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Aggregatibacter actinomycetemcomitans QseBC is activated by catecholamines and iron and regulates genes encoding proteins associated with anaerobic respiration and metabolism

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SUMMARY

Aggregatibacter actinomycetemcomitans **QseBC regulates its own expression and is essential for biofilm growth and virulence. However, the signal that activates the QseC sensor has not been identified and the qseBC regulon has not been defined. In this study, we show that QseC is activated by catecholamine hormones and iron but not by either component alone. Activation of QseC requires an EYRDD motif in the periplasmic domain of the sensor and site-specific mutations in EYRDD or the deletion of the periplasmic domain inhibits catecholamine/iron-dependent induction of the *ygiW-qseBC* operon. Catecholamine/iron-dependent induction of transcription also requires interaction of the QseB response regulator with its binding site in the *ygiW-qseBC* promoter. Whole genome microarrays were used to compare gene expression profiles of *A. actinomycetemcomitans* grown in a chemically defined medium with and without catecholamine and iron supplementation. Approximately 11.5% of the *A. actinomycetemcomitans* genome was differentially expressed by at least two-fold upon exposure to catecholamines and iron. The expression of ferritin was strongly induced, suggesting that intracellular iron storage capacity is increased upon QseBC activation. Consistent with this, genes encoding iron binding and transport proteins were down-regulated by QseBC. Strikingly, 57% of the QseBC up-regulated genes (56/99) encode proteins associated with anaerobic metabolism and respiration. Most of these up-regulated genes were recently reported to be induced during *in vivo* growth of *A. actinomycetemcomitans*. These results suggest that detection of catecholamines and iron by QseBC may alter the cellular metabolism of *A. actinomycetemcomitans* for increased fitness and growth in an anaerobic host environment.**

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a Gram-negative opportunistic oral pathogen that is strongly associated with aggressive forms of periodontitis and other systemic diseases and infections such as cardiovascular diseases (Yew *et al.*, 2014), atherosclerosis (Zhang *et al.*, 2010), urinary tract infections (Townsend & Gillenwater, 1969) and brain abscesses (Rahamat-Langendoen *et al.*, 2011). The organism resists killing mediated by neutrophils (Permpanich

et al., 2006; Ji *et al*., 2007) and produces a variety of potential virulence factors including a cytolethal distending toxin (Cdt), a leukotoxin (LtxA) of the RTX family of bacterial toxins, and a collagenase (Robertson *et al*., 1982; Kachlany *et al*., 2010; Jinadasa *et al*., 2011). *Aggregatibacter actinomycetemcomitans* also produces autoinducer-2 (AI-2) and this quorum-sensing mechanism has been shown to be essential for virulence and growth under iron-limiting conditions (Fong *et al*., 2001, 2003; Shao *et al*., 2007). Novak *et al.* (2010) have also shown that AI-2 regulates *qseBC* and that this two-component system is essential for *A. actinomycetemcomitans* biofilm growth and virulence. However, the mechanism that links the detection of AI-2 to the activation of QseBC, or the signal(s) that activate the QseC sensor have not yet been determined.

In *Eschericha coli* and *Salmonella enterica*, QseBC is activated by catecholamine hormones such as epinephrine (Ep) and norepinephrine (Ne) and these hormones regulate the expression of the genes involved in the formation of the attaching and effacing lesion, the Type Three Secretion System contained on the locus of enterocyte effacement, and flagella and motility genes (Sperandio *et al*., 2003). Catecholamine hormones have also been suggested to control virulence-associated genes in *Actinobacillus pleuropneumoniae* (Li *et al*., 2012). However, the QseBC paralogue of *Haemophilus influenza* (designated FirRS) is not activated by catecholamines, but instead responds to ferrous iron (Steele *et al*., 2012). Interestingly, Ep and Ne have been shown to increase virulence and stimulate the growth of *Bordetella*, *E. coli* and *S. enterica* by functioning as pseudo-siderophores (Freestone *et al*., 2000, 2008). Ep and Ne are capable of extracting iron from host proteins such as transferrin and lactoferrin (Freestone *et al*., 2000) which is subsequently imported into the cell by the enterobactin transporter complex (Burton *et al*., 2002; Freestone *et al*., 2003; Anderson & Armstrong, 2006). Catecholamine hormones have also been suggested to stimulate the growth of some periodontal pathogens (Jentsch *et al*., 2013) but their mechanism of action is not known. In addition, recent studies show that activated phagocytic cells (e.g. neutrophils, polymorphonuclear cells and macrophages) release catecholamine hormones and lactoferrin in response to inflammatory stimuli (Brown *et al*., 2003; Flierl *et al*., 2007, 2008, 2009) and can produce local concentrations of catecholamines in the millimolar range (Brown *et al*., 2003). This suggests that the inflamed gingival pocket may be an environment that is rich in both catecholamines and lactoferrin.

In *A. actinomycetemcomitans*, *qseBC* is co-expressed with *ygiW*, which encodes a periplasmic solute binding protein in the bacterial OB-fold family (Juárez-Rodríguez *et al*., 2013b). The functional outcomes of QseBC activation have only been broadly defined as influencing biofilm growth and virulence. Little is known about how QseBC activation influences these complex phenotypes, in part because the *qseBC* regulon has not been defined and the signals (ligands) detected by QseC have not been identified. In this report, we show that *qseBC* expression is maximally induced in the presence of both iron and catecholamine hormones, and that activation requires the periplasmic domain of QseC. This suggests that iron and catecholamines may be signals that activate the QseC sensor. In addition, using genome microarrays, we show that exposure of *A. actinomycetemcomitans* to catecholamines and iron significantly induces genes that are involved in anaerobic respiration, anaerobic metabolism and iron storage, and downregulates genes involved in iron uptake. Together, these results suggest that the detection of catecholamines and iron by the QseBC two-component system may play an important role in the adaptation of *A. actinomycetemcomitans* to the host cell environment.

METHODS

Bacterial strains, plasmid and media

The bacterial strains and plasmids used in this study are listed in the Supplementary material (Table S1). Luria–Bertani (LB) broth and LB agar (LB broth plus 1.5% agar), or brain–heart infusion (BHI) broth and BHI agar (all from Difco, BD Biosciences, Franklin Lakes, NJ) were routinely used for the propagation and plating of *E. coli* or *A. actinomycetemcomitans* strains, respectively. The *A. actinomycetemcomitans* (afimbriated, smooth-colony-morphotype strain 652, serotype c) was grown at 37°C under microaerophilic conditions in a candle jar. For some experiments, *A. actinomycetemcomitans* strains were grown in a chemically defined medium (CDM) essentially as described by Socransky *et al.* (1985) or in CDM supplemented with 100 μM $FeCl_2$ or $FeCl_3$ and/or 50 μM

Ep or Ne. The composition of CDM is shown in detail in the Supplementary material (Table S2) and after combining the individual components, CDM was adjusted to pH 7.3 with HCl, supplemented with 0.2% glucose and sterilized by filtration through a 0.22-μm filter before use. If necessary for plasmid maintenance, medium was also supplemented with 25 μg ml^{-1} kanamycin or 50 μg ml^{-1} spectinomycin.

Aggregatibacter actinomycetemcomitans growth kinetics

To determine the effect of catecholamines and iron on cell growth, a frozen stock of *A. actinomycetemcomitans* 652 was inoculated into BHI and was grown to an optical density at 600 nm (OD_{600}) of 0.3–0.4. This culture was then inoculated into fresh BHI at a 1 : 30 dilution. At an OD_{600} of 0.5–0.6, cells were washed with CDM and inoculated into freshly prepared CDM at a 1 : 30 dilution. At various timepoints, aliquots were removed from each of triplicate cultures and the OD_{600} was measured using a Bio-Rad SmartSpec Plus uV-vis spectrophotometer (Bio-Rad, Hercules, CA). Growth kinetics were determined for two independently grown cultures for each condition tested.

DNA procedures

DNA manipulations were carried out as described by Sambrook & Russell (2001). Transformation of *E. coli* and *A. actinomycetemcomitans* was carried out by electroporation as previously described (Juárez-Rodríguez *et al.*, 2013a). Transformants containing plasmids were selected on LB agar plates supplemented with the appropriate antibiotics and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Restriction digestions were carried out following the protocols as recommended by the manufacturer (New England Biolabs, Ipswich, MA). All primers used in this study were synthesized by Integrated DNA Technology, Inc. (Coralville, IA) and are shown in Table S3. Restriction enzyme sites to facilitate cloning of the resulting polymerase chain reaction (PCR) products are underlined in the primer sequences. All primer sequences were designed based on the genome sequence of *A. actinomycetemcomitans* D11S-1, serotype c, which is available from the Pathosystems Resource Integration Center (http://www.genome.jp/kegg-bin/show_organism?org=aat). All constructs were verified by DNA sequencing (University of Louisville Core Sequencing Facilities).

Site-specific mutagenesis of the putative EYRDD iron-binding motif of QseC

The conserved amino acid residues tyrosine (Y) and arginine (R) of the putative iron-binding motif of QseC at positions 155 and 156, respectively, were individually substituted by alanine (A). The *qseC* codon substitutions for Y155A (TAT to GCT) and R156A (CGC to GCC) were achieved by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using plasmid pDJR28 as the template and primer set MDJR-163F/MDJR-164R. The successful incorporation of the desired mutations was verified by sequencing the resulting plasmid construct, pDJR28-M1-2.

Integration of QseC mutant alleles in single-copy into *A. actinomycetemcomitans qseBC*

To integrate a single copy of the *qseC* mutant allele encoding $QseC_{Y155A,\ R156A}$ into the *A. actinomycetemcomitans* chromosome, an approach that was used previously to integrate a single copy of a *ygiW* promoter-*lacZ* fusion into the *A. actinomycetemcomitans* genome was used (Juarez-Rodriguez *et al.*, 2014). Briefly, the *qseC* wild-type or mutant alleles along with the upstream *ygiW* and *qseB* genes were amplified by PCR from the chromosome of *A. actinomycetemcomitans* 652 and pDJR28-M1-2, respectively, using the primer sets MDJR-124F/MDJR-125R. The resulting 3135-base-pair PCR fragments were digested with *Not*I–*Pst*I and cloned into the *Not*I–*Pst*I-digested suicide vector pJT1. The recombinant suicide plasmids (20 μg) were then introduced individually into *A. actinomycetemcomitans* Δ*qseBC* by electroporation. Subsequently, 10 Sp^r colonies containing a single-copy of the suicide vector inserted into the chromosome were selected and verified for the appropriate insertion event by PCR using primer sets MDJR-63F/MDJR-61R and MDJR-54F/MDJR-77R. One of the PCR-verified Sp^r colonies was designated *A. actinomycetemcomitans* Δ*qseBC*::61 and contained the mutated *qseC* allele (*ygiW-qseB-*$qseC_{Y155A,\ R156A}$). A second colony containing the

wild-type *qseC* allele was also selected for further study and was designated *ΔqseBC*::46. The pDJR29 *lacZ* reporter plasmid was subsequently introduced into each strain by electroporation.

β-Galactosidase assays

β-Galactosidase (β-gal) activity was qualitatively assessed on BHI agar plates that were supplemented with 50 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Quantitative evaluation of β-gal activity was carried out using permeabilized cells incubated with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate (Sigma, St Louis, MO) as previously described (Miller, 1972). Briefly, a primary culture of the desired strain (OD_{600} of 0.3–0.4) was diluted 1 : 30 into 1.5 ml of BHI in a 1.7-ml propylene centrifuge tube and grown standing for 24 h at 37°C. Subsequently, an aliquot of the secondary overnight culture was diluted 1 : 30 into 1.5 ml of CDM in 1.7-ml propylene centrifuge tubes and grown standing for 24 h at 37°C. An aliquot of 0.1 ml was then used to determine the OD_{600} of the culture and triplicate aliquots of 0.1 ml were used to measure β-galactosidase activity. Average values (± the standard deviations) for activity units were routinely calculated from three independent experiments using GraphPad Prism v5 software (GraphPad, San Diego, CA).

Microarray analysis of the *A. actinomycetemcomitans* transcriptome

A custom *A. actinomycetemcomitans* gene expression microarray was printed by Agilent Technologies (Santa Clara, CA; GE 8 × 15,000 grids per slide, 60-mer oligonucleotide probes were used). Oligonucleotide probe sequences were obtained from the University of Michigan OligoArray Database that is available for *A. actinomycetemcomitans* D11S-1 (http://berry.engin.umich.edu/oligoarraydb/organismPage.php?ORG=Aggregatibacter%20actinomycetemcomitans%20D11S-1). This database comprises 5638 oligonucleotide probes representing 2062 transcripts encoded by the *A. actinomycetemcomitans* D11S-1 genome. Of the 2062 transcripts, 1552 are represented by three independent oligonucleotide probes, 215 are represented by two probes and 221 by a single specific oligonucleotide. Each oligonucleotide was printed twice on each 15,000-spot grid; hence the majority of genes are represented by six separate spots on the array and no transcript is represented by fewer than two spots. Seventy-two putative transcripts of *A. actinomycetemcomitans* D11S-1 are not represented in the oligonucleotide database; these mostly comprise hypothetical proteins ($n = 37$) and transposases/integrases of mobile genetic elements ($n = 11$).

Aggregatibacter actinomycetemcomitans 652 was cultured in CDM or in CDM supplemented with 50 μM Ne and 100 μM $FeCl_2$ for 14 h as described previously. The late exponential phase cultures were then adjusted to equivalent OD_{600} and labeling of RNA, microarray hybridization, array scanning and data analysis were carried out by the MicroArray Core Facility at the University of Louisville. Briefly, RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the protocols supplied by the manufacturer. The quality of the isolated RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). RNA samples were considered to have little or no degradation if they exhibited an RNA integrity number >9 (Schroeder *et al.*, 2006). Total RNA (100 ng) was labeled with Cyanine 3-CTP using the Low Input Quick Amplification WT Labeling Kit for one color (Agilent Technologies). This kit uses random nucleotide-based T7 promoter primers to first synthesize cDNA, which is then reacted with T7 RNA polymerase in the presence of NTPs and Cyanine 3-CTP to generate labeled cRNA. The labeled cRNA was purified using the RNeasy Mini Elute kit (Qiagen) and total yield and Cy3 incorporation efficiency was determined using a NanoDrop Technologies spectrophotometer.

Array hybridizations were carried out using the Agilent Gene Expression Hybridization kit as described by the manufacturer. Briefly, 600 ng of each labeled cRNA sample was incubated at 60°C for 30 min and then hybridized to the custom *A. actinomycetemcomitans* gene expression array at 65°C for 17 h. After hybridization, the microarray slides were washed with Agilent gene expression wash buffer 1 at 37°C for 5 min followed by wash buffer 2 at 37°C for 1 min. After hybridization and washing, the slides were scanned using an Agilent microarray scanner (Model G2505C) set for one-color using the green channel and 5-μm resolution. The one-color microarray images (.tif) were extracted with the aid of Feature Extraction software (v 9.5.1; Agilent Technologies). The raw data files (.txt) were imported into GeneSpring (GX 11.1) and the data

were transformed to bring any negative value or value <0.01–0.01. Normalization was then performed using a per-chip 75 centile method that normalizes each chip on its 75th centile to facilitate comparison among chips. A per-gene on median normalization was then performed to normalize the expression of every gene on its median among samples. An expression threshold of at least two-fold was applied and the differentially expressed genes were evaluated using a pair-wise *t*-test and genes with *P*-values < 0.05 were considered to be significantly upregulated or downregulated. The array data have been submitted to the Gene Expression Omnibus Database with the accession number GSE68749.

Determination of formate concentration

Extracellular formate concentration was determined using the formate assay kit supplied by r-BioPharm (Darmstadt, Germany). This kit measures formate concentration by the conversion of formate and NAD^+ to bicarbonate and NADH through the action of formate dehydrogenase. The stoichiometric production of NADH is measured by absorbance at 365 nm. Culture supernatants from mid-exponential phase cultures (6–8 h post inoculation) were collected and treated with 30 mM trichloroacetic acid (Sigma-Aldrich) at a ratio of 1 part supernatant to 2 parts trichloroacetic acid to precipitate proteins. After neutralizing with 1 M KOH, the samples were filtered (0.22 μM) to remove insoluble proteins and 100 μl of the filtered product was used in a formic acid assay following the protocol supplied by the manufacturer. Formate concentration was calculated from the levels of NADH produced as determined by measuring the OD_{365} and the data were normalized for cell density in the CDM culture. Assays were performed in triplicate and results are presented from two independent experiments.

RESULTS

Catecholamines and iron activate the *A. actinomycetemcomitans* QseBC two component system

We previously showed that the *ygiW-qseBC* operon of *A. actinomycetemcomitans* is auto-regulated by QseB (Juárez-Rodríguez *et al.*, 2014) and is essential for biofilm growth and virulence (Novak *et al.*, 2010). However, the signal that activates the *A. actinomycetemcomitans* QseBC two-component system was not previously identified. In *E. coli* and *S. enterica*, *qseBC* is activated by catecholamine hormones. In contrast, the *qseBC* paralog of *H. influenzae* is activated by ferrous iron and does not appear to respond to catecholamines or ferric iron (Steele *et al.*, 2012). To determine if the *ygiW-qseBC* operon is induced by catecholamines and/or iron, *A. actinomycetemcomitans* 652 was transformed with pDJR29 containing *lacZ* fused to the *ygiW-qseBC* promoter (Juárez-Rodríguez *et al.*, 2013b) and cultures were grown in CDM supplemented with catecholamines (50 μM Ep or Ne) and/or 100 μM $FeCl_2$ or $FeCl_3$. As shown in Fig. 1, the presence of Ep, Ne, Fe^{2+} or Fe^{3+} alone did not significantly induce *lacZ* expression. However, the addition of Ep or Ne together with either Fe^{2+} or Fe^{3+} induced *lacZ* expression by approximately five-fold over the control (CDM alone), suggesting that QseBC activation in *A. actinomycetemcomitans* requires both components.

To determine if catecholamines and iron function as signals that directly activate the QseC sensor,

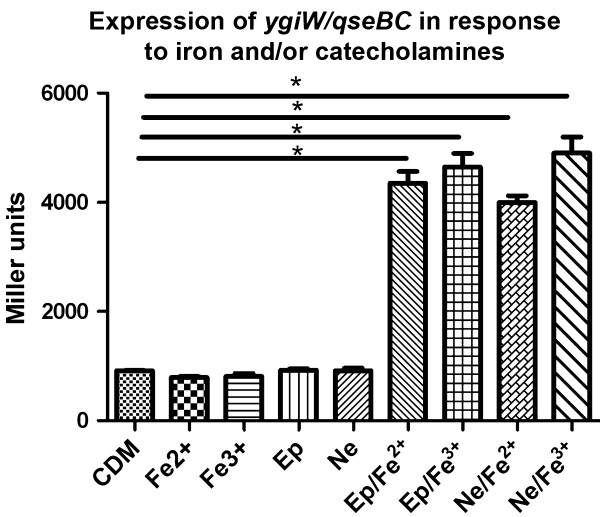


Figure 1 Expression of the *ygiW-qseBC* operon in *Aggregatibacter actinomycetemcomitans* cultures exposed to catecholamines and iron. *Aggregatibacter actinomycetemcomitans* 652 harboring the *ygiW-qseBC* promoter-*lacZ* reporter plasmid pDJR29 was grown in chemically defined medium (CDM) broth or in CDM supplemented with either ferrous or ferric chloride (Fe^{2+} or Fe^{3+}; 100 μM), epinephrine (Ep; 50 μM), norepinephrine (Ne; 50 μM, or a combination of both catecholamine (50 μM) and iron (100 μM). β-galactosidase activity was determined after 24 h of growth. Significant differences ($P < 0.05$) are indicated by asterisks.

pJDR29 was transformed into non-polar gene deletion mutants of *A. actinomycetemcomitans* that lacked *qseC* or *qseB* (*ΔqseC* and *ΔqseB*, respectively), or into a strain in which *qseC* was replaced by a single copy of the *qseC* gene that did not encode the periplasmic region of the sensor kinase (*qseCΔ*p, see Juárez-Rodríguez *et al.*, 2013b). As shown in Fig. 2, *lacZ* expression in CDM alone was reduced by approximately four-fold in the *ΔqseC* and *ΔqseB* strains and no induction of *lacZ* expression occurred in the presence of Ep/$FeCl_2$. These results are consistent with our previous finding that *ygiW-qseBC* is auto-regulated by QseC-mediated activation of QseB (Juárez-Rodríguez *et al.*, 2014). Importantly, no Ep/$FeCl_2$-dependent induction of *lacZ* occurred in *A. actinomycetemcomitans* that expressed a QseC protein that lacked the periplasmic domain. These results suggest that Ep and Fe^{2+} function as signals that activate the QseC sensor and that activation requires the periplasmic domain.

To confirm that Ep/$FeCl_2$-dependent induction of *lacZ* was mediated by QseB, a family of reporter plasmids containing a nested series of *ygiW-qseBC* promoter deletions was tested (Fig. 3A). These constructs have been previously described and were used to map the –10, –35 and QseB binding sites in the *ygiW-qseBC* promoter (Juárez-Rodríguez *et al.*, 2014). As shown in Fig. 3(B), *ygiW-qseBC* promoter activity was reduced by approximately 35% when nucleotides –94 to –138 were deleted (compare pDJR63, pDJR57 and pDJR56), consistent with our previous results (Juárez-Rodríguez *et al.*, 2014). However, Ep/$FeCl_2$-dependent induction of *lacZ* expression still occurred in each of these constructs. Ep/$FeCl_2$-dependent induction of *lacZ* was significantly reduced only after deletion (pDJR58) or site-specific mutation (pDJR86) of the QseB binding site. This indicates that the interaction of QseB with its binding site is essential for Ep/$FeCl_2$-dependent induction of the operon and is consistent with a model where catecholamines and iron represent signals that are recognized by and activate QseC, which in turn activates the QseB response regulator.

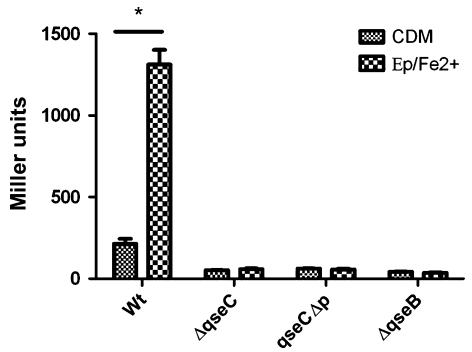
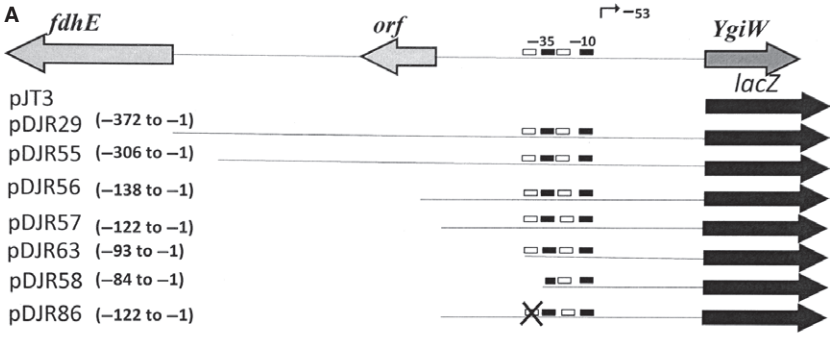


Figure 2 Catecholamine/iron-dependent induction of *ygiW-qseBC* requires the periplasmic domain of QseC and the QseB response regulator. *Aggregatibacter actinomycetemcomitans* 652 (WT), isogenic non-polar gene deletion strains lacking *qseC* (*ΔqseC*) or *qseB* (*ΔqseB*), or a strain expressing the QseC sensor without the periplasmic domain (*qseCΔ*p; see Methods) each harboring plasmid pDJR29 were grown in chemically defined medium (CDM) broth alone or in CDM supplemented with both Ep and FeCl2 (Ep/Fe^{2+}; 50 μM and 100 μM, respectively). β-galactosidase activity was determined after 24 h of growth. Significant differences ($P < 0.05$) are indicated by asterisks.

A putative iron responsive motif regulates the activation of QseC

The periplasmic region of *A. actinomycetemcomitans* QseC contains the sequence EYRDD (residues 154–157) that resembles the DYRED motif previously shown to be important for the sensing of iron by the QseC paralogs of *H. influenzae* (Steele *et al.*, 2012) and *Salmonella typhimurium* (Merighi *et al.*, 2009). To determine if this motif in *A. actinomycetemcomitans* is important for Ep/$FeCl_2$-dependent activation of QseC, EYRDD was altered to EAADD by site-directed mutagenesis and the mutant (encoding $QseC_{Y155A,\ R156A}$) or wild-type *qseC* alleles together with *ygiW* and *qseB* were integrated by homologous recombination into the chromosome of *A. actinomycetemcomitans ΔqseBC* to generate strains *ΔqseBC*::61 and *ΔqseBC*::46, respectively. As shown in Table 1, *lacZ* expression from pDJR29 in *ΔqseBC*::61 was not significantly induced when cells were cultured in CDM supplemented with Ep and $FeCl_2$. In contrast, *ΔqseBC*::46 exhibited an approximately five-fold increase in *lacZ* activity, similar to the wild-type strain. However, *lacZ* expression in *ΔqseBC*::61 was approximately 90-fold higher than in the wild type even when cultured in CDM alone. These results suggest that $QseC_{Y155A,\ R156A}$ cannot sense Ep/$FeCl_2$ and that mutations in EYRDD may lock QseC in an activated conformation that results in constitutive activation of QseB and high expression of the *ygiW-qseBC* operon.



B

Reporter Plasmid	LacZ activity in: CDM	CDM + Ep/$FeCl_2$	
pJT3	4.9 ± 0.3	4.2 ± 0.6	n.s.
pDJR29	207.4 ± 6.2	1201 ± 42.4	*
pDJR55	233.7 ± 9.5	1299 ± 9.2	*
pDJR56	207.2 ± 4.1	1095 ± 15.9	*
pDJR57	149.4 ± 10.9	841.6 ± 24.9	*
pDJR63	136.9 ± 10.6	432.5 ± 7.5	*
pDJR58	15.7 ± 0.7	17.5 ± 1.1	n.s.
pDJR86	26.8 ± 3.2	24.5 ± 1.0	n.s.

Figure 3 Catecholamine/iron-dependent induction of *ygiW-qseBC* requires a functional QseB binding site. (A) Schematic diagram of the *ygiW-qseBC* promoter region and transcriptional fusion constructs pDJR29, pDJR55, pDJR56, pDJR57, pDJR63, pDJR58, pDJR85 and pDJR86 (Juárez-Rodríguez *et al.*, 2013b, 2014) showing the binding regions for QseB (white boxes), the −10 and −35 promoter elements (black boxes) and the primary transcriptional start site (bent arrow). Site-specific mutations in the QseB binding site are indicated with the × symbol. The numbering of the nucleotides is relative to the *ygiW* translational start codon. (B) β-galactosidase activity in *A. actinomycetemcomitans* 652 transformed individually with each reporter plasmid. Cultures were grown in chemically defined medium (CDM) or CDM supplemented with epinephrine (Ep; 50 μM) and $FeCl_2$ (100 μM) and β-galactosidase activity was determined after 24 h of growth. Values are means of results from three independent experiments ± standard deviations. Statistical analysis was performed by using one-way analysis of variance followed by Tukey's multiple-comparison test. Significant differences ($P < 0.05$) are indicated by asterisks; n.s., not significant.

Table 1 The EYRDD motif regulates the QseC response to Ep/$FeCl_2$

	LacZ activity in		
Strain	CDM	CDM + Ep/$FeCl_2$	Fold induction
WT	280.6 ± 44.0	1240.0 ± 207.6	4.4 ($P < 0.05$)
ΔqseBC::46	273.2 ± 26.7	1422.0 ± 234.2	5.2 ($P < 0.05$)
ΔqseBC::61	25,066 ± 11,041	17,246 ± 10,247	n.s.[1]

[1]n.s., No significant difference; CDM, chemically defined medium; Ep, epinephrine; WT, wild-type.

Catecholamines and iron stimulate *A. actinomycetemcomitans* growth

Exposure to catecholamine stress hormones has recently been reported to stimulate the growth of several potential periodontal pathogens such as *Fusobacterium nucleatum* and *Tannerella forsythia* but had little effect on the growth of *Porphyromonas gingivalis* (Jentsch *et al.*, 2013). To determine if exposure to catecholamines and iron influence *A. actinomycetemcomitans* growth, cells were cultured in CDM alone, CDM supplemented with 50 μM Ne or 100 μM $FeCl_2$, or in CDM supplemented with both Ne and $FeCl_2$. As shown in Fig. 4, there was no significant difference in the growth of *A. actinomycetemcomitans* in CDM or medium supplemented with Ne. However, addition of $FeCl_2$ resulted in a significant increase in growth over CDM alone and a further significant stimulation of *A. actinomycetemcomitans* growth was observed when CDM was supplemented with both Ne and $FeCl_2$. Similar results were observed when medium was supplemented with Ep or Ep/$FeCl_2$ (not shown).

Exposure to catecholamines and $FeCl_2$ induces genes involved in anaerobic respiration/metabolism and decreases expression of genes involved in iron acquisition

The role of the QseBC two component system in *A. actinomycetemcomitans* has only been broadly

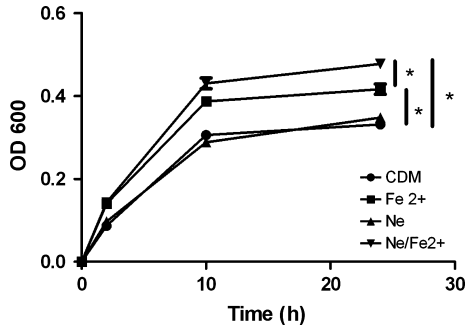


Figure 4 Catecholamine/iron-mediated induction of *Aggregatibacter actinomycetemcomitans* growth. The *A. actinomycetemcomitans* 652 was grown in chemically defined medium (CDM) alone or in CDM supplemented either with $FeCl_2$ (Fe^{2+}), norepinephrine (Ne), or both norepinephrine and iron (Ne/Fe^{2+}). At the designated times, growth was measured by determining the optical density at 600 nm (OD_{600}). Significant differences ($P < 0.05$) are indicated by asterisks.

described as regulating complex phenotypes such as biofilm formation and virulence. To better understand how this regulation occurs, we generated custom microarrays based on the genome sequence of *A. actinomycetemcomitans* D11S-1 (see Methods) and used these arrays to compare the transcriptomes of cultures grown in CDM and CDM supplemented with Ne and $FeCl_2$. As shown in Supplementary material (Table S4), 235 genes (approximately 11.5% of the *A. actinomycetemcomitans* genome) were differentially expressed at greater than or equal to two-fold in cells cultured in Ne/Fe^{2+}-supplemented medium relative to cells grown in CDM alone. Ninety-nine genes (~4.9% of the genome) were upregulated 2- to 18.5-fold and 135 genes (~6.7% of the genome) were down-regulated 2- to 9.5-fold in cells exposed to $Ne/FeCl_2$. Consistent with the results presented in Fig. 1, *ygiW-qseBC* was one of the most strongly induced operons detected in cells grown in supplemented medium and this was further confirmed by reverse transcription PCR (see Table S4). Strikingly, 56 of the 99 upregulated genes encoded proteins associated with anaerobic metabolism and respiration, as shown in Table 2. Six operons that were induced encode components associated with anaerobic electron transport, e.g. the *nap* operon (D11S_205–210), an operon involved in the biogenesis of c-type cytochromes (D11S_2000–1990), the hydrogenase-4 complex and *hydN* (D11S1735–1747 and D11S_1092/1093) involved in electron transport from formate to hydrogen and the oxidation of hydrogen, D11S_1412/1413 involved in trimethylamine *N*-oxide anaerobic respiration, and D11S_493/494 encoding a dimethylsulfoxide terminal reductase. At least four additional induced operons and several other single genes encode proteins that may be associated with anaerobic metabolism. D11S_303 and D11S_1771 each encode a C4 dicarboxylate transporter involved in the utilization of aspartate and fumarate. Consistent with this, D11S_597 encodes aspartate ammonia lyase, which converts aspartate to fumarate, D11S_810–812 encodes a fumarate reductase, and D11S_1061 encodes fumarate hydratase. Other upregulated operons code for oxaloacetate decarboxylase (D11S_1379-1381) which converts oxaloacetate to pyruvate, and D11S_1749/1748 and D11S_1989-1986, which encode formate dehydrogenase and catalyze the formate-dependent reduction of nitrite to ammonia, respectively. To confirm the microarray results, 15 genes representative of many of the operons listed above were selected for verification of induction using reverse transcription PCR. The results obtained, shown in the Supplementary material (Table S4) were consistent with the array data. As formate dehydrogenase was induced 10-fold and several of the metabolic pathways that were predicted to be induced from the array data use formate, the array results were further confirmed by determining the levels of formate in cultures grown in CDM alone or in supplemented media. As shown in Fig. 5, formate levels were significantly increased when cells were cultured in medium containing $FeCl_2$ and a further significant increase occurred when the medium was supplemented with Ne/Fe^{2+}. Together, these data suggest that the activation of QseBC may function to prime *A. actinomycetemcomitans* for growth in an anaerobic niche by upregulating genes involved in anaerobic metabolism and electron transport.

The genes that were downregulated were more diverse in their putative functions. However, 20 of the 135 downregulated genes (~15%) encode proteins associated with iron transport (see Table 3). Within this group, four operons encode putative ABC-type iron transporters (e.g. D11S_621/622, D11S_815-818, D11S_1128-1131 and D11S_1557/1558). Other downregulated genes encode putative outer membrane iron receptors (D11S_1630 and D11S_1864) and consistent with this, the expression of *tonB* components (D11S_487–489) is also reduced by

Table 2 Differentially expressed genes associated with anaerobic respiration and metabolism

ID tag	Product	Fold change	Direction	Putative function
D11S_0205	Cytochrome c-type protein TorC	3.05	Up	Trimethylamine *N*-oxide anaerobic respiration
D11S_0206	Periplasmic nitrate reductase, diheme cytochrome	2.94	Up	Diheme cytochrome c subunit
D11S_0207	Ferredoxin-type protein NapH	3.36	Up	Quinol dehydrogenase membrane component
D11S_0208	Quinol dehydrogenase periplasmic component	2.74	Up	Nitrate respiration electron transport
D11S_0209	Periplasmic nitrate reductase, large subunit	4.92	Up	Nitrate redutase periplasmic component
D11S_0210	NapD protein	6.69	Up	Assembly of D11S_208 and D11S_209
D11S_0303	Anaerobic C4-dicarboxylate membrane transporter	2.41	Up	Anaerobic utilization of aspartate/fumarate
D11S_0383	Alkylhydroperoxidase AhpD core	4.03	Up	Anti-oxidant protein
D11S_0493	Anaerobic dimethyl sulfoxide reductase chain A	2.26	Up	Anaerobic terminal reductase
D11S_0494	Anaerobic dimethyl sulfoxide reductase chain B	2.23	Up	Anaerobic terminal reductase
D11S_0597	Aspartate ammonia-lyase	6.86	Up	Converts aspartate to fumarate
D11S_0810	Fumarate reductase subunit C (Fumarate reductase)	2.39	Up	Anaerobic conversion of fumarate to succinate
D11S_0811	Fumarate reductase iron-sulfur subunit	2.39	Up	Anaerobic conversion of fumarate to succinate
D11S_0812	Fumarate reductase flavoprotein subunit	3.86	Up	Anaerobic conversion of fumarate to succinate
D11S_1061	Fumarate hydratase, class II	2.43	Up	Reversible hydration/dehydration of fumarate to malate
D11S_1092	Hydrogenase accessory protein HypB	1.81	Up	GTP hydrolase; assembly of hydrogenase
D11S_1093	Hydrogenase expression/formation protein HypD	1.57	Up	Hydrogenase maturation protein
D11S_1376	Hydrogenase assembly chaperone HypC/HupF	1.88	Up	Hydrogenase maturation protein
D11S_1379	Oxaloacetate decarboxylase γ chain 3	4.74	Up	Conversion of OAA to pyruvate
D11S_1380	Oxaloacetate decarboxylase α subunit	4.44	Up	Conversion of OAA to pyruvate
D11S_1381	Oxaloacetate decarboxylase β chain	4.31	Up	Conversion of OAA to pyruvate
D11S_1412	Cytochrome c-type protein TorY	7.66	Up	Trimethylamine *N*-oxide anaerobic respiration
D11S_1413	Trimethylamine-n-oxide reductase 2	4.67	Up	Trimethylamine *N*-oxide anaerobic respiration
D11S_1676	Cytochrome c peroxidase	5.05	Up	Metabolism of reducing equivalents from cytochrome c
D11S_1735	[NiFe] hydrogenase maturation protein HypF	2.58	Up	Hydrogenase maturation protein
D11S_1736	Electron transport protein HydN	18.44	Up	Electron transport from formate to hydrogen
D11S_1737	Hydrogenase-4 component B	14.93	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1738	Hydrogenase-4 component B	15.02	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1739	Hydrogenase-4 component C	10.68	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1740	Hydrogenase-4 component D	10.59	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1741	Hydrogenase-4 component E	7.95	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1742	Hydrogenase-4 component F	7.76	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1743	Hydrogenase-4 component G	5.89	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1744	Hydrogenase-4 component H	5.87	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1745	Hydrogenase-4 component I	5.4	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1746	Hydrogenase-4 component J	4.62	Up	Oxidation of hydrogen; coupled to electron transport
D11S_1747	Hydrogenase maturation peptidase Hyc	4.79	Up	C-terminal processing of hydrogenase
D11S_1748	Formate dehydrogenase H	10.42	Up	Anaerobic metabolism of formate to H_2 and CO_2
D11S_1749	Formate dehydrogenase, α subunit	10.0	Up	Oxidation of formate to CO_2
D11S_1771	C4-dicarboxylate membrane transporter	1.73	Up	Anaerobic utilization of aspartate/fumarate
D11S_1811	Bifunctional acetaldehydeCoA/alcohol dehydrogenase	2.72	Up	Oxidation aldehydes and alcohols
D11S_1888	TorCAD operon transcriptional regulatory protein	1.86	Up	Regulator of trimethylamine *N*-oxide respiration genes
D11S_1984	Cytochrome c-type biogenesis protein CcmF	3.87	Up	Assembly of c-type cytochromes
D11S_1986	NrfD protein	11.59	Up	Electron transfer from quinone to type c cytochromes
D11S_1987	Cytochrome c nitrite reductase, Fe-S protein	13.63	Up	Reduction of nitrite to ammonia
D11S_1988	Cytochrome c nitrite reductase, pentaheme	17.08	Up	Formate dependent reduction of nitrite to ammonia
D11S_1989	Nitrite reductase (cytochrome; ammonia-forming)	18.43	Up	Reduction of nitrite to ammonia

Table 2 (continued)

ID tag	Product	Fold change	Direction	Putative function
D11S_1993	Cytochrome c-type biogenesis protein CcmH	1.57	Up	Biogenesis of cytochrome c
D11S_1994	Thiol-disulfide interchange protein	2.04	Up	Biogenesis of cytochrome c
D11S_1995	Cytochrome c-type biogenesis protein CcmF	2.39	Up	Assembly of c-type cytochromes
D11S_1996	Cytochrome c-type biogenesis protein CcmE	2.44	Up	Heme chaperone; assembly of c-type cytochromes
D11S_1997	Heme exporter protein D (CcmD)	2.65	Up	Heme transporter
D11S_1998	CcmC	3.15	Up	Cytochrome c biosynthesis
D11S_1999	Heme exporter protein CcmB	2.69	Up	Heme exporter; cytochrome c biosynthesis
D11S_2000	Heme ABC exporter, ATP-binding protein CcmA	3.3	Up	Heme exporter; cytochrome c biosynthesis
D11S_2128	Anaerobic ribonucleoside-triphosphate reductase	2.7	Up	Reduction of CTP to dCTP

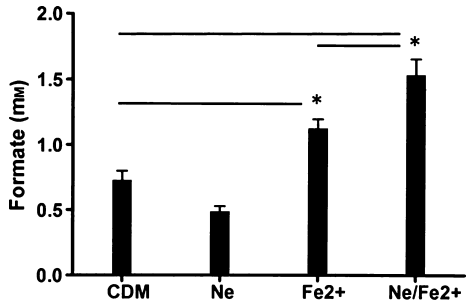


Figure 5 Formate concentration in spent media from *Aggregatibacter actinomycetemcomitans* 652 cultures. Cells were grown in chemically defined medium (CDM) alone or CDM supplemented with iron (Fe^{2+}), norepinephrine (Ne) or both norepinephrine and iron (Ne/Fe^{2+}). Formate concentration was determined by measuring the production of NADH after the enzymatic conversion of formate to bicarbonate by formate dehydrogenase. Significant differences ($P < 0.05$) are indicated by asterisks.

approximately five-fold. In contrast to the downregulation of iron acquisition genes, the expression of ferritin (D11S_1330/1331) was significantly increased (~10-fold), suggesting that intracellular iron storage is increased when QseBC is activated.

DISCUSSION

In *A. actinomycetemcomitans*, QseBC regulates biofilm growth and virulence (Shao *et al.*, 2007; Novak *et al.*, 2010) but little is known about how this two-component system controls these complex phenotypes, in large part because the signal that activates the QseC sensor has not been identified and the *qseBC* regulon has not been defined. A variety of stimuli have been shown to activate the QseBC two-component system in other organisms. In *E. coli* and *S. enterica*, the QseC sensor is activated by catecholamine hormones and/or AI-3, an autoinducer of unknown structure (Clarke *et al.*, 2006; Bearson *et al.*, 2008;). In contrast, the *qseBC* paralog in *H. influenzae* (*firRS*) is activated by cold shock or by Fe^{2+} (but not Fe^{3+}) and does not respond to catecholamines (Steele *et al.*, 2012). Using the AI-3 purification scheme described by Sperandio *et al.* (2003), we were unable to detect AI-3 produced by *A. actinomycetemcomitans* and furthermore, cold shock at either 25 or 4°C did not induce *qseBC* expression (data not shown). Our results indicate that *A. actinomycetemcomitans* QseC is activated by catecholamine hormones, but only in the presence of iron. Furthermore, activation of QseC occurs in the presence of either Fe^{2+} or Fe^{3+} and so appears to be distinct from FirS of *H. influenzae*, which is not activated by Fe^{3+} (Steele *et al.*, 2013). This suggests that a complex of catecholamines and iron may function as the signal to activate QseC and consistent with this, catecholamines have been reported to function as pseudosiderophores (Freestone *et al.*, 2000; Anderson & Armstrong, 2008; Bearson *et al.*, 2008; Sandrini *et al.*, 2010). However, we cannot exclude the possibility that catecholamines and iron interact individually with the sensor and that activation of QseC only occurs when both are bound.

Interestingly, *qseBC* expression in *E. coli* is also induced by elevated levels of Fe^{3+} but the QseC sensor itself is not directly activated by iron. Instead, iron activates the PmrB sensor, which in turn phosphorylates the PmrA response regulator. Activated PmrA binds to the *qseBC* promoter and induces expression

Table 3 Differentially expressed genes associated with iron/heme acquisition

ID Tag	Product	Fold change	Direction
D11S_0487	TonB-system energizer ExbB	0.208	Down
D11S_0488	TonB system transport protein ExbD	0.223	Down
D11S_0489	Protein TonB	0.229	Down
D11S_0621	Iron(III)-transport system permease protein FbpB	0.182	Down
D11S_0622	Ferric iron binding protein	0.195	Down
D11S_0815	Iron(III) dicitrate transport ATP-binding	0.123	Down
D11S_0816	ABC transporter, iron chelate uptake transporter	0.108	Down
D11S_0817	Iron(III) dicitrate transport system permease	0.166	Down
D11S_0818	Iron(III) dicitrate-binding periplasmic protein	0.157	Down
D11S_1128	Iron(III) dicitrate transport ATP-binding	0.215	Down
D11S_1129	Iron(III) transport system permease protein	0.331	Down
D11S_1130	Putative iron/heme permease	0.23	Down
D11S_1131	Putative periplasmic siderophore binding protein	0.143	Down
D11S_1330	Nonheme iron-containing ferritin	9.87	Up
D11S_1331	Ferritin	10.59	Up
D11S_1557	High-affinity Fe^{2+}/Pb^{2+} permease	0.245	Down
D11S_1558	High affinity Fe^{2+} transporter	0.122	Down
D11S_1559	Putative Fe^{2+} permease	0.138	Down
D11S_1630	Putative TonB-dependent iron receptor	0.48	Down
D11S_1643	AfeC periplasmic iron binding protein	0.403	Down
D11S_1809	Heme acquisition system receptor	0.463	Down
D11S_1864	OMP 64 (heme/hemogobin receptor)	0.106	Down

of the operon. However, PmrB also phosphorylates the non-cognate QseB response regulator, which autoregulates the *qseBC* operon (Guckes *et al.*, 2013). The QseB binding site in the *ygiW-qseBC* promoter of *A. actinomycetemcomitans* matches the consensus PmrA binding sequence in the *pmrAB* operon of *E. coli* (Juárez-Rodríguez *et al.*, 2014), but the *pmrAB* genes are not present in the *A. actinomycetemcomitans* genome. Hence, it is possible that the *A. actinomycetemcomitans* QseC sensor integrates the iron and catecholamine sensory functions of the PmrAB and QseBC two component systems of *E. coli*. Finally, although QseC is activated by catecholamines in both *A. actinomycetemcomitans* and enteric organisms, the virulence strategies employed by pathogenic strains of *E. coli* differ significantly from *A. actinomycetemcomitans.* Many of the virulence genes that are induced by QseBC in *E. coli* (Clarke *et al.*, 2006; Hughes *et al.*, 2009) are not present in the *A. actinomycetemcomitans* genome. This suggests that the regulons controlled by the QseBC two-component system may have evolved in a species-specific manner.

The periplasmic domain of *A. actinomycetemcomitans* QseC is required for interacting with catecholamines and iron as deleting this region of the protein prevented the induction of *ygiW-qseBC* in cells grown in the presence of catecholamine and iron. Deleting the periplasmic domain also significantly reduced the overall expression of *ygiW-qseBC* in cells that were cultured in CDM alone, consistent with our previous results showing that QseBC regulates its own expression (Juárez-Rodríguez *et al.*, 2013b). *Aggregatibacter actinomycetemcomitans qseCΔ*p was also previously shown to exhibit defective biofilm growth (Juárez-Rodríguez *et al.*, 2013b). The periplasmic region of QseC contains the EYRDD motif that is related to the iron-responsive elements that are present in the periplasmic domains of the *E. coli* PmrB (Wosten *et al.*, 2000) and *H. influenza* FirS (Steele *et al.*, 2013) sensors. Site-specific mutation of this motif significantly reduced iron-dependent expression of *firRS* in *H. influenzae* (Steele *et al.*, 2013) and reduced *lacZ* expression of a *pmrAB*-regulated promoter–reporter construct in *E. coli* (Wosten *et al.*, 2000). In *A. actinomycetemcomitans*, mutations in EYRDD not only prevented catecholamine/Fe^{2+}-dependent induction of *qseBC*, but also resulted in constitutively high expression of *qseBC*

(approximately 90-fold greater than the wild-type grown in CDM and ~12-fold higher than wild-type grown in CDM supplemented with catecholamines and iron). Hence, although EYRDD is important for catecholamine/Fe^{2+}-dependent induction of *qseBC*, mutations in this motif may also induce a conformational change in QseC that locks the protein in an active state which constitutively phosphorylates QseB, resulting in high-level constitutive expression of the *ygiW-qseBC* operon. In contrast, deletion of the entire periplasmic domain completely uncouples the sensory and kinase regions of QseC, preventing activation of QseB and reducing transcription of the *ygiW-qseBC* operon.

A variety of virulence-associated genes are differentially expressed after activation of the *E. coli* QseBC system (Clarke *et al.*, 2006; Hughes *et al.*, 2009) but although *qseBC* has been clearly shown to be required for virulence of *A. actinomycetemcomitans* in an animal model of periodontitis (Novak *et al.*, 2010), the specific genes that potentially contribute to this process have not been identified. Our results indicate that a significant portion of the *A. actinomycetemcomitans* genome (>11%) is differentially expressed when QseBC is activated by catecholamines and iron. Sixty-three percent of the differentially expressed genes (147/235) were organized in operons where at least one other gene in the operon was also identified from the array data as being differentially expressed. Interestingly, none of the genes encoding the well characterized virulence factors of *A. actinomycetemcomitans*, e.g. the leukotoxin, cytolethal distending toxin, tad fimbriae, autotransporter epithelial cell adhesins, EmaA or the *pga* matrix biogenesis components, were upregulated by QseBC when a threshold of at least two-fold difference in expression was applied. Indeed, the leukotoxin (*ltxC* and *ltxA*) and *pgaB* were downregulated by ~2.1-fold upon activation of QseBC. The striking result was that 57% of the upregulated genes (56/99) that were identified encode proteins associated with anaerobic metabolism or respiration. This group includes electron transport components such as a hydrogenase complex and proteins involved in the reduction of nitrate, dimethyl sulfoxide, trimethylamine-*N*-oxide, fumarate and formate. In addition, enzymes associated with the metabolism of aspartate, fumarate, malate, oxaloacetate, pyruvate and formate were significantly induced upon activation of QseBC. Also striking is that many of the genes induced by catecholamine/iron activation of QseBC were identified by Jorth *et al.* (2013) as being upregulated during *in vivo* growth in a study that used RNAseq to compare *A. actinomycetemcomitans* gene expression during subcutaneous growth *in vivo* (mouse abscess model) and biofilm growth *in vitro*. Similar to the results reported here, Jorth *et al.* (2013) showed that ~14% of the *A. actinomycetemcomitans* genome was differentially expressed during *in vivo* growth and a preponderance of these genes encoded proteins involved in anaerobic respiration and metabolism. In light of these data, our results suggest that the detection of catecholamines and iron by the QseBC two component system may alter *A. actinomycetemcomitans* gene expression to prime cellular metabolism for growth in an anaerobic environment, such as that which occurs in the subgingival pocket *in vivo*. Interestingly, recent studies suggest that activated phagocytic cells (e.g. neutrophils, polymorphonuclear cells and macrophages) release catecholamines in response to inflammatory stimuli (Brown *et al.*, 2003; Flierl *et al.*, 2007, 2008, 2009) and can produce local concentrations of catecholamines in the millimolar range (Brown *et al.*, 2003). Hence, the inflamed gingival pocket may be an environment that is rich in catecholamines, which *A. actinomycetemcomitans* may detect via QseBC as a stimulus to increase its fitness to survive in this niche. Consistent with this, our results also showed that catecholamines and iron stimulate *A. actinomycetemcomitans* growth. It is also possible that the pattern of gene expression observed by Jorth *et al.* (2013) in the mouse abscess arose from the response of QseBC to catecholamines produced by phagocytic cells responding to *A. actinomycetemcomitans* infection. A model that illustrates this potential QseBC signaling cascade is shown in Fig. 6. Finally, we can speculate that the shift in cellular metabolism and energy production may be the primary factor that links the QseBC two-component system with *A. actinomycetemcomitans* virulence as QseBC does not appear to be a major regulator of virulence gene expression but is essential for *A. actinomycetemcomitans* virulence (Novak *et al.*, 2010).

The genes that are down-regulated when QseBC is activated are associated with a variety of metabolic processes. However, 15% of the downregulated genes code for iron binding and transport proteins, including the AfuABC ferric iron transporter

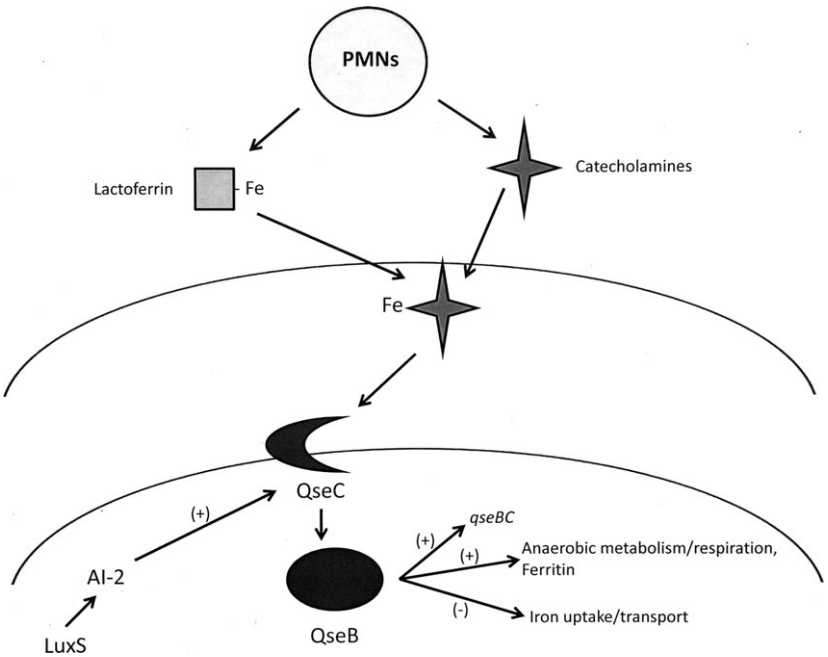


Figure 6 Schematic model of the QseBC signaling cascade of *Aggregatibacter actinomycetemcomitans*. Catecholamines released by phagocytic cells responding to *A. actinomycetemcomitans* infection may function as pseudosiderophores and extract iron from host proteins such as lactoferrin or transferrin. Iron and catecholamines, either in complex or individually function to activate QseC by interacting with the periplasmic domain of the sensor. Activated QseC then phosphorylates QseB, which induces the expression of the *qseBC* operon and numerous genes encoding proteins involved in anaerobic metabolism, electron transport and intracellular iron storage. Activated QseB also downregulates several operons encoding putative inner and outer membrane iron acquisition and transport proteins. The QseBC two-component system is also influenced by autoinducer-2-dependent quorum sensing but the mechanism that links the detection of the autoinducer with induction of *qseBC* expression has not yet been determined.

(D11S_0622-0620) and the FecBCDE operon (D11S_0818-0815). In addition, the expression of several putative outer membrane iron/heme-binding proteins was reduced and consistent with this, genes encoding TonB and its accessory energizer proteins ExbB and ExbD were also downregulated. These results are not surprising because CDM supplemented with catecholamines and $FeCl_2$ represents an iron-rich medium and under these growth conditions, high-level expression of high-affinity iron binding and transport proteins is not likely to be required. Consistent with this, the expression of ferritin was induced by ~10-fold, suggesting that intracellular iron storage capacity is being increased when QseBC is activated. As lactoferrin is also released by neutrophils, the inflamed gingival pocket may represent an iron replete environment for *A. actinomycetemcomitans* as catecholamines are known to function as pseudosiderophores that can extract iron from transferrin and lactoferrin (Freestone *et al.*, 2000; Anderson & Armstrong, 2008; Bearson *et al.*, 2008; Sandrini *et al.*, 2010). Indeed, host catecholamines are used by *Bordetella* and other micro-organisms to acquire iron (Freestone *et al.*, 2000; Burton *et al.*, 2002; Anderson & Armstrong, 2008; Bearson *et al.*, 2008) and it is possible that inter-kingdom signaling mediated by QseBC allows *A. actinomycetemcomitans* to detect and exploit the production of catecholamines by host cells to facilitate the acquisition of iron from lactoferrin or other host iron-binding proteins during infection.

In summary, we have shown that a combination of catecholamines and iron function as a signal to activate QseBC and that this response requires the EY-RDD motif in the periplasmic domain of the QseC sensor and the QseB response regulator. Activation of QseC stimulates the expression of numerous gene products that are involved in anaerobic metabolism and respiration and may prime the organism for growth in an anaerobic niche in the host. Hence, QseBC may represent a therapeutic target, as suggested for QseBC of *E. coli* (Curtis *et al.*, 2014) to control *A. actinomycetemcomitans* infections.

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