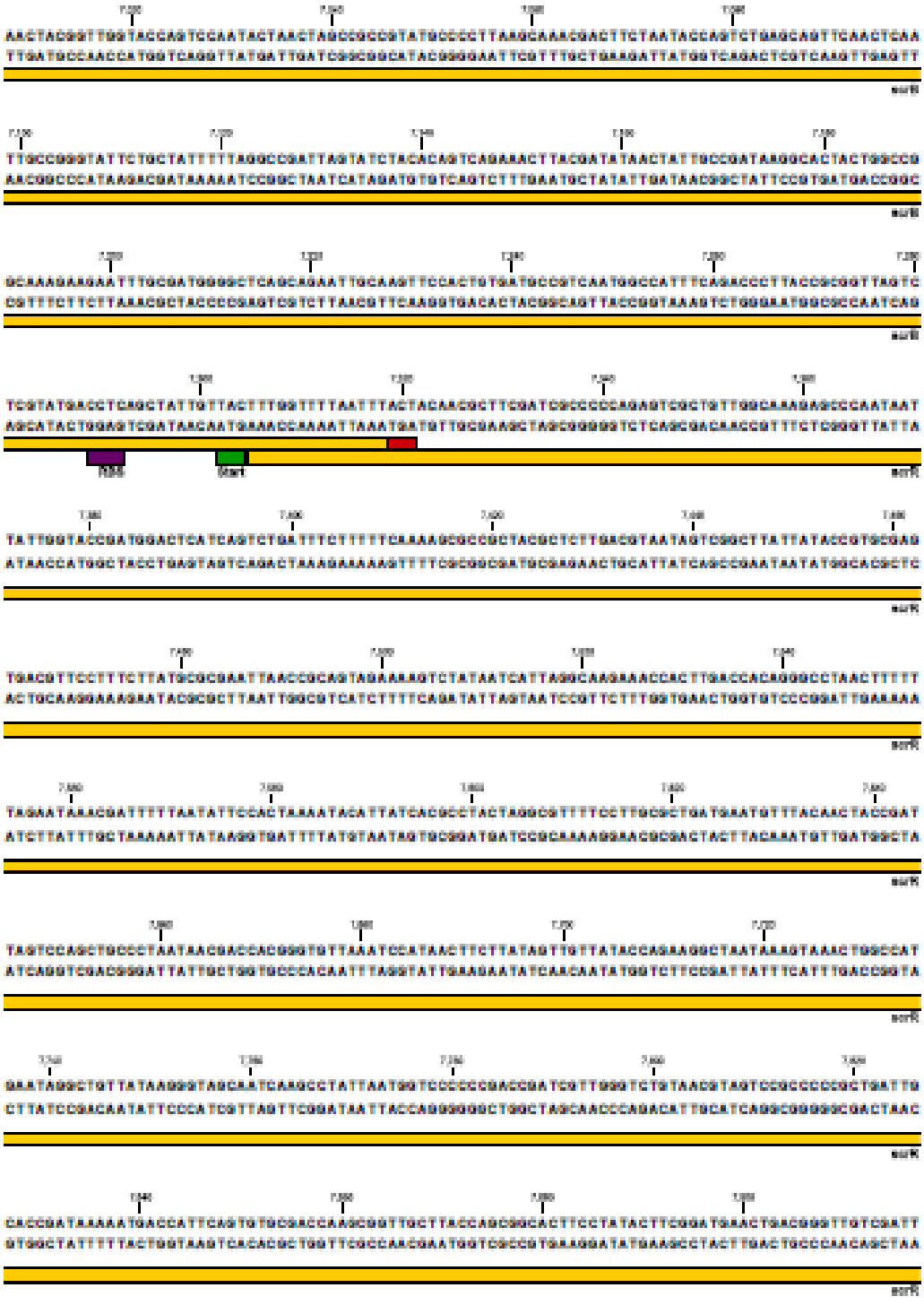
Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



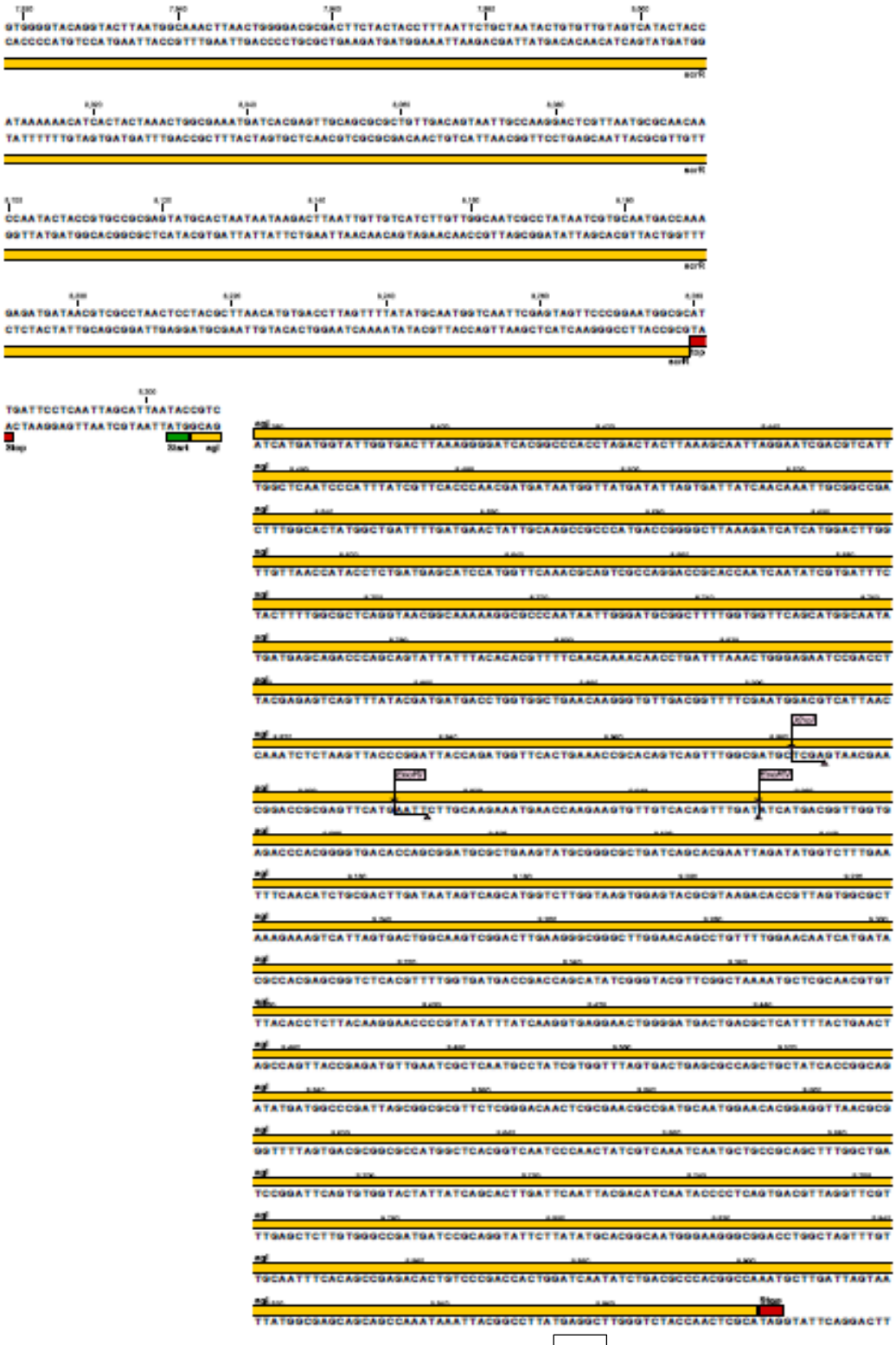
Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.



B

Continued: Figure 24. The Sucrose Operon of *Pediococcus acidilactici* D3.

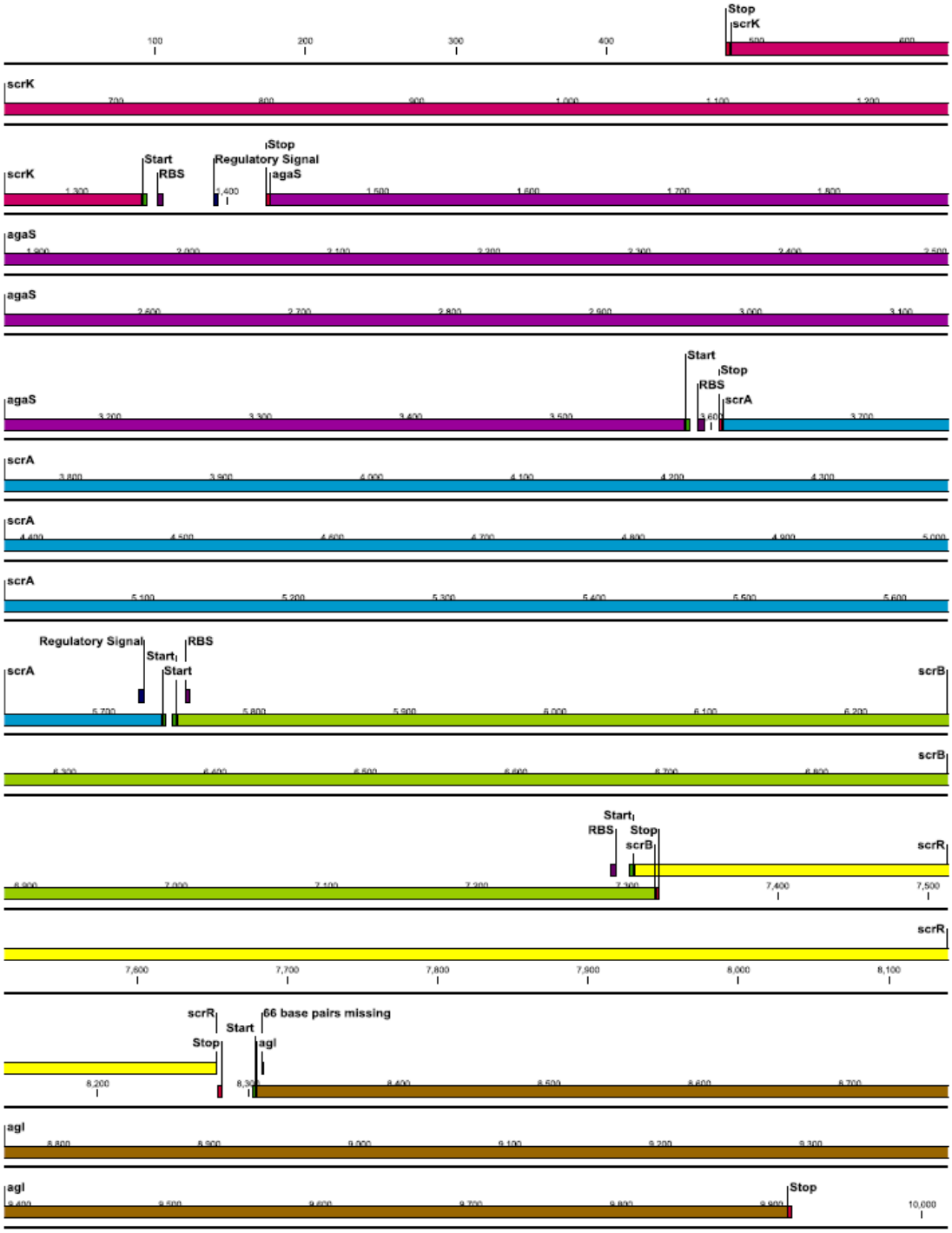


Figure 25. Condensed illustration of the sucrose operon. The genes are: *scrK* (29), *agaS* (pink), *scrA* (blue), *scrB* (green), *scrR*(yellow), and *agl* (brown). The 66bp gap in the *agl* gene is shown in orange.

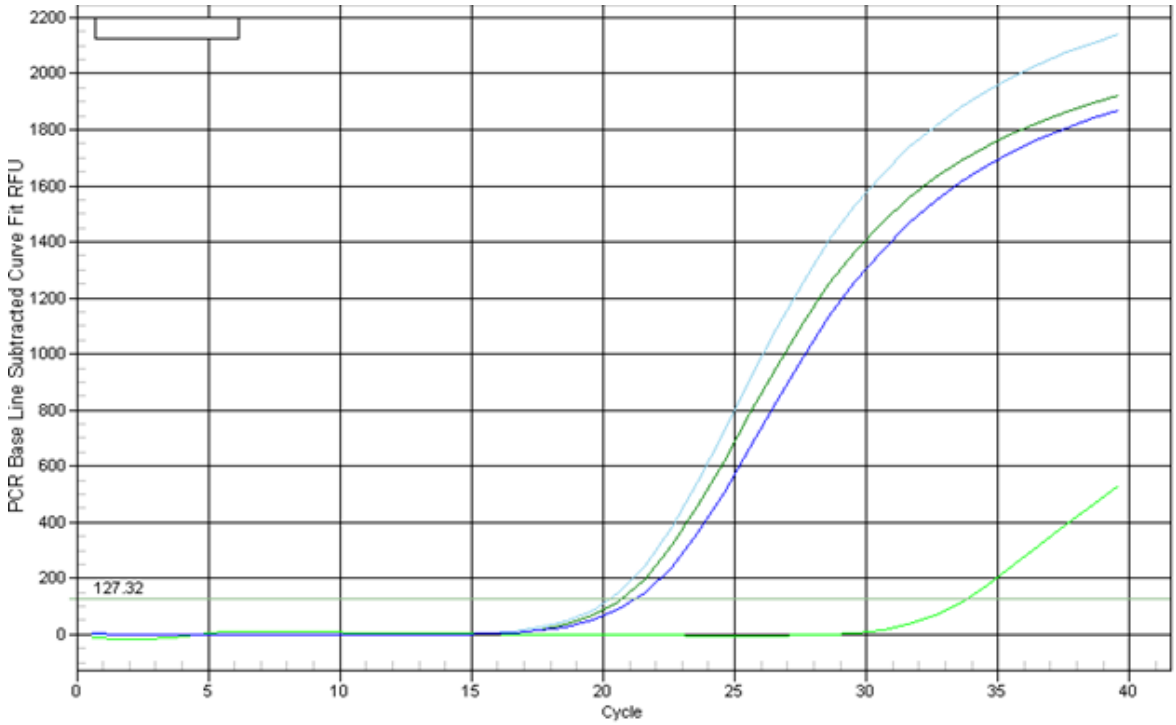


Figure 26. qPCR amplification of *P. acidilactici* D3 with probe pair 'pedjms'. The experiment was done in technical replicates. 1 ng of genomic DNA was amplified for all three replicates of *P. acidilactici* D3. At cycle 30, the non-template control (light green) started showing non-specific amplification which could be due to the production of primer-dimers.

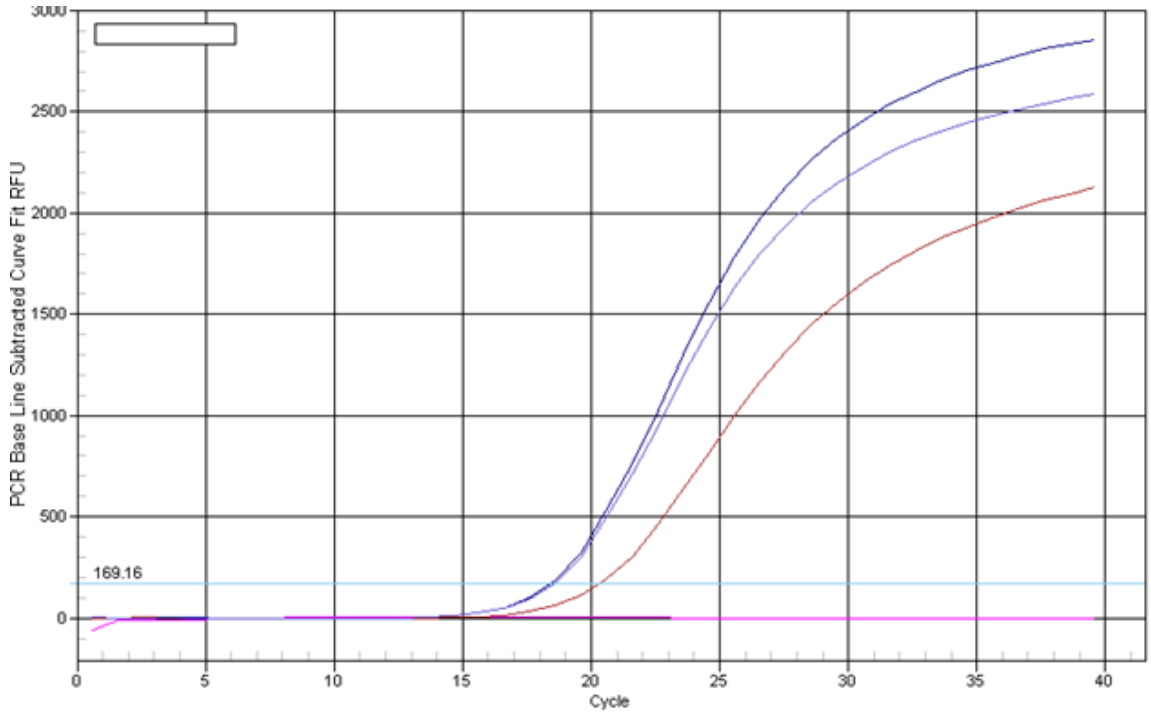


Figure 27. qPCR amplification of *P. acidilactici* D3 with probe pair 1SUCD3F and 1SUCD3R. 1 ng of genomic DNA was amplified for all three replicates of *P. acidilactici* D3 (blue, purple, brown). The non-template control is shown in pink.

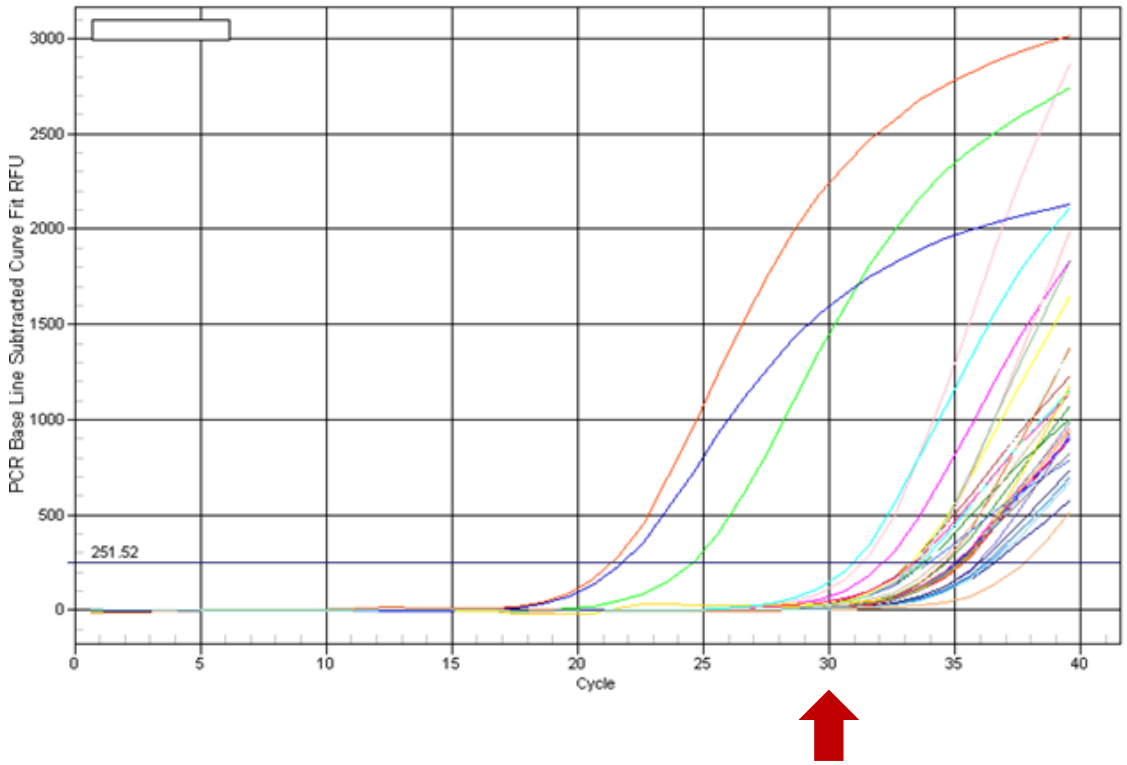


Figure 28. qPCR amplification with probe pair '1SUCD3F_N and 1SUCD3R'. qPCR amplification of *P. acidilactici* D3 (orange, light green and dark blue). Related strains and non-template controls – each done in triplicates (all other colors). At cycle 30, only the *P. acidilactici* D3 operon is amplified. Cycles > 30 show non-specific amplification.

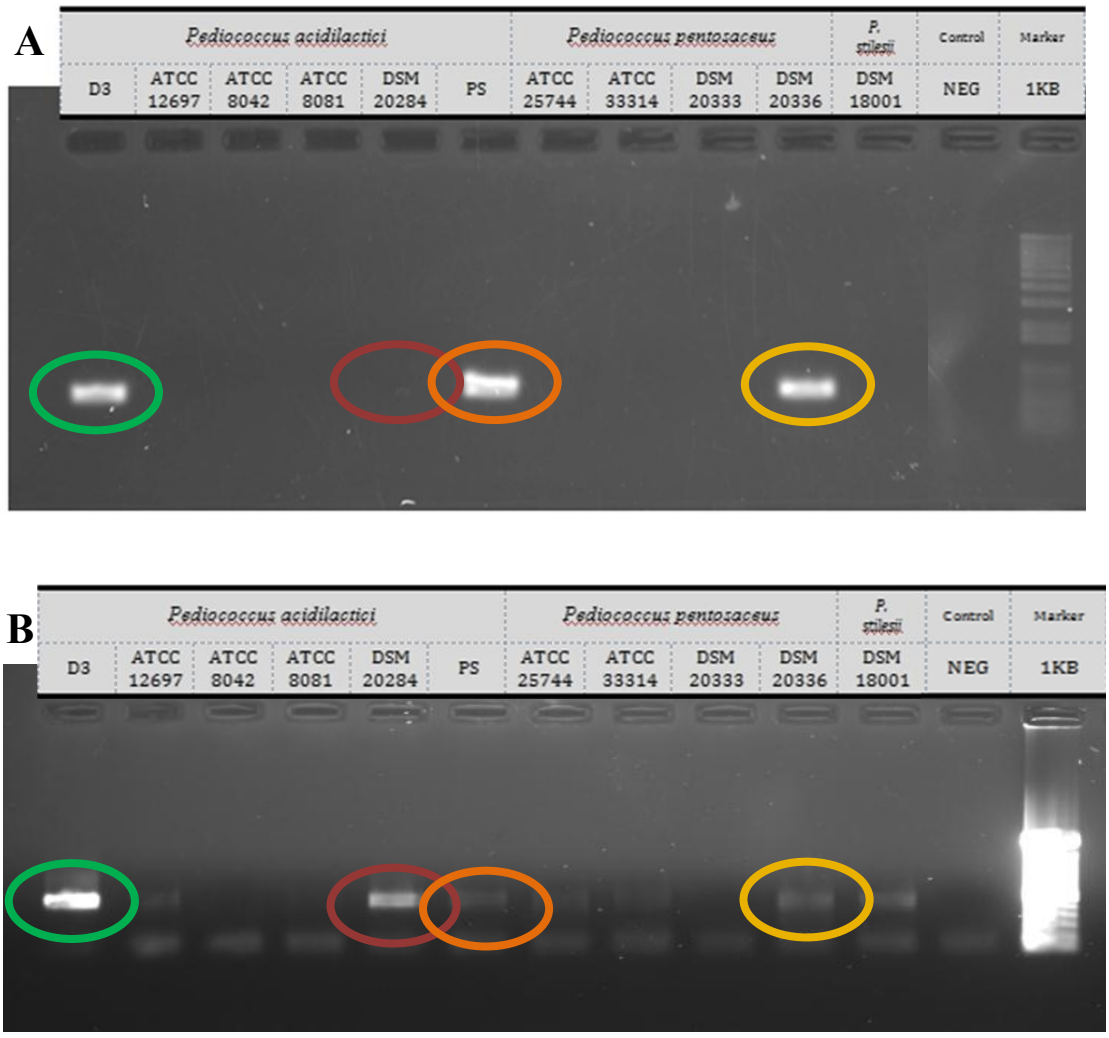


Figure 29. A combination approach using probe pairs to detect the pediocin and sucrose-utilization operons enabled the identification of *P. acidilactici* D3. Panel A, PCR products generated using primers pedjmsF and pedjmsR for *P. acidilactici* D3 and related strains. When the pediocin operon was used as a target, amplification was observed in a few related strains due to high sequence conservation of the pediocin operon. **Panel B,** PCR products generated using primers 1SUCD3F_N and 1SUCD3R for *P. acidilactici* D3 and related strains. *P. acidilactici* D3 showed amplification when both the pediocin and sucrose probe targets were used (green). In contrast, the related strains showed specific amplification, represented by a bright band on the gel, for either the pediocin target, sucrose target, or neither target (red, orange, and yellow).

Primer Name	5' to 3' Sequence	Target/Specificity
pedjms_F	ATTCCTGCTCTGTTGACT	pedA
pedjms_R	CTTAGACAACTACAATATCCCC	pedB

Primer Name	5' to 3' Sequence	Target/Specificity
1SUCD3F	CGTAAGTCAAATGCACCCT	scrA (PTS EII transport)
1SUCD3F_N	ACGTAAGTCAAATGCACCC	scrA (PTS EII transport)
1SUCD3R	CGCTACGGGTCAAAAGAAAA	scrA
2SUCD3F	AGTAACCACGCAACAACG	agaS (α-galactosidase)
2SUCD3R	TTACACAGGTTGGCAGCAC	agaS

Figure 30. Final list of primers and their target gene. Nucleotides shown in red are SNPs.