

ORIGINAL ARTICLE

Iron-dependent gene expression in *Actinomyces oris*

Matthew P. Mulé¹, David Giacalone¹, Kayla Lawlor¹, Alexa Golden¹,
Caroline Cook¹, Thomas Lott¹, Elizabeth Aksten¹, George A. O'Toole² and
Lori J. Bergeron^{1*}

¹Department of Biology, New England College, Henniker, NH, USA; ²Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, NH, USA

Background: *Actinomyces oris* is a Gram-positive bacterium that has been associated with healthy and diseased sites in the human oral cavity. Most pathogenic bacteria require iron to survive, and in order to acquire iron in the relatively iron-scarce oral cavity *A. oris* has been shown to produce iron-binding molecules known as siderophores. The genes encoding these siderophores and transporters are thought to be regulated by the amount of iron in the growth medium and by the metal-dependent repressor, AmdR, which we showed previously binds to the promoter of proposed iron-regulated genes.

Objective: The purpose of this study was to characterize siderophore and associated iron transport systems in *A. oris*.

Design: We examined gene expression of the putative iron transport genes *fetA* and *sidD* in response to low- and high-iron environments. One of these genes, *sidD*, encoding a putative Fe ABC transporter protein, was insertionally inactivated and was examined for causing growth defects. To gain a further understanding of the role of iron metabolism in oral diseases, clinical isolates of *Actinomyces* spp. were examined for the presence of the gene encoding AmdR, a proposed global regulator of iron-dependent gene expression in *A. oris*.

Results: When *A. oris* was grown under iron-limiting conditions, the genes encoding iron/siderophore transporters *fetA* and *sidD* showed increased expression. One of these genes (*sidD*) was mutated, and the *sidD*::Km strain exhibited a 50% reduction in growth in late log and stationary phase cells in media that contained iron. This growth defect was restored when the *sidD* gene was provided in a complemented strain. We were able to isolate the AmdR-encoding gene in seven clinical isolates of *Actinomyces*. When these protein sequences were aligned to the laboratory strain, there was a high degree of sequence similarity.

Conclusions: The growth of the *sidD*::Km mutant in iron-replete medium mirrored the growth of the wild-type strain grown in iron-limiting medium, suggesting that the *sidD*::Km mutant was compromised in iron uptake. The known iron regulator AmdR is well conserved in clinical isolates of *A. oris*. This work provides additional insight into iron metabolism in this important oral microbe.

Keywords: *siderophore*; *oral*; *transport*

*Correspondence to: Lori J. Bergeron, 98 Bridge Street, Henniker, NH 03242, USA,
Email: lbergeron@nec.edu

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Iron is essential for most pathogenic bacteria to survive and persist. In the human host, most iron is scavenged by host-binding proteins such as lactoferrin. In order to acquire iron, bacteria possess iron transporters as well as iron-scavenging molecules known as siderophores. When iron is low, organisms produce siderophores that solubilize the iron. Bacteria then transport the iron-siderophore complex into the cell by transport proteins such as ATP-binding cassette (ABC) transporters (1). However, too much iron can lead to damaging oxidative stress. Therefore, the concentration of iron within the bacterium is tightly regulated. Both Gram-positive and

Gram-negative bacteria possess transcriptional regulators that control expression of the genes involved in iron uptake. Gram-positive organisms, which make up a significant proportion of oral bacteria, possess regulators similar to the diphtheria toxin repressor (DtxR). The DtxR protein in *Corynebacterium diphtheriae* is a global iron-dependent repressor that regulates transcription from multiple promoters including the phage-encoding *tox* gene and at least seven other genes (2). In *C. diphtheriae* and other pathogens, low iron modulates the expression of surface carbohydrates, adherence to human red blood cells, and slime production (3–5).

Actinomyces oris (formerly *A. naeslundii*) belongs to the genus *Actinomyces*, which includes several species of Gram-positive, facultatively anaerobic, rod-shaped bacteria. *A. oris* has been referred to as a pioneer of the oral cavity, because this microbe is one of the first organisms to colonize the mouth after birth and is among the first to recolonize newly cleaned tooth surfaces (6, 7). *A. oris* is a numerically dominant member of the oral flora throughout life and is found in significant numbers in dental plaque, as well as on mucosal surfaces in adults (8). These organisms have been found at both healthy and diseased sites in the mouth (9). For example, *A. oris* peptidoglycan has been shown to contribute to bone loss in periodontal disease (10), and *Actinomyces* species have been isolated from bisphosphonate-related osteonecrosis of the jaw (11–13).

Despite all of these roles in important human illnesses, there is comparatively little known about *A. oris* due to the difficulties of conducting molecular studies with this organism. Our laboratory has shown that *A. oris* possesses a transcriptional repressor AmdR, which appears to mediate the metal-dependent gene expression of a broad number of genes, including those coding for known or suspected virulence factors (14). Furthermore, we have shown that *A. oris* produces siderophores when cells are grown in low-iron medium and that siderophore production decreased when the iron was added to the medium (14). It is likely that the siderophores produced by *Actinomyces* contribute to the ecology of the plaque biofilm. D'onofrio et al. (15) have shown that siderophores are important molecules in complex microbial communities and, furthermore, siderophores from one organism may be used by another microbe. The siderophores produced by *A. oris* may play an important role in the survival of other organisms in the dental plaque that lack siderophores, such as *Streptococcus mutans*.

The acquisition of metal plays a major role in microbial virulence and disease process. Our hypothesis is that the low-metal-ion environment in the human oral cavity is used as a signal to induce expression of *A. oris* genes involved in metal ion acquisition and transport. The purpose of our study was to characterize metal transport in *A. oris*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strain *A. oris* MGI and 10 *Actinomyces* clinical isolates (from Dr. Robert Burne at the University of Florida) were maintained on brain–heart infusion agar at 5% CO₂ at 37°C. The chemically defined *Actinomyces* medium (ADM) was used to cultivate *A. oris* (16). This medium was a modified version of the ‘HP 6/A’ medium omitting iron, hemin, and manganese to allow for low-metal conditions (16). The base ADM medium was shown to have ~8 µM iron as assessed by inductively coupled

plasma mass spectrometry (ICP-MS), as described below. Ferric chloride was used to supplement the medium to allow for a high-iron condition (100 µM to 1 mM). In addition, to achieve iron-limiting conditions the iron-binding compound dipyrindyl (DP) was added to the growth medium at 75 and 150 µM; given that there is 8 µM iron in the base medium, this concentration of DP is sufficient to scavenge the free iron in the medium.

The *Escherichia coli* strain DH5α from New England Biolabs was maintained on lysogeny broth (LB) solidified with 1% agar as appropriate. The medium was supplemented with ampicillin (Ap^R; 100 µg mL⁻¹), kanamycin (Km^R; 50 µg mL⁻¹), or streptomycin (Sm^R; 50 µg mL⁻¹), as indicated. For growth curves, bacteria were grown in 96-well plates and optical density (OD) at 630 nm was recorded using a spectrophotometer.

RNA isolation and qRT-PCR studies

Total RNA was isolated from mid-log stage *A. oris* and an *A. oris* *sidD*::Km mutant using the RNA mini prep kit (Qiagen, Hilden, Germany). RNA was quantified using Nanodrop lite (Thermo Scientific, Waltham, MA). Samples were treated with DNase (Qiagen, Hilden, Germany). For qRT-PCR experiments, 2 µg of cDNA was synthesized from RNA using the cDNA kit from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. qRT-PCR was performed using Applied Biosystems Step One applying the recommended Taqman protocol. The experiments were conducted in triplicate. The cycle threshold (Ct) value was recorded and the relative quantification of the specific gene expression was calculated using the 2^{-ΔΔCt} method, with the 16S rRNA as the reference gene. ANOVA analysis was conducted on the biological replicates to determine if there was a significant difference in the gene expression in the different iron concentrations, and the results were analyzed using Dunnett's multiple comparisons test.

Construction of a *sidD*::Km mutant strain of *A. oris*

An *A. oris* mutant containing an insertion in the *sidD* (*anae_c_1_5343*) gene was constructed as follows: Primers *sidF* (5'-CCGAAGAAGGGATCCGTGCC-3') and *sidR* (5'-GGGCAGCGCGGATCCCTACCGGC-3') containing engineered *Bam*HI sites (underlined) were used to amplify a 1 kb fragment containing the *sid* open reading frame (ORF). This ORF was subcloned into pGEM-7 (Promega, Madison, WI), which does not replicate in *A. oris* (17). A kanamycin-resistant cassette Km^R with *Nhe*I generated ends was PCR amplified from pJRD215 (ATCC) with the primers *kanF* *Nhe*I (5'-TGGGCGT CGCTTGCTAGTCATTTTCGC-3') and *kanR* *Nhe*I (5'-CCCTGCAGCCAAGCTAGCTTACGC-3'). The Km^R gene was introduced into a unique *Nhe*I site in the *sidD* ORF. The resulting plasmid (psidKm^R) was electroporated into *A. oris* MGI as described previously (18).

Chromosomal DNA was isolated from Km^R colonies and was screened for the presence of the kanamycin-resistant marker via PCR. Primers KmF71114 (5'-CAGACAATCGGCTGCTCTGA-3') and KmR71114 (5'-GATGTTTCGCTTGGTGGTTCG-3'), which corresponded to the sequences of the Km^R gene and *sid* ORF, were used for PCR confirmation of insertion of the Km cassette.

Generation of the complemented strain

The ORF of the *sidD* gene was synthesized by Genewiz (South Plainfield, NJ) with the promoter (corresponding to regions 1567709-1567928 on the chromosome) located upstream of the operon driving its expression. The promoter and *sidD* gene were isolated by restriction digest with *Bam*HI and *Eco*RI from a plasmid carrying the *sidD* gene and promoter (pUC57, which was synthesized by Genewiz), and subcloned into *Bam*HI and *Eco*RI restriction-digested pJRD215 (18). Plasmid pJRD215 replicates in *A. oris*. This resulting plasmid, *psidD*, was electroporated into the *A. oris sidD::Km* mutant strain and plated onto BHI supplemented with streptomycin (50 µg/ml) and kanamycin (50 µg/ml). Plasmid pJRD215 was also electroporated into the *A. oris sidD::Km* mutant to serve as the vector-only control. Km^R and Sm^R colonies were selected, and plasmids were isolated to confirm complementation. The *sidD::Km/pJRD215* and *sidD::Km/psidD* strains were used in the growth experiments.

Identification of the *amdR* gene in clinical isolates

Clinical isolates of *Actinomyces* were obtained from Dr. Robert Burne (University of Florida). 16S rRNA sequence analysis was used to confirm that the strains were *Actinomyces* species. These strains may be either *A. naeslundii* (now *oris*) genospecies 1 or genospecies 2. These two strains cannot be separated based on 16S rRNA analysis alone (19). Gram staining was used to confirm the morphology. To determine if metal-dependent repressors were conserved in the clinical isolates, primers corresponding to the *amdR* gene (5'-AGAAACACTCCTCAAACCCCC-3' and 5'-ATCACAGGGTGACGCCTATC-3') were used for PCR amplification from chromosomal DNA isolated from these clinical isolates. Chromosomal DNA was isolated from the *Actinomyces* clinical isolates using the Wizard Genomic Purification System (Promega). PCR products were subcloned into the pCR2.1 vector (Life Technologies, Carlsbad, CA) and sequenced at the Dartmouth Sequencing Core. Deduced amino acid sequences were compared using CLUSTAL alignment analysis.

Measurement of iron in the ADM medium

Iron concentration in the *Actinomyces* defined medium was measured by ICP-MS as described by Moreau-Marquis (20).

Results

The Actinomyces metal-dependent repressor possesses a metal-dependent DNA-binding domain

As a first step in investigating iron-dependent gene regulation in *A. oris*, we examined the published genome of *A. oris* MG1 for the presence of a metal-dependent regulator with sequence similarity to the well-characterized DtxR family of regulators in Gram-positive organisms. The ORF encoding *Actinomyces* metal-dependent repressor, AmdR, was identified. The deduced amino acid sequence of AmdR contained domains found in other metal-dependent repressors, including SloR of *S. mutans* and DtxR of *Corynebacterium diphtheriae* (Fig. 1). These domains include the DNA-binding helix-turn-helix domain and the iron-dependent repressor superfamily domain, and have been described for both SloR from *S. mutans* (21) and DtxR from *C. diphtheriae* (22). Unlike *S. mutans* SloR, the AmdR protein does not possess a 'FeoA' domain (Fig. 1); this short domain is found at the C-terminus of a variety of metal-dependent transcriptional regulators (23). A recent study suggests that the FeoA domain may mediate metal binding (21). AmdR has more sequence similarity and identity to DtxR (64%/52%) and shares less similarity and identity with SloR (47%/26%).

fetA and sidD show increased expression in iron-limited growth medium

Our laboratory has previously determined that *Actinomyces* metal-dependent repressor AmdR binds to the promoter regions upstream of the operon containing the *sidD* gene, which codes for a component of an iron transport system (14). These studies were conducted using purified AmdR in electrophoretic mobility shift assays. We hypothesized that a number of *A. oris* genes were likely subject to regulation by changing iron concentrations, due to binding of the AmdR to promoter sequences when abundant iron is present. We identified genes that were likely to be involved in iron acquisition by examining the genome of *A. oris*, which is available via the Bioinformatics Resources for Oral Pathogens (BROP). At the time of this publication, BROP refers to *A. oris* MG1 as *A. naeslundii* MG1.

As outlined here, sequence analyses revealed that there are over a dozen genes that may be involved in iron acquisition. Two of these genes identified, *fetA* and *sidD*, are located in operons with a high degree of sequence similarity (>98%) to transporters involved in iron uptake (Fig. 2). The *sidC* gene encodes a predicted 37 kDa, iron-siderophore ABC transporter substrate-binding protein, whose periplasmic component is 99% identical to iron-siderophore ABC transporter substrate-binding protein from *A. viscosus*. The *sidD* gene likely encodes the iron-siderophore ATP-binding protein of the ABC transporter component containing an ATP-binding site and the ABC transporter signature motif. The SidD protein is

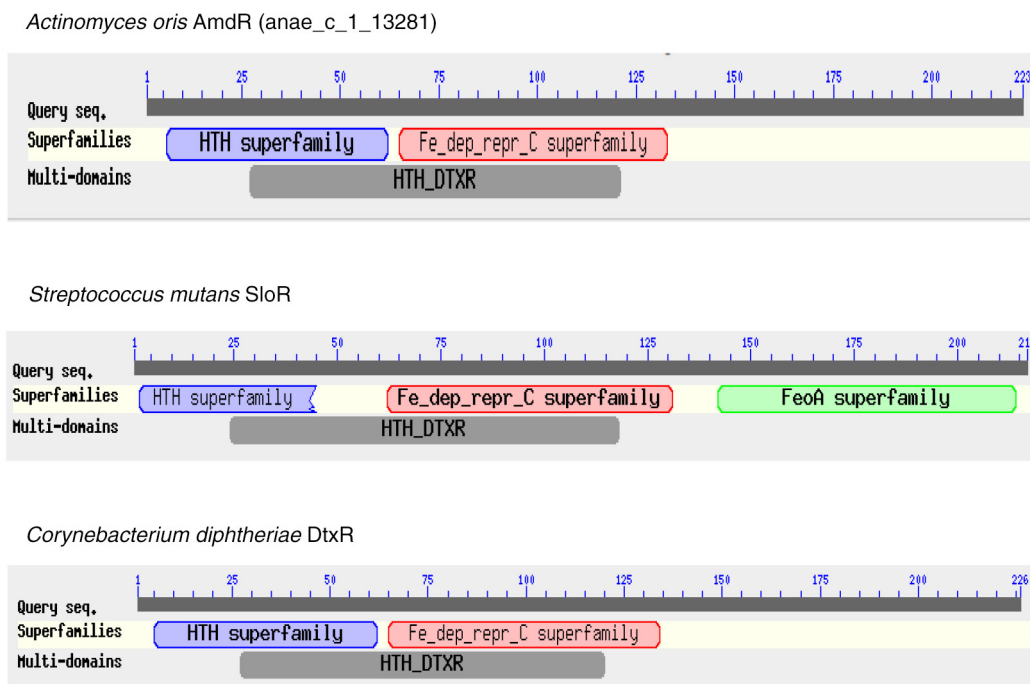


Fig. 1. AmdR shares conserved domains with other metal-dependent repressors. Predicted domains of the deduced amino acid sequence of *Actinomyces* metal-dependent repressor AmdR, *S. mutans* SloR, and *C. diphtheriae* DtxR from NCBI.

95% identical to the iron dicitrate ABC transporter ATP-binding protein from *A. viscosus* (accession number WP_009406982.1; Fig. 2a). The first gene in the *fetA* (*anae_c_1_8278*) operon encodes a predicted 32 kDa protein with 98% identity and 99% similarity to a predicted *A. oris* high-affinity iron transporter (accession number WP_010612841.1; Fig. 2b) with 65% identity and 78% similarity to predicted ferrous iron permease EfeU from *Propionibacterium acidipropionici* ATCC 4875 (accession number = WP_015071892.1). The second gene in the operon *fetB* (*anae_c_1_8283*) encodes a 46.8 kDa protein with 99% similarity and 99% identity to a predicted peptidase M75 from *A. oris*. The peptidase M75 family includes an insulin-cleaving membrane protease, an iron-regulated protein A (IrpA) and an iron transporter EfeO-like alginate-binding protein (NCBI). The FetB protein also shows 67% identity and 75% similarity to a predicted PbrT family lead (Pb2+) uptake porter of *Actinomyces urogenitalis* S6-C4.

Using qRT-PCR analysis, we explored the regulation of *fetA* and *sidD* genes as representative genes in each of the operons described above to determine if their expression was indeed regulated by iron availability. To perform these studies, RNA was extracted from cells grown in minimal medium (ADM) or the same medium treated with the chemical iron-chelator dipyriddy (DP), or ADM supplemented with iron chloride.

The transcripts of both the *fetA* and *sidD* genes are increased when the wild-type *A. oris* is grown in metal-limited medium (Fig. 2c and d), which supports our

hypothesis that these genes are regulated in response to iron limitation in the growth medium. Interestingly, addition of iron chloride did not significantly change the expression of these genes compared to the unamended medium control, suggesting the ADM is an iron-replete medium for this microbe. Consistent with this idea, we showed that ADM contains $\sim 8 \mu\text{M}$ iron. In addition, we examined the *sidD*::Km mutant strain for both *fetA* and *sidD* gene expression. We were unable to detect a *sidD* transcript in the *sidD*::Km mutant strain, as expected (data not shown). However, the *fetA* transcript was detected, but in much lower levels than observed for the wild-type strain (Fig. 2d).

Generating an iron transport mutant of *A. oris*

To determine if the *sidD* locus contributes to iron transport in *A. oris*, we built an insertional loss-of-function mutant carrying a kanamycin resistance marker (see Materials and Methods for details). The kanamycin resistance insertion was verified using primers for the kanamycin marker and for the *sidB* gene, as described in Materials and Methods.

The growth of *sidD*::Km was compared to a wild-type strain on both iron-replete and iron-limited media. The final growth yield of *sidD*::Km was reduced when compared to the wild type in the iron-depleted medium and the lag phase was extended (Fig. 3a). The *sidD*::Km growth in iron-replete media was very similar to the growth of the wild-type strain in the DP-treated, iron-limited medium. To determine if supplementing the growth medium with additional iron would rescue the growth defect of the of *sidD*::Km strain, iron chloride was added

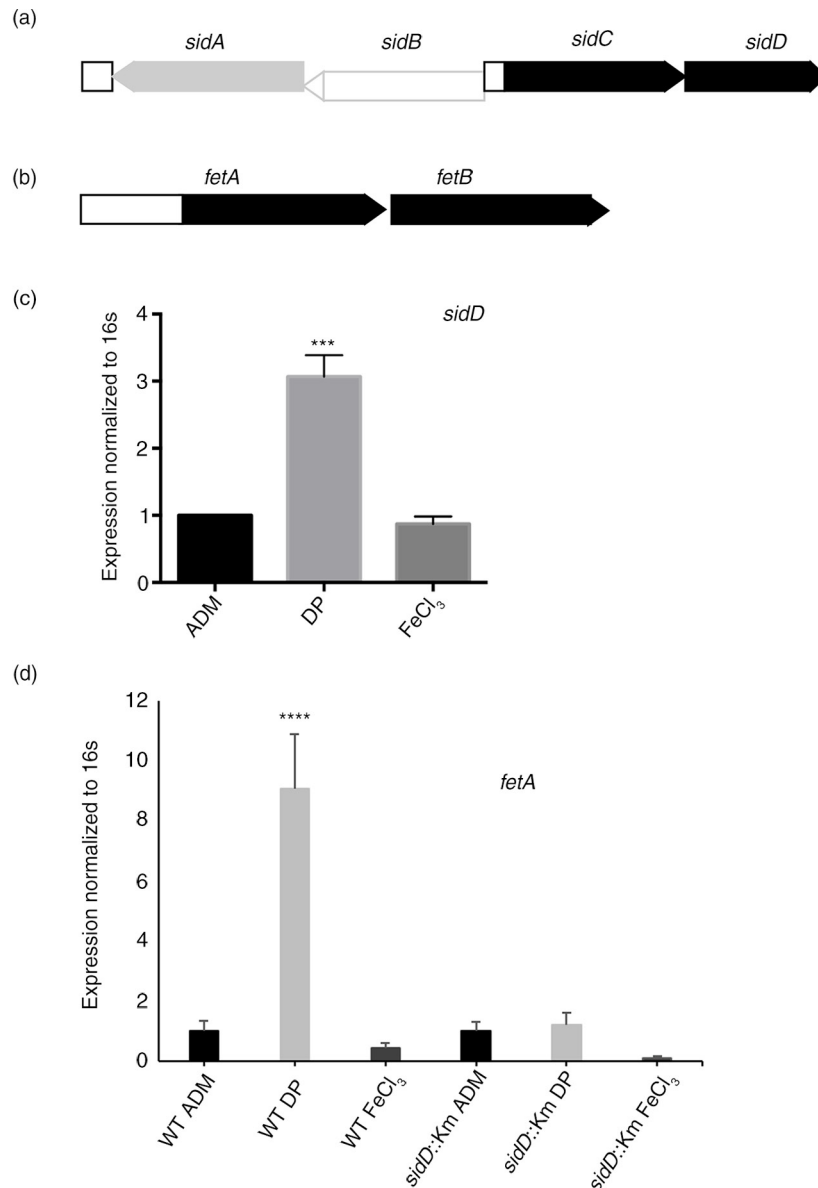


Fig. 2. Iron transport genes are regulated in response to iron. (a) Diagram of the ABC operon, the proposed regulatory region is underlined. *sidA* (anae_c_1_15012) and *sidB* (anae_c_1_15008), ABC transporter permease; *sidC* (anae_c_1_5341), ABC substrate-binding component; *sidD* (anae_c_1_5343), ABC ATP-binding component. (b) Diagram of the operon containing high-affinity iron transporter *fetA* (anae_c_1_8278) and a putative peptidase M75, *fetB* (anae_c_1_8283). (c) Shown is the relative quantity of transcript of the *sidD* gene in *Actinomyces* defined medium (ADM), ADM with 75 μ M dipyrindyl (DP; a strong iron chelator) or ADM plus 100 μ M FeCl₃ (FeCl₃). (d) Relative quantity of transcript of the *fetA* gene in wild-type *A. oris* ADM (WT ADM), wild-type *A. oris* in ADM with 75 μ M dipyrindyl (WT DP), or *A. oris* in ADM plus 100 μ M FeCl₃ (WT FeCl₃). Also shown is the relative quantity of transcript of the *fetA* gene in the *sidD* mutant (*sidD*::Km ADM), *sidD* mutant in ADM with 75 μ M dipyrindyl (*sidD*::Km DP), or *sidD* mutant in ADM plus 100 μ M FeCl₃ (*sidD*::Km FeCl₃). ‘****’ indicates significant difference ($p < 0.05$) in gene expression as determined by ANOVA and Dunnett’s multiple comparisons test. ‘*****’ indicates significant difference ($p < 0.02$) in gene expression.

to the medium at a final concentration of up to 1 mM; the additional iron did not restore the growth of the *sidD*::Km mutant (Fig. 3b). The growth profile of the *sidD*::Km mutant in iron-replete medium was reduced to about 50% of that of the wild-type strain in late log and stationary phases.

When the *sidD* gene on a plasmid was added back to the *sidD*::Km mutant, growth was restored in the iron-replete media to almost wild-type levels. However, when the conditions were severely iron-limited, the complemented strain only partially rescued growth; this strain did not reach the same final growth yield as well as the wild type.

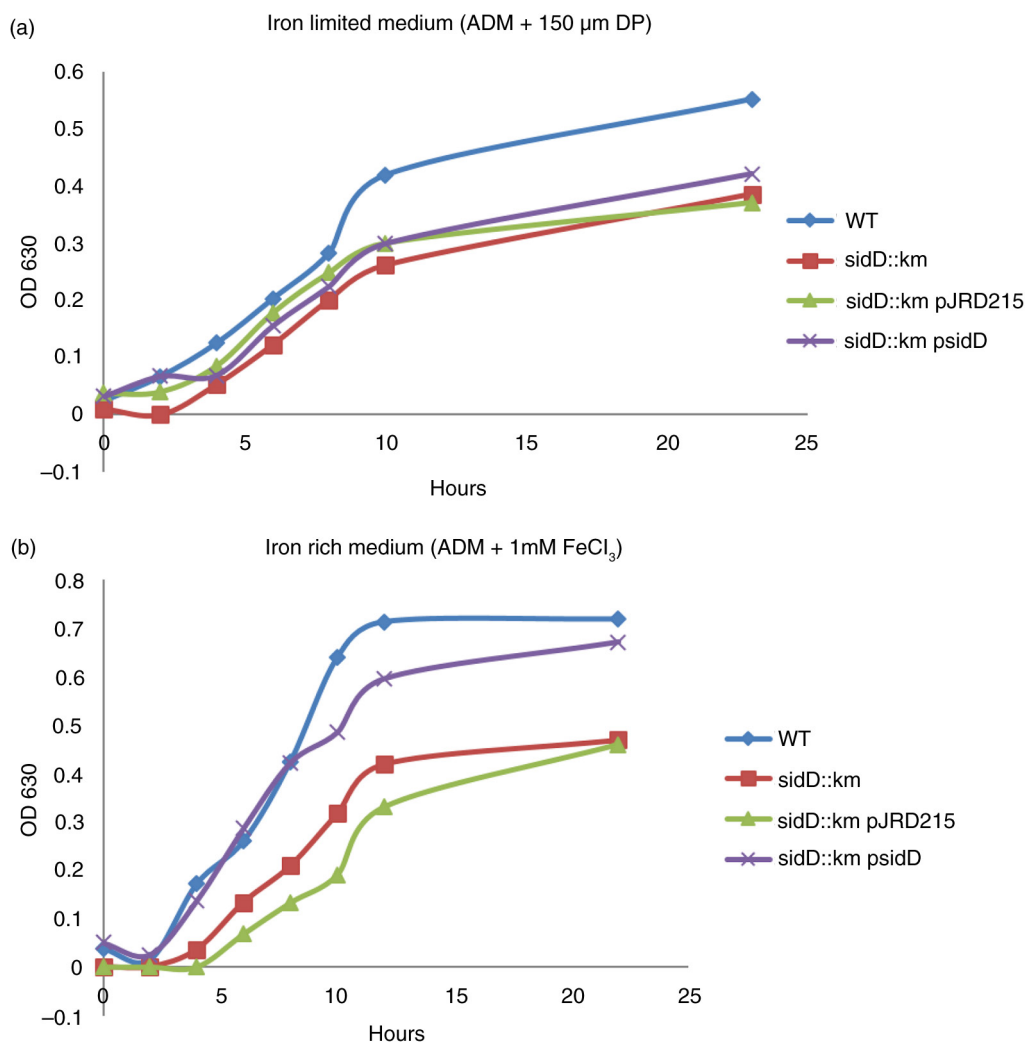


Fig. 3. Growth of *sidD::Km* mutant is reduced when compared to wild type in the iron-depleted medium. Strains were grown in *Actinomyces* defined medium (ADM) in low iron (addition of 150 µM dipyrindyl, DP) or high iron (addition of 1 mM FeCl₃) in 96-well plates at 5% CO₂, WT = wild-type *A. oris* MG1.

Actinomyces metal-dependent repressor is conserved in clinical isolates

There is often a high degree of gene variation in microorganisms in the oral cavity (24), and as such, we wanted to explore whether the key iron regulator required to control expression of the siderophore systems outlined above, *AmdR*, was conserved in clinical isolates of *Actinomyces* spp. found in the oral cavity. Ten *Actinomyces* spp. strains isolated from individual patient's oral cavity at the University of Florida were kindly provided by Dr. Burne. *amdR* was successfully amplified by PCR (see Material and Methods for primers) from 7 of the 10 clinical isolates. The reason that we were unable to amplify the *amdR* ORF from the remaining isolates, even after repeated attempts, was not clear. Deduced amino acid identity varied from 92 to 99% among these *AmdR* homologs (Fig. 4), indicating that this gene and its product are highly

conserved among this collection of isolates. GenBank Accession numbers for clinical isolates UF5–UF45 are KU162964–KU162970.

Discussion

ABC transporters are responsible for the uptake of iron in many bacteria (1). *A. oris* contains multiple putative iron transport systems (BROP.org) including the operons containing the *sidABCD* genes as well as the *fetAB* genes. Iron ABC transporters have been shown to be expressed differentially depending on the amount of iron in the environment. In a low-iron environment, expression of iron transporters in many other organisms increases due to the need of scavenging iron in the environment using siderophores (1, 26). In a high-iron environment, expression of iron ABC transporters is typically low since iron is readily available through low-affinity transporters (26).

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UF14      MTELIDTTEMYLKTVYELEEDGLPPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
UF10      MTELIDTTEMYLKTVYELEEDGLPPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
UF5       MTELIDTTEMYPKTVYELEEDGVPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
UF22      MTELIDTTEMYPKTVYELEEDGVPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
UF28      MTELIDTTEMYLKTVYELEEDGLPPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
UF12      MTELIDTTEMYLKTVYELEEDGLPPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
UF45      MTELIDTTEMYPKTVYELEEDGVPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
LAB       MTELIDTTEMYLKTVYELEEDGVPLRARIVERLDHSGPTVSQTVARMERDGLIKVAEDR
          *****
UF14      SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHDEACRWEHVMSEQVEERLADIL
UF10      SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHDEACRWEHVMSEQVEERLVDIL
UF5       SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHDEACRWEHVMSEQVEERLVDIL
UF22      SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHDEACRWEHVMSEQVEERLADIL
UF28      SLELTDEGR-RATDVIRKHRLAERLLPDVIGIERRFVHEEACRWEHVMSEQVEERLADIL
UF12      SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHEEACRWEHVMSEQVEERLADIL
UF45      SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHEEACRWEHVMSEQVEERLADIL
LAB       SLELTDEGRRRATDVIRKHRLAERLLLDVIGIERRFVHEEACRWEHVMSEQVEERLADIL
          *****
UF14      DDVSTDPFGNPVPSRTAEHPRPSADEVSADRFAGRDTVTAVVSRIGEPIQADAEI IAGPE
UF10      DDISTDPFGNPVPSRTAEHPRPSADEVSADRFAGRDTVTAVVSRIGEPIQADAEI IAGLE
UF5       DDISTDPFGNPVPSRTAEHPRPSADEVSADRFAGRDTVTAVVSRIGEPIQADAEI IAGLE
UF22      DDISTDPFGNPVPSRTAEHPRPSADEVSADRFAGRDTVTAVVSRIGEPIQADAEI IAGLE
UF28      DDVSTDPFGNPVPPRTAEHPRPSADEVSVDVFAGRETTTAVVSRIGEPIQADAEI IAGLE
UF12      DDASTDPFGNPVPPRTAEHPRPSADEVSVDVFAGRETTTAVVSRIGEPIQADAEI IAGLE
UF45      DDVSTDPFGNPVPPRTAEHPRPSADEVSVDVFAGRETTTAVVSRIGEPIQADAEI IAGLE
LAB       DDVSTDPFGNPVPPRTAEHPRPSADEVSVDVFAGRETTTAVVSRIGEPIQADAEI IAGLE
          ** *****
UF14      DAQIVAGAEVELRVS
UF10      DAQIVAGAEVELRVS
UF5       DAQIVAGAEVELRVS
UF22      DAQIVAGAEVELRVS
UF28      DAQIVAGAEVELRVS
UF12      DAQIVAGAEVELRVS
UF45      DAQIVAGAEVELRVS
LAB       DAQIVAGAEVELRVS
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Fig. 4. Alignment of *AmdR* sequences from clinical isolates. Alignment of deduced amino acid sequences of cloned *amdR* genes from seven *Actinomyces* clinical isolates was performed using Clustal Omega (25). The laboratory strain *A. oris* MG1 (BROP.org) is labeled 'LAB'. Percent amino acid identity and similarity to *A. oris* MG1 is as follows: UF5 (95/97), UF 10 (95/97), UF 12 (98/98), UF 14 (96/97), UF 22 (96/97), UF 28 (98/98), UF 45 (99/99). '*' indicates identical amino acids; '.' and ':' indicate conservative substitution.

Here, we found that the genes *fetA* and *sidD* showed significantly increased gene expression in iron-limited medium in the wild-type strain. In order to further characterize the role of these transporters in the survival of *A. oris*, we sought to mutate the *sidD* gene. This *sidD*::Km mutant showed a longer lag phase and lower yield in iron-depleted medium compared to the wild type; however, the addition of iron to the growth media did not restore growth of the mutant to wild-type levels. Other studies that have generated mutants of iron transport, including one report in *C. diphtheriae*, have found similar growth defects. That is, a mutation in the *C. diphtheriae* *ciuA* gene, encoding a putative lipoprotein component of an iron transport system, resulted in a severe defect in iron uptake and reduction of *C. diphtheriae* siderophore uptake (27). When the *sidD* gene was added back to the *sidD*::Km mutant in a complementation experiment, the growth

was restored to wild-type levels in iron-replete medium; however, when the conditions were severely iron-limited, the complemented strain did not reach quite the same final growth yield as well as the wild type, although the growth rates of these strains were similar. The *sidD*::Km mutant was examined for a *sidD* transcript, and as predicted, no transcript was detected. We found that the *fetA* gene transcript in the *sidD*::Km mutant was decreased compared to the wild type. As noted above, the *sidD*::Km mutant strain grows much slower than the wild type and does not reach the same final OD, perhaps explaining the reduced *fetA* gene expression. Alternatively, the *sidD*::Km mutant may be defective in responding to iron limitation.

Mutants of iron transporters have been linked with virulence, speaking to the key role of iron acquisition during infection (28). Mutations in *S. mutans* *sloC* (part of an ABC transporter involved in iron transport)

resulted in decreased virulence in an endocarditis model (29, 30). Future studies could examine the effects of the *sidD* defective strain of *A. oris* in an animal model for periodontal disease.

A. oris is an important colonizer of the oral cavity. By the age of 1 year, 90% of infants evaluated are colonized with *A. oris* (31). In addition, this organism is a prominent part of the periodontal flora (32). Laboratory strains are useful model organisms, and clearly metal transport is important for optimal growth of the *A. oris* MG1 strain in our laboratory studies; however, not all genes that are found in laboratory strains are conserved across clinical isolates. To further illustrate how important metal transport genes are, studies characterizing the genomes of clinical isolates of *S. mutans* have found that most of the genome varies widely; however, metal transporters and other key virulence factors are conserved (24). The 73 ‘core genes’ of *S. mutans* are thought to contribute to conditions that allow *S. mutans* to colonize and persist in the oral cavity (24). In order to gain a better understanding of the role of iron uptake and regulation in clinically relevant strains, we obtained 10 clinical isolates of *Actinomyces*, including *A. oris*. The identity of these isolates was confirmed by sequencing the 16S rRNA gene. We were able to PCR-amplify, sequence, and subclone a gene encoding AmdR from 7 out of the 10 clinical isolates. We saw a high degree of deduced amino acid sequence identity (between 92 and 99%), indicating that these metal-dependent regulators may be conserved across at least a subset of clinical isolates. Taken together with our previously published work (14), our data indicate that AmdR, and the AmdR- and iron-regulated genes, including siderophore systems, are keys for growth of this microbe in low-iron environments, and thus likely contribute to their survival in the oral cavity.

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There is no conflict of interest in the present study for any of the authors.

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