molecular oral microbiology

molecular oral microbiology

A galactose-specific sugar: phosphotransferase permease is prevalent in the non-core genome of *Streptococcus mutans*

L. Zeng[1], P. Xue[1,*], M.J. Stanhope[2] and R.A. Burne[1]

1 Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, USA
2 Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Correspondence: Robert A. Burne, Department of Oral Biology, University of Florida, College of Dentistry, P.O. Box 100424, Gainesville, FL 32610, USA Tel.: +1 352 273 8850; fax: +1 352 273 8829; E-mail: rburne@dental.ufl.edu
*Present address: School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA.

Keywords: biofilm; dental caries; galactose-phosphotransferase system; sugar : phosphotransferase system; tagatose pathway
Accepted 18 January 2013
DOI: 10.1111/omi.12025

SUMMARY

Three genes predicted to encode the A, B and C domains of a sugar : phosphotransferase system (PTS) permease specific for galactose\ (EII^{Gal}) were identified in the genomes of 35 of 57 recently sequenced isolates of *Streptococcus mutans*, the primary etiological agent of human dental caries. Mutants defective in the EII^{Gal} complex were constructed in six of the isolates and showed markedly reduced growth rates on galactose-based medium relative to the parental strains. An EII^{Gal}-deficient strain constructed using the invasive serotype *f* strain OMZ175 (OMZ/IIGal) expressed significantly lower PTS activity when galactose was present as the substrate. Galactose was shown to be an effective inducer of catabolite repression in OMZ175, but not in the EII^{Gal}-deficient strain. In a mixed-species competition assay with galactose as the sole carbohydrate source, OMZ/IIGal was less effective than the parental strain at competing with the oral commensal bacterium *Streptococcus gordonii*, which has a high-affinity galactose transporter. Hence, a significant proportion of *S. mutans* strains encode a galactose PTS permease that could enhance the ability of these isolates to compete more effectively with commensal streptococci for galactose in salivary constituents and the diet.

INTRODUCTION

Galactose is one of the major constituents of the glycoconjugates produced by eukaryotes (Caldwell & Pigman, 1966), and is abundant in the glycoproteins and other secretions that bathe the surfaces of the oral cavity. When liberated via bacterial glycosidases or through other processes, galactose can be catabolized by many oral bacteria, so this carbohydrate helps to shape the composition and function of the oral microbiome in health and disease (Van der Hoeven & Camp, 1991; Hojo *et al.*, 2009). As the abundant streptococcal species that colonize the mouth and nasopharynx, including the viridans and mitis streptococci as well as group A and B streptococci, rely on fermentation of carbohydrates for generation of energy, the ability to efficiently uptake and metabolize galactose is believed to enhance the persistence and ability of these organisms to compete with other members of the microbial flora.

Our present knowledge about galactose use in the dental caries pathogen *Streptococcus mutans* comes primarily from the reference strain UA159 and indicates that this organism does not use galactose as efficiently as many other oral streptococci, in part because of the lack of a high-affinity galactose

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

transporter (Zeng *et al.*, 2010). Like many bacteria, *S. mutans* depends mainly on the phosphoenol pyruvate-energized sugar : phosphotransferase systems (PTS) to internalize and concomitantly phosphorylate a variety of carbohydrates that exist in the oral cavity. The PTS is usually composed of two general enzymes (Enzyme I and the phospho-carrier protein HPr) and a series of substrate-specific, membrane-associated Enzyme II (EII) permeases that are directly responsible for sugar transport (Postma *et al.*, 1993). The *S. mutans* strain UA159 has been reported to harbor as many as 14 PTS permeases in its genome (Ajdic *et al.*, 2002). However, previous studies have indicated an overall low affinity in substrate acquisition and slower growth on galactose compared with PTS sugars, such as glucose or fructose (Ajdic *et al.*, 1996; Ajdic & Ferretti, 1997; Abranches *et al.*, 2004). Furthermore, a recent publication from our group has suggested that this deficiency is likely due to the absence of a high-affinity galactose-specific PTS permease (Zeng *et al.*, 2010), which is often present in related Gram-positive bacteria, including *Streptococcus pneumoniae* (Kaufman & Yother, 2007), *Streptococcus pyogenes* (Loughman & Caparon, 2007) and *Streptococcus gordonii* (Zeng *et al.*, 2012). Instead, the major glucose-PTS permease EII^{Man} and a lactose-PTS (EII^{Lac}) permease are the primary transporters for galactose in UA159, but they require relatively high concentrations of galactose to allow for optimal growth (Zeng *et al.*, 2010).

Using next-generation high-throughput sequencing technology, an international collection of 57 clinical isolates of *S. mutans* has been subjected to whole genome shotgun sequencing (Do *et al.*, 2010; Cornejo *et al.*, 2012). This effort has resulted in the identification of a core genome of approximately 1490 open reading frames for the species *S. mutans.* Also identified were over 1900 genes that are present in only a subset of the strains, highlighting considerable genomic heterogeneity in the *S. mutans* population. Among the non-core genes, we identified a PTS permease complex bearing homology to galactose-PTS permeases previously identified in some Gram-positive bacteria. In this report we initiated the characterization of this new PTS permease and began to explore whether it could potentially impact the ecology of the oral microbiome.

METHODS

Bacterial strains and culture conditions

Wild-type strains of *S. mutans*, including strains UA159 (serotype *c*) and OMZ175 serotype *f*, 10 clinical isolates (Table 1) and *S. gordonii* DL1 were routinely maintained on brain–heart infusion (BHI; Difco Laboratories, Detroit, MI) agar plates and grown in BHI liquid medium. Antibiotic-resistant mutants were kept on BHI agar plates supplemented with erythromycin 10 μg ml^{-1} or kanamycin 1 mg ml^{-1} (Sigma-Aldrich, St Louis, MO). Tryptone-vitamin (TV) base medium (Burne *et al.*, 1999) supplemented with various carbohydrates was used to test bacterial growth phenotypes and to grow cells for enzymatic assays. All bacterial cultures were incubated without agitation in a 5% CO_2 aerobic atmosphere at 37°C, unless specified otherwise.

DNA manipulation and construction of mutants

Ligation transformation

The deletion mutant of OMZ175 defective in the putative EII^{Gal} complex was constructed via a ligation transformation procedure described previously (Lau

Table 1 Bacterial strains used in this study

Strains	Serotype	Source or reference
Streptococcus mutans		
UA159	*c*	ATCC 700610
OMZ175	*f*	(Scholler *et al.*, 1981)
OMZ/IIGal	*f*	This study
11SSST2	*c*	(Cornejo *et al.*, 2012)
11SSST2/IIGal	*c*	This study
SM6	*c*	(Do *et al.*, 2010)
SM6/IIGal	*c*	This study
ST1	*c*	(Do *et al.*, 2010)
ST1/IIGal	*c*	This study
3SN1	*e*	(Cornejo *et al.*, 2012)
3SN1/IIGal	*e*	This study
U2A	*e*	(Do *et al.*, 2010)
U2A/IIGal	*e*	This study
15JP3	*c*	(Cornejo *et al.*, 2012)
11VS1	*e*	(Cornejo *et al.*, 2012)
NFSM2	*c*	(Cornejo *et al.*, 2012)
T4	*c*	(Do *et al.*, 2010)
NLML4	*e*	(Cornejo *et al.*, 2012)
Streptococcus gordonii		
DL1		(LeBlanc & Hassell, 1976)

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

et al., 2002). Primers specific for the regions flanking the EII^{Gal} complex were designed to include restriction enzyme sites that facilitated fusion with an antibiotic-resistance cassette: for the region 5′ of EII^{Gal}, OMZ-gal-1 (5′-GGT GAT GAA GAA GTT AAC CAG CAA-3′) and OMZ-gal-2 *Hin*dIII (5′-GCA ATC TCT TTG AAA GCT TCT TCT TTT TCT T-3′); and for region 3′ of EII^{Gal}, OMZ-gal-3 *Eco*RI (5′-TGC TGT GAA TTC ACT TGC TAT TTA TAC TCT-3′) and OMZ-gal-4 (5′-GTT CCC ATA AAG GCA CTC AAG AAA-3′). The polymerase chain reaction (PCR) products were treated with the respective restriction enzymes and ligated with an erythromycin cassette released from plasmid NPEM1 (Zeng & Burne, 2009) via digestion using *Hin*dIII and *Eco*RI, and subsequently used to transform the wild-type strain OMZ175. The transformation was carried out in BHI medium in the presence of 10% horse serum and 100 nM competence-stimulating peptide (Petersen & Scheie, 2010). Erythromycin-resistant transformants were further confirmed by PCR and sequencing, including sequencing the flanking regions to ensure no unwanted mutations were introduced.

Deletion of the EII^{Gal} complex in five additional isolates of S. mutans

Five clinical isolates of *S. mutans* (3SN1, U2A, 11SSST2, SM6, ST1; Cornejo *et al.*, 2012) were incubated for 3 h with 1 μg chromosomal DNA extracted from strain OMZ/IIGal, and then plated on BHI agar plates containing erythromycin. The erythromycin-resistant transformants were subsequently confirmed by PCR using primers that target the *lacD-lacF* region of the genome.

Growth tests

Growth phenotypes of the wild-type strain UA159, OMZ175 and its isogenic EII^{Gal} mutant OMZ/IIGal were studied at 37°C in a 5% CO_2 aerobic environment in TV base medium supplemented with various carbohydrates. The optical density at 600 nm (OD_{600}) of the cultures was monitored hourly using a spectrophotometer. Conversely, a Bioscreen C automated growth monitor (Oy Growth Curves Ab Ltd., Helsinki, Finland) was employed to assess the growth characteristics of 10 clinical isolates and their isogenic EII^{Gal} mutants (when applicable) in TV-galactose medium at 37°C. When the Bioscreen C was employed, 40 μl of sterile mineral oil was overlaid on top of the cultures to minimize the inhibitory impact of air on the growth of *S. mutans* (Ahn *et al.*, 2007).

Mixed-species liquid culture competition assay

Following a protocol employed previously (Zeng *et al.*, 2012), *S. mutans* and *S. gordonii* strains were cultured in BHI medium to mid-exponential phase, mixed in a 1 : 1 ratio, and diluted 100-fold into TV medium supplemented with 0.5% galactose. The *S. mutans* strains each contained a kanamycin-resistance marker to facilitate enumeration. The colony-forming units (CFU) of each bacterial strain were immediately measured by serial dilution and plating on both BHI and BHI-kanamycin plates. The mixed cultures were then incubated at 37°C in a 5% CO_2 atmosphere for 24 h, before the pH was measured and CFU of each bacterial species were again determined by plating and incubation for 48 h. The CFU from BHI plates represented the total of the two bacterial species, whereas CFU on BHI-kanamycin plates represented that of *S. mutans* alone. Data were derived from three biological replicates.

Enzymatic assays

Chloramphenicol acetyltransferase (CAT; Shaw, 1975) and phosphoenolpyruvate-dependent PTS sugar transport assays (LeBlanc *et al.*, 1979) were carried out according to previously published protocols (Zeng *et al.*, 2006).

RESULTS AND DISCUSSION

Presence of genes for the EII^{Gal} complex in clinical isolates of *S. mutans*

From a total of 57 strains sequenced (Cornejo *et al.*, 2012), the coding sequences of a putative galactose-PTS EII complex, with open reading frames for EIIA^{Gal}, EIIB^{Gal} and EIIC^{Gal}, were identified in the genomes of 35 isolates, but not in the completely sequenced reference strain UA159. Using the same query protein sequences, a BLAST search (http://blast.ncbi.nlm.nih.gov/) identified only one *S. mutans* strain (JL23), out of the four stains for which complete genome sequence information is available, that

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

contained the same EII^{Gal} complex. Searches using nucleic acid sequences resulted in no additional hits in *S. mutans* (data not shown). Among these EII^{Gal}-positive strains was OMZ175, a Bratthall serotype *f* strain that was chosen for further study on the basis of our previous demonstration that it is capable of invading human coronary artery endothelial cells (Abranches *et al.*, 2009) and has markedly enhanced virulence in the greater waxworm model than *S. mutans* UA159 (Abranches *et al.*, 2011).The sequences of the genes and proteins from OMZ175 are presented in the Supplementary material (Fig. S1) and the sequences can be accessed at http://strep-genome.bscb.cornell.edu/cgi-bin/hgBlat using the *S. mutans* pull-down menu under 'Genomes'. Further, the genome sequences of all isolates examined in this study have been deposited with GenBank (Cornejo *et al.*, 2012).

As depicted in Fig. 1, the EII^{Gal} complex in OMZ175 is organized as part of the *lac* operon, which also includes the tagatose-6-phosphate pathway genes (*lacABCD*), a lactose-PTS EII complex (*lacFE*) and a phospho-β-galactosidase (*lacG*; Rosey & Stewart, 1992; Zeng *et al.*, 2010). Similarly organized *lac* operons have been detected in all EII^{Gal}-positive strains analysed so far (data not shown). When the EII^{Gal} gene products were aligned against known galactose-PTS EII genes of *S. pneumoniae* (Kaufman & Yother, 2007), significant similarity in primary sequences was evident: EIIA^{Gal} 60%, EIIB^{Gal} 82% and EIIC^{Gal} 75%. Comparable levels of similarity were also found between these open reading frames and their apparent homologues in *S. gordonii*, *Streptococcus bovis*, *Streptococcus gallolyticus*, *Enterococcus faecalis*, *Streptococcus mitis*, *Streptococcus agalactiae*, *S.pyogenes* and other Gram-positive bacteria (data not shown). Further comparison indicated largely identical primary sequences in the rest of the operon between the genomes of UA159 and OMZ175, with only LacR, LacD and LacG containing a single amino acid replacement in each protein.

The genetic divergence between these two groups of *S. mutans* strains may be the result of gene acquisition by horizontal transfer or from loss of the EII^{Gal} genes. More importantly, the possession or the lack of a high-affinity galactose transport system could represent a fundamental difference within the species *S. mutans* related to the ability of isolates to adapt to particular niches in the oral cavity. For example, strains with the high-affinity galactose porter may have retained the capacity to better compete with commensal streptococci that generally have the same system, perhaps at the expense of caries-associated traits. In fact, a preliminary analysis of the distribution patterns of 1900 non-core genes within the 57 isolates suggests that there may be a small subset of non-core genes that show positive or negative correlations with the presence of the EII^{Gal} locus (Table S1). We are currently exploring the significance of these associations, but here we undertook experimentation to begin to characterize the function of the putative permease and how it could affect traits of *S. mutans* that could be related to competitive fitness or virulence.

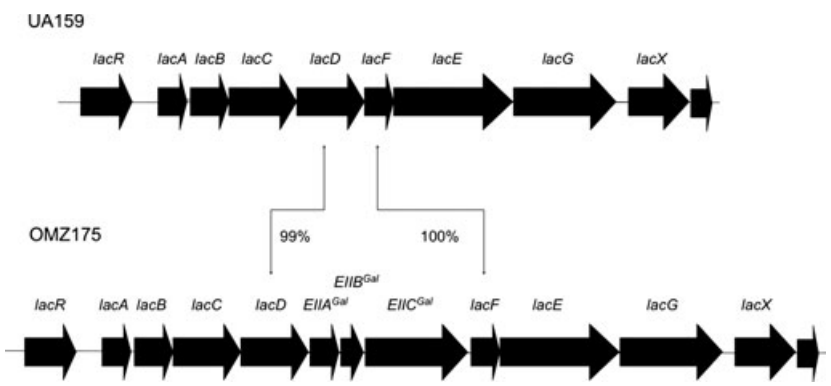

Figure 1 Diagrams depicting the tagatose-6-phosphate pathway clusters in *Streptococcus mutans* strains UA159 and OMZ175. A three-gene cluster encoding a putative galactose phosphotransferase (PTS) Enzyme II–*EIIA^{Gal}, EIIB^{Gal}* and *EIIC^{Gal}*–exists in strain OMZ175, but not UA159. A single amino acid replacement was identified in each of the LacR, LacD and LacG primary sequences between OMZ175 and UA159. Otherwise 100% sequence identity was observed among all homologous open reading frames. Other *lac* clusters similar to that in OMZ175 have been detected in all other EII^{Gal}-containing isolates analysed so far (data not shown).

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Contribution of EII^{Gal} to utilization of galactose by *S. mutans*

To investigate the function of the putative galactose-PTS permease, a deletion mutant was constructed using OMZ175 as the parental strain by replacing the entire coding region of $EIIABC^{Gal}$ with a non-polar erythromycin-resistance marker (Zeng & Burne, 2009). Growth of the mutant strain, OMZ/IIGal, in TV-base medium supplemented with 0.5% of glucose, fructose, galactose or lactose was compared with the parental strain OMZ175 and the standard laboratory strain UA159. When compared with the wild-type strain OMZ175 growing on galactose, OMZ/IIGal displayed a dramatically slower growth rate, with a minimum doubling time of 253 vs. 117 min for OMZ175 (Table 2). The mutant also achieved a lower OD_{600} after 24 h of incubation. However, OMZ/IIGal maintained largely the same growth phenotypes as that of the parental strain when tested on TV medium containing 0.5% glucose, fructose or lactose (Table 2). Notably, the growth rate of strain OMZ175 on 0.5% galactose was moderately faster than that of *S. mutans* UA159 (135 min), which lacks the EII^{Gal} complex. Furthermore, when the pH of stationary phase cultures grown in TV with 0.5% galactose was measured, OMZ175 produced a final pH of 4.79 ± 0.06, while OMZ/IIGal had a pH of 5.25 ± 0.06. These data add further support to the notion that the EII^{Gal} complex contributes to improved assimilation of galactose by OMZ175.

To further characterize the contribution of the EII^{Gal} complex and other galactose-transporting EII permeases to growth on galactose, the same manual growth tests were repeated using TV containing 2% galactose. As shown in Table 2, both OMZ175 and OMZ/IIGal displayed more rapid growth on 2% galactose compared with 0.5% galactose, although OMZ/IIGal still grew significantly more slowly than the parental strain. Interestingly, out of three strains tested, UA159 grew the fastest on 2% galactose. These findings are consistent with our previous reports demonstrating the presence of lower-affinity galactose transporters in *S. mutans* (Zeng *et al.*, 2010), including EII^{Man} and EII^{Lac}, which are also present in the genome of OMZ175.

To rule out the possibility that the contribution of the EII^{Gal} complex to galactose utilization by OMZ175 was an isolated phenomenon, additional EII^{Gal} mutants were constructed in five clinical isolates of *S. mutans*. These strains were selected based on (i) the presence of a homologous EII^{Gal} cluster in the genome; (ii) their ability to become competent for genetic transformation; and (iii) the fact that they were representatives of serotypes *c* and *e* (serotype *c*, 11SSST2, SM6, ST1; and *e* 3SN1, U2A). Chromosomal DNA was extracted from strain OMZ/IIGal and used to transform these isolates, the mutation was verified by PCR and sequencing, and the resultant mutants were compared with their parental strains for their ability to grow on galactose. Five additional isolates that lack a homologous EII^{Gal} locus (serotype *c*, NFSM2, T4, 15JP3; and serotype *e*, 11VS1, NLML4) were also included in this test. Due to the large number of samples to be tested, a Bioscreen C monitor was used to automate the process. Cells were incubated at 37°C in an aerobic environment, but with mineral oil overlaid to mitigate the inhibitory effects of oxygen on *S. mutans* growth (Ahn *et al.*, 2007). As shown in Table 3, various degrees of reduction in growth rate were evident in all five mutants lacking

Table 2 Doubling time (T_d in min) and final optical density (OD_{600}) of strains OMZ175, OMZ/IIGal and UA159 growing in various carbohydrate conditions

	OMZ175		OMZ/IIGal		UA159	
TV with	T_d	OD_{600}	T_d	OD_{600}	T_d	OD_{600}
0.5% glucose	80.7 ± 3.7	0.84	77.3 ± 5.2	0.85	ND	ND
0.5% fructose	71.5 ± 2.8	0.78	74.9 ± 1.2	0.82	ND	ND
0.5% galactose	117.1 ± 16	0.83	253.3 ± 19	0.68	135.3 ± 11	0.93
2% galactose	97.1 ± 14	0.80	106.6 ± 15	0.73	83.5 ± 2.4	0.91
0.5% lactose	67.2 ± 0.1	0.82	69.7 ± 3.1	0.81	ND	ND

Results (average $\pm$ SD) are based on manual growth curve analyses using Tryptone-vitamin (TV) medium containing various carbohydrates, in the presence of 5% of CO_2.
ND, not determined.

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Table 3 Doubling time ($T_d \pm$ SD in min) of various clinical isolates, and their EII^{Gal} deletion mutants if applicable, obtained using a Bioscreen monitor in Tryptone-vitamin medium with 0.5 or 2% of galactose

Strain	Serotype	T_d (0.5% galactose)	T_d (2% galactose)
U2A	*e*	272.3 ± 16	167.6 ± 12
U2A/IIGal	*e*	305.5 ± 3.1	209.5 ± 7.3
3SN1	*e*	189.3 ± 22	129.3 ± 4.5
3SN1/IIGal	*e*	403.3 ± 40	212.5 ± 5.3
11SSST2	*c*	172.5 ± 2.3	144.8 ± 9.5
11SSST2/IIGal	*c*	312.5 ± 40	175.7 ± 16
SM6	*c*	202.5 ± 20	127.1 ± 4.4
SM6/IIGal	*c*	250.5 ± 40	206.1 ± 36
ST1	*c*	172.0 ± 0.3	156.6 ± 9.3
ST1/IIGal	*c*	256.0 ± 2.1	172.5 ± 4.1
15JP3	*c*	331.1 ± 17	181.4 ± 7.2
11VS1	*e*	228.1 ± 6.3	153.7 ± 6.1
NFSM2	*c*	348.0 ± 59	202.3 ± 9.8
T4	*c*	239.1 ± 11	147.2 ± 7.6
NLML4	*e*	266.4 ± 4.7	151.7 ± 6.4

the EII^{Gal} complex when growing in TV-galactose. Based on these observations, this novel galactose-PTS could be contributing in significant ways to galactose use by a majority of the *S. mutans* population colonizing humans.

When tested in 2% galactose, all the isolates demonstrated improved growth, as compared with that displayed in 0.5% galactose; this was also true in the 5 EII^{Gal} mutants. At the same time, strains that contained the EII^{Gal} complex showed a somewhat modest improvement in growth rate on 2% galactose, as opposed to strains without the galactose permease. One exception was strain U2A, which had markedly faster growth on 2% galactose, but showed the smallest reduction of growth rate when its EII^{Gal} genes were deleted. Importantly, though, the presence of EII^{Gal} consistently improved the growth of strains in lower concentrations of galactose, but when galactose was present in high concentrations, arguably higher than might be encountered in the oral cavity, EII^{Gal} became less important, likely due to the activity of lower-affinity, secondary systems that can internalize galactose (Zeng *et al.*, 2010). Thus, EII^{Gal} could improve the competitive fitness of *S. mutans* strains *in vivo*, where free galactose would be present in low steady-state levels as it is liberated from glycoconjugates or when it is presented transiently as part of the human diet.

Transport of galactose by EII^{Gal+} isolates

To better assess the role of the EII^{Gal} complex in galactose metabolism, PTS assays were employed to compare the rate of galactose transport by both the wild-type strain OMZ175 and its isogenic mutant OMZ/IIGal. Bacterial strains were cultured to the exponential phase in TV medium supplemented with 0.5% galactose before the cells were washed in phosphate buffer, permeablized with toluene : acetone (1 : 9) and subjected to phosphoenol pyruvate-dependent sugar phosphorylation assay. The results (Fig. 2) showed significantly lower PTS activities in the EII^{Gal}-deficient mutant when compared with that of the wild-type strain grown under the same conditions. Given the limited supply of galactose in the oral cavity under normal circumstances (Caldwell & Pigman, 1966), a high-affinity EII^{Gal} complex could significantly improve the ability of its host to compete for this carbohydrate against oral commensal bacteria.

Role of EII^{Gal} in competition against *S. gordonii* DL1

To begin to explore the potential role of the EII^{Gal} permease in microbial ecology in the human oral cavity, a liquid culture, mixed-species competition assay was performed using either OMZ175 or OMZ/IIGal competing against *S. gordonii* strain DL1. We previously

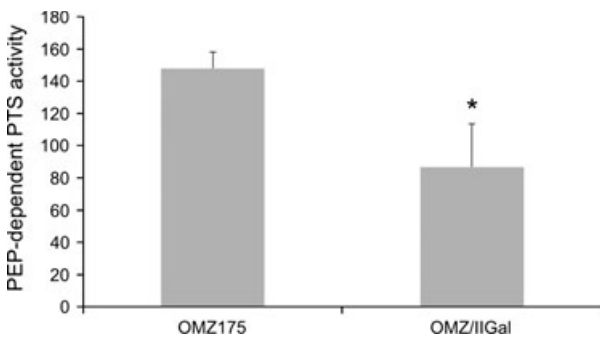

Figure 2 Results of the phosphoenolpyruvate galactose-phosphorylation assay. Strains OMZ175 and OMZ/IIGal were grown to exponential phase in Tryptone-vitamin with 0.5% galactose, washed with phosphate buffer, permeabilized with toluene : acetone, and then subjected to phosphotransferase (PTS) assays as described previously (Zeng *et al.*, 2006). The data [where units are nmol of NADH oxidized (mg of protein)$^{-1}$ min^{-1}] were the averaged results of three independent biological replicates each assayed in triplicate. The error bars represent SD. The asterisk represents a *P*-value of < 0.005 as determined by Student's *t*-test.

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

showed that *S. gordonii* DL-1, which has a high-affinity galactose PTS (Zeng *et al.*, 2012), had a strong competitive advantage over UA159 when galactose was the growth carbohydrate. To facilitate CFU enumeration, both *S. mutans* strains were engineered to contain a kanamycin-resistance marker in the coding sequence of the *gtfA* gene, which is involved only in sucrose metabolism (Russell *et al.*, 1988) and does not affect the competitive fitness of *S. mutans* in an animal model (Yamashita *et al.*, 1993). Both *S. mutans* and *S. gordonii* strains were cultured in BHI medium to mid-exponential phase before being mixed in 1 : 1 ratios and diluted 1 : 100 into fresh TV medium supplemented with 0.5% galactose. The mixed cultures were incubated statically for 24 h, then the pH was measured and CFU for both bacterial species were enumerated by plating. After 24 h of co-culturing, the wild-type strain OMZ175 made up 24.5% ($\pm$ 3%)of the viable counts of the mixed culture, whereas strain OMZ/IIGal accounted for only 11% ($\pm$ 2%) of the total bacterial population. Measurement of the mixed-species cultures indicated that the OMZ175 : DL1 co-cultures had significantly lower pH (4.82 $\pm$ 0.02) than those of the OMZ/IIGal : DL1 co-cultures (4.91 $\pm$ 0.02). These results indicated that the presence of the EII^{Gal} complex enables *S. mutans* to better compete against an antagonistic commensal species not only by allowing more efficient use of galactose, but perhaps by helping the organism to lower the environmental pH to a greater extent, which would be beneficial to the survival of an aciduric bacterium like *S. mutans.*

Ability of galactose to induce carbon catabolite repression in the EII^{Gal+} background

Bacteria have the capacity to repress the expression of secondary catabolic genes/pathways when presented with preferred metabolizable sugars through a process known as carbon catabolite repression (CCR). In Gram-positive bacteria, this regulation generally requires the activity of the catabolite control protein CcpA and its cognate binding partner HPr-Ser46-PO_4 (Deutscher, 2008). It has long been observed that galactose is not very effective at eliciting CCR in *S. mutans* UA159. This is partly because *S. mutans* metabolizes galactose mainly through the tagatose-6-phosphate pathway (de Vos & Vaughan, 1994; Zeng *et al.*, 2010), which bypasses the production of glucose-6-phosphate and fructose-1,6-bisphosphate that helps to trigger the production of HPr-Ser46-PO_4 (Deutscher, 2008). It was demonstrated in our previous studies on regulation of the fructan hydrolase gene *fruA*, which harbors two CcpA-binding catabolite response elements (*cre*) in its promoter region, that CCR was much less efficient in cells growing on galactose compared with cells growing on glucose or fructose (Abranches *et al.*, 2008). Importantly, in *S. mutans*, CCR initiated via CcpA-independent mechanisms exerted by PTS permeases and HPr appear to play dominant roles in controlling carbon metabolism in this bacterium, as opposed to other low-GC Gram-positive bacteria (Zeng & Burne, 2010).

To ascertain whether the presence of the EII^{Gal} permease could affect the response of catabolite repressible operons to the presence of galactose, we examined expression of the CcpA-independent *levD* gene using a *levD-cat* gene fusion (Zeng *et al.*, 2006) integrated in single copy into the chromosome of strains UA159, OMZ175 and OMZ/IIGal via a double cross-over recombination. Each strain was grown to exponential phase in TV medium supplemented with galactose, the inducing polysaccharide inulin, or a combination of both inulin and galactose, before being harvested for CAT assays to assess the *levD* promoter activity. When tested in strain UA159, the results (Fig. 3) indicated that the addition of galactose lowered the expression of the *levD* promoter from 2021 $\pm$ 98 units when growing in inulin alone to 418 $\pm$ 31 units when growing on a combination of galactose and inulin. For comparison, we noted that the ratio of CAT activity on inducer vs. inducer + galactose as a CCR index of 4.8. In contrast, when the promoter fusion was tested in the background of strain OMZ175, the CCR index was 75, so galactose was much more effective at triggering CCR in the EII^{Gal}-positive OMZ175 compared with UA159. However, when the same comparison was made in strain OMZ/IIGal, the CCR index for the promoter fusion was a mere 1.7, indicating significant alleviation of CCR. Also of note was the elevated expression of this promoter in strain OMZ/IIGal when growing in TV-galactose or TV-inulin, compared with OMZ175 growing in the same conditions. Therefore, it appears that the EII^{Gal} complex in strain OMZ175 can contribute to CcpA-independent CCR when galactose is present, indicating that galactose is a preferred

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

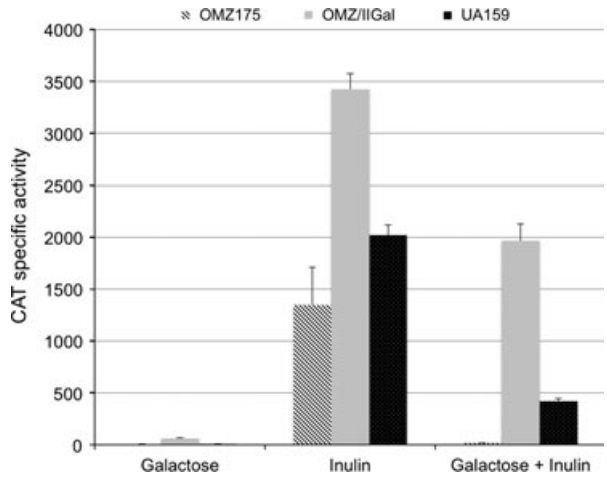

Figure 3 Results of the chloramphenicol acetyltransferase (CAT) assays representing the expression of a P*levD-cat* fusion. The reporter fusion was tested in the background of OMZ175, OMZ/IIGal and UA159, while growing exponentially in Tryptone-vitamin media supplemented with 0.5% of galactose, inulin or a combination of both carbohydrates. Specific CAT activities are expressed as nmol of chloramphenicol acetylated (mg of protein)$^{-1}$ min^{-1}. The error bars represent SD calculated using three independent biological replicates.

carbohydrate in OMZ175, unlike in strain UA159. These new findings add to the support for the notion that CCR in *S. mutans* is mediated directly through multiple components of the PTS, bypassing the requirement of CcpA and certain metabolic intermediates that are essential for CCR in other low G+C Gram-positive bacteria.

CONCLUDING REMARKS

A galactose-PTS was identified in more than half of a collection of 57 newly sequenced *S. mutans* clinical isolates and its functionality was confirmed by growth and sugar transport assays. Although it is clear that the presence of this EII^{Gal} gene cluster affords its hosts an enhanced capacity for assimilating galactose, especially when the carbohydrate is present in lower concentrations, it remains to be determined if such a trait correlates with altered cariogenicity, or better colonization, persistence or competition with commensals; or perhaps even enhanced survival or virulence when the organism enters the bloodstream. Nevertheless, as galactose is known to be an important carbohydrate used as an energy source and structural component of many oral bacterial species, the ability to efficiently uptake this hexose by a well-characterized caries agent could have important implications in our understanding of the microbial ecology that affects the balance between healthy and cariogenic oral biofilms. It will also be of interest to determine whether the possession of particular non-core genes that are positively or negatively associated with the presence of EII^{Gal} influences the physiology or virulence of *S. mutans* isolates.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institute of Dental and Craniofacial Research (DE12236) to R.A.B. and a grant from the National Institute of Allergy and Infectious Diseases (AI73368) to M.J.S and R.A.B. We thank Tristan Lefébure for the initial analysis of gene content and Vince Richards for assistance with analysing gene content associated with the EII^{Gal} locus.

REFERENCES

Abranches, J., Chen, Y.Y. and Burne, R.A. (2004) Galactose metabolism by Streptococcus mutans. *Appl Environ Microbiol* **70**: 6047–6052.

Abranches, J., Nascimento, M.M., Zeng, L. *et al.* (2008) CcpA regulates central metabolism and virulence gene expression in Streptococcus mutans. *J Bacteriol* **190**: 2340–2349.

Abranches, J., Zeng, L., Belanger, M. *et al.* (2009) Invasion of human coronary artery endothelial cells by Streptococcus mutans OMZ175. *Oral Microbiol Immunol* **24**: 141–145.

Abranches, J., Miller, J.H., Martinez, A.R., Simpson-Haidaris, P.J., Burne, R.A. and Lemos, J.A. (2011) The collagen-binding protein Cnm is required for Streptococcus mutans adherence to and intracellular invasion of human coronary artery endothelial cells. *Infect Immun* **79**: 2277–2284.

Ahn, S.J., Wen, Z.T. and Burne, R.A. (2007) Effects of oxygen on virulence traits of Streptococcus mutans. *J Bacteriol* **189**: 8519–8527.

Ajdic, D. and Ferretti, J.J. (1997) Regulation of the galactose operon of Streptococcus mutans. *Adv Exp Med Biol* **418**: 1015–1018.

Ajdic, D., Sutcliffe, I.C., Russell, R.R. and Ferretti, J.J. (1996) Organization and nucleotide sequence of the Streptococcus mutans galactose operon. *Gene* **180**: 137–144.

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Ajdic, D., McShan, W.M., McLaughlin, R.E. *et al.* (2002) Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A* **99**: 14434–14439.

Burne, R.A., Wen, Z.T., Chen, Y.Y. and Penders, J.E. (1999) Regulation of expression of the fructan hydrolase gene of Streptococcus mutans GS-5 by induction and carbon catabolite repression. *J Bacteriol* **181**: 2863–2871.

Caldwell, R.C. and Pigman, W. (1966) Changes in protein and glycoprotein concentrations in human submaxillary saliva under various stimulatory conditions. *Arch Oral Biol* **11**: 437–450.

Cornejo, O.E., Lefebure, T., Bitar, P.D. *et al.* (2012) Evolutionary and population genomics of the cavity causing bacteria Streptococcus mutans. *Mol Biol Evol* In press.

Deutscher, J. (2008) The mechanisms of carbon catabolite repression in bacteria. *Curr Opin Microbiol* **11**: 87–93.

Do, T., Gilbert, S.C., Clark, D. *et al.* (2010) Generation of diversity in Streptococcus mutans genes demonstrated by MLST. *PLoS One* **5**: e9073.

Hojo, K., Nagaoka, S., Ohshima, T. and Maeda, N. (2009) Bacterial interactions in dental biofilm development. *J Dent Res* **88**: 982–990.

Kaufman, G.E. and Yother, J. (2007) CcpA-dependent and -independent control of β-galactosidase expression in *Streptococcus pneumoniae* occurs via regulation of an upstream phosphotransferase system-encoding operon. *J Bacteriol* **189**: 5183–5192.

Lau, P.C., Sung, C.K., Lee, J.H., Morrison, D.A. and Cvitkovitch, D.G. (2002) PCR ligation mutagenesis in transformable streptococci: application and efficiency. *J Microbiol Methods* **49**: 193–205.

LeBlanc, D.J. and Hassell, F.P. (1976) Transformation of *Streptococcus sanguis* Challis by plasmid deoxyribonucleic acid from *Streptococcus faecalis*. *J Bacteriol* **128**: 347–355.

LeBlanc, D.J., Crow, V.L., Lee, L.N. and Garon, C.F. (1979) Influence of the lactose plasmid on the metabolism of galactose by *Streptococcus lactis*. *J Bacteriol* **137**: 878–884.

Loughman, J.A. and Caparon, M.G. (2007) Comparative functional analysis of the *lac* operons in *Streptococcus pyogenes*. *Mol Microbiol* **64**: 269–280.

Petersen, F.C. and Scheie, A.A. (2010) Natural transformation of oral streptococci. *Methods Mol Biol* **666**: 167–180.

Postma, P.W., Lengeler, J.W. and Jacobson, G.R. (1993) Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* **57**: 543–594.

Rosey, E.L. and Stewart, G.C. (1992) Nucleotide and deduced amino acid sequences of the *lacR*, *lacABCD*, and *lacFE* genes encoding the repressor, tagatose 6-phosphate gene cluster, and sugar-specific phosphotransferase system components of the lactose operon of *Streptococcus mutans*. *J Bacteriol* **174**: 6159–6170.

Russell, R.R., Mukasa, H., Shimamura, A. and Ferretti, J.J. (1988) *Streptococcus mutans gtfA* gene specifies sucrose phosphorylase. *Infect Immun* **56**: 2763–2765.

Scholler, M., Klein, J.P. and Frank, R.M. (1981) Common antigens of streptococcal and non-streptococcal oral bacteria: immunochemical studies of extracellular and cell-wall-associated antigens from *Streptococcus sanguis*, *Streptococcus mutans, Lactobacillus salivarius*, and *Actinomyces viscosus*. *Infect Immun* **31**: 52–60.

Shaw, W.V. (1975) Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol* **43**: 737–755.

Van der Hoeven, J.S. and Camp, P.J. (1991) Synergistic degradation of mucin by *Streptococcus oralis* and *Streptococcus sanguis* in mixed chemostat cultures. *J Dent Res* **70**: 1041–1044.

de Vos, W.M. and Vaughan, E.E. (1994) Genetics of lactose utilization in lactic acid bacteria. *FEMS Microbiol Rev* **15**: 217–237.

Yamashita, Y., Bowen, W.H., Burne, R.A. and Kuramitsu, H.K. (1993) Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. *Infect Immun* **61**: 3811–3817.

Zeng, L. and Burne, R.A. (2009) Transcriptional regulation of the cellobiose operon of *Streptococcus mutans*. *J Bacteriol* **191**: 2153–2162.

Zeng, L. and Burne, R.A. (2010) Seryl-phosphorylated HPr regulates CcpA-independent carbon catabolite repression in conjunction with PTS permeases in *Streptococcus mutans*. *Mol Microbiol* **75**: 1145–1158.

Zeng, L., Wen, Z.T. and Burne, R.A. (2006) A novel signal transduction system and feedback loop regulate fructan hydrolase gene expression in *Streptococcus mutans*. *Mol Microbiol* **62**: 187–200.

Zeng, L., Das, S. and Burne, R.A. (2010) Utilization of lactose and galactose by *Streptococcus mutans*: transport, toxicity, and carbon catabolite repression. *J Bacteriol* **192**: 2434–2444.

Zeng, L., Martino, N.C. and Burne, R.A. (2012) Two gene clusters coordinate galactose and lactose metabolism in *Streptococcus gordonii*. *Appl Environ Microbiol* **78**: 5597–5605.

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The nucleic acid and amino acid sequences of the EII^{Gal} locus in strain OMZ175.

Table S1. Prevalence of non-core genes in association with EII^{Gal} complex.

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Copyright of Molecular Oral Microbiology is the property of Wiley-Blackwell and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.