Published in final edited form as:

Mol Oral Microbiol. 2015 August ; 30(4): 280–294. doi:10.1111/omi.12093.

Regulation of *Streptococcus mutans* PTS^{Bio} by the transcriptional repressor NigR

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SUMMARY

Streptococcus mutans is implicated in human dental caries, and the carbohydrate metabolism of this organism plays an important role in the formation of this disease. Carbohydrate transport and metabolism are essential for the survival of *S. mutans* in the oral cavity. It is known that a unique phosphoenolpyruvate-sugar phosphotransferase system PTS^{Bio} of *S. mutans* UA159 is expressed in sucrose-grown biofilms (*Mol Oral Microbiol* 28: 2013; 114). In this study we analyzed the transcriptional regulation of the operon (O^{Bio}) encoding the PTS^{Bio} and showed that it was repressed by NigR, a LacI-like transcriptional regulator. Using electromobility shift assay, we described two operators to which NigR bound with different affinities. We also identified the transcriptional start site and showed that one of the operators overlaps with the promoter and presumably represses initiation of transcription. Mutational analyses revealed the key nucleotides in the operators required for high-affinity binding of NigR. PTS^{Bio} is expressed in *S. mutans* biofilms so understanding its regulation may provide improved strategies for caries treatment and prevention.

Keywords

dental caries; phosphotransferase system; *Streptococcus mutans*; transcriptional regulation

INTRODUCTION

Streptococcus mutans is a major organism implicated in human dental caries. The carbohydrate metabolism of *S. mutans* plays an important role in the formation of caries. *Streptococcus mutans* is able to use numerous carbohydrates. If the human diet is rich in sugars, the final product of the sugar metabolism is mostly lactic acid, which can lead to demineralization of tooth enamel (Loesche, 1986). Therefore, sugar transport and metabolism by this bacterium are directly related to development of dental caries.

The phosphoenolpyruvate-sugar phosphotransferase system (PTS) is the most common type of carbohydrate-transporter in bacteria including *S. mutans*. PTSs are responsible for recognition, binding, transmembrane transport, and phosphorylation of sugar substrates

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(Deutscher *et al.*, 2006). Each PTS consists of two non-specific components, enzyme I (EI) and a heat-stable protein (HPr), and a sugar-specific permease known as enzyme II (EII). The EII is a protein complex consisting of several subunits (EIIA, EIIB, EIIC and occasionally EIID) that can be proteins or protein domains. Subunits EIIA and EIIB are located in the cytoplasm, while EIIC and EIID form a membrane channel (Postma *et al.*, 1993; Vadeboncoeur & Pelletier, 1997). In the process of carbohydrate transport, PTS acts as a phospho-relay in which each subunit exhibits a specific function. The donor of the phosphate group is the energy molecule phosphoenolpyruvate. The first step of the phosphorylation cascade is auto-phosphorylation of the EI, which then transfers the phosphoryl group to a histidine residue on HPr. P-HPr phosphorylates the sugar-specific EIIA subunit which then phosphorylates an EIIB molecule of the same carbohydrate specificity. Finally, P-EIIB phosphorylates carbohydrate as it crosses the plasma membrane through the transmembrane EIIC (and EIID if present), forming sugar-phosphate (Postma *et al.*, 1993; Deutscher *et al.*, 2006). There are multiple PTSs in *S. mutans* (Abranches *et al.*, 2003, 2006; Ajdic & Pham, 2007; Zeng *et al.*, 2010; Moye *et al.*, 2014a). We have shown the presence of 14 PTSs in *S. mutans* UA159 that are responsible for transport of multiple mono-, di- and oligosaccharides (Ajdic *et al.*, 2002; Ajdic & Pham, 2007).

Carbohydrate transport and metabolism are essential for the survival of numerous bacterial species including *S. mutans* (Tanzer *et al.*, 2006). Also, they are directly related to acid production, which is one of the virulence attributes of this cariogenic bacterium (Loesche, 1986; Tanzer *et al.*, 2006).

The organism is capable of expressing and coordinating the regulation of multiple uptake systems in response to the specific sugar. Expression of PTSs is regulated by different mechanisms that include two component systems (Zeng *et al.*, 2006; McKessar & Hakenbeck, 2007; Qian *et al.*, 2010), transcriptional repressors (Ajdic & Ferretti, 1998; Porcheron *et al.*, 2011), transcriptional activators, carbon catabolite repression, including carbon catabolite repression mediated by catabolite control protein A (Titgemeyer & Hillen, 2002; Abranches *et al.*, 2008; Tong *et al.*, 2011; Zeng *et al.*, 2013; Moye *et al.*, 2014a,b), anti-terminators and direct protein–protein interactions between various regulatory elements and different PTS components (Moon *et al.*, 2005; Zeng & Burne, 2009, 2013; Joyet *et al.*, 2013; Deutscher *et al.*, 2014). In addition, the EII^{Man/Glu} component of PTS has been shown to play a vital role in the regulation of carbohydrate metabolism and uptake in different bacterial species including *S. mutans* (Tong *et al.*, 2011; Joyet *et al.*, 2013; Deutscher *et al.*, 2014; Moye *et al.*, 2014b).

LacI family is a distinct group of transcriptional regulators that mainly control sugar utilization pathways (Ravcheev *et al.*, 2014). The *nigBCDAER* locus in *S. mutans* UA159 has been predicted to encode a putative repressor (NigR) that belongs to the LacI family of transcriptional regulators (Ajdic & Chen, 2013). LacI regulators harbor a helix-turn-helix DNA-binding motif and a periplasmic sugar-binding domain. They mediate the direct regulation of a cognate PTS by binding to the gene/operon promoter region in the absence of an effector. Hence, they occlude RNA polymerase binding or destabilize the open complex resulting in the repression of the PTS in the absence of the specific sugar (Weickert & Adhya, 1992).

Dental plaque, the biofilm that develops on the tooth surface, is a complex community of microorganisms. When carbohydrates, particularly sucrose, predominate in the human diet, the most prevalent organisms present are acidogenic and aciduric bacteria such as *S. mutans* (Bowden *et al.*, 1979; Bradshaw *et al.*, 1989; Burne, 1998; Bowden, 1999). Following initial colonization of the hard surfaces in the oral cavity, many bacteria, including *S. mutans*, synthesize exopolysaccharides and exooligosaccharides by specific extracellular enzymes known as glucosyltransferases (Loesche, 1986; Kuramitsu, 1993; Kuramitsu *et al.*, 1995). In the previous study, we showed that the unique PTS^{Bio} of *S. mutans* is expressed in biofilm mode of growth in the presence of sucrose. It is responsible for the transport and metabolism of disaccharide nigerose (3-*O*- α -D-glucopyranosyl-D-glucose) and might be involved in the transport and metabolism of exooligosaccharides synthesized by glucosyltransferase B (GtfB) and glucosyltransferase C (GtfC) of *S. mutans* (Ajdic & Chen, 2013).

In this study we analyzed the transcriptional regulation of the operon encoding PTS^{Bio}. Here we show that this operon is repressed by NigR, a LacI-like transcriptional regulator. Furthermore, we defined the operators for NigR, identified the transcriptional start site of the operon and showed that one of the operators overlaps with the promoter. NigR binding to this operator presumably represses the operon by blocking the initiation of transcription.

METHODS

Bacterial strains and growth conditions

Streptococcus mutans UA159 was routinely cultured in Todd–Hewitt broth (BD Biosciences, San Jose, CA) supplemented with 0.3% yeast extract (BD Biosciences) (THY). For RNA isolation, *S. mutans* were grown in chemically defined medium (CDM) (Terleckyj *et al.*, 1975; Ajdic & Pham, 2007) supplemented with glucose or sucrose at 0.5% concentration. Ultrapure sugars from Sigma-Aldrich (St Louis, MO) were used in this study. *Escherichia coli* strains Top10 (Life Technologies, Grand Island, NY), DH5 α (Invitrogen, Carlsbad, CA) and BL21 (DE3) (Agilent Technologies, Santa Clara, CA) were propagated in LB Broth (BD Biosciences). The strains used in this study are listed in Table 1. For *S. mutans* antibiotic selection, kanamycin (Kan) at a concentration of 800 $\mu\text{g ml}^{-1}$ or erythromycin (Erm) at 5 $\mu\text{g ml}^{-1}$ was used. Streptomycin (Str) (Sigma-Aldrich) was used at a concentration of 50 $\mu\text{g ml}^{-1}$ for *E. coli* DH5 α and BL21. Kanamycin was used at a concentration of 30 $\mu\text{g ml}^{-1}$ for *E. coli* Top10 selection when needed.

Construction of *nigR* mutant

The *nigR* mutant was constructed by overlap extension polymerase chain reaction (PCR) as published previously (Chalker *et al.*, 2001; Ajdic & Chen, 2013). The primers used in this study are listed in Table 2. The replacement of *nigR* with erythromycin cassette (Erm) was confirmed by PCR amplification of the cassette using primer pair ErmF and ErmR. Additionally, correct location of the cassette in the genome was confirmed by PCR amplification using primers *nigR*-P1erm and ErmR as well as primers *nigR*-P4erm and ErmF. The Erm cassette did not exert a polar effect on the downstream gene (data not shown).

Complementation of *nigR* deletion

The *nigR* deletion in *S. mutans* UA159 genome was complemented *in trans* with wild-type (WT) *NigR*. To do so, we cloned *nigR* in the complementation vector that harbors the constitutive lactate dehydrogenase (*Pldh*) promoter of *S. mutans* UA159. Cloning of the promoter and the gene were performed as follows. *Pldh* was amplified from *S. mutans* UA159 genome using primer pair *Pldh*-5' *EcoRI*/*Pldh*-3' *SmaI* and cloned into a *EcoRI*/*SmaI*-digested pDL276 shuttle vector (Dunny *et al.*, 1991) to generate pVS08. The *nigR* complement insert was amplified from the UA159 genome using primer pair *nigR*-5' *SmaI*/*nigR*-3' *BamHI*, digested with *SmaI*/*BamHI* and ligated with pVS08 digested with the same enzymes to generate the *nigR* complementation plasmid, pVS09. The plasmids were propagated in *E. coli* Top10 cells (Life Technologies) in the presence of kanamycin (30 µg ml⁻¹). The integrity of the plasmid was confirmed by restriction mapping. The *nigR* mutant was transformed with plasmid pVS09 to construct the strain *nigR*/pVS09 for complementation. The *nigR* mutant was independently transformed with pVS08 (the strain *nigR*/pVS08) and used as a control. The transformants were selected on THY plates containing kanamycin and confirmed by PCR using primer pair *Pldh*-5' *EcoRI*/*nigR*-3' *BamHI*.

RNA isolation and cDNA synthesis

RNA isolation was performed as previously described (Ajdic & Pham, 2007). Briefly, two biological replicates of the WT, *nigR* mutant, *nigR*/pVS08 and *nigR*/pVS09 strains were grown in CDM supplemented with 0.5% glucose or 0.5% sucrose at 37°C. Cells were harvested in mid-exponential phase of growth (optical density at 600 nm ~0.64) and resuspended in TrizolTM (Ambion Life Technologies, Grand Island, NY). RNA was isolated using the Ambion RiboPureTM Bacteria Kit (Ambion Life Technologies) and treated with DNase I from the same manufacturer. Absence of DNA was confirmed by PCR and the integrity of the isolated RNA was assessed by electrophoresis. Complementary DNA was synthesized from 10 µg of RNA with Superscript III using random primers [for quantitative real-time PCR (qPCR)] or gene-specific primers [for reverse transcription (RT-) PCR and rapid amplification of cDNA ends (5' RACE)]. The gene-specific primers used are listed in Table 2.

Quantitative real-time PCR

The cDNA synthesized using RNA isolated from glucose-grown cultures were used for qPCR as previously described (Ajdic & Pham, 2007). The qPCR was performed with 10 ng of cDNA (synthesized with random primers) using IQTM SYBR Green supermix (Bio-Rad, Hercules, CA) on a CFX96 Real-time PCR system (Bio-Rad) using primers specific for *nigB*. Following denaturation at 95°C for 90 s, the qPCR was set for 40 cycles with 95°C for 10 s and 60°C for 15 s. The relative quantification of qPCR data was calculated using previously published mathematical models (Pfaffl, 2001; Malke *et al.*, 2006; Ajdic & Pham, 2007). The housekeeping gene gyrase A (*gyrA*) was used for normalization. All the reactions, including the no-template control, were performed in triplicates.

Microarray analysis of *nigR*

Microarray analysis was performed as previously described (Ajdic & Pham, 2007), using a whole-genome custom GeneChip antisense expression microarray chip designed in collaboration with Affymetrix (Santa Clara, CA). As per the manufacturer's instructions and our previously published protocol (Ajdic & Pham, 2007), hybridization, washing, and scanning of the microarray chips were performed. The EXPRESSION AND TRANSCRIPTION ANALYSIS CONSOLE software from Affymetrix was used for normalization and gene expression analysis. The cut-off score for the analysis was three-fold. Relevant microarray data were confirmed by qRT-PCR.

The microarray data are available under platform and series accession numbers GPL4769 and GSE63213, respectively, at the National Center for Biotechnology Information Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>).

RT-PCR

Total RNA was isolated as described above. The cDNA synthesized from total RNA of sucrose grown WT culture was used for RT-PCR. The cDNA was synthesized with gene-specific primers and used as a template in the PCR. The PCR analysis of the cDNA was performed with three sets of primers designed to encompass the entire putative operon. Specifically, primer pairs P1–P2 and P3–P4 were used in the PCR when cDNA synthesized with RT-1 primer was used as the template. Primer pair P5–P6 was used when cDNA was synthesized using RT-2 primer. Two gene-specific reverse transcription primers were used to circumvent the degradation of single long cDNA product. The PCR products were separated by electrophoresis on 1% agarose gel. Genomic DNA from *S. mutans* UA159 was used as the template in control reactions with the same set of primers as described above.

5' RACE

The 5' RACE system from Invitrogen (Life Technologies) was used to identify the transcription start site of the O^{Bio} operon by following the manufacturer's instructions. Total RNA isolated from *S. mutans* UA159 grown on sucrose was used to generate cDNA with the gene-specific primer 1 (GSP1) (Table 2). The cDNA was dC tailed and then amplified using the 5' RACE abridged anchor primer supplied in the kit and GSP2 (Table 2). The resulting PCR product was used in a nested PCR with manufacturer-supplied abridged universal amplification primer and GSP3 (Table 2). The amplified DNA fragment of about 350 bp was purified and sequenced.

Cloning, expression and purification of NigR

The *nigR* gene was PCR amplified from *S. mutans* UA159 genomic DNA using Q5 High-Fidelity Polymerase (New England Biolabs, Ipswich, MA) and primer pair NigR-F/*SalI* and NigR-R/*NotI* (Table 2). PCR product was purified using PCR purification kit (Qiagen, Valencia, CA), digested with *SalI/NotI* and cloned into the *SalI/NotI*-digested pCDFDuet-1/3C expression vector containing an N-terminal hexahistidine tag and an engineered rhinovirus 3C protease cleavage site (Vujanac & Stebbins, 2013), generating the NigR expression construct pMV1. *Escherichia coli* DH5 α competent cells (Invitrogen) were

transformed with the ligation mix. The positive clones were verified by sequencing. For expression, the *nigR*-construct (pMV1) was transferred into *E. coli* BL21 cells (Agilent Technologies). The cells were grown at 37°C with aeration in LB broth supplemented with 50 µg ml⁻¹ of streptomycin to an optical density at 600 nm of 0.6. NigR expression was induced with 1 mM IPTG and the cells were harvested after 6 h of incubation. Purification of NigR was performed using Ni-NTA agarose-resin (Qiagen) as described previously (Vujanac & Stebbins, 2013). For removal of the histidine tag, purified NigR was incubated overnight at 4°C with the site-specific rhinovirus 3C protease. The 3C protease was then removed using an Ni-NTA agarose column (Qiagen). The protein concentration was determined with a Bicinchoninic acid Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

Construction of the biotin-labelled electrophoretic mobility shift assay probes

The DNA fragments used as electrophoretic mobility shift assay (EMSA) probes (probes 1, 2 and 3) were made by PCR using Q5 High-Fidelity Polymerase (New England Biolabs) according to the manufacturer's instructions. *Streptococcus mutans* UA159 genomic DNA was used as the template in these experiments. The primers used to generate probes are listed in Table 2. All forward primers were biotin-labeled at the 5' end (IDT Technologies, Coralville, IA). The PCR products were purified using a PCR purification kit (Qiagen). Probes 4–11 were made by annealing synthesized complementary oligonucleotides where one of the oligonucleotides was 5' labeled with biotin (IDT Technologies). The sequences of the oligonucleotides are listed in Table 2.

EMSA

EMSA was performed using a LightShift™ EMSA Optimization & Control Kit (Thermo-Fisher Scientific, Waltham, MA) with biotin-labeled DNA probes and purified NigR according to the manufacturer's instructions. Poly(dI-dC) was used in all reactions as a non-specific competitor at a concentration of 1 µg per reaction (Ajdic & Ferretti, 1998; Nieto *et al.*, 2001; Chawla *et al.*, 2010). Unlabeled specific competitor was added where indicated. Both non-specific and specific competitors were used as described previously (Ajdic & Ferretti, 1998; Gaigalat *et al.*, 2007; Nentwich *et al.*, 2009). An unrelated protein InvB (Type III secretion chaperone from *Salmonella enterica*; Lilic *et al.*, 2006) was used as a negative control. The 20-µl binding reactions were incubated at room temperature for 20 min and the DNA–protein complex was separated from the free probe by electrophoresis on 4 or 5% native polyacrylamide gel in TBE buffer. The material was transferred to positively charged Hybond nylon membrane (GE Healthcare, Pittsburgh, PA) using Trans-Blot Semi Dry Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The membrane was cross-linked for 10 min using UV Crosslinker (UVP HL-200 HybriLinker) and developed using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) following the manufacturer's instructions.

Computational tools used in this study

The primers and EMSA probes used in this study were designed using PRIMER3 (Rozen & Skaletsky, 2000). The DNA-palindrome finder (<http://emboss.bioinformatics.nl/cgi-bin/>

[emboss/palindrome](#)) and [BPROM](#) software (Solovyev & Salamov, 2011) were used to identify promoter elements of the *nigBCDAER* locus. [REGPRECISE](#) database (Novichkov *et al.*, 2013) was used to create an ScrR operator consensus sequence. DNA sequence logos were generated using [WEBLOGO](#) (Crooks *et al.*, 2004).

RESULTS

The O^{Bio} locus (*nigBCDAER*) is transcribed as a single operon

In silico analysis of the *S. mutans* UA159 *nigBCDAER* locus was performed in our previous study. This locus was designated O^{Bio} (Ajdic & Chen, 2013). The O^{Bio} locus was shown to be highly expressed in sucrose-grown cultures. The annotation data (Ajdic *et al.*, 2002) and *in silico* analysis suggested that O^{Bio} is organized as a putative operon consisting of six open reading frames (Fig. 1A). To confirm that these genes indeed constitute an operon, reverse transcription analysis (RT-PCR) was performed on the total RNA isolated from sucrose grown *S. mutans* UA159 culture. In the reverse transcription reaction, two operon-specific primers (Table 2) were used to synthesize cDNA (Fig. 1). Subsequent PCR were performed using this cDNA as a template with three primer sets (Table 2) designed such that their products overlap. The result showed the presence of DNA bands of 1, 1.3 and 2.4 kb (Fig. 1B). The sizes corresponded to the expected sizes of the overlapping products. This suggests that the genes *nigB,C,D,A,E,R* are co-transcribed and they form an operon.

Identification of transcription start site

Having confirmed that O^{Bio} is indeed an operon, 5' RACE analysis was used to identify its transcriptional start site. We synthesized cDNA from the RNA template using the *nigB*-specific internal primer (GSP1). Because O^{Bio} is transcribed as a single operon (Fig. 1), the specific cDNA included a transcriptional start at the 5' end. The 5' end of the cDNA was C-tailed to provide a sequence for primer annealing. We used abridged primer complementary to C-tail along with a gene-specific primer (GSP2) to PCR amplify the tailed cDNA. This product was used as a template and PCR were performed using gene-specific nested primers. A single product of approximately 350 bp was purified and sequenced. The result showed that an adenine nucleotide located 73 bp upstream from the *nigB* start codon is the site of transcription initiation (Fig. 2).

NigR is the transcriptional repressor of O^{Bio}

Computational analysis of the O^{Bio} locus was performed previously and the last gene of the operon, *nigR*, was predicted to encode a LacI-like putative transcriptional repressor harboring a helix-turn-helix motif at the N-terminus and a ligand-binding domain at the C-terminus (Ajdic & Chen, 2013). To determine the role of NigR in the transcription of O^{Bio}, we constructed a *nigR* deletion mutant (*nigR*) in *S. mutans* by replacement with an erythromycin resistance cassette. O^{Bio} is expressed in the presence of sucrose, but not of glucose. Therefore we compared expression of the *nigB* gene (the first gene of O^{Bio}) in *nigR* and WT under non-inducing condition (glucose-grown cultures) using qPCR analysis. The transcription of *nigB* in *nigR* was approximately 72-fold upregulated in comparison to that of WT (Fig. 3). The expression of *nigB* in the *nigR/pVS09* strain harboring the complementation plasmid was 73-fold downregulated compared with that of the strain

nigR/pVS08 (carrying an empty vector) (Fig. 3). Therefore, complementation of *nigR* with NigR expressed *in trans* restored the WT phenotype, confirming that the increased expression of O^{Bio} in the *nigR* strain occurred as a result of the deletion of *nigR*. Taken together the results confirmed that NigR is a transcriptional repressor of O^{Bio}.

Microarray analysis of the *nigR*

To elucidate the role of NigR in the regulation of *S. mutans* carbohydrate transporters we performed full genome expression microarrays using custom Affymetrix chip. Expression microarray analysis was performed with *nigR* grown in CDM supplemented with glucose and compared with that of WT grown under identical conditions. Deletion of the *nigR* gene did not exert broad transcription changes in *S. mutans* under the tested conditions. O^{Bio} was the only operon that was significantly upregulated in the *nigR* strain (Table 3). These data confirmed that NigR is the repressor of O^{Bio} and that O^{Bio} is its only PTS target under tested condition.

Specific binding of NigR to the O^{Bio} promoter region

Transcriptional repressors usually exert their effect by binding to the operator regions of the operons/genes they regulate (Madan Babu & Teichmann, 2003). To assess if NigR exhibits similar mechanism of action we tested the binding of NigR to the O^{Bio} promoter region. For this purpose, NigR carrying an N-terminal histidine tag was overexpressed and purified from *E. coli* using affinity chromatography. The histidine tag was subsequently removed using the 3C site-specific protease. EMSA was performed using purified NigR to address its binding ability to the O^{Bio} promoter region. The assay was carried out using a specific 209-bp DNA fragment spanning the upstream region of O^{Bio} (Probe 1, Fig. 2). As shown in Fig. 4A, lanes 1–5, the mobility of Probe 1 was retarded upon addition of the purified NigR in a concentration dependent manner indicating the formation of a specific DNA–protein complex. As a negative control we used an unrelated protein, InvB that was overexpressed and purified under identical conditions. The control protein did not bind to the labeled probe (Fig. 4A, lanes 6–9). Next, we performed competition gel mobility shift experiments to confirm specificity of NigR DNA-binding. Addition of the identical unlabeled DNA fragment reduced complex formation as compared with the labeled probe (Fig. 4B, lanes 3–7). Furthermore, NigR binding was completely abolished if the concentration of the specific competitor was 100-fold higher (Fig. 4B, lane 7). In contrast, non-specific competitor Poly(dI-dC) had no effect on NigR binding even at a 370-fold higher concentration than the labeled probe (Fig. 4B, lane 2). These results clearly show that NigR specifically binds to the promoter region of the O^{Bio} *in vitro*.

Transcriptional repressor NigR binds to two operator sites

Using DNA-palindrome finder we identified two DNA motifs with dyad symmetry (palindromes) upstream of *nigB* gene, each consisting of 18 bp (Fig. 2). On the basis of their proximity to the operon they were named as proximal and distal palindrome, respectively. The distal palindrome is located upstream of the –10 promoter site, whereas the proximal palindrome is just upstream of the predicted ribosome binding site. Based on the dyad symmetry and the position of the identified palindromes we hypothesized that NigR-binding

sites might be located within these DNA regions because palindromes appear to be a common feature of the binding sites for many DNA-binding proteins (Gilbert & Maxam, 1973; Sadler *et al.*, 1983; Schleif, 1988; Rhodes *et al.*, 1996). To check our hypotheses, we PCR amplified the two DNA fragments of 108 bp (Probe 2, Fig. 2) and 101 bp (Probe 3, Fig. 2) containing the distal or proximal palindrome, respectively and used them in EMSA analyses. Under the conditions tested, both fragments bound NigR but with varying affinities (Fig. 5A). NigR bound Probe 2 even at lower protein concentration (10 ng per reaction), whereas higher concentration (20–40 ng per reaction) of the protein was required for binding Probe 3 (Fig. 5A).

To better define the NigR-binding site, 31-bp and 28-bp probes containing the distal (Probe 4, Fig. 2) and proximal (Probe 5, Fig. 2) palindromes, respectively were used in EMSA. NigR bound to both probes (Fig. 5B), suggesting that these sequences act as the operators for O^{Bio}. We named the distal and proximal palindromes Operator 1 (O1) and Operator 2 (O2), respectively. At the protein concentration of 20 ng per reaction, there was almost complete shift of Probe 4, whereas a small amount of the DNA–protein complex formed with Probe 5 even at a protein concentration of 40 ng per reaction suggesting the lower affinity of NigR for O2 (Fig. 5B).

Mutagenesis of the NigR operators

To address the role of the inverted repeats within O1 in NigR binding, we introduced three independent mutations in it (Fig. 6A). Specifically, we replaced the TGGAA sequence within the left inverted repeat with GATCG (Probe 6) or changed TTCCA into CGATC in the right inverted repeat (Probe 7). As shown in Fig. 6B,C, each mutation completely abolished the binding of NigR, suggesting that the five bases on each side of the symmetry center are required for NigR binding. On the contrary, changing five bases (TATAA to GGATC) that included terminal T of O1 along with 4 bp located just outside the palindrome (Probe 8) did not alter protein–DNA complex formation (Fig. 6A,D). Hence, these bases appear to be dispensable for NigR binding.

O1 and O2 sequences differ by four bases (Figs 2, 6 and 7). To address the importance of this sequence difference in NigR binding, mutations were introduced in O2 (substitutions: A to T; T to G; A to C; A to G) converting it to O1 (Probe 9, Fig. 6E). The probe sequence outside O2 remained unchanged. EMSA results revealed that NigR bound Probe 9 with an affinity comparable to that seen with native O1 (Fig. 6F). However, substitutions of the two bases closer to the O2 center of symmetry (T to G and A to C; Probe 10; Fig. 6G) resulted in higher NigR binding affinity than substitution of the bases further away (A to T and A to G; Probe 11; Fig. 6H). The results indicate that sequence differences between O1 and O2 play a role in NigR binding affinity.

DISCUSSION

The novel carbohydrate transporter PTS^{Bio} of *S. mutans* UA159 responsible for uptake of a disaccharide nigerose and possibly, oligosaccharides with α -1,3 linkage produced by GtfC and GtfB, has been previously described (Ajdic & Chen, 2013). *In silico* analysis of the operon (*O^{Bio}*) encoding the PTS^{Bio} showed the presence of four genes for EII-PTS

components (EIIBACD) responsible for sugar uptake, a putative enzyme glucosidase/glycosyl hydrolases of the GH31 superfamily and a putative transcriptional regulator NigR, that belongs to the LacI family of repressors (Ajdic & Chen, 2013). In this study we have addressed the regulation of the PTS^{Bio} by NigR. We hypothesized that the transcription of the operon *O^{Bio}* was regulated by NigR, a putative transcriptional regulator encoded by the last gene of the *O^{Bio}*. We designed experiments to verify this hypothesis by addressing the following questions: Is *O^{Bio}* really an operon and if yes, is the transcription of the *O^{Bio}* regulated by NigR? Reverse transcription analysis of the putative *O^{Bio}* proved that this gene-cluster was indeed transcribed as a single operon.

Our previous results showed that the transcription of *O^{Bio}* was induced in the presence of sucrose as well as nigerose and was repressed in glucose (Ajdic & Chen, 2013). To determine the role of NigR in regulation of *O^{Bio}*, we deleted it from the *S. mutans* UA159 genome. The constructed *nigR* mutant (*nigR*) showed highly increased *O^{Bio}* transcription when grown in the media supplemented with glucose, a non-inducing carbohydrate. In contrast, *O^{Bio}* was not transcribed in the WT under the same growth conditions. The *nigR* phenotype was complemented by expressing NigR *in trans*, confirming that NigR is indeed the transcriptional repressor of *O^{Bio}*. Furthermore, microarray data mirrored this finding and showed that *O^{Bio}* was the only operon regulated by NigR under the tested condition.

It is interesting to note that *S. mutans nigR* is transcribed from the same NigR-regulated promoter as shown by our transcriptional analysis, suggesting that autoregulation of *nigR* plays a vital role in the functioning of this operon. In the LacI/GalR family of transcriptional repressors, it has been shown that the binding of an effector molecule to the C-terminal domain of the protein most probably results in its conformational change. This leads to the dissociation of the protein from the DNA (Nguyen & Saier, 1995), so alleviating repression. Deletion of NigR resulted in the loss of stimulus specificity as seen by a 72-fold increase *in transcription* in the presence of glucose. Our previous studies have shown that the products of GtfB and GtfC relieved repression of *O^{Bio}* operon (Ajdic & Chen, 2013). It is likely that the same product (s), α -1,3-linked carbohydrate(s), may act as effector molecules that dictate the regulation of *O^{Bio}* as well as autoregulation of NigR.

Transcriptional regulators involved in uptake and metabolism of carbohydrates in bacteria are usually grouped into four families: LacI, IclR, DeoR and GntR (Perez-Rueda & Collado-Vides, 2000). NigR belongs to the LacI family named after *lac* repressor (LacI) of *E. coli*, which regulates transcription of the genes responsible for lactose metabolism (Jacob & Monod, 1961). Most LacI-family transcription regulators control carbohydrate transport and utilization pathways (Ravcheev *et al.*, 2014). Repressors belonging to the LacI-family, including NigR, harbor a helix-turn-helix DNA-binding motif at their N-terminus and a ligand-binding domain at the C-terminus (Mauzy & Hermodson, 1992; Weickert & Adhya, 1992; Nguyen & Saier, 1995). NigR also showed high sequence similarity (30% identity and 53% similarity) to the ScrR sub-group of LacI-family. ScrR is a repressor of sucrose utilization in numerous bacterial families including Streptococcaceae (Ravcheev *et al.*, 2014).

Transcriptional repressors bind to specific DNA sequences that are called operators. These are palindromes consisting of highly conserved inverted repeats (Francke *et al.*, 2008; Camas *et al.*, 2010; Milk *et al.*, 2010) usually found in the promoter regions (Schleif, 1988; Rhodes *et al.*, 1996). Sequence analyses of the O^{Bio} promoter region revealed the presence of two sets of inverted repeats. Both displayed similarity to ScrR binding sites of Streptococcaceae (Fig. 7) (Ravcheev *et al.*, 2014). The two inverted repeats appear to be 18-bp palindromes. The signature feature of LacI-family palindromes is the presence of CG nucleotides in the center (Ravcheev *et al.*, 2014). This characteristic was found in both ScrR and NigR (Fig. 7). By using multiple EMSA probes we confirmed that the two palindromes were indeed the NigR-binding sites (operators O1 and O2) as predicted by bioinformatic tools. NigR formed complex with both operators *in vitro*; however, its binding affinity was higher for O1. The difference in NigR-binding affinity was seen with both, longer and shorter probes carrying O1, indicating that the length of the probes did not influence the formation of NigR-operator complex. Lower affinity of NigR for O2 can be attributed to the four-base difference in the two palindromes. A change that converted O2 into O1 resulted in stronger NigR binding in comparison to the native O2, suggesting the importance of these bases in NigR–DNA interaction. Several studies of LacI, PurR and CytR protein–DNA recognition have shown that the 7 bp closest to the center of the operator interact with the protein residues (Schumacher *et al.*, 1994; Barbier *et al.*, 1997; Kalodimos *et al.*, 2002). Based on this knowledge and our data, nucleotides located at the position 4–8 and 11–15 of O1 (Fig. 7) are most likely indispensable for NigR binding. Furthermore, bases at the position 5 (G) and 14 (C) (Fig. 7) exert a stronger influence on NigR binding *in vitro* than nucleotides at the position 2 and 17.

The 5-bp mutation in either the left or the right inverted repeat of O1 completely abolished DNA–NigR interaction. Altering these sequences also abrogated the symmetry of O1, emphasizing its role in NigR binding. The importance of sequence symmetry has been previously demonstrated for the binding of LacI-family transcription regulators to their operators (Gilbert & Maxam, 1973; Sadler *et al.*, 1983; Falcon & Matthews, 2000; Francke *et al.*, 2008; Camas *et al.*, 2010; Milk *et al.*, 2010). The members of LacI repressor family usually form homo-dimers where the helix-turn-helix domain of each monomer recognizes and makes contact with one half of the operator (Weickert & Adhya, 1992; Dong *et al.*, 1999). In the light of this knowledge and our data, we speculate that NigR might bind to its operator in the similar manner.

O1 overlaps with the O^{Bio} promoter, whereas O2 is located between transcriptional and translational start sites. The position of O1 suggests that NigR could exert its role as a transcriptional repressor by occluding the binding site of the RNA polymerase, so blocking the initiation of transcription in the absence of the effector molecule. This would imply that O2 is dispensable *in vivo*. Alternatively, both O1 and O2 might be functional *in vivo* such that NigR could exert its activity through cooperative binding. The binding would presumably happen sequentially, first to O1 (primary operator) and then to O2 (ancillary operator). Occupying both operators on single DNA molecule could in turn allow subsequent interaction between DNA-bound NigR molecules and formation of stable loop structures. Formation of such structures might be necessary to achieve maximal repression

in vivo (Pfahl *et al.*, 1979; Whitson *et al.*, 1987; Sasse-Dwight & Gralla, 1988; Oehler *et al.*, 1990).

ACKNOWLEDGEMENTS

We thank Dr. Erec Stebbins, The Rockefeller University for pCDFDuet-1/3C vector. This work was supported by grant DE021424 (to D.A.) from the National Institute of Dental and Craniofacial Research.

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Figure 1.

Reverse transcription analysis of *O^{Bio}*. (A) Genetic organization of *O^{Bio}* operon. Primers used in the experiment are shown as arrows. (B) Photograph of the agarose gels showing RT-PCR products. In the RT reaction, total RNA isolated from *Streptococcus mutans* grown in 0.5% sucrose was used as the template to generate cDNA with primer RT-1 or RT-2. The cDNA generated using RT-1 primer was used as a template in the PCR with primer pairs P1/P2 and P3/P4 (lanes 2 and 3). The cDNA generated using RT-2 primer was used as the template in the PCR with primer pair P5/P6 (lane 5). Molecular weight markers are presented in lanes 1 and 4. The expected product sizes are indicated on the right-hand side of the image. All the PCR products generated have overlapping regions (A).

Figure 3.

Transcription of *O^{Bio}* operon in the *nigR* mutant and the complementation strain.

Transcription of the first gene (*nigB*) in the operon was analyzed in the wild-type (WT), *nigR*, the complemented strain *nigR/pVS09* and *nigR/pVS08* (control). Mid-exponential phase cultures grown in CDM media supplemented with 0.5% glucose were used for the analysis. Transcription was analyzed by quantitative PCR and gyrase A (*gyrA*) was used as the normalization gene.

Figure 4.

Specific binding of NigR to the promoter region of *O^{Bio}* (A) EMSA was performed using Probe 1 and the purified NigR protein (lanes 1–5) or negative control protein InvB (lanes 6–9). Increasing amounts of NigR were used in the assay: lane 1, 0 ng; lane 2, 5 ng; lane 3, 10 ng; lane 4, 20 ng; lane 5, 40 ng. Amount of the purified InvB was: lane 6, 5 ng; lane 7, 10 ng; lane 8, 20 ng; lane 9, 40 ng. The amount of labeled DNA probe was 20 fmol per reaction. (B) Competition gel mobility shift assay. Amount of purified NigR was: 0 ng in lane 1 or 20 ng in lanes 2–7. The amount of labeled DNA probe was 20 fmol in all reactions. The concentrations of identical but unlabeled specific DNA fragment (added 5 min before addition of labeled DNA) were: lane 3, 350 fmol; lane 4, 700 fmol; lane 5, 1400 fmol; lane 6, 2000 fmol; and lane 7, 4000 fmol per reaction. Specific competitor DNA was not added in lane 2.

Figure 5.

Identification of NigR-binding sites. (A) Binding of NigR to the probes carrying either distal (Probe 2) or proximal (Probe 3) palindrome. EMSA was performed with Probe 2 (lanes 1–4) or Probe 3 (lanes 5–8). The amount of purified NigR was 0 ng (lanes 1 and 5); 10 ng (lanes 2 and 6); 20 ng (lanes 3 and 7) and 40 ng (lanes 4 and 8). The amount of the labeled probe was 20 fmol per reaction. (B) Gel mobility shift assay with O1-containing Probe 4 (lanes 1–3) or O2-containing Probe 5 (lanes 4–6). The amount of purified NigR: lane 1, 0 ng; lanes 2 and 4, 10 ng; lanes 3 and 5, 20 ng; lane 6, 40 ng. Labeled probes were used at a concentration of 4 fmol per reaction.

Figure 6. Mutagenesis of O1 and O2 and its effect on NigR binding. (A and E) Various mutations introduced into probes carrying operators O1 (Probes 6, 7, 8) or O2 (Probes 9, 10, 11), respectively; (B–D) EMSA with Probes 6, 7 or 8, respectively; (F–H) EMSA with Probes 9, 10 or 11, respectively. O1 and O2 are indicated by arrows on Probes 4 and 5, respectively. The amount of purified NigR: lanes 1, 0 ng; lanes 2, 10 ng; lanes 3, 20 ng; lanes 4, 40 ng. The amount of labeled probe was 10 fmol per reaction. Asterisk indicates center of symmetry.

Figure 7.

Comparison of ScrR and NigR operators. NigR operator consensus sequence was created by aligning O1 and O2 from *Streptococcus mutans* UA159. ScrR consensus derived from 27 operators found in 13 streptococcus species (Novichkov *et al.*, 2013). DNA sequence logos were created using W_{EB}LOGO (Crooks *et al.*, 2004), a graphical representation of a nucleic acid multiple sequence alignment.

Table 1

Bacterial strains and plasmids used in this study

Strain	Description	Reference
WT	<i>Streptococcus mutans</i> UA159, serotype c strain, wild-type	P. Caufield
<i>nigR</i>	<i>nigR</i> ⁻ ; Erm ^R	
<i>nigR/pVS08</i>	<i>nigR</i> ⁻ harboring vector pVS08; Erm ^R , Kan ^R	This study
<i>nigR/pVS09</i>	<i>nigR</i> ⁺ harboring plasmid pVS09; Erm ^R , Kan ^R	This study
Plasmids		
pDL276	Kan ^R ; Lac ⁺	Dunny <i>et al.</i> (1991)
pVS08	pDL276 harboring the promoter (<i>PldH</i>) of constitutively expressed <i>S. mutans</i> lactate dehydrogenase; Kan ^R	This study
pVS09	pVS08 constitutively expressing <i>nigR</i> under the control of <i>Pldh</i> ; Kan ^R	This study
pCDFDuet-1/3C	pCDFDuet-1 (Novagen) His-tag protein overexpression vector harboring 3C protease cleavage site; Str ^R	Vujanac & Stebbins (2013)
pMV1	pCDFDuet-1/3C harboring full length <i>nigR</i> ; Str ^R	This study

Table 2

Primers and oligonucleotides used in this study

Primer	Sequence (5'–3')	Application
nigR-P1	GATTAATCGTCTGCATTCTTCAGAT	These primer sets were used for the
nigR-P2ermF	CAAATCAAACAAATTTTGGGCCCGGACATTAGCCAGTTGAGCTACATCAT	construction of <i>nigR</i> . The same primers
nigR-P3ermR	ATAATTCTATGAGTCGCTGCCGACTCGAGGAGGTTCCAAGTCATTATA G	were used for PCR confirmation of the
nigR-P4	CCAAATGAACGACTTAAATGATACC	mutant
ErmF	CCGGGCCCAAATTTGTTTGATTT G	
ErmR	AGTCGGCAGCGACTCATAGAATTAT	
PldH-R/SmaI	ATATCCCGGGGTTCTAAACATCTCCTTATAATTTATTAAGT	Used to amplify <i>ldh</i> promoter region
Pldh-F/EcoRI	ATATGAATTCCCGAGCAACAATAACACTCAT	
nigR-F/SmaI	CTCTCCCGGGATGAAAAGAAATCCTACAATCAATGATGT	Used to amplify <i>nigR</i> complementation
nigR-3R/BamHI	CTCTGGATCCTTACACTAACCTTTTCAGGCTCAACAAAAC	insert
NigR-F/SalI	ATAAGAATGTCGACATGAAAAGAAATCCTACAATCAATG	Used for cloning of <i>nigR</i> gene
NigR-R/NotI	ATAAGAATGCGGCCGCTTACACTAACCTTTTCAGGCTCAACAAAAC	
Probe-1F	BioTAAGGCTTGAAATCTAGAACT	Used to generate EMSA Probes 1-3 to
Probe-1R	TCCATGGATTAATCTTTGATCTACTCT	test specific binding of NigR to its
Probe-2F	BioTAAGGCTTGAAATCTAGAACT	operators
Probe-2R	GTGTAAGCGTTTTAGTGATTATATTATACATGG	
Probe-3F	BioTTAAAGTAGTTTTTTGAGAGAAATTGAACG	
Probe-3R	TCCATGGATTAATCTTTGATCTACTCT	
qPCR primers		
nigB-F	GATATTGTAAGCAATCAGCCAGAG	Used to analyze <i>nigB</i> transcription
nigB-R	TTTTTGAAATTTGCAATAGCCTTT	
gyrA-F	TTC CAA ATC TTC TGG TTA ATG GA	Used to analyze <i>gyrA</i> transcription
gyrA-R	TCC ATA ACT AAT TTG ACC GCA TC	
RT-PCR primers		
RT-1	GATTTAGTACTGTTGTTGAAGGACGAT	Used in reverse transcription reaction for the operon verification
P1	TATTCGGATGATTCTGTAATTGTC	Used for PCR amplification to verify that
P2	TTTAGCTAAGGACCAAACTGAG	<i>O^{Bio}</i> is an operon. cDNA synthesized
P3	GTGATCGTCACAATGCTATTCTCAG	using RT-1 was the template
P4	TGAAGGACGATCTTCAAATACTTTT	
RT-2	GTCGATGTTTTTGAIGTTTTTAACCTCC	Used in reverse transcription reaction for the operon verification.
P5	TGGAAGAAGAACAGGTAAGTTATATGG	Used for PCR amplification to verify that

Primer	Sequence (5'-3')	Application
P6	TCTAGCATTGGGAACATATTTCAAC	<i>O^{Bio}</i> is an operon. cDNA synthesized using RT-2 was the template
5'-RACE primers		
GSP-1	CCTCCAATTAAGTCCTGTATTGA	Used to synthesize cDNA for transcription start site identification.
GSP-2	GACAATTACAGAATCATCCGAATA	Used in PCR with GSP-1 generated cDNA
GSP-3	AAGCAAAGTAGAAGGTTCTTTGACA	as the template
Oligonucleotide		
Probe-4F	BioACAATATGGAACGTTCCATGTATAATATAAT	Used to make EMSA Probes 4 and 5 to
Probe-4R	ATTATATTATACATGGAACGTTCCATATTGT	test specific binding of NigR to its
Probe-5F	BioAGAAATTGAACGTTCAATA TTTTAAGGA	operators
Probe-5R	TCCTTAAATATTGAACGTTCAATTTCT	
Probe-6F	BioACAATAGATCGCGTTCCATGTATAATATAAT	Used to generate EMSA Probes 6–11 for
Probe-6R	ATTATATTATACATGGAACGCGATCTATTGT	mutational analysis of NigR operators
Probe-7F	BioACAATAGATCGCGTTCCATGGGATCTATAAT	
Probe-7R	ATTATAGATCCCATGGAACGCGATCTATTGT	
Probe-8F	BioACAATATGGAACGTTCCATGGGATCTATAAT	
Probe-8R	ATTATAGATCCCATGGAACGTTCCATATTGT	
Probe-9F	BioTTTTTGAGAGAAATTGAACGTTCAATATTTTAAGGAGGA	
Probe-9R	TCCTCCTTAAATATTGAACGTTCAATTTCTCTCAAAAA	
Probe-10F	BioTTTTTGAGAGAAATGGAACGTTCCATATTTTAAGGAGGA	
Probe-10R	TCCTCCTTAAATATTGAACGTTCCATTTCTCTCAAAAA	
Probe-11F	BioTTTTTGAGAGATATTGAACGTTCAATG TTTT AAGGAGGA	
Probe-11R	TCCTCCTTAAACATTGAACGTTCAATATCTCTCAAAAA	

Bio indicates 5'-biotin labeled primer/oligo. Restriction sites are shown in bold.

Table 3

The *nigR* transcriptome analysis. Differentially transcribed genes in *nigR* compared with the wild-type following growth in CDM supplemented with glucose

Gene number (NCBI database)	Gene name	Fold change	ANOVA <i>P</i> -value	<i>P</i> -value	Function
SMU.100	<i>nigB</i>	7.68	0.006618	0.24755	PTS ^{Bio} EIIB component
SMU.101	<i>nigC</i>	6.04	0.001173	0.15857	PTS ^{Bio} EIIC component
SMU.102	<i>nigD</i>	5.53	0.004307	0.23765	PTS ^{Bio} EIID component
SMU.103	<i>nigA</i>	9	0.007843	0.25211	PTS ^{Bio} EIIA component
SMU.104	<i>nigE</i>	4.26	0.000819	0.15857	Putative α -glucosidase; glycosyl hydrolase
SMU.105	<i>nigR</i>	-89.92	0.00088	0.15857	Transcriptional repressor NigR

ANOVA, analysis of variance.