

# Metabolism of sugars by genetically diverse species of oral *Leptotrichia*

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## SUMMARY

*Leptotrichia buccalis* ATCC 14201 is a gram-negative, anaerobic rod-shaped bacterium resident in oral biofilm at the tooth surface. The sequenced genome of this organism reveals three contiguous genes at loci: *Lebu\_1525*, *Lebu\_1526* and *Lebu\_1527*. The translation products of these genes exhibit significant homology with phospho- $\alpha$ -glucosidase (PagI), a regulatory protein (GntR) and a phosphoenol pyruvate-dependent sugar transport protein (EIICB), respectively. In non-oral bacterial species, these genes comprise the *sim* operon that facilitates sucrose isomer metabolism. Growth studies showed that *L. buccalis* fermented a wide variety of carbohydrates, including four of the five isomers of sucrose. Growth on the isomeric disaccharides elicited expression of a 50-kDa polypeptide comparable in size to that encoded by *Lebu\_1525*. The latter gene was cloned, and the expressed protein was purified to homogeneity from *Escherichia coli* TOP10 cells. In the presence of two cofactors, NAD<sup>+</sup> and Mn<sup>2+</sup> ions, the enzyme readily hydrolyzed *p*-nitrophenyl- $\alpha$ -glucopyranoside 6-phosphate (pNP $\alpha$ G6P), a chromogenic analogue of the phosphorylated isomers of sucrose. By comparative sequence alignment, immunoreactivity and signature motifs, the enzyme can be assigned to the phospho- $\alpha$ -glucosidase (PagI) clade of Family 4 of the glycosyl hydrolase super family. We

suggest that the products of *Lebu\_1527* and *Lebu\_1525*, catalyze the phosphorylative translocation and hydrolysis of sucrose isomers in *L. buccalis*, respectively. Four genetically diverse, but 16S rDNA-related, species of *Leptotrichia* have recently been described: *L. goodfellowii*, *L. hofstadii*, *L. shahii* and *L. wadei*. The phenotypic traits of these new species, with respect to carbohydrate utilization, have also been determined.

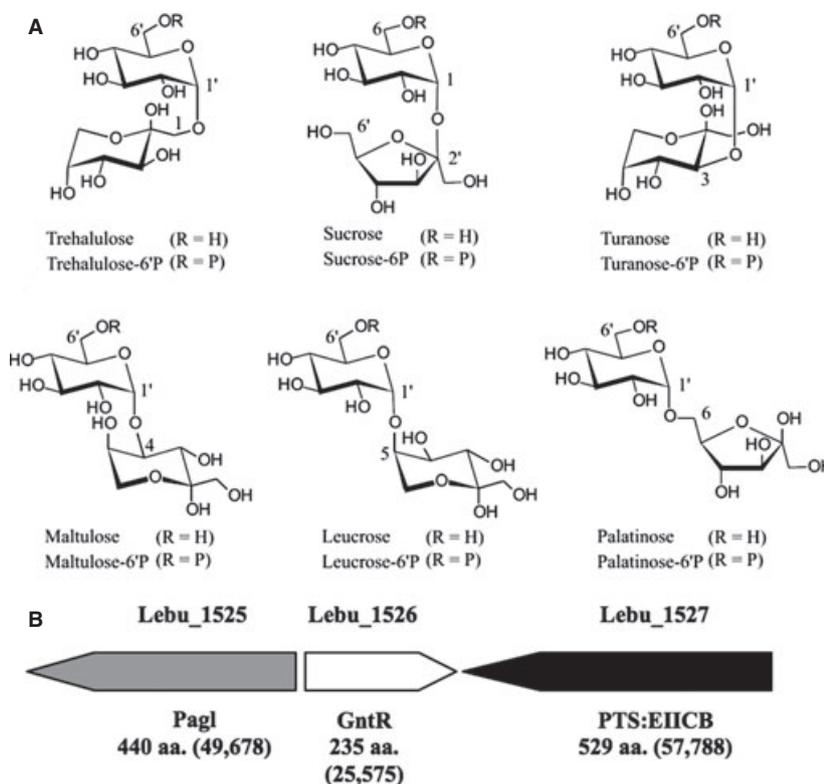
## INTRODUCTION

Of the many microbial species resident in oral biofilms (plaque) at the tooth surface (Becker *et al.*, 2002; Jenkinson & Lamont, 2005; Paster *et al.*, 2006; Dewhirst *et al.*, 2010), mutans streptococci have long been recognized as a primary contributor in the etiology of dental caries (Loesche, 1986; van Houte, 1994; Banas, 2004). The pathogenicity of organisms such as *Streptococcus mutans* and *Streptococcus sobrinus*, is attributable in part to: (i) the capacity of these species to produce, from dietary sucrose, extracellular glycan(s) that facilitate microbial adherence to the tooth surface, and (ii) the transport and fermentation of the disaccharide to lactic acid, the causative agent in the demineralization of tooth enamel (Slee & Tanzer, 1979; St. Martin & Wittenberger, 1979).

Remarkably, mutans streptococci are unable to metabolize the five structural isomers of sucrose designated: trehalulose, turanose, maltulose, leucrose and palatinose (Fig. 1A; Ziesenič *et al.*, 1989; Ooshima *et al.*, 1983, 1991; Matsuyama *et al.*, 2003). Consequently, these comparatively sweet, and ostensibly non-fermentable disaccharides are increasingly used as ‘non-cariogenic’ substitutes for dietary sucrose in Japan, Europe and the United States (Hamada, 2002; Matsukubo & Takazoe, 2006). In contrast with mutans streptococci, numerous non-oral species including *Bacillus subtilis*, *Klebsiella pneumoniae*, *Clostridium acetobutylicum*, *Lactobacillus casei* (Thompson *et al.*, 1998, 2001a,b, 2004, 2008) and *Fusobacterium mortiferum* (Thompson *et al.*, 1995; Pikiš *et al.*, 2002) readily use all five isomers of sucrose as energy sources for growth. In these bacteria, the isomers are translocated via an  $\alpha$ -glucoside-specific phosphoenolpyruvate-dependent: phosphotransferase system (PEP-PTS; Postma *et al.*, 1993) and the phosphorylated derivatives are hydrolyzed intracellularly to

glucose-6P and fructose by an NAD<sup>+</sup>- and Mn<sup>2+</sup>-dependent phospho- $\alpha$ -glucosidase (Thompson *et al.*, 1998; Rajan *et al.*, 2004; Yip *et al.*, 2007; Vocadlo & Davies, 2008). This catalytically unique enzyme has been assigned to Family GH4 of the glycosyl hydrolase superfamily (Henrissat & Davies, 1997; Cantarel *et al.*, 2009; Hall *et al.*, 2009).

The inability of mutans streptococci to ferment sucro-disaccharides is well established, but whether this deficiency is characteristic of other oral microorganisms is the subject of debate (Peltroche-Llacsahuanga *et al.*, 2001; Matsuyama *et al.*, 2003). Among the ~ 700 bacterial species presently catalogued in the Human Oral Microbiome Database (Chen *et al.*, 2010; Dewhirst *et al.*, 2010), *Leptotrichia buccalis* (Human Oral Taxon 563) occurs as a minor constituent of the total flora of plaque (Hofstad, 1992; Hofstad & Olsen, 2007; Eribe & Olsen, 2008). However, *L. buccalis* is highly saccharolytic (Kasai, 1965; Thompson, 2002) and, because it ferments a wide variety of monosaccharides and disaccharides to



**Figure 1** (A) Structural representations of sucrose and its five linkage-isomeric  $\alpha$ -D-glucosyl-D-fructoses. In the free form R at C6' = H, and in the phosphorylated derivatives R = phosphate. (B) Genetic composition and organization of the putative sucrose isomer metabolic (*sim*) operon of *Leptotrichia buccalis* 14201.

lactic acid, the bacterium has evident cariogenic potential (Brown & Gross, 1981; Hofstad, 1992). For many years, *L. buccalis* was believed to be the sole member of the genus *Leptotrichia*, but four novel oral species (*L. goodfellowii*, *L. hofstadii*, *L. shahii* and *L. wadei*) have been assigned to the genus (Eribe *et al.*, 2004). Equally significant was the discovery, in the recently sequenced genome of the type strain *L. buccalis* ATCC 14201, of a locus of three genes (Fig. 1B) with extensive similarity to those previously shown to encode the requisite proteins for dissimilation of sucro-disaccharides by non-oral bacteria, including *Escherichia coli* (Pikis *et al.*, 2006). These *in silico* findings prompted us to hypothesize that *L. buccalis* 14201 (and related species) might also metabolize the isomers of sucrose. We have tested this hypothesis using growth studies, enzymatic assays and proteomic analyses. Our findings are presented herein.

## METHODS

### Reagents and materials

High-purity sugars including glucose, sucrose, maltose and 1-*O*-methyl- $\alpha$ -D-glucopyranoside were purchased from Pfanstiehl Laboratories, Inc., Waukegan, IL, USA. The five isomers of sucrose were obtained from the following sources: trehalulose was a generous gift from Südzucker, Mannheim, Germany; turanose was obtained from Pfanstiehl; maltulose from TCI America, Portland, OR, USA; leucrose from Fluka (Fluka Chemie AG, Buchs, Switzerland) and palatinose was purchased from Wako Chemicals, Richmond, VA, USA. Phosphorylated chromogenic derivatives, including *p*-nitrophenyl- $\alpha$ -D-glucopyranoside-6-phosphate (pNP $\alpha$ G6P), were synthesized in this laboratory (Thompson *et al.*, 1995). Tris-Acryl M-DEAE, Ultrogel AcA-44 chromatography media and other materials, were obtained from Sigma-Aldrich, St. Louis, MO, USA. Polyclonal rabbit antibody to phospho- $\alpha$ -glucosidase (MalH) from *F. mortiferum* ATCC 25557 was prepared by Covance Research Laboratories, Denver, PA (Thompson *et al.*, 1995). Glucose-6-phosphate dehydrogenase (G6PDH, baker's yeast, E.C. 1.1.1.49) and phosphoglucose isomerase (PGI, baker's yeast, E.C. 5.3.1.9) were obtained from Boehringer Ingelheim, Ridgefield, CT, USA.

### Bacterial strains

*Leptotrichia buccalis* ATCC 14201 (DSM 1135/C-1013-b<sup>T</sup>) was purchased from the American Type Culture Collection (Manassas, VA). The four novel *Leptotrichia* species: *L. goodfellowii* CCUG 32286<sup>T</sup> (DSM 19756/LB 57<sup>T</sup>), *L. shahii* CCUG 47503<sup>T</sup> (LB 37<sup>T</sup>), *L. hofstadii* CCUG 47504<sup>T</sup> (LB 23<sup>T</sup>) and *L. wadei* CCUG 47505<sup>T</sup> (LB 16<sup>T</sup>), were obtained from the Culture Collection University of Göteborg, Sweden. *Leptotrichia goodfellowii* FO264 was kindly provided by Dr. F. Dewhirst from the Forsyth Institute Culture Collection (FICC), Boston, MA, USA. Chromosomal DNA from *L. goodfellowii* CCUG 32286<sup>T</sup>/DSM 19756 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany.

### Maintenance of *Leptotrichia* species

Organisms were grown under anaerobic conditions (BD GasPak) at 37°C in 10 ml Reinforced Clostridial Medium (Difco, Franklin Lakes, NJ, USA). At mid-log stage, the cells were collected by centrifugation and supernatants were decanted. The white cell pellets were resuspended in 0.5 ml of filter sterilized Clostridial Medium containing 20% glycerol and dispensed into sterile 1 ml tubes (Sarstedt, Newton, NC, USA). The samples were immediately frozen in dry ice, and maintained at -80°C until required.

### Growth studies and large-scale preparation of cells

To determine their carbohydrate specificities, *Leptotrichia* species were grown anaerobically (24–48 h) at 37°C in 10 ml of modified Todd–Hewitt broth supplemented with salts and vitamins (MTH, Robrish & Thompson, 1990) containing 0.4% (weight/volume) of desired sugar. For large-scale preparations, the organisms were grown anaerobically in 800 ml volumes of appropriate MTH. Stationary-phase cells were harvested by centrifugation (13,000 *g* for 20 min at 5°C), and washed by resuspension and centrifugation from 25 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM NAD<sup>+</sup>, 1 mM Mn<sup>2+</sup> and 1 mM dithiothreitol (TMND buffer). The yield of cells was ~ 1.1 g wet weight l<sup>-1</sup>.

### Cloning and expression of *L. buccalis* ATCC 14201 gene *Lebu\_1525* in *E. coli* TOP10

From the complete genome sequence of *L. buccalis* ATCC 14201 (Ivanova *et al.*, 2009; GenBank Accession no. NC\_013192) the following primers were designed to amplify the *Lebu\_1525* gene. Forward primer: 5'-GTGCACGCCATGGGTATGAAGAAATTTCAATTGTAGTAGC-3' (*Lebu\_1525* is shown in bold type and the *NcoI* restriction site is underlined). Reverse primer: 5'-GCGGTCCCGAATTCCTACTATTCAACTGGCCAGAAGTC-3' (the sequence complementary to the downstream region of *Lebu\_1525* is shown in bold type and the *EcoRI* restriction site is underlined). Polymerase chain reaction (PCR) amplification was carried out with a thermal cycler (GeneAmp 9700; PE Applied Biosystems, Carlsbad, CA, USA) using *Pfu* high-fidelity DNA polymerase from Stratagene, Santa Clara, CA, USA. The components of the amplification mixture (100  $\mu$ l) were as follows: 5 U *Pfu* high-fidelity DNA polymerase, 1 $\times$  reaction buffer provided by the manufacturer, 20 mM each of the four deoxynucleotide triphosphates, 250 ng of each primer and 100 ng *L. buccalis* ATCC 14201 chromosomal DNA. After an initial 2-min incubation at 95°C, the mixture was subjected to 30 cycles of amplification under the following conditions: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min kb<sup>-1</sup> of insert. This was followed by a 10-min runoff at 72°C. The amplicon was digested with restriction endonucleases *NcoI* and *EcoRI*, electrophoresed in 1% agarose gel, and purified using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). The PCR product (~1.3 kbp) was ligated to a similarly digested and purified pTrcHis2B expression vector (Invitrogen, Carlsbad, CA, USA) to form the recombinant plasmid pLBpagl. In this construct, the *Lebu\_1525* gene is under the control of the *trc* promoter, which is also regulated by the *lacO* operator and the product of the *lacI<sup>f</sup>* gene. Therefore, expression of the *Lebu\_1525* gene is fully induced in the presence of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Plasmid pLBpagl was transformed into *E. coli* TOP10 competent cells (Invitrogen), and colonies were selected on Luria–Bertani agar plates containing 150  $\mu$ g ml<sup>-1</sup> ampicillin. It should be noted that in the pTrcHis2B vector the fusion peptide is located at the C terminus. However, the stop codon included in the reverse primer prevents the expression of the fusion peptide.

### Purification of phospho- $\alpha$ -glucosidase (Pagl) from *E. coli* TOP10 (pLBpagl)

The plasmid-containing cells were grown in 800 ml Luria–Bertani broth containing ampicillin (150  $\mu$ g ml<sup>-1</sup>) in 2-l baffled flasks at 37°C on a rotary shaker. At an absorbance at 600 nm of ~0.6, IPTG (0.5 mM) was added, and growth was continued for 3–4 h. The cells were harvested by centrifugation (13,000 *g* for 10 min at 5°C) and washed by centrifugation from TMND buffer. Washed cells (16 g wet weight) of *E. coli* TOP10 (pLBpagl) were resuspended in 40 ml TMND buffer, and the organisms were disrupted at 0°C by sonic oscillation. The extract was clarified by high-speed centrifugation (180,000 *g* for 2 h at 5°C), and the supernatant was dialyzed for 16 h in a cold room against 4 l TMND buffer. The purification procedure for LB Pagl, was essentially that used previously for the purification of Pagl from *Lactobacillus casei* ATCC 334 (Thompson *et al.*, 2008). This two-stage process, involving Tris–Acryl M-DEAE (anion exchange) and Ultrogel AcA-44 (gel filtration) chromatography, yielded 9.8 mg of highly purified enzyme.

### Enzyme assays

#### Phospho- $\alpha$ -glucosidase (Pagl) activity

Enzyme activity was determined using a discontinuous assay and a 2-ml mixture that contained Tris–HCl buffer (50 mM, pH 7.5), 1 mM MnCl<sub>2</sub>, 0.1 mM NAD<sup>+</sup>, 25 mM dithiothreitol and the requisite amount of extract or purified Pagl. After 5 min of incubation at 37°C, reactions were initiated by addition of the chromogenic substrate (0.5 mM pNP $\alpha$ G6P). Samples of 0.25 ml were removed at 20-s intervals, and immediately injected into 0.75 ml of a solution of 0.5 M Na<sub>2</sub>CO<sub>3</sub> containing 0.1 M EDTA to stop the reaction. The absorbance at 400 nm was measured, and the rates of *p*-nitrophenol (pNP) formation were calculated from progress plots, assuming a molar extinction coefficient at pH 10.2 for the yellow *p*-nitrophenolate anion  $\epsilon = 18,000 \text{ M}^{-1} \text{ cm}^{-1}$ . Pagl activity was expressed in nmol or  $\mu$ mol of pNP $\alpha$ G6P hydrolyzed min<sup>-1</sup> mg<sup>-1</sup> of protein.

#### Fructokinase activity

In this assay fructose is first phosphorylated to fructose-6P by ATP-dependent fructokinase present in

the cell extract, and subsequently is converted to G6P by PGI. Oxidation of G6P is coupled to the reduction of NADP<sup>+</sup> by G6PDH, and the increase in absorbance at 340 nm was followed in a Beckman DU640 recording spectrophotometer. The standard 1-ml assay contained: 0.1 M potassium phosphate buffer (pH 7.0), 5 mM ATP, 10 mM Mg<sup>2+</sup>, 1 mM NADP<sup>+</sup>, 10 mM fructose, 5 U G6PDH, 5 U PGI and the reaction was initiated by addition of 50  $\mu$ l of cell extract. The initial rates of NADPH formation (equivalent to fructose-6P) were determined using the kinetics program installed in the instrument. In all calculations, a molar extinction coefficient  $\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$  was assumed for NADPH.

#### **Amplification and characterization of a region encoding the PagI and EII(CB) genes of *L. goodfellowii* CCUG 32286/DSM 19756**

Based on the incomplete genome sequence of *L. goodfellowii* CCUG 32286/DSM 19756, the following two pairs of primers were designed to amplify and characterize the DNA fragment encoding the putative PagI and EII(CB) genes (LepgoDRAFT\_1708 and LepgoDRAFT\_1710, respectively). The two primer sets were constructed as follows: F1 5'-CCGGTGTAATGTAATTCCTCCTC-3' and R1 5'-ATACCCTCCTAAAAAGCGAAAAA-3' for the amplification of PagI; F2 5'-GCCGAGACTGAGAAGT-TAGTATTAATAAAGGG-3' and R2 5'-GAATAA-CTGTGCGACAAGACTTAGAGTAAGTG-3' for the amplification of the EII(CB) gene. Amplifications were carried out with a thermal cycler (GeneAmp 9700; PE Applied Biosystems) using Phusion high-fidelity DNA polymerase (New England Biolabs, Inc., Ipswich, MA, USA). For the amplification of LepgoDRAFT\_1708 the following PCR settings were used: after an initial 1-min incubation at 98°C, the mixture was subjected to the following cycling parameters: two cycles of amplification under the following conditions: denaturation at 98°C for 20 s, annealing at 65°C for 20 s, and extension at 72°C for 2 min; two cycles with denaturation at 98°C for 20 s, annealing at 63°C for 20 s, and extension at 72°C for 2 min; and two cycles with denaturation at 98°C for 20 s, annealing at 61°C for 20 s, and extension at 72°C for 2 min. These cycles were followed by 29 cycles with the following conditions: denaturation at 98°C for 20 s, annealing at 59°C for 20 s, and extension at 72°C for

2 min. This procedure was followed by a 10-min runoff at 72°C. For the amplification of the region encoding LepgoDRAFT\_1710, the PCR parameters used were as follows: after an initial 1 min of incubation at 95°C, the mixture was subjected to 32 cycles of amplification. Each cycle consisted of 20 s of denaturation at 95°C, 30 s of annealing at 60°C, and extension at 72°C for 2 min. These were followed by a 10-min runoff at 72°C. All sequencing was performed by OriGene Technologies Inc. (Rockville, MD, USA). The MACVECTOR sequence analysis package (Version 11.0; Genetics Computer Group, Madison, WI, USA) was used to assemble, edit and analyze the results.

#### **Analytical procedures**

The molecular weight of denatured PagI was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the Novex XCell mini-cell system (Invitrogen). Novex NUPage (4–12% acrylamide) bis-Tris gels and morpholine-ethanesulfonic acid (MES)-SDS running buffer (pH 7.3) were used together with All Blue Precision Plus Protein Standards (BioRad, Hercules, CA, USA). Polypeptides were visualized by staining with Coomassie blue R-250. For Western blots, proteins and See-Blue pre-stained standards were transferred to nitrocellulose membranes using NuPage transfer buffer. Immunodetection of PagI was performed by incubation of the membrane with polyclonal antibody to MalH from *F. mortiferum*, and subsequently with goat anti-rabbit horseradish peroxidase-conjugated antibody as described previously (Thompson *et al.*, 1995).

The native  $M_r$  of PagI from *L. buccalis* was estimated chromatographically in an Ultrogel AcA-44 filtration column (1.6 by 94 cm) previously calibrated with TMND buffer containing the standards: alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and Blue Dextran. Protein concentrations of cell extracts were determined with the BCA Assay Kit (Pierce, Rockford, IL, USA), and the concentration of purified PagI was determined spectrophotometrically (absorbance at 280 nm) assuming a theoretical molar absorption coefficient of  $62,340 \text{ M}^{-1} \text{ cm}^{-1}$ . The N-terminal sequence of PagI was determined with an ABI 477A protein sequencer (Applied Biosystems, Foster

City, CA) connected online to an ABI 120A phenylthiohydantoin analyzer.

## RESULTS

### Growth of *L. buccalis* 14201 on sucrose isomers

*Leptotrichia buccalis* 14201 showed excellent growth on all isomers of sucrose with the exception of leucrose (Table 1). SDS-PAGE analyses of cell extracts revealed expression of a unique polypeptide during growth of *L. buccalis* on trehalulose, turanose, maltulose and palatinose (Fig. 2A, white arrows). The  $M_r$  of this polypeptide (~ 50 kDa), was the size expected for the product encoded by Lebu\_1525. Importantly, the results of a Western blot (Fig. 2B) showed that the induced polypeptide cross-reacted strongly with polyclonal antibody prepared against the NAD<sup>+</sup>-dependent and Mn<sup>2+</sup>-dependent phospho- $\alpha$ -glucosidase (MalH) from *F. mortiferum* (Thompson *et al.*, 1995). Data from enzymatic analyses (Table 2) provided convincing evidence that the ~ 50-kDa protein induced by growth on sucrose isomers also exhibited NAD<sup>+</sup>-dependent and Mn<sup>2+</sup>-dependent phospho- $\alpha$ -glucosidase (PagI) activity. The latter cytoplasmic enzyme catalyzes the hydrolysis of phosphorylated sucrose isomers to yield G6P and fructose. It seemed

likely that to facilitate the fermentation of the ketohexose, an ATP-dependent fructokinase might also be induced by growth on the sucro-disaccharides. Surprisingly, enzymatic analyses (Table 2) revealed constitutively high levels of fructokinase in all cell extracts. Inspection of the *L. buccalis* genome suggests that this ATP-dependent kinase may reside at locus Lebu\_0629, the translation product of which predicts a polypeptide of 295 residues and  $M_r = 32,626$  (GenBank Accession no. YP\_003163528).

### Purification and properties of PagI from *L. buccalis* 14201

Lebu\_1525 from *L. buccalis* 14201 was cloned, and the phospho- $\alpha$ -glucosidase (PagI) was expressed in *E. coli* TOP10. The enzyme was purified by two-stage chromatography (anion exchange and gel filtration), in 25 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM NAD<sup>+</sup> and 1 mM MnCl<sub>2</sub>. During purification, enzyme activity was monitored by hydrolysis of pNP $\alpha$ G6P, a chromogenic analogue of the phosphorylated *O*- $\alpha$ -linked isomers of sucrose. The specific activity of purified PagI was 0.74  $\mu$ mol pNP $\alpha$ G6P hydrolyzed min<sup>-1</sup> mg<sup>-1</sup> protein. The molecular weight of the denatured enzyme determined by SDS-PAGE (~ 50 kDa, Fig. 3, inset), was in excellent agreement

**Table 1** Growth of *Leptotrichia* species on various sugars<sup>1</sup>

Sugar added <sup>2</sup>	<i>L. buccalis</i> ATCC14201	<i>L. goodfellowii</i> CCUG 32286	<i>L. goodfellowii</i> FICC F0264	<i>L. shahii</i> CCUG 47503	<i>L. hofstadii</i> CCUG 47504	<i>L. wadei</i> CCUG 47505
None	NG	0.33 <sup>3</sup>	0.30 <sup>3</sup>	NG	NG	NG
Glucose	1.24	1.04	1.05	1.02	0.90	0.82
$\alpha$ -MGlc <sup>4</sup>	0.19	0.13	1.00	0.61	NG	NG
Maltitol	NG	0.24	0.77	0.32	NG	NG
Maltose	1.21	0.75	1.02	0.96	0.86	0.91
Lactose	1.29	0.40	0.76	0.99	0.71	NG
Sucrose	1.26	0.93	1.12	1.00	0.87	NG
<b>Trehalulose<sup>5</sup></b>	<b>1.08</b>	0.30 (NG) <sup>6</sup>	<b>1.09</b>	<b>1.20</b>	<b>0.77</b>	<b>0.94</b>
<b>Turanose</b>	<b>0.95</b>	0.23 (NG)	<b>1.08</b>	<b>1.15</b>	<b>0.91</b>	<b>0.96</b>
<b>Maltulose</b>	<b>0.92</b>	0.26 (NG)	<b>1.05</b>	<b>0.95</b>	<b>0.82</b>	(NG)
<b>Leucrose</b>	(NG)	0.23 (NG)	<b>0.83</b>	<b>1.04</b>	<b>0.22</b>	(NG)
<b>Palatinose</b>	<b>0.91</b>	0.29 (NG)	<b>1.01</b>	<b>1.09</b>	<b>0.89</b>	<b>0.81</b>

<sup>1</sup>Organisms were grown anaerobically at 37°C, and after 48 h, growth was recorded (absorbance at 600 nm) using a Beckman DU 640 spectrophotometer. Results are mean values from two experiments.

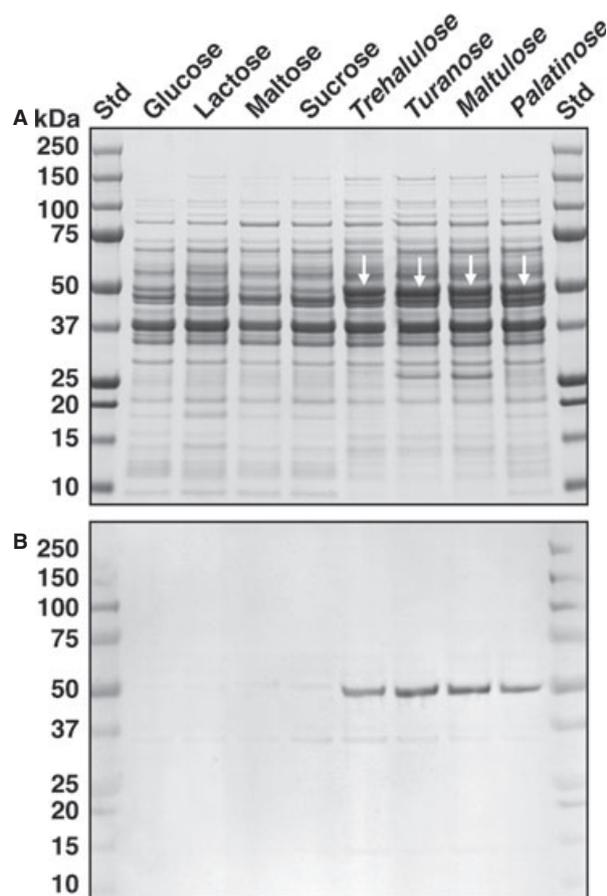
<sup>2</sup>All sugars were present at a final concentration of 0.4% (weight/volume).

<sup>3</sup>Note growth of *L. goodfellowii* strains in absence of sugar.

<sup>4</sup> $\alpha$ -MGlc: methyl- $\alpha$ -D-glucopyranoside.

<sup>5</sup>Sugars in bold face are sucrose isomers.

<sup>6</sup>(NG), no growth on designated sucrose isomer.



**Figure 2** Proteomic analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Western blot assay of extracts from cells of *Leptotrichia buccalis* 14201 grown on various sugars. (A) SDS–PAGE and visualization of proteins by staining with Coomassie blue R-250. Approximately 30  $\mu\text{g}$  of protein was applied per lane. Note the expression (*white arrowheads*) of a 50-kDa protein induced by growth of the organism on the four isomers of sucrose. (B) Western blot of a duplicate gel showing the immuno-cross-reactivity of the induced 50-kDa protein with polyclonal antibody raised against the  $\text{NAD}^+$ -dependent and  $\text{Mn}^{2+}$ -dependent phospho- $\alpha$ -glucosidase (MalH) from *Fusobacterium mortiferum*.

with the theoretical  $M_r$  of 49,678. However, the molecular weight of the native enzyme ( $110 \pm 10$  kDa) estimated by calibrated gel filtration chromatography (Fig. 3), suggests that in the catalytically active solution state, PagI assumes a homodimeric conformation. The homogeneity of the preparation was confirmed by the unambiguous micro-sequence determination of the first 25 residues from the N-terminus: MKKFSIVVAGGGSTFTP-GIVLMLLE. Optimum conditions for PagI activity were found in the pH range 6.9–7.3, at an assay tem-

**Table 2** Enzyme activities in extracts from cells of *Leptotrichia buccalis* 14201 grown on various sugars

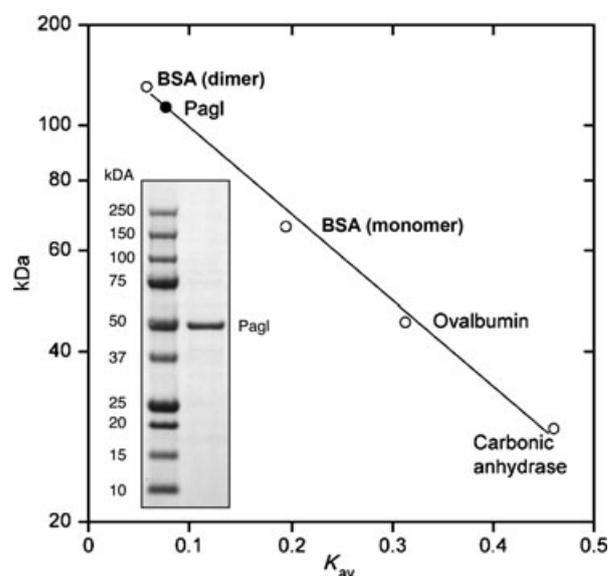
Growth sugar	P- $\alpha$ -glucosidase (PagI) <sup>1</sup>	ATP-dependent <sup>2</sup> fructokinase
Glucose	NDA <sup>3</sup>	36
Maltose ( $\alpha$ , 1 <sub>g</sub> -4 <sub>g</sub> )	0.52	116
Lactose ( $\beta$ , 1 <sub>gal</sub> -4 <sub>g</sub> )	NDA	102
<b>Trehalulose</b> <sup>4</sup> ( $\alpha$ , 1 <sub>g</sub> -1 <sub>t</sub> )	8.41	185
Sucrose ( $\alpha$ , 1 <sub>g</sub> -2 <sub>t</sub> )	NDA	159
<b>Turanulose</b> ( $\alpha$ , 1 <sub>g</sub> -3 <sub>t</sub> )	7.72	187
<b>Maltulose</b> ( $\alpha$ , 1 <sub>g</sub> -4 <sub>t</sub> )	3.28	75
<b>Palatinulose</b> ( $\alpha$ , 1 <sub>g</sub> -6 <sub>t</sub> )	5.26	212

<sup>1</sup>Specific activity: nmol pNP $\alpha$ G6P hydrolyzed  $\text{min}^{-1} \text{mg}^{-1}$  protein.

<sup>2</sup>Specific activity: nmol F6P formed  $\text{min}^{-1} \text{mg}^{-1}$  protein.

<sup>3</sup>NDA, no detectable activity: all values are average rates from three separate assays.

<sup>4</sup>Sugars in bold face are sucrose isomers.



**Figure 3** Estimation of the solution-state molecular weight of  $\text{NAD}^+$ -dependent and  $\text{Mn}^{2+}$ -dependent phospho- $\alpha$ -glucosidase (PagI) of *Leptotrichia buccalis* 14201 by gel filtration chromatography. Inset: demonstration of the purity, and estimated  $M_r$ ,  $\sim 50$  kDa of the denatured PagI monomer.

perature of 36–38°C. The enzyme was stable during prolonged incubation ( $> 0.5$  h) at 37°C in the presence of  $\text{NAD}^+$  and  $\text{Mn}^{2+}$  ions, but was irreversibly inactivated within 8 min when the two cofactors were omitted from the assay. Whereas pNP $\alpha$ G6P was readily hydrolyzed by PagI, neither the C2 nor C4 chromogenic stereomers (pNP $\alpha$ -mannopyranoside-6P and pNP $\alpha$ -galactopyranoside-6P, respectively) were substrates for the enzyme.

### Sugar utilization and metabolic diversity of *Leptotrichia* species

Data presented in Table 1 show considerable variation in the range of carbohydrates able to support growth of *Leptotrichia* species. In the absence of sugar(s), there is no growth of *L. buccalis*, *L. shahii*, *L. hofstadii* and *L. wadei*. However, there is significant growth of both strains of *L. goodfellowii* in sugar-free MTH medium. Arginine dihydrolase activity (GenBank accession no. ZP\_06011002) has been reported in this organism (Eribe *et al.*, 2004), and it is likely that arginine catabolism provides the requisite ATP for growth of *L. goodfellowii* in this medium. By extrapolation, we may assume that the enzymes comprising the arginine fermentation pathway are either absent or non-functional in other species. All species grew readily on glucose, maltose (and, with the exception of *L. wadei*) on lactose and sucrose also. Examination of the published DNA sequence of *L. buccalis* 14201 (Ivanova *et al.*, 2009) reveals genetic loci for the PEP-PTS for glucose (Lebu\_1946), sucrose (Lebu\_1906) and lactose (Lebu\_0588). Similar PEP-PTS operons are likely present in the genomes of *L. goodfellowii*, *L. shahii* and *L. hofstadii*, but presumably are absent in *L. wadei*. The results from growth experiments involving sucrose isomers revealed considerable diversity in those compounds able to support growth of the *Leptotrichia* species. For example, *L. shahii* and *L. hofstadii* showed excellent growth on all five isomers, whereas *L. buccalis* 14201 failed to grow on leucrose and *L. wadei* was unable to metabolize either maltulose or leucrose.

### Metabolism of sucrose isomers by *L. goodfellowii* strains FO264 and 32286/DSM 19756

Two strains have been assigned to the species *L. goodfellowii*. The complete chromosome of *L. goodfellowii* FO264 has been sequenced by the J. Craig Venter Institute (Rockville, MD, USA) as part of the Human Microbiome Project. A draft (incomplete) sequence of the chromosome of *L. goodfellowii* CCUG 32286/DSM 19756 was released on 10 January 2010 by the US Department of Energy, Joint Genome Institute (<http://www.jgi.doe.gov>). As reported in Table 1, *L. goodfellowii* FO264 showed excellent growth on all five sucrose isomers whereas,

unexpectedly, strain CCUG 32286 failed to grow on any of the sucro-disaccharides. To explain the latter findings, we hypothesized that *L. goodfellowii* 32286 might lack (or had incurred mutational inactivations in) the requisite genes for metabolism of the isomers. To obtain support for this hypothesis, an *in silico* analysis was undertaken of the contigs available from the incomplete sequence of the *L. goodfellowii* CCUG 32286/DSM 19756 genome. Contig 00026 encoded a gene, *LepgoDRAFT\_1708*, whose translation product was of similar size (440 residues,  $M_r = 49,672$ ) and with 90% identity to PagI from *L. buccalis* 14201 (Fig. 1). Contig 00026 also contained a presumptive pseudogene, *LepgoDRAFT\_1710*, whose predicted polypeptide (302 amino acids,  $M_r = 33,020$ ) was considerably smaller, but with 67% identity to the  $\alpha$ -glucoside-specific EII(CB) PTS transporter encoded by *Lebu\_1527* in *L. buccalis* 14201 (Fig. 1). We have confirmed these results by re-sequencing of the appropriate region of chromosomal DNA from *L. goodfellowii* CCUG 32286/DSM 19756 containing contig 00026 (see Methods). Importantly, we have found that a glutamate residue at position 302 in *LepgoDRAFT\_1710* is immediately followed by a tag stop codon. The presence of this codon presumably causes premature termination, and production of a functionally inactive EII(CB) transporter of the sucrose isomers.

### DISCUSSION

Growth of non-oral bacteria on the five isomers of sucrose has been well documented for species from gram-negative and gram-positive genera. However, to our knowledge, this report is the first to document the genetic organization and enzymatic basis for growth of a common resident of the oral microflora on sucro-disaccharides. Our findings show that for both *Leptotrichia* and non-oral species, the phosphorylative transport and hydrolysis of the isomeric derivatives are mediated by the products of chromosomal genes encoding an  $\alpha$ -glucoside-specific PTS transport system and a unique  $\text{NAD}^+$ -dependent and  $\text{Mn}^{2+}$ -dependent phospho- $\alpha$ -glucosidase. Notably, PagI is only the second phospho-glycosidase to have been isolated and characterized from *L. buccalis* 14201. Previously, a phospho- $\beta$ -galactosidase (Pbgal; EC. 3.2.1.85; gene locus *Lebu\_0590*) was purified to electrophoretic homogeneity from cells of

*L. buccalis* grown on lactose or lactulose (Thompson, 2002). Phospho- $\beta$ -galactosidase (UniProtKB/Swiss-Prot C7N8L9; 467 residues,  $M_r = 54,271$ ) hydrolyzes lactose 6'-phosphate to yield galactose-6P and glucose, but in contrast with PagI, Pbgal has no cofactor requirements for catalytic activity. Importantly, by the sequence-based CAZy classification (Henrissat & Davies, 1997; Cantarel *et al.*, 2009) Pbgal from *L. buccalis* 14201 is included in Family GH1, whereas PagI is assigned to GH4 of the glycosyl hydrolase superfamily.

Non-oral species, including *B. subtilis*, *Lactobacillus casei*, *C. acetobutylicum*, *F. mortiferum* and *K. pneumoniae*, invariably grow on all five sucrose isomers. However, in the present study we find considerable variation in the number(s) of isomers that support the growth of a particular species of *Leptotrichia*. For example, *L. shahii* metabolizes all five isomers, *L. buccalis* uses four of the sucro-disaccharides whereas, under the same conditions, *L. wadei* fails to grow on maltulose or leucrose. At the molecular level, such isomeric discrimination presumably reflects subtle species-specific mutations or conformational changes in either the transport or intracellular PagI proteins. The genetic basis for the variability of metabolic traits may be revealed by comparative analyses when the genomes of the different species of *Leptotrichia* have been sequenced in their entirety. Earlier surveys have reported the generation of acid from selected isomers of sucrose by some (but not all) oral species of *Actinomyces* and lactobacillae (Peltroche-Llacsahuanga *et al.*, 2001; Matsuyama *et al.*, 2003). Interestingly, *Actinomyces israelii* ATCC12102 and *Actinomyces gerencseriae* ATCC 23860 produced acid from all five sucrose isomers, but other species including *Actinomyces odontolyticus* ATCC 17929, were unable to ferment any of the disaccharides. The recently sequenced genomes of *A. odontolyticus* strains ATCC 17982 and F0309 encode a gene whose predicted polypeptide (460 residues,  $M_r \sim 49,430$ ) shows 28% identity with PagI from *L. buccalis* 14201, but a BLAST search (Altschul *et al.*, 1997) showed that a comparable gene was not present in the genomes of *Actinomyces viscosus* C505 or *Actinomyces oris* K20. Of the oral lactobacillae investigated, *Lactobacillus crispatus* strains JV-V01, ST1 and 214-1 encode a gene whose translation product (445 amino acids,  $M_r = 50,639$ ) exhibits 57% identity with PagI from *L. buccalis* 14201, but this

gene is absent from the genomes of *Lactobacillus salivarius* strains ATCC 11741 and GJ-24. The metabolic pathway(s) involved in the fermentation of sucrose isomers by *Actinomyces* and lactobacillae have yet to be elucidated. However, as genetic information accumulates in the Human Oral Microbiome Database, it is highly likely that genetic loci similar to the *sim* operon in *Leptotrichia* and *Lactobacillus casei* ATCC 334 will be discovered in currently unsequenced genomes of oral bacteria. In this context, the fermentation of presumed 'non-cariogenic' disaccharides by oral bacteria may be more widespread than presently envisaged. It remains to be seen whether continued commercial-scale use of the various sucrose isomers will impact upon the species composition or ecological distribution of the oral microflora (Hamada, 2002; Matsukubo & Takazoe, 2006).

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