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Deficiency of PdxR in *Streptococcus mutans* affects vitamin B₆ metabolism, acid tolerance response and biofilm formation

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Summary

Streptococcus mutans, a key etiological agent of the human dental caries, lives primarily on the tooth surface in tenacious biofilms. The SMU864 locus, designated *pdxR*, is predicted to encode a member of the novel MocR/GabR family proteins, which are featured with a winged helix DNA-binding N-terminal domain and a C-terminal domain highly homologous to the pyridoxal phosphate-dependent aspartate aminotransferases. A *pdxR*-deficient mutant, TW296, was constructed using allelic exchange. PdxR deficiency in *S. mutans* had little effect on cell morphology and growth when grown in brain heart infusion. However, when compared with its parent strain, UA159, the PdxR-deficient mutant displayed major defects in acid tolerance response and formed significantly fewer biofilms ($P < 0.01$). When analyzed by realtime polymerase chain reaction, PdxR deficiency was found to drastically reduce expression of an apparent operon encoding a pyridoxal kinase (SMU865) and a pyridoxal permease (SMU866) of the salvage pathway of vitamin B₆ biosynthesis. In addition, PdxR deficiency also altered the expression of genes for ClpL protease, glucosyl-transferase B and adhesin SpaP, which are known to play important roles in stress tolerance and biofilm formation. Consistently, PdxR-deficiency affected the growth of the deficient mutant when grown in defined medium with and without vitamin B₆. Further studies revealed that although *S. mutans* is known to require vitamin B₆ to grow in defined medium, B₆ vitamers, especially pyridoxal, were strongly inhibitory at millimolar concentrations, against *S. mutans* growth and biofilm formation. Our results suggest that PdxR in *S. mutans* plays an important role in regulation of vitamin B₆ metabolism, acid tolerance response and biofilm formation.

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Keywords

dental caries; MocR/GabR family proteins; *Streptococcus mutans*; vitamin B₆

Introduction

Streptococcus mutans, a key aetiological agent of dental caries, lives primarily on the tooth surface in a dynamic environment that has frequent, often drastic, fluctuations in conditions, such as the source and availability of nutrients, pH, temperature, oxygen tension and antimicrobial agents (Burne, 1998). This bacterium is known for its ability to colonize the tooth surface, to survive and adapt to various environmental conditions, and to become numerically significant under certain conditions, leading to carious lesions (Burne, 1998; Lemos & Burne, 2008). Besides molecular chaperones and the Clp proteases (Lemos *et al.*, 2001; Lemos & Burne, 2008; Kajfasz *et al.*, 2009), multiple networks including two-component signal transduction systems (Ahn *et al.*, 2006; Biswas *et al.*, 2008; Senadheera *et al.*, 2009; Suntharalingam *et al.*, 2009; Burne *et al.*, 2011; Stipp *et al.*, 2013), the Com system (Li *et al.*, 2002b), AI-2 mediated signaling (Wen & Burne, 2004; Wen *et al.*, 2011), and cell envelope associated transcriptional regulator BrpA (Wen *et al.*, 2006; Bitoun *et al.*, 2012a), have been shown to play integral roles in survival and adaptation to low pH, reactive oxygen species and cell envelope stresses and are required for biofilm formation by *S. mutans*.

The MocR/GabR family proteins belong to the GntR super-family of transcriptional regulators (Rigali *et al.*, 2002). They are chimeric proteins that incorporate a short N-terminal helix-turn-helix-containing domain with DNA-binding properties (Rigali *et al.*, 2002; Lee *et al.*, 2003; Wiethaus *et al.*, 2008) and a long C-terminal domain closely related in amino acid sequence to full-length aminotransferases or to the pyridoxal-5'-phosphate (PLP)-binding domain of aminotransferases (Schneider *et al.*, 2000; Belitsky & Sonenshein, 2002; Wiethaus *et al.*, 2008; Bramucci *et al.*, 2011; Edayathumangalam *et al.*, 2013). GabR in *Bacillus subtilis* is the first member of the family shown to function as a transcriptional regulator (Belitsky & Sonenshein, 2002; Belitsky, 2004; Edayathumangalam *et al.*, 2013). In the presence of γ -aminobutyric acid, GabR activates transcription of the *gabTD* operon. It also negatively regulates the divergently transcribed *gabR* gene. In vitro transcription reactions showed that GabR alone repressed expression from the *gabR* promoter but activated expression from the *gabT* promoter only in the presence of gamma-aminobutyric acid and PLP (Belitsky, 2004). MocR in *Sinorhizobium meliloti* is known to be required for rhizopine utilization (Rossbach *et al.*, 1994), although no further information is available. PdxR, a MocR/GabR homologue in *Corynebacterium glutamicum*, controls expression of divergently oriented *pdxST* genes coding for the subunits of PLP synthase whose deficiency caused vitamin B₆ auxotrophy in this bacterium (Jochmann *et al.*, 2011). Similar results were also recently reported in *Listeria monocytogenes* (Belitsky, 2014). However, the presence and the possible role of the aminotransferase domain in MocR/GabR-like proteins remain unclear (Belitsky, 2004; Bramucci *et al.*, 2011).

Pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) are collectively referred to as vitamin B₆, although PLP is considered as the only active form (Fitzpatrick *et al.*, 2007; El Qaidi *et al.*, 2013). PLP is an essential cofactor of numerous enzymes catalyzing a variety of biochemical reactions primarily in amino acid metabolism (Fitzpatrick *et al.*, 2007; El Qaidi *et al.*, 2013). Besides, PLP is also shown to function as an inhibitor of PdxR-mediated activation of the deoxyxylulose 5-phosphate (DXP)-independent pathway of vitamin B₆ biosynthesis in *Streptococcus pneumoniae* and *Corynebacterium glutamicum* (Fitzpatrick *et al.*, 2007; Jochmann *et al.*, 2011; El Qaidi *et al.*, 2013) and GabR-regulated utilization of γ -aminobutyrate in *B. subtilis* (Belitsky & Sonenshein, 2002; Belitsky, 2004).

The SMU864 locus, herein designated *pdxR*, is annotated as a member of the GntR superfamily of regulatory proteins. As revealed by BLAST search, the *pdxR* product contains, in its N-terminal region, a GntR-type of winged helix-turn-helix DNA-binding domain and a large C-terminal domain that is similar to the PLP-dependent aspartate aminotransferase (AST). It was found recently by DNA microarray analysis to be down-regulated more than twofold in response to deficiency of LuxS, an enzyme known to be responsible for AI-2-mediated signaling in *S. mutans* and many other bacterial species (Wen & Burne, 2004; Wen *et al.*, 2011). In this study, a PdxR-deficient mutant was constructed by allelic replacement. Characterization of the resulting mutant revealed that PdxR is required for the expression of PL kinase, PdxK and PL permease, PdxU. Deficiency of PdxR affected growth of the deficient mutant when grown in defined medium and caused major defects in acid tolerance response and biofilm formation. These results suggest that PdxR in *S. mutans* plays an important role in vitamin B₆ and amino acid metabolism and in regulation of stress tolerance response and biofilm formation.

Methods

Plasmids, bacterial strains and growth conditions

Streptococcus mutans UA159 and its derivatives used in this study are listed in Table 1. They were maintained in brain heart infusion (BHI, Difco Laboratories, Detroit, MI). For growth characterization, chemically defined medium FMC was also used (Terleckyj *et al.*, 1975). For biofilm growth, a modified semi-defined biofilm medium (BM) was used with glucose (20 mM, BMG), sucrose (10 mM, BMS), and glucose plus sucrose (18 and 2 mM, respectively) (BMGS) as the supplemental carbohydrate and energy sources (Loo *et al.*, 2000; Li *et al.*, 2002a; Wen & Burne, 2002b). All solid media were prepared similarly, but agar (Difco Laboratories) was added at a concentration of 1.5% (w/v). When needed, kanamycin (1 mg ml⁻¹) and/or spectinomycin (1 mg ml⁻¹) were added. Unless stated otherwise, *S. mutans* strains were grown at 37°C in an aerobic chamber with 5% CO₂. For growth studies, Bioscreen C (Oy Growth Curves AB Ltd, Finland) was also used to culture cells at 37°C with and without an overlay of mineral oil, and the optical density (OD) at 600 nm was monitored automatically every 30 minutes following moderate shaking for 10 seconds. *Escherichia coli* strains were grown in Luria–Bertani medium, with or without the addition of kanamycin (40 µg ml⁻¹) and/or ampicillin (100 µg ml⁻¹) (Table 1).

Construction of mutants and complement strains

Streptococcus mutans strains deficient for *pdxR* were generated using a polymerase chain reaction (PCR)-ligation-mutation strategy as described elsewhere (Lau *et al.*, 2002; Wen & Burne, 2004). Briefly, a 990-bp fragment 5' of *pdxR* and a 30 fragment of 1010 bp were amplified by PCR using high-fidelity DNA polymerase Phusion (New England Biolabs, Ipswich, MA) with gene specific primers (Table 1). Following proper digestions to generate compatible ends, the PCR amplicons were ligated with a non-polar kanamycin resistance cassette (*aphA* encoding aminoglycoside 3'-phosphotransferase) (Zeng *et al.*, 2006), and the resulting ligation mix was used to transform *S. mutans* UA159. Putative mutants were selected on BHI-kanamycin plates, and further analyzed by PCR and DNA sequencing to verify the deficiency and sequence accuracy. For mutant complementation, the coding sequence plus its putative promoter region were amplified by PCR and directly cloned into shuttle vector pDL278 (LeBanc & Lee, 1991). Following sequence confirmation, the resulting construct, pDL278:*pdxR*, was transformed into the mutant, and transformants carrying with the wild-type *pdxR* were isolated from BHI-Spc.

Biofilm formation and microscopic analyses

For biofilm assays, *S. mutans* was grown in biofilm medium BMG, BMS and BMGS as described above (Loo *et al.*, 2000; Li *et al.*, 2002a; Wen & Burne, 2002b). For confocal laser scanning microscopy (CLSM), biofilms were grown on hydroxylapatite (HA) disks (Clarkson Chromatography Products Inc., South Williamsport, PA) that were vertically deposited in 24-well culture plates (Corning, NY) (Lemos *et al.*, 2010) or plain glass slides deposited in 50-ml Falcon tubes (Wen *et al.*, 2010) for 24 and 48 h, and were then stained with *Live/Dead BacLight* fluorescent dye (Invitrogen, Carlsbad, CA) before optical dissection using an Olympus Fluoview BX61 confocal laser scanning microscope (Olympus, Tokyo, Japan) at 6009 magnification (Bitoun *et al.*, 2011; Wen *et al.*, 2011). At least five random areas were scanned for each sample. For post-acquisition analysis, simulated xyz three-dimensional images were generated using SLIDEBOOK 5.0 (Olympus) (Bitoun *et al.*, 2011; Wen *et al.*, 2011). Quantitative analysis, such as biovolume, biomass, surface area and mean height, was carried out using COMSTAT 2.0 with at least five independent scans for each sample (Heydorn *et al.*, 2000; Wen *et al.*, 2011). The biovolume is defined as the volume (μm^3) of the biomass per μm^2 of substratum area. Field-emission-scanning electron microscopy (FE-SEM) was carried out similarly as described previously (Wen & Burne, 2004; Bitoun *et al.*, 2011).

Acid killing and hydrogen peroxide challenge assays

To evaluate the ability of *S. mutans* strains to withstand acid and oxidative stress, planktonic cultures were prepared from mid-exponential phase ($\text{OD}_{600\text{ nm}} \approx 0.5$) cultures grown in BHI broth. For sessile populations, BMGS was used to support bacterial growth and glass slides were used as substratum, as detailed elsewhere (Wen *et al.*, 2010, 2011). Acid and hydrogen peroxide killing assays were carried out as described previously (Wen & Burne, 2004; Wen *et al.*, 2006).

Glycolytic pH drop

The glycolytic pH drop assay was carried out as described by Belli & Marquis (1991). Briefly, *S. mutans* strains were grown in BHI broth until mid-exponential phase ($OD_{600\text{ nm}} \approx 0.5$), washed twice with ice-cold de-ionized water by centrifugation at $2737\times g$ at 4°C for 10 min, and the cells were then resuspended in 50 mM KCl and 1 mM MgCl_2 . The pH was adjusted to 7.2 with 0.1 M KOH before the addition of 50 mM glucose. The pH drop was monitored continuously for a period of 30 min.

Expression and purification of recombinant PdxR

For recombinant protein rPdxR, *pdxR*-coding sequence was PCR amplified with gene-specific primers (Table 1) and then directly cloned in expression vector pMAL-c2X, generating an in-frame fusion behind *E. coli malE* sequence that encodes an engineered maltose-binding protein domain (New England Biolabs) (Zeng *et al.*, 2006). The resulting construct was transformed to and amplified in *E. coli* BL21 (New England Biolabs). Following sequence confirmation by sequencing, the fusion protein was expressed by Isopropyl β -D-1-thiogalactopyranoside induction and purified using amylose affinity columns by following the manufacturer's recommendation (New England Biolabs) (Zeng *et al.*, 2006).

Aspartate aminotransferase assay

For AST assay, purified rPdxR was washed and reconstituted in 50 mM sodium phosphate buffer, pH 7.4, using Protein Desalting Spin Columns (#89849, Pierce Biotechnology, Rockford, IL). The *S. mutans* whole cell lysates were prepared from mid-exponential phase cultures grown in BHI and homogenized using a glass bead beater (Biospec, Bartlesville, OK) as described previously (Burne *et al.*, 1999; Wen & Burne, 2002a). AST activity was measured in a coupled assay with malate dehydrogenase and aspartate plus 2-oxoglutarate as substrates (Karmen *et al.*, 1955; Ziak *et al.*, 1990). Malate dehydrogenase catalyzed oxidation of NADH, which is proportional to AST product, oxaloacetate, was monitored by the change of absorbance at 340 nm using a Bio-Tek microplate reader (Synergy IITM; Bio-Tek, Winooski, VT). Porcine AST (Sigma, St Louis, MO) was used as a positive control, and maltose-binding protein domain (MalE) (New England Biolabs) expressed and purified using similar strategies, was used as a negative control. Protein concentration was assayed using a BCATM protein assay kit (Pierce) as recommended by the manufacturer. AST activity was further normalized by protein concentration and is expressed as units per milligram of protein.

RNA isolation and real-time PCR assay

For RNA extraction, mid-exponential phase ($OD_{600\text{ nm}} \approx 0.5$) *S. mutans* cultures were treated with RNAProtect (Qiagen, Inc., Hilden, Germany), and total RNAs were extracted using hot phenol as described previously (Browngardt *et al.*, 2004; Wen *et al.*, 2006, 2011). To remove residual DNA, RNA samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) and total RNA was retrieved with the RNeasy purification kit (Qiagen, Inc.). For real-time PCR analysis, cDNA was synthesized with 1 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) by following the procedures

recommended by the supplier. Real-time PCR was carried out with a Bio-Rad iCycler using procedures detailed elsewhere (Ahn *et al.*, 2006; Wen *et al.*, 2006).

Statistical analysis

Comparisons between different strains and different conditions were carried out using paired Student's *t*-test. A difference at $P < 0.05$ or less is considered statistically significant.

Results

PdxR-deficiency affects vitamin B₆ metabolism

Genetic analysis revealed that the *pdxR* (SMU864) locus is flanked by genes encoding proteins with potential roles in amino acid and vitamin B₆ metabolism (see Supporting information, Fig. S1). Downstream of *pdxR* in opposite orientation are *mmuM* (SMU863) for a putative homocysteine *S*-methyltransferase and *ybgF* (SMU862) for a putative histidine permease. Upstream of *pdxR* are two divergently transcribed genes in an apparent operon coding for a putative PL kinase (PdxK, SMU865) and a putative PL transporter (PdxU, SMU866), respectively. A PdxR-deficient mutant, TW296, was constructed by allelic exchange using a non-polar kanamycin resistance marker (Table 1). PdxR-deficiency did not have a major effect on cell morphology under the optical microscope (data not shown) and growth rate during growth in regular BHI (Fig. 1A). Relative to the parent strain, UA159, the deficient mutant only slightly extended its lag phase and slightly reduced its final OD, as measured using Bioscreen C. However, when incubated in BHI adjusted to pH 6.0 (Fig. 1A) and lower (data not shown), the growth rate of the deficient mutant was significantly reduced when compared with the parent strain ($P < 0.05$). Inclusion of methyl viologen (also paraquat, Sigma) in the growth medium also slightly reduced the growth rate of the PdxR-deficient mutant compared with parent strain, UA159 (data not shown), although the differences were not statistically significant. Similar trends were also obtained when grown in chemically defined medium FMC, although the impact of PdxR-deficiency on the culture OD was much more significant ($P < 0.05$) (Fig. 1B). Complementation in TW296C via a shuttle vector carrying a wild-type copy of *pdxR* plus its cognate promoter was able to restore the phenotypes to a level similar to the wild-type during growth in both BHI (Fig. 1A) and in FMC (Fig. 1B).

PL kinase and PL permease are part of the salvage pathway of vitamin B₆ biosynthesis (Fitzpatrick *et al.*, 2007; Mukherjee *et al.*, 2011). To further investigate the role of the *pdxKU* operon in vitamin B₆ metabolism, mutants deficient for PdxK, PdxU, and both PdxK and PdxU were generated using similar strategies as detailed in Methods, and further evaluated using modified FMC medium with or without inclusion of B₆ vitamers PL, PN, PM or PLP (all from Sigma) at 5 μ M (final concentration). The results showed that mutants deficient for PdxK, PdxU or both PdxK and PdxU grew as well as the parent strain, UA159 when PL, PM, PN or PLP was the B₆ vitamin (data not shown). In FMC without inclusion of B₆ vitamers, both *S. mutans* UA159 and the PdxR mutant were able to continue to grow albeit at extremely low rates, yielding very limited increases in optical density (Fig. 1B and see Supporting information, Fig. S2). Similar, but slightly better performance was seen with

the complement strain. In contrast, however, all three PdxK-, PdxU- and PdxKU-deficient mutants displayed significantly better growth rates and final ODs ($P < 0.001$) (Fig. S2).

The PdxR-deficiency affects acid tolerance response

Reduction of growth rate during growth in BHI adjusted to lower pH indicates a reduced glycolytic activity and/or weakened acid tolerance. When analyzed using glycolytic pH drop experiments, it was shown that relative to wild-type, the rate (slope) of glucose-induced pH drop of the PdxR-deficient mutant was significantly reduced and the resting pH of the PdxR mutant was significantly higher ($P < 0.05$) (Fig. 2). As expected, complementation of the mutant with the wild-type coding sequence plus its cognate promoter region in TW296C was able to partly restore these phenotypes to levels more resembling the wild-type. When analyzed by acid-killing by incubation in a buffer of pH 2.8, PdxR-deficient mutant, TW296, displayed more than 1-log reduction in survival rate ($P < 0.001$) compared with the wild-type, UA159 (Fig. 3). Complementation with the wild-type copy of *pdxR* in *trans* increased the resistance moderately, but not to a level similar to the wild-type. When subjected to oxidative challenge by incubation in buffer containing 0.2% (or 58 mM) of hydrogen peroxide, no significant differences were measured between UA159 and TW296 (data not shown), which is consistent with results of the growth study with inclusion of paraquat (data not shown).

PdxR-deficiency reduces biofilm formation

No significant differences in growth rate were measured when grown planktonically in BM medium (data not shown). However, when compared with the parent strain, UA159, the PdxR-deficient mutant, TW296, showed a significant reduction in its ability to form biofilms. During biofilm growth in BMG, the PdxR-deficient mutant, TW296 formed $< 43\%$ biofilms ($P < 0.05$) after 24 h compared with the parent strain, with TW296 averaging $1.9E7$ colony-forming-units (CFU) vs. $3.4E7$ CFU for the parent strain, UA159 ($P < 0.05$). When grown in BMGS, the mutant biofilms decreased by $>62\%$ averaging $2.35E7$ CFU, compared with $6.15E7$ CFU for the parent strain ($P < 0.05$). When analyzed using FE-SEM, *S. mutans* UA159 developed substantial biofilms after 24 h (Fig. 4A). In contrast, the mutant biofilms were flat and tight and contained little or no glucans (Fig. 4A). Similar results were also obtained using CLSM (Fig. 4B). CLSM also showed that complementation with a wild-type copy of the genes plus its cognate promoter was able to restore the ability to form biofilms to a level similar to the wild-type. As revealed by comstat analysis, the wild-type and the PdxR-deficient mutant biofilms did not have major differences in surface areas (data not shown), suggesting that the PdxR-deficient mutant colonized similarly well on the HA disks. The biovolume of the PdxR-deficient mutant biofilms was reduced, averaging only $1.14 (\pm 1.04) \mu\text{m}^3 \mu\text{m}^{-2}$, compared with $5.73 (\pm 3.9) \mu\text{m}^3 \mu\text{m}^{-2}$ for the parent strain, although the differences were not statistically significant ($P = 0.06$). When analyzed using colorimetric assay (Bitoun *et al.*, 2012b), the PdxR-deficient mutant, TW296, had only $11.49 (\pm 0.46) \text{ ng } \mu\text{l}^{-1}$ of glucans (Fig. 4C), compared with $49.18 (\pm 5.42) (P < 0.01) \text{ ng } \mu\text{l}^{-1}$ of the wild-type, UA159, and $31.67 (\pm 5.09) \text{ ng } \mu\text{l}^{-1} (P < 0.05)$ by the complement strain, TW296C. These results corroborate well with what were seen under both FE-SEM and CLSM.

PdxR-deficiency affects expression of genes in vitamin B₆ metabolism

Real-time PCR was used to analyze the effect of PdxR-deficiency on the expression of flanking genes and those known to be associated with biofilm formation. Of the genes downstream *pdxR*, *pdxK* and *pdxU* were found to be down-regulated by 15.30-fold and 3.80-fold, respectively. Relative to the wild-type, transcription of *clpL* (SMU867), for a ClpL protease, was also down-regulated by more than seven-fold. Of the upstream genes analyzed, *ybgF* (SMU861) for an S-methyl methionine transporter, and *mmuM* (SMU863) for a putative homocysteine S-methyltransferase were upregulated by 1.87-fold and 3.37-fold, respectively. Of the genes known to be critical to *S. mutans* biofilm formation, *spaP*, for high-affinity adhesin SpaP, was found to be upregulated fourfold, and *gtfB*, for glucosyltransferase B, was increased by 3.94-fold in the PdxR-deficient mutant, relative to the wild-type, UA159 (Table 2). No differences were measured in expression of *gtfC* and *gfpB*.

B₆ vitamers affect growth and biofilm formation

It is well documented that micromolar vitamin B₆ is required for *S. mutans* to grow in chemically defined medium (Carlsson, 1970; Terleckyj *et al.*, 1975). However, there is also evidence that pyridine analogs, including B₆ vitamers, could reduce glucose uptake and inhibit glucosyltransferase activity (Thaniyavarn *et al.*, 1982). In an effort to further investigate the effects of vitamin B₆ on growth and biofilm formation by *S. mutans*, various concentrations of PL, PM and PN were included in BHI for growth studies and in BMGS for biofilm formation on 96-well plates. Our studies so far have shown that PL, PM and PN all displayed inhibitory effects against *S. mutans* growth and biofilm formation, and such effects were concentration dependent. Of the B₆ vitamers tested, PL was the most potent with a minimal inhibitory concentration (MIC) at 10 mM (Fig. 5A). No effect on growth was measured at 0.75 mM and below. No biofilms were measured when *S. mutans* was grown with PL at 5 mM or above (Fig. 5B). However, relative to the control that received no PL, more than 40% ($P < 0.05$) reduction of biofilms was still measured at 0.16 mM. Similar effects on growth and biofilm formation were also seen with PM and PN, albeit to a much lesser degree (see Supporting information, Fig. S3–S6).

Discussion

Unlike *S. pneumoniae* and many others, *S. mutans* lacks the DXP-independent *de novo* pathway of vitamin B₆ biosynthesis, but does contain the salvage pathway(s) and two genes encoding enzymes of the DXP-dependent *de novo* pathway can be identified in the genome (http://www.genome.jp/kegg-bin/show_pathway?smu00750) (Ajdic *et al.*, 2002; El Qaidi *et al.*, 2013). In the salvage pathway, PL kinase catalyzes the transfer of a phosphate group from ATP to the 5-hydroxymethyl group of B₆ vitamers, leading to the generation of PLP (Fitzpatrick *et al.*, 2007; Mukherjee *et al.*, 2011). It is apparent that PdxR-deficiency drastically reduces the expression of PdxK (SMU865) and PdxU (SMU866), although the deficient mutant was still capable of growing in defined medium FMC with the B₆ vitamers tested, albeit in a reduced growth rate. Interestingly and surprisingly, the PdxK and PdxU single and double mutants grew similarly well compared with the wild-type in FMC with inclusion of B₆ vitamers but grew a lot better than the wild-type in medium without addition

of B₆ vitamers. A BLAST search of the genome database (www.theseed.org) revealed that *S. mutans* possesses at least another two-gene operon in loci SMU77 and SMU78 that are predicted to code for a PL kinase, PdxK2 (SMU77) of the ThiD family and a PL permease, PdxU2 (SMU78), respectively. It is possible that the expression of PdxK2 and PdxU2 is PLP-dependent and becomes de-repressed in response to the diminished intracellular pool of PLP in the PdxK and PdxU mutants. Such a de-repression could compensate the deficiency of PdxK and PdxU in the respective mutants, and at least partly explain why the PdxK and PdxU mutants were able to grow like the wild-type when B₆ vitamers were included in the growth medium. On the other hand, when grown in FMC without inclusion of any vitamin B₆, the intracellular PLP became quickly depleted in the PdxK-, PdxU-, and PdxKU-deficient mutants. It is possible that a DXP-dependent pathway exists in *S. mutans* and that PLP depletion triggered the activation of this not-yet characterized alternative *de novo* pathway, enabling the respective mutants to grow in the absence of exogenous B₆ vitamers.

Widespread in both gram-positive and gram-negative bacteria (Belitsky & Sonenshein, 2002; Belitsky, 2004, 2014; Bramucci *et al.*, 2011), the GabR/MocR sub-family proteins all possess a C-terminal domain with high similarity to AST. The AST catalyzes the interconversion of aspartate and α -ketoglutarate to oxaloacetate and glutamate. It contains PLP as a co-factor essential for catalytic activity. As revealed recently by crystal structure analysis, the C-terminal region of *Bacillus subtilis* GabR shows high similarity to many aminotransferases, and PLP appears to be a tightly bound covalently linked component of the protein, similar to the case of aminotransferases and other PLP-dependent enzymes (Edayathumangalam *et al.*, 2013). While the GabR/MocR family proteins have been well-documented for their roles as transcriptional activators and/or repressors and for their dependence on PLP for regulatory activities (Belitsky & Sonenshein, 2002; Belitsky, 2004, 2014; Wiethaus *et al.*, 2008; Jochmann *et al.*, 2011; El Qaidi *et al.*, 2013), there is currently no report that these proteins actually possess any aminotransferase activity. In an effort to see whether PdxR in *S. mutans* contains any aminotransferase activity, the recombinant protein, rPdxR was expressed, purified and analyzed for transaminase activity using a coupled assay (Karmen *et al.*, 1955; Ziak *et al.*, 1990). The results so far indicated that rPdxR possessed little or no detectable transaminase activity (data not shown). Protease Factor Xa (New England Biolabs) was also used to cleave the fusion domain, MalE, but such cleavage did not seem to cause any major differences in enzyme activity (data not shown). These results suggest that PdxR in *S. mutans* does not possess aminotransferase activity under the conditions studied, consistent with several other MocR/GabR homologues in other bacterial species (Belitsky, 2004; Bramucci *et al.*, 2011). It is worth noting that when analyzed using whole cell lysates, the transaminase activity of the PdxR-deficient mutant was decreased by more than 40% ($P < 0.05$) when compared with the wild-type, UA159 (data not shown), and that complementation with the wild-type *pdxR* plus its cognate promoter in a shuttle vector restored the level to be similar to that of the wild-type. The mechanism that underlies the modulated transaminase activity in response to PdxR-deficiency awaits further investigation.

Streptococcus mutans, a well recognized key etiological agent of human dental caries, has evolved a variety of adaptive mechanisms that contribute to its abilities to tolerate and

survive various detrimental conditions such as low pH and nutrient deficiency (Lemos & Burne, 2008; Lemos *et al.*, 2013). LuxS/AI-2-mediated signaling is an intercellular communication system that has been shown to play an important role in *S. mutans* pathophysiology, regulating carbohydrate and amino acid metabolism, acid and oxidative stress tolerance and biofilm formation (Wen & Burne, 2004; Sztajer *et al.*, 2007; Wen *et al.*, 2011). Consistent with DNA microarray analysis, SMU864 is confirmed by real-time PCR as part of the LuxS-regulon (Wen *et al.*, 2011). Our results presented here showed that PdxR-deficiency caused alteration in expression of genes known to be involved in the stress tolerance response and biofilm formation.

Glucosyltransferase GtfB produces primarily α 1,3-linked water-insoluble glucans that are known to play an essential role in *S. mutans* adherence, biofilm accumulation and structure (Bowen & Koo, 2011; Gregoire *et al.*, 2011). Our results showed that PdxR-deficiency reduced *S. mutans* biofilm formation. Consistently, both FE-SEM analysis and colorimetric assay demonstrated that the PdxR-deficient mutant had significantly less glucans than the parent strain, UA159, which could be at least in part attributed to the structure and quantitative characteristics of the deficient mutant biofilms. In contrast, however, realtime PCR revealed that *gtfB* transcription, but not *gtfC*, was elevated in response to PdxR-deficiency. The *gtfB* and *gtfC* genes are known to be linked, but as indicated in multiple reports (Goodman & Gao, 2000; Wen *et al.*, 2005; Bitoun *et al.*, 2013), our results again showed that these two genes could be differentially transcribed under the conditions studied. PLP at 10 mM was shown by Thaniyavarn *et al.* (1982) to reduce Gtf activity in vitro (see below for more), although whether it affects GtfB activity and glucan production at micromolar levels remains unclear. It is also possible that PdxR-deficiency influences the conformation and/or stability of GtfB protein, which consequently can affect the assembly of glucose polymer and biofilm formation of the deficient mutant. Of the genes/proteins altered in TW296, ClpL is an ATP-dependent Clp protease that has been shown to play a role in the stress tolerance response (Kajfasz *et al.*, 2009). Major reduction in expression of ClpL is likely a part of the contributing factors to the weakened tolerance to low pH, which in turn likely contributes to the reduced biofilm formation. Considering the role of PLP as an essential cofactor of numerous enzymes, dysregulation of vitamin B₆ metabolism as a result of PdxR-deficiency will also likely cause defects that can be attributed to the phenotypes observed with the deficient mutant.

Results presented here also show that vitamin B₆, especially PL at higher concentrations, can strongly inhibit *S. mutans* growth. This is consistent with the finding by Thaniyavarn *et al.* (1982) that *S. mutans* significantly reduced its D-glucose uptake following pre-incubation of *S. mutans* cells with several other pyridine analogs, including PL, PM, PN and PLP (Thaniyavarn *et al.*, 1982). Besides, pyridine analogs were also shown to inhibit glucan production by purified Gtf enzyme *in vitro* (Thaniyavarn *et al.*, 1982). Consistently, our studies showed that vitamin B₆, especially PL, are potent inhibitors of *S. mutans* biofilm formation. Besides growth defects, the influences on Gtf enzymes and glucan production are likely part of the underlying mechanisms. It awaits further investigation if any other factors are involved. However, it is unlikely that cytotoxicity played a role in the growth defects observed with the respective mutants. First, the concentration (5 μ M) used in the growth

studies, such as those shown in Fig. 1B and the supporting information, Fig. S2, is well below the threshold of toxicity measured, especially for PM and PN. Second, the fact that the PdxR-deficient mutant had a decreased growth rate relative to the wild-type also supports this notion. It is also interesting to note that PL was effective against *S. mutans*' biofilm formation at concentrations higher and lower than 2.5 mM, but not as significant at 2.5 mM tested. The underlying mechanism awaits further investigation.

In summary, PdxR in *S. mutans* is a member of the MocR/GabR family of proteins and regulates expression of genes in vitamin B₆ metabolism and those known to play a role in stress tolerance response and biofilm formation. However, it remains unclear whether other genes are affected by PdxR and how exactly PdxR regulates gene expression in response to environmental conditions in *S. mutans*. Current effort is being directed to elucidation of the roles of PdxK2/U2 in vitamin B₆ metabolism and to determination of the DXP-dependent *de novo* pathway in PLP biosynthesis in *S. mutans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Growth studies in brain–heart infusion (BHI) (A) and chemically defined medium FMC (B). (A) *Streptococcus mutans* UA159, its PdxR-deficient mutant, TW296, and the complement strain, TW296C were grown in BHI adjusted to pH 7.0 and 6.0, and the culture optical density at 600 nm was continuously measured using Bioscreen C. Our results showed that PdxR-deficiency significantly affects the growth rate of the mutant, TW296 at pH 6.0. (B) *Streptococcus mutans* UA159, the PdxR-deficient mutant, TW296 and its complement strain, TW296C were grown in defined medium FMC with (solid symbols) and without (open symbols) inclusion of vitamin B₆ at 5 μM. Results showed that *S. mutans* requires vitamin B₆ for growth and PdxR-deficiency causes major growth defects under the conditions studied. Data presented here are representatives of three independent experiments. Symbol # indicates differences in growth rate between the wild-type, UA159 and the PdxR mutant, TW296 at $P < 0.05$.

Figure 2.

Glycolytic pH drop. Results of pH drop experiments showed that relative to *Streptococcus mutans* UA159, the PdxR-deficient mutant, TW296, had a slower pH drop and a higher resting pH after 20 min ($\#P < 0.05$). Complementation with wild-type *pdxR* in TW296C was able to partly restore the phenotype to the wild-type, UA159.

Figure 3. Acid killing assay. Panel shows survival rates of sessile populations of *S. mutans* wild-type UA159, PdxR-deficient mutant TW296, and its complement strain, TW296C, after incubation in buffer of pH 2.8 for periods of time indicated. Results showed that PdxR-deficiency significantly reduced the ability of the deficient mutant to survive low pH challenge (** $P < 0.001$), compared with the parent strain, UA159.

Figure 4.

Field-emission-scanning electron microscopy (FE-SEM) (A) and confocal laser scanning microscopy (CLSM) (B) analysis of biofilms and Glucan measurement (C). (A) For FE-SEM analysis, *Streptococcus mutans* wild-type UA159 and the PdxR mutant, TW296 were grown in biofilm medium (BM) sucrose on hydroxylapatite (HA) disks for 24 h. Relative to the wild-type, biofilms of the PdxR-deficient mutant appeared to be tight and flat, and contained little or no extracellular glucans. Images were taken at 5000 $\times$. (B) CLSM images show 24-h biofilms grown on HA disks with BM glucose and stained with Live/Dead BacLight bacterial viability kit (Invitrogen). Results showed PdxR-deficiency significantly decreased biofilm formation. Images presented here are representatives of xyz, xz and yz images (512 $\times$ 512). (C) Glucans in the biofilms of different strains were measured using a phenol-sulfuric acid assay. The results showed that relative to the wild-type, PdxR-deficiency in TW296 significantly reduced glucan production ($*P < 0.01$). Complementation in TW296C partly restored the production ($\#P < 0.05$).

Figure 5.

Effects of pyridoxal (PL) on growth (A) and biofilm formation (B). For growth effects, *Streptococcus mutans* was grown in brain heart infusion broth with different concentrations (mM) of PL, and the culture optical density was continuously monitored using Bioscreen C. For biofilm formation, *S. mutans* was grown in biofilm medium with glucose and sucrose (BMGS) with different concentrations of PL (mM) using 96-well plates. After 24 h, biofilms were stained with 0.1% crystal violet and then measured by spectrophotometry. Symbols **, * and # indicate differences in growth rate (A) or biofilms (B) at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively, when compared with controls that received no PL.

Table 1
Bacterial strains, plasmids and primers used in this study

Strains/plasmid	Relevant characteristics		References/sources
<i>S. mutans</i> UA159	Wild-type		Ajdic <i>et al.</i> (2002)
<i>S. mutans</i> TW296	SMU864, Kan ^r		This study
<i>S. mutans</i> TW296C	TW296/pDL278: <i>pdxR</i> , Kan ^r and Spc ^r		This study
<i>S. mutans</i> TW372	<i>pdxK</i> , Kan ^r		This study
<i>S. mutans</i> TW373	<i>pdxU</i> , Spc ^r		This study
<i>S. mutans</i> TW374	<i>pdxKU</i> , Spc ^r		This study
<i>E. coli</i> BL2			New England Biolabs
pDL278	Shuttle vector, Spc ^r		LeBanc & Lee (1991)
pMALc2x	Expression vector		New England Biolabs
Primers	Sequence (5'–3')	Sequence (5'–3')	Application
<i>pdxR</i> 5F	55-atccaagtatcattcaaacgattc	53-agaaaactggcagaattcgctaac	5', <i>pdxR</i> for mutation
<i>pdxR</i> 3F	35-tgcagtgggaattcttgctcagttg	33-tcaagtaaatgcggtcataacaaagac	3', <i>pdxR</i> for mutation
<i>pdxR</i> C	C5-actagactgaggatccttctc	C3-agtaagattgaattctcacaaagagc	For complementation
<i>pdxR</i> E	E5: ggacggatccgtagtaaatatcatgaa	E3: tcaccaagagctctagtagttttaag	For SMU.953 expression
16S-1 rRNA	Fw: cacaccgccctcacacc	Rv: cagccgcaccttcgatacg	16S rRNA qPCR, 160 bp
<i>pdxR</i>	Fw: cagccaacaagccctttacatcc	Rv: caaggtgttcgtaggtagg	<i>pdxR</i> qPCR, 100 bp
<i>gtfB</i>	Fw: agcaatgcagccatctacaat	Rv: acgaacttgcggttattgtca	<i>gtfB</i> qPCR, 98 bp
<i>gtfC</i>	Fw: atggcgacaatatgatta	Rv: cggatgaaggaataagaa	<i>gtfC</i> qPCR, 172 bp
<i>gtfD</i>	Fw: tgacttctgttcgttatg	Rv: gggtattgctggaatga	<i>gtfD</i> qPCR, 98 bp
SMU860	Fw: attatccgtgaagaacta	Rv: tgattatagacagcaact	SMU860 qPCR, 143 bp
SMU861	Fw: acaatgcttctattctctt	Rv: cctaacagcgtattgataa	SMU861 qPCR, 124 bp
SMU862	Fw: ttgttgtagtctggtgt	Rv: atagcataatcgtaatgtaagg	SMU862 qPCR, 135 bp
SMU863	Fw: atagaagaagtggctgaa	Rv: tatctgttatatcggaatc	SMU863 qPCR, 118 bp
<i>pdxK</i>	Fw: ttgaagcagataagattggtt	Rv: taagcgggaagacaggatt	<i>pdxK</i> qPCR, 133 bp
<i>pdxU</i>	Fw: ttagaatcactgctgtt	Rv: attgagaataatcttcgttac	<i>pdxU</i> qPCR, 126 bp
SMU867	Fw: cggctatgatgataacag	Rv: ttgaaggagaagagtgtat	SMU867 qPCR, 118 bp

Kan^r and Spc^r, for kanamycin and spectinomycin resistance, respectively. Sequences underlined are restriction sites engineered for cloning.

Table 2
Analysis of selected genes by real-time polymerase chain reaction

Gene/locus	Description and putative function ¹	UA159 ²	TW296 ²	Ratio (TW/UA) ³	P value
SMU860	Putative ATP-dependent Clp protease, subunit X	1.17E+06	9.88E+05	1.18	>0.05
SMU861	Putative GTP-binding protein	8.61E+05	9.89E+05	1.15	>0.05
SMU862	Putative methionine permease	3.70E+06	1.96E+06	1.87	<0.01
SMU863	Putative homocysteine S-methyltransferase	7.18E+05	2.46E+06	3.37	<0.01
SMU865	Putative pyridoxal kinase, PdxK	2.80E+06	1.83E+05	-15.3	<0.001
SMU 866	Putative pyridoxal transporter, PdxU	1.75E+06	4.60E+05	-3.80	<0.001
SMU867	ATP-dependent Clp protease, ATP-binding subunit	2.08E+06	2.96E+5	-7.01	<0.001
GbpB	Glucan binding protein B	6.22E+07	5.8E+07	1.07	>0.05
SpaP	Multi-function adhesin, also Agl/II and P1	4.42E+06	1.76E+07	4.0	<0.001
GtfC	Glucosyltransferase C	3.16E+07	3.95E+07	1.25	>0.05
GtfB	Glucosyltransferase B	1.86E+06	7.33E+06	3.94	<0.001

¹ Description and putative function of the selected genes are based upon the published *Streptococcus mutans* database.

² Expressed as copy number of respective transcripts per lg of total RNA. Real-time PCR were repeated at least three times using total RNA from four separate sets of cultures. Data presented here are average of different sets of experiments.

³ Defined as levels of expression in the SMU864-deficient mutant, TW296, relative to those of the wild-type, UA159, with “-” indicating downregulation.