ygiW and *qseBC* are co-expressed in *Aggregatibacter actinomycetemcomitans* and regulate biofilm growth

María Dolores Juárez-Rodríguez,[†] Ascensión Torres-Escobart and Donald R. Demuth

Correspondence

Donald R. Demuth
drdemu01@louisville.edu

Research Group in Oral Health and Systemic Disease, University of Louisville School of Dentistry, 501 S. Preston Street, Louisville, KY 40202, USA

The quorum-sensing *Escherichia coli* regulators B and C (QseBC) two-component system were previously shown to regulate biofilm growth of the oral pathogen *Aggregatibacter actinomycetemcomitans* and to be essential for virulence. In this study, we use RT-PCR to show that an open reading frame, *ygiW*, residing upstream of *qseBC* and encoding a hypothetical protein is co-expressed with *qseBC*. In addition, using a series of *lacZ* transcriptional fusion constructs and 5'-rapid amplification of cDNA Ends (RACE), the promoter that drives expression of the *ygiW-qseBC* operon and the transcriptional start site was mapped to the 372 bp intergenic region upstream from *ygiW*. No internal promoters drive *qseBC* expression independently from *ygiW*. However, *qseBC* expression is attenuated by approximately ninefold by a putative attenuator stem-loop ($\Delta G = -77.0$ KJ/mol) that resides in the 137 bp intergenic region between *ygiW* and *qseB*. The QseB response regulator activates expression of the *ygiW-qseBC* operon and transcription from the *ygiW* promoter is drastically reduced in $\Delta qseB$ and $\Delta qseBC$ mutants of *A. actinomycetemcomitans*. In addition, transcriptional activity of the *ygiW* promoter is significantly reduced in a mutant expressing an in-frame deletion of *qseC* that lacks the sensor domain of QseC, suggesting that a periplasmic signal is required for QseB activation. Finally, a non-polar in-frame deletion in *ygiW* had little effect on biofilm depth but caused a significant increase in surface coverage relative to wild-type. Complementation of the mutant with a plasmid-borne copy of *ygiW* reduced surface coverage back to wild-type levels. Interestingly, deletion of the sensor domain of QseC or of the entire *qseC* open reading frame resulted in significant reductions in biofilm depth, biomass and surface coverage, indicating that the sensor domain is essential for optimal biofilm formation by *A. actinomycetemcomitans*. Thus, although *ygiW* and *qseBC* are co-expressed, they regulate biofilm growth by distinct mechanisms.

Received 9 January 2012

Accepted 19 March 2013

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a Gram-negative, facultative anaerobe of the *Pasteurellaceae* family and is the primary aetiological agent of localized aggressive periodontitis and severe non-oral infections, including endocarditis, soft tissue abscesses, meningitis, pneumonia, septicaemia, urinary tract infections and osteomyelitis (Haffajee & Socransky, 1994; Hyvärinen *et al.*, 2012; Henderson *et al.*,

2003; Nørskov-Lauritsen & Kilian, 2006; Paturel *et al.*, 2004; Rahamat-Langendoen *et al.*, 2011; Wang *et al.*, 2010). The molecular mechanisms involved in *A. actinomycetemcomitans* virulence and pathogenesis are not well defined, but *A. actinomycetemcomitans* expresses two autotransporter adhesins (Aae and ApiA) and fimbriae (encoded by the *tad* locus) that are involved in the colonization and formation of biofilms on oral tissues (Fine *et al.*, 2010; Perez *et al.*, 2006; Saito *et al.*, 2010; Yue *et al.*, 2007). In addition, the organism expresses virulence factors that target the innate immune response to infection, including a leukotoxin (LtxA) that kills leukocytes expressing lymphocyte function-associated antigen I (LFA-I) and a cytolethal distending toxin (CdtABC) that induces cell cycle arrest and apoptosis in lymphocytes and other mammalian cells (Kachlany, 2010; Jinadasa *et al.*, 2011).

[†]These authors contributed equally to this study.

Abbreviations: AI-2, autoinducer-2; AI-3, autoinducer-3; EHEC, enterohaemorrhagic *E. coli*; GST, glutathione S-transferase; LtxA, leukotoxin; QseBC, quorum-sensing *Escherichia coli* regulators B and C; RACE, rapid amplification of cDNA ends; UPEC, uropathogenic *E. coli*.

Two supplementary figures are available with the online version of this paper.

Adhesion and biofilm formation are essential for *A. actinomycetemcomitans* colonization and persistence within the oral cavity (Perez *et al.*, 2006; Amarasinghe *et al.*, 2009), and we previously showed that biofilm formation of both fimbriated and afimbriated strains of *A. actinomycetemcomitans* is regulated by a quorum-sensing (QS) system responding to autoinducer-2 (AI-2) (Demuth *et al.* 2011; Novak *et al.*, 2010). In addition, iron acquisition and virulence of *A. actinomycetemcomitans* are regulated by AI-2 (Fong *et al.*, 2001, 2003; Novak *et al.*, 2010). One component of this QS system is the quorum-sensing *Escherichia coli* regulators B and C (QseBC) two-component system, whose expression is regulated by AI-2 (Novak *et al.*, 2010). However, it is currently unclear how regulation of *qseBC* is linked to the detection or importation of AI-2 by *A. actinomycetemcomitans*.

The QseBC two-component system is present in several Gram-negative pathogens, and in *E. coli* it has been suggested to mediate inter-kingdom communication by responding to a hormone-like signal (AI-3), as well as to epinephrin and norepinephrin (Clarke *et al.* 2006). The sensing of these signals by the QseC sensor kinase initiates a signalling cascade that activates transcription of *flhDC* genes, which encode the master regulator for the flagella and motility genes (Sperandio *et al.*, 2002; Clarke & Sperandio, 2005). In enterohaemorrhagic *E. coli* (EHEC), QseC phosphorylates three response regulators, including QseB, KdpE and QseE (which is also phosphorylated by its cognate sensor kinase, QseF, in response to ephrine), which induces transcription of several additional genes implicated in pedestal formation (Reading *et al.*, 2007; Hughes *et al.*, 2009). QseBC also has been reported to influence the virulence properties of uropathogenic *E. coli* (UPEC) (Kostakioti *et al.*, 2009), *Salmonella* serovar Typhimurium (Bearson & Bearson, 2008; Merighi *et al.*, 2009; Moreira *et al.*, 2010), *Edwardsiella tarda* (Wang *et al.*, 2011) and *Aeromonas hydrophila* (Khajanchi *et al.*, 2012). In UPEC, activation of the QseC sensor results in dephosphorylation of QseB (Kostakioti *et al.*, 2009), and deletion of *qseC* causes dysregulation of nucleotide, amino acid and carbon metabolism, suggesting that QseC also controls the bacterial central metabolic circuit (Hadjifrangiskou *et al.*, 2011). However, while some of the metabolic pathways controlled by QseC are conserved in *A. actinomycetemcomitans*, the regulon and function of QseBC in *A. actinomycetemcomitans* are also distinct from *E. coli* because this organism is non-motile. Furthermore, a protein BLAST search (BLASTP) of the *A. actinomycetemcomitans* genome using the *E. coli* sequences as probes failed to identify paralogues of the QseEF or KdpDE two-component systems, whereas a similar search using the sensor domain of *E. coli* QseC readily detected its *A. actinomycetemcomitans* counterpart.

In this study, we show that the *A. actinomycetemcomitans* *qseBC* operon contains a third gene, *ygiW*, and that transcription of the *ygiW-qseBC* operon is directed by a promoter (P_{ygiW}) that resides in the 372 bp intergenic

region upstream of *ygiW*. In addition, QseB and the QseC periplasmic sensor domain are necessary for the expression of *ygiW-qseBC*. Furthermore, expression of functional YgiW and QseC proteins is necessary for the optimal biofilm growth of *A. actinomycetemcomitans*.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. LB (Luria–Bertani) broth, LB agar (1.5 % agar) and brain–heart infusion (BHI) broth and BHI agar (Becton Dickinson Co., Sparks, Md) (Difco) were used to propagate and plate bacteria. *A. actinomycetemcomitans* 652 serotype c (afimbriated, smooth-colony-morphotype) was routinely grown at 37 °C in BHI medium supplemented with 40 mg NaHCO₃ l⁻¹. When required, the medium was supplemented with 25 µg kanamycin (Km) ml⁻¹, 12.5 µg ml⁻¹ tetracycline or 100 µg ampicillin (Ap) ml⁻¹. However, for the selection of markerless gene deletion mutants, *A. actinomycetemcomitans* strains were cultured in 1 % tryptone supplemented with 0.5 % yeast extract and 1.5 % agar.

DNA procedures. DNA manipulations were carried out as previously described (Sambrook & Russell, 2001). Transformation of *E. coli* and *A. actinomycetemcomitans* was done by electroporation (Bio-Rad). Transformants containing plasmids were selected on agar plates supplemented with the appropriate antibiotics. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). Restriction enzymes were used as recommended by the manufacturer (New England Biolabs). All primers used in this study (synthesized by Integrated DNA Technology) were flanked with restriction enzyme recognition sites (underlined in the primer sequence) and are shown in Table 2. Primer sequences were designed based on the genome sequence of serotype c *A. actinomycetemcomitans* D11S-1 strain, available from the Pathosystems Resource Integration Center (<http://patricbrc.vbi.vt.edu>). All constructs were verified by DNA sequencing (University of Louisville Core Facilities).

Reverse transcriptase (RT)-PCR. *A. actinomycetemcomitans* 652 was grown in BHI broth to the late-exponential phase (OD₆₀₀ of 0.5), and total RNA was extracted using the ZR Fungal/Bacteria RNA MiniPrep kit (Zymo Research) as specified by the manufacturer. RNA samples were treated with RNase-free DNase I (Promega) to eliminate contaminated DNA and quantified by measurements of OD₂₆₀ and gel electrophoresis. RNA was transcribed using SuperScript III enzyme (Invitrogen) following the protocol of the manufacturer. The cDNA obtained was amplified with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) using primer sets: for *ygiW* (MDJR-56F and MDJR-57R) and (MDJR-140F and MDJR-57R), for *qseB* (MDJR-58F and MDJR-59R), for *qseC* (MDJR-60F and MDJR-61R), from *ygiW* to *qseB* (MDJR-63F and MDJR-64R) and (MDJR-140F and MDJR-59R), from *qseB* to *qseC* (MDJR-65F and MDJR-66R), from *ygiW* to *qseC* (MDJR-140F and MDJR-61R) and for 16S rRNA (MDJR-75F and MDJR-76R) (see Table 2). The PCR amplification conditions were 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 68 °C for 1 min and a final cycle of elongation at 68 °C for 5 min. Control reactions without RT were used to determine RNA template purity from DNA. Resulting PCR products were separated by electrophoresis in a 1.5 % agarose gel.

Identification of the *ygiW-qseBC* transcriptional start site. The transcriptional start site of *ygiW-qseBC* was determined using a Gene

Table 1. Strains and plasmids used in this work

In the descriptions of the genotype, P represents promoter, and the subscript refers to a composite insertion of the indicated gene. Amp^r Ampicillin resistance, Km^r kanamycin resistance, Tc^r tetracycline resistance, Sp^r spectinomycin resistance.

Strain or plasmid	Derived, relevant genotype or characteristics	Source or reference
<i>Escherichia coli</i>		
XL1-Blue MRF'	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1}$ $\text{supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB}$ $\text{lacI}^{\Delta}Z\Delta M15 \text{ Tn10 (Tc}^r\text{)]}$	Stratagene
<i>E. coli</i> BL21	(DE3 ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻)gal dcm rne131</i> BL21(DE3)	Novagen
<i>Aggregatibacter actinomycetemcomitans</i>		
652	Wild-type, serotype c, minimally leukotoxic	Laboratory stock
652-JR25	652 if $\Delta ygiW$	This study
652-JR37	652 $\Delta qseB$	Juárez-Rodríguez <i>et al.</i> (2013)
652-JR38	652 $\Delta qseC$	This study
652-JR39	652 $\Delta qseBC$	This study
652-JR23	652 $qseC\Delta pr$	This study
Plasmids		
pJT1	Sp ^r ; suicide vector	Juárez-Rodríguez <i>et al.</i> (2013)
pJT3	Km ^r ; pYGK*, promoterless <i>lacZ</i> reporter vector	Juárez-Rodríguez <i>et al.</i> (2013)
Plasmids derived from pJT3		
pDJR28	pJT3, operon <i>ygiW-qseB-qseC-ΔlacZ</i>	This study
pDJR29	pJT3, P _{<i>ygiW</i>372} - <i>lacZ</i>	This study
pDJR30	pJT3, <i>ygiW-qseB</i> intergenic region- <i>lacZ</i>	This study
pDJR31	pJT3, <i>ygiW</i> and <i>ygiW-qseB</i> intergenic region- <i>lacZ</i>	This study
pDJR32	pJT3, P _{<i>ygiW</i>372} - <i>ygiW</i> and <i>ygiW-qseB</i> intergenic region- <i>lacZ</i>	This study
pDJR33	pJT3, <i>ygiW-qseB</i> intergenic region and <i>qseB-lacZ</i>	This study
pDJR34	pJT3, P _{<i>ygiW</i>372} - <i>ygiW</i> , <i>ygiW-qseB</i> intergenic region, and <i>qseB-lacZ</i>	This study
Plasmids derived from suicide vector pJT1		
pDJR23	pJT1, $qseC\Delta pr$	This study
pDJR25	pJT1, if $\Delta ygiW$	This study
pDJR38	pJT1, $\Delta qseC$	This study
pDJR39	pJT1, $\Delta qseBC$	This study
Plasmids for protein expression		
pET28a+	Km ^r ; pBR322 expression vector	Novagen
pDJR3	Km ^r ; pET28a+ <i>ygiW</i> -AU1-6 × His	This study
pGEX-4T-1	Amp ^r ; expression vector	GE Healthcare
pDJR1	pGEX-4T-1; GST:: <i>qseC</i> periplasmic region	This study

*pYGK is a low copy number plasmid (3–4 copies per cell) initially described by Brogan *et al.* (1996).

Racer kit (Invitrogen). *A. actinomycetemcomitans* 652 was grown in BHI broth to mid-exponential phase (OD₆₀₀ of 0.3), and total RNA was extracted and digested with DNase I as described above for RT-PCR. A 44-base 5' RNA adaptor oligonucleotide was ligated to the 5' ends of the total RNA (1 mg) using T4 DNA ligase. RT was performed with AMV RT and random hexamers that were included in the Gene Racer kit. PCR was performed using the Gene Racer 5' primer and the *ygiW*-specific primer, MDJR-57R. The PCR product was subsequently cloned into a pCR4-TOPO vector and transformed into *E. coli* Top10 (Invitrogen). Five positive independent clones were sequenced.

Construction of *ygiW* and *qseB/lacZ* fusion plasmids. Various fragments containing portions of the upstream region of *ygiW* and surrounding coding region of the *ygiW-qseBC* locus were amplified by PCR as follows using *A. actinomycetemcomitans* genomic DNA as a template. The typical amplification profile used was: 94 °C for 2 min

for 1 cycle and then 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min for 25 cycles. For the *ygiW* promoter fusion construction, the fragment encompassing the upstream region of *ygiW* and its ATG start codon was amplified using primer set MDJR-67F and MDJR-70R. The 375 bp PCR product was then digested with *KpnI*–*Bam*HI and cloned into *KpnI*–*Bam*HI-digested pJT3 (Juárez-Rodríguez *et al.*, 2013) to create pDJR29 (P_{*ygiW*372}-*lacZ*). A similar approach was followed to create the transcriptional fusion plasmids pDJR30, pDJR31, pDJR32, pDJR33 and pDJR34. The structures of these plasmids are shown in Fig. 2. Primer sets used to amplify the appropriate fragment for these constructs were MDJR-69F and MDJR-71R, MDJR-68F and MDJR-71R, MDJR-67F and MDJR-71R, MDJR-69F and MDJR-72R and MDJR-67F and MDJR-72R, respectively.

Construction of *YgiW* and *QseC* expression plasmids. The periplasmic sensor domain of *QseC* resides between the two

Table 2. Oligonucleotides used in this work

Related product and oligonucleotide	Sequence (5'→3')
RT-PCR primers	
<i>ygiW</i>	
MDJR-56F	CGAATGCGCCGAAAATGGAACACCACAAAAAGGTG3
MDJR-57R	CAATTTTTCTACGCGTTCAATATCCACTTCGGTCTTATTCC
MDJR-140F	ATTTAATTGAGGATCCTTCACATGAAGAAACGCAGTTTAGCAAC
<i>qseB</i>	
MDJR-58F	GCAGTGGTGCTGGATTTAACCTTGCCGAAAATGG
MDJR-59R	GCGTTACTGCTCACTTCGTTATCCAGTTATAAAAGTTTGTC
<i>qseC</i>	
MDJR-60F	GGTGTCGGTGTCGTGTGGCTGATTTCCAC
MDJR-61R	CGTACGTTTTGCCACTTGTTACCGATTTGTTTCAGG
<i>ygiW-qseB</i>	
MDJR-63F	CCAAGTGGAATCGAACGCAAAGCCTGGC
MDJR-64R	CGTCGGTAAACCAATCCACGGCGAAACC
MDJR-140F	ATTTAATTGAGGATCCTTCACATGAAGAAACGCAGTTTAGCAAC
MDJR-59R	GCGTTACTGCTCACTTCGTTATCCAGTTATAAAAGTTTGTC
<i>qseB-qseC</i>	
MDJR-65F	GCACGTTTGCAGGCACTGATTCGTCGTCG3
MDJR-66R	GTGGAAATCAGCCACACGCACACCGACACC
<i>ygiW-qseC</i>	
MDJR-140F	ATTTAATTGAGGATCCTTCACATGAAGAAACGCAGTTTAGCAAC
MDJR-61R	CGTACGTTTTGCCACTTGTTACCGATTTGTTTCAGG
16S RNA	
MDJR-75F	GCTTGGAATCTGTCTTATGGAGGGGATAACG
MDJR-76R	CGAAGGCCTTCTTCATTCACGCGGCATGG
<i>lacZ</i> fusion constructions	
MDJR-67F <i>KpnI</i>	GGAAGAATTCGGATGGTACCAGTTGTGTTGTTAATAGAAAGTG
MDJR-68F <i>KpnI</i>	GAGGAGATTTGGTACCAAGAAACGCAGTTTAGCAACAATTTTAG
MDJR-69F <i>KpnI</i>	GAAAAAATTGTAGGTACCTCCAAACCGAAAAACAGAAAGTGGC
MDJR-70R <i>BamHI</i>	GCTAAACTGCGGGATCCCATGTGAAATCTCCTCAATTAATTC
MDJR-71R <i>BamHI</i>	CGATTAATAAGGATCCCATAAAAAACCTACCGCACTTTTAAAGTC
MDJR-72R <i>BamHI</i>	ATTCCACTTACTGGATCCCATCATTTTTTCCAGTGCATAGC
GST-QsCper	
MDJR-1F <i>EcoRI</i>	TTCCACGGAATTCGGTGGCGCGCAGGTGCGTAAAGAAGTGAACC
MDJR-12R <i>XhoI</i>	CCGGCAAACCTCGAGCCACATTTGCCAAACACCATTTCATTAC
YgiW-AU1-6×His	
MDJR-10F <i>NcoI</i>	ATTGAGGAGATTTCCCATGGGCAAACGCAGTTTAGCAAC
MDJR-11R <i>XhoI</i>	CAAAAAGCGCTCGAGGATGTAGCGGTACGTGTCCGCGCCACCACC
	TTTTACAATTTTTTCTACGCGTTCAATATCC
pDJR28	
MDJR-67F <i>KpnI</i>	GGAAGAATTCGGATGGTACCAGTTGTGTTGTTAATAGAAAGTG
MDJR-77R <i>XbaI</i>	CGCAATCCTCTAGAGAAAACGGTCGGGTATTGATC
Construction of chromosomal deletions	
<i>qseC</i> deletion	
Upstream region:	
MDJR-85F <i>NotI</i>	CTTAGACGCGCGCCGCGATATAAAATTTTTTCGGATTGGAG
MDJR-86R <i>XhoI</i>	CAAAACGAAACTCGAGGCTGTAATTATCCACTTACTCAGTTTC
MDJR-87F <i>XhoI</i>	GATCTGAATCTCGAGCGAATGGGTTTGTGGCAGAGATTTCAG
MDJR-88R <i>PstI</i>	CCAAAACGCTGCAGGAAGAAAAGGACAATTTGGGCAAATGG
PCR verification:	
MDJR-54F	GGAAGTGCATATTTACAACCTGCGCCAAAAGCTCGG
MDJR-77R	CGCAATCCTCTAGAGAAAACGGTCGGGTATTGATC
<i>qseBC</i> deletion	
MDJR-49F <i>NotI</i>	GATTTGTTGCGGCCGCTGTTCCATTGCTTTTCGCACAAG
MDJR-82R <i>XhoI</i>	GCCGTTACCTCGAGAGGATCATCTTCGATTAATAAAATCCGC
MDJR-87F <i>XhoI</i>	GATCTGAATCTCGAGCGAATGGGTTTGTGGCAGAGATTTCAG

Table 2. cont.

Related product and oligonucleotide	Sequence (5'→3')
MDJR-88R <i>Pst</i> I	CCAAAACGCTGCAGGAAGAAAAGGACAATTTGGGCAAATGG
PCR verification:	
MDJR-63F	CCAAGTGGAATCGAACGCAAAGCCTGGC
MDJR-77R	CGCAATCCTCTAGAGAAAACGGTCGGGTATTGATC
<i>ifA-ygiW</i>	
MDJR-49F <i>Not</i> I	GATTTGTTGCGGCCGCTGTTCCATTGCTTTTCGCACAAG
MDJR-51R <i>Xho</i> I	CGTTTCCTCGAGTGAAATCTCCTCAATTAAATTCATCTTATAAG
MDJR-50F <i>Xho</i> I	GCGTGTTTGCTCGAGTAGGTTTCTAGACCCGAATG
MDJR-48R <i>Pst</i> I	CAATAAGCCTGCAGACACCAGCGACAAGGTGACAATCAAAC
PCR verification:	
MDJR-52F	GTTCCGCCAGCCATTGCTTAATGCGATTTACGCCTTC
MDJR-53R	CCTACCGCACTTTTAAAGTCAATCTCTTTACGCCTCC
<i>qseC</i> Δpr	
Upstream region:	
MDJR-46F <i>Not</i> I	GTGTTTGACACGCGGCCGCTTTCAAGACCCGAATGCG
MDJR-13R <i>Bam</i> HI	CAAACACTTGGGATCCTTCTTTACGCACCTGAAACCAAGC
MDJR-14F <i>Bam</i> HI	GTAATGAAAGGATCCTTTGGGCAAATGTGGATTGGTTTGCCGG
MDJR-5R <i>Pst</i> I	TGCGAAGCCTGCAGCATCGCCCCACAAAAACG
PCR verification	
MDJR-54F	GGAAGTGCATATTTACAACCTGCGCCAAAAGCTCGG
MDJR-55R	GTGCGGTCAAAAACTGATTCAAACCTTGAATGAGCGG

transmembrane regions of the sensor kinase. The DNA fragment encoding the QseC periplasmic domain (amino acids 37–169) and the structural *ygiW* gene were PCR-amplified from *A. actinomycetemcomitans* genomic DNA using primer sets MDJR-1F and MDJR-12 and MDJR-10F and MDJR-11R, respectively. The 419 bp PCR product containing the region encoding the QseC periplasmic domain was digested with *Eco*RI–*Xho*I and cloned downstream in-frame with the sequence encoding the glutathione S-transferase (GST), into *Eco*RI–*Xho*I-digested pGEX-4T-1 vector (GE Healthcare) to create pDJR1. The primer MDJR-11R was used to amplify the structural *ygiW* that was flanked by the sequence encoding the AU1 epitope tag DTYRYI (Torres-Escobar *et al.*, 2010) with the purpose of introducing this epitope tag at the carboxy-terminal end of YgiW to facilitate its detection. The 452 bp PCR product was digested with *Nco*I–*Xho*I and cloned into *Nco*I–*Xho*I-digested pET28a+ vector (Novagen) to obtain the pDJR3.

Generation of *A. actinomycetemcomitans* markerless deletion mutants. *A. actinomycetemcomitans* 652 strain deletion mutants (Table 1) used in this study were constructed by allelic replacement of the target gene by double homologous recombination using a suicide vector pJT1 (Juárez-Rodríguez *et al.*, 2013). Briefly, the flanking regions of the *qseC* were amplified by PCR using *A. actinomycetemcomitans* chromosomal DNA as a template with two primer sets (MDJR-85F and MDJR-86R) and (MDJR-87F and MDJR-88R). Each primer was flanked with a restriction site as stated above (Table 2). The 1743 and 1348 bp PCR products were digested with *Not*I–*Xho*I and *Xho*I–*Pst*I respectively, and both PCR fragments were cloned adjacent to each other (joined by the *Xho*I restriction site) into *Not*I–*Pst*I-digested pJT1 suicide vector to create pDJR38 (Table 1). A similar approach was used to generate the *A. actinomycetemcomitans* markerless deletion mutants for *qseBC* (double deletion), harbouring an in-frame deletion in *ygiW* (*ifΔygiW*) and the deletion of the sequence encoding the *qseC* periplasmic region (*qseC*Δpr) by constructing and using the suicide plasmids pDJR39, pDJR25 and pDJR23. Primer sets used to amplify the upstream and downstream flanking regions of each target to be deleted were for *qseBC* (MDJR-49

and MDJR-82) and (MDJR-87 and MDJR-88), for *ifΔygiW* (MDJR-49 and MDJR-51) and (MDJR-50 and MDJR-48), and for *qseC*Δpr (MDJR-46 and MDJR-13) and (MDJR-14 and MDJR-5). Each recombinant suicide plasmid was introduced into *A. actinomycetemcomitans* by electroporation. Then, the cells were incubated for 5 h at 37 °C and plated onto BHI agar containing 50 µg spectinomycin (Sp) ml⁻¹ to select for cells with single recombinant crossover events. Sp-resistant (Sp^r) colonies were selected and grown statically in BHI broth for 24 h at 37 °C. On the next day, each culture was diluted 1:200 in tryptone–yeast-extract (TYE) broth and grown for 24 h at 37 °C. This step was repeated for 3 consecutive days, except that the cultures on day 3 were grown in the presence of 1 mM IPTG. Afterwards, to select bacteria with a second recombination event resulting in the deletion of the target gene, cells were diluted 10-fold and spread onto TYE agar supplemented with 1 mM IPTG and 10% sucrose and grown for 24 h at 37 °C. Sucrose-resistant (Suc^r) colonies were selected and replica plated onto TYE agar supplemented with sucrose and onto BHI agar supplemented with spectinomycin. Spectinomycin-sensitive (Sp^s) colonies were selected to perform PCR to confirm the deletion of the target gene. Sp^s colonies that were PCR-positive for the appropriate gene deletion were chosen for further analysis. Primer sets used to verify by PCR amplification the expected fragment and nucleotide sequence for each isogenic mutant were: for *ΔqseC* (MDJR-54F and MDJR77R), for *ΔqseBC* (MDJR-63F and MDJR-77R), for *ifΔygiW* (MDJR-52F and MDJR53R) and for *qseC*Δpr (MDJR-54F and MDJR-55R). For complementation experiments, the *ygiW-qseBC* operon containing its promoter region was PCR-amplified from *A. actinomycetemcomitans* chromosomal DNA using the primer set (MDJR-67F and MDJR-77R). The resulting 3111 bp PCR product was digested with *Kpn*I–*Xba*I and was cloned with *Kpn*I–*Xba*I-digested pJT3 to create the pDJR28.

Growth kinetics. A single colony of *A. actinomycetemcomitans* 652 wild-type or its isogenic mutants harbouring each reporter plasmid was independently inoculated into 10 ml BHI medium, supplemented with 25 µg Km ml⁻¹ and grown statically for 24 h at 37 °C in BHI medium supplemented with 40 mg NaHCO₃ l⁻¹. The overnight

culture (OD₆₀₀ of 0.6) was then diluted 1:30 to inoculate 20 ml BHI medium (20 ml in a 50 ml conical centrifuge tube) with 25 µg Km ml⁻¹ and grown as described above. For the first 12 h of growth, a 1.1 ml aliquot [1 ml to read OD₆₀₀ and 0.1 ml for the β-galactosidase (β-gal) activity assay] was removed each hour. Additional aliquots were taken from each culture for analysis at 24 h. β-gal activity was also determined for each aliquot as described below.

β-gal assays. β-gal activity was qualitatively assessed on LB 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 was carried out using permeabilized cells incubated with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate (Sigma-Aldrich) as previously described by Miller (1972). Mean values (±SD) for activity units were routinely calculated from three independent assays.

Biofilm formation and analysis. *A. actinomycetemcomitans* biofilms were grown in an FC81 polycarbonate flow chamber (Biosurface Technologies) (chamber dimensions are 50.5 × 12.7 × 2.54 mm) at a flow rate of 10 ml h⁻¹ at 25 °C, essentially as described by Shao *et al.* (2007b) with two exceptions (see below). Briefly, a frozen glycerol stock of *A. actinomycetemcomitans* 652 WT, mutants or complemented mutant strains was streaked onto a BHI agar plate (supplemented with 25 µg Km ml⁻¹ when required) and incubated at 37 °C. A single colony from each strain from the plates was inoculated in BHI (10 ml in a 15 ml polypropylene tube) and was grown overnight. Each culture was used to inoculate BHI (45 ml in a 50 ml polypropylene tube) at a dilution of 1:20 and was grown to an OD₆₀₀ of 0.4. Each culture (45 ml) was then inoculated for 3 h into the polycarbonate flow chamber at a flow rate of 10 ml h⁻¹. After removing non-adhered cells by washing with PBS (136.8 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄ and 1.76 mM KH₂PO₄; pH 7.4) for 30 min at a flow rate of 10 ml h⁻¹ using a peristaltic pump (Manostat Sarah cassette; Thermo Fisher Scientific), bound cells were fed with BHI medium supplemented with 40 mg l⁻¹ NaHCO₃ for 62 h at a flow rate of 10 ml h⁻¹. The resulting biofilm was stained with 0.2 mg fluorescein isothiocyanate (FITC, Sigma-Aldrich) ml⁻¹ for 1 h in the dark and then washed with PBS for 2 h. Biofilms were visualized using an Olympus Fluoview FV500 confocal scanning laser microscope (Olympus) at a magnification of 600× using an argon laser. Confocal images were captured from 20 randomly chosen frames from each flow chamber, and z-plane scans from 0 to 100 µm at 1 µm intervals were performed for each frame. The z-stacks were analysed using Volocity image analysis software (PerkinElmer) to generate simulated three-dimensional images. Biofilm depth (average and maximal), total biomass and total surface coverage were determined using the Volocity software package. The values reported are means of data from the different frames obtained. Biofilm assays were repeated independently three times with each strain. Differences among biofilms were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Differences of *P* < 0.05 were considered significant. Data were analysed with GraphPad Prism v5 software (GraphPad Software).

RESULTS

The *ygiW* gene is co-transcribed with *qseBC* in *A. actinomycetemcomitans*

Examination of the *A. actinomycetemcomitans* D11S genome sequence (<http://www.ncbi.nlm.nih.gov/gene?term=d11s%20qseB>) showed that an open reading frame designated D11S_1135 and annotated as *ygiW* was present just

upstream from *qseBC* and was potentially transcribed in the same direction (Fig. 1a). To determine if *ygiW* is co-transcribed along with the *qseBC* genes, total RNA was extracted and analysed by RT-PCR using primers designed to amplify fragments within each of the structural genes, as well as fragments spanning each of the intergenic regions of the putative operon. Reactions of 16S rRNA served as an internal control. Transcription of *ygiW*, *qseB* and *qseC* was detected by amplification of fragments corresponding to internal coding regions of *ygiW* (fragment 1 of 324 bp and fragment 6 of 430 bp), *qseB* (fragment 3 of 428 bp) and *qseC* (fragment 5 of 550 bp) and the control 16S rRNA (fragment of 302 bp) (Fig. 1b and 1c). Products spanning the intergenic regions between *ygiW* and *qseB* (fragment 2 of 373 bp) and between *qseB* and *qseC* (fragment 4 of 423 bp) (Fig. 1b) were also detected, indicating that *ygiW* and *qseBC* are co-transcribed in a single primary transcript and organized in an operon. In addition, products from *ygiW* to *qseB* (fragment 7 of 1101 bp) and from *ygiW* to *qseC* (fragment 8 of 1845 bp) were detected. No amplification products were observed when RT was not added to the transcription reactions (Fig. 1b and 1c).

The promoter region of the *ygiW-qseBC* operon resides within the 372 bp intergenic region upstream of *ygiW*

To map the promoter that directs expression of the *ygiW-qseBC* operon, several DNA fragments encompassing portions of the operon with or without the 372 bp upstream region of *ygiW* were amplified by PCR and cloned into the low copy promoterless *lacZ* plasmid pJT3 (Fig. 2a). The recombinant reporter plasmids and a control vector without a promoter were individually introduced into *A. actinomycetemcomitans* 652 by electroporation, and β-gal activity was determined. All of the recombinant strains exhibited similar growth kinetics (see Fig. 2b). However, as shown in Fig. 2(c), β-gal activity was detected only in *A. actinomycetemcomitans* harbouring pDJR29 which contains the 372 bp intergenic region. However, pDJR32 and pDJR34 expressed approximately ninefold lower β-gal activity than pDJR29. Examination of the 137 bp intergenic region that resides between *ygiW* and *qseB* identified a putative attenuator region (see Fig. S1, available in *Microbiology* Online), consistent with the structure of the *ygiW-firRS* operon recently reported in *Haemophilus influenzae* (Steele *et al.*, 2012). Strains that were transformed with pDJR30, pDJR31, pDJR33 and the promoterless control plasmid pJT3 expressed no measurable β-gal activity. These results indicate that the *ygiW-qseBC* promoter (*P*_{*ygiW*}) resides in the 372 bp region upstream of *ygiW* and that no internal promoters exist in the operon. 5'-RACE mapped the transcriptional start site to 15 bp upstream from the *ygiW* start codon and putative -10 and -35 elements are located at -26 to -31 and -48 to -54, respectively (see Fig. S2). However, *qseBC* transcription is significantly attenuated relative to *ygiW*. Overall, transcriptional activity of the *ygiW* promoter

Fig. 1. Transcriptional analysis of the *ygiW-qseBC* locus. (a) Schematic representation of *ygiW-qseBC* genes on the *A. actinomycetemcomitans* 652 chromosome. Boxes 1, 3, 5, 6, 7 and 8 and thick lines 2 and 4 represent the products obtained from RT-PCR using the primers described in Methods. (b) Ethidium bromide-stained agarose gel showing the RT-PCR products represented by boxes 1, 3 and 5 and thick lines 2 and 4 in (a) obtained using cDNA as the template in the presence (+) or absence (–) of RT. Additional control reactions were conducted using genomic DNA as the template (d) or using primers specific for the 16S rRNA gene. Product sizes are as follows: 1 (324 bp), 2 (373 bp), 3 (428 bp), 4 (423 bp), 5 (550 bp) and 16S RNA (302 bp). (c) Ethidium bromide-stained agarose gel showing the RT-PCR products represented by boxes 6, 7 and 8 in (a) obtained using cDNA as the template in the presence (+) or absence (–) of RT. Additional control reactions were conducted using genomic DNA as the template (d) or using primers specific for the 16S rRNA gene. Product sizes are as follows: 6 (430 bp), 7 (1101 bp), 8 (1845 bp) and 16S RNA (302 bp).

increased approximately 2.5-fold during early and mid-exponential phases of growth and decreased slightly as the culture entered stationary phase.

QseBC are required for expression of the *ygiW-qseBC* operon

To determine if the *ygiW-qseBC* operon is auto-regulated by the QseB response regulator and/or the QseC sensor, *lacZ* expression from pDJR29 and pDJR32 was measured in a series of markerless mutant strains that lacked one or both of the genes. For *qseB*, a non-polar deletion of the structural *qseB* gene (designated $\Delta qseB$) was constructed previously (Juárez-Rodríguez *et al.*, 2013). For *qseC*, two mutants were constructed; one contained an in-frame deletion of the periplasmic domain of the sensor

corresponding to amino acids 37–169 (designated *qseC* Δ pr) and the entire *qseC* gene was deleted in the second. Additionally, a double-deletion mutant that lacks both the *qseB* and *qseC* coding regions was constructed ($\Delta qseBC$). The growth kinetics of each of the mutant strains were similar to the wild-type strain harbouring pDJR29 (Fig. 3a, b). As shown in Fig. 3(c), β -gal activity expressed by $\Delta qseB$ (pDJR29) and *qseC* Δ pr(pDJR29) was significantly reduced relative to the wild-type (25, 26 and 730 Miller units at the 6 h time point, respectively), suggesting that the QseB response regulator is indispensable for the transcriptional activity of P_{ygiW} and that the sensory function of the QseC sensor is required for activation of the response regulator. A similar reduction of β -gal activity was observed in the *qseBC* double-deletion mutant. A similar pattern of expression was observed using

Fig. 2. Transcriptional reporter plasmids. (a) Schematic representation of the fusions of different DNA fragments containing the *ygiW-qseBC* operon to the promoterless *lacZ* gene. (b) Growth of all strains was monitored by measuring OD₆₀₀ at the indicated time intervals. (c) β -Gal activity for each construct is expressed as Miller units and was measured in *A. actinomycetemcomitans* transformed individually with each plasmid and grown in BHI medium as described in Methods. The values are means $\pm$ SD of results from three independent experiments.

pDJR32 (not shown), but overall expression levels were attenuated, which is consistent with the results shown in Fig. 2.

ygiW* influences biofilm formation by *A. actinomycetemcomitans

We previously showed that *A. actinomycetemcomitans* biofilm formation is regulated by AI-2 (Shao *et al.*, 2007a,b) and that *qseBC* may be part of the AI-2 response circuitry (Novak *et al.*, 2010). Because *ygiW* is co-expressed with *qseBC*, we next determined if *ygiW* contributed to biofilm formation. To accomplish this, we compared biofilms formed by the wild-type strain with several isogenic deletion mutants, including $\Delta ygiW$, which contains a non-polar in-frame deletion of 72 nt coding for the *ygiW* start codon, signal peptide and two codons of the mature protein (and thus cannot express YgiW protein): *qseC* Δ pr (described above) and $\Delta qseC$, which lacks the *qseC* structural gene. Representative three-dimensional images of the biofilms rendered by the Volocity software package are shown for

each strain in Fig. 4(a–e), and quantification of biofilm depth, biomass and surface coverage is summarized in Table 3. Interestingly, the in-frame deletion in *ygiW* had little effect on biofilm depth but caused a significant increase in surface coverage relative to the wild-type. Complementation of the mutant with pDJR28 reduced surface coverage back to wild-type levels. Deleting the periplasmic sensor domain of QseC resulted in significant reductions in biofilm depth, biomass and surface coverage, indicating that the sensor domain is essential for optimal biofilm formation by *A. actinomycetemcomitans*. The control $\Delta qseC$ strain also exhibited reduced biofilm biomass and surface coverage, consistent with the phenotype previously reported by Novak *et al.* (2010), who also showed that complementing $\Delta qseC$ with a plasmid-borne copy of *qseC* increased biomass and surface coverage to levels similar to wild-type. Together, these results suggest that both the sensor domain of QseC and a functional YgiW protein regulate biofilm growth of *A. actinomycetemcomitans*.

Fig. 3. β -gal activity of wild-type *A. actinomycetemcomitans* 652 and isogenic ifΔ*ygiW*, Δ*qseB*, Δ*qseBC* and *qseC*Δpr mutants harbouring pDJR29. (a) Graphic representation of the transcriptional reporter plasmids pDJR29. (b) Growth of each strain was monitored by measuring OD₆₀₀ at the indicated time intervals. (c) β -gal activity for each construct is expressed as Miller units and was measured in *A. actinomycetemcomitans* transformed individually with each plasmid and grown in BHI medium as described in Methods. The values are means $\pm$ SD of results from three independent experiments.

DISCUSSION

A. actinomycetemcomitans possesses an AI-2 dependent quorum-sensing system that controls biofilm formation and iron acquisition (Demuth *et al.*, 2011; Fong *et al.*, 2003; Shao *et al.*, 2007b). AI-2 also regulates the expression of *qseC*, which in turn contributes to biofilm growth and controls the expression of at least some of the AI-2-regulated iron acquisition genes (Novak *et al.*, 2010; E. Novak & D. Demuth, unpublished data). However, the link between the detection and importation of AI-2 by LsrB and the Lsr transporter (James *et al.*, 2006; Shao *et al.*, 2007b) and the QseBC two-component system remains to be determined. While expression of the *lsr* operon has been shown to be negatively regulated by the transcriptional regulator LsrR (Li *et al.*, 2007; Torres-Escobar *et al.*, 2013),

LsrR does not appear to affect *qseBC* gene expression (A. Torres-Escobar & D. Demuth, unpublished data). Thus, it was of interest when we recognized an additional potential open reading frame upstream of *qseBC* in the genome sequence of *A. actinomycetemcomitans*, designated D11S_1135 and annotated as *ygiW*, which may be co-transcribed with *qseBC*.

Two independent approaches demonstrated that *ygiW* is co-expressed with *qseBC*. RT-PCR indicated that all three genes were transcribed in a primary transcript, and this was further confirmed by mapping the promoter of the operon to the 372 bp intergenic region (IGR) upstream of *ygiW*. Although an IGR of 137 bp resides between *ygiW* and *qseBC*, a *lacZ* reporter plasmid containing this region expressed no β -gal activity. Similarly, a reporter in which *qseC* was replaced by *lacZ* exhibited no activity. Together, these results demonstrate that no internal promoters exist that are capable of expressing *qseBC* independently of *ygiW*. However, plasmids pDJR32 and pDJR34 containing the 372 bp promoter, the *ygiW* coding sequence and the 137 bp IGR exhibited a roughly ninefold reduction of *lacZ* expression relative to pDJR29. This could be explained by the presence of a putative attenuator stem-loop in the 137 bp IGR. The calculated ΔG for the putative attenuator downstream from *ygiW* is -77.0 KJ/mol, suggesting that it is more stable than the transcriptional attenuators that exist between *glyA* and *ltxC* ($\Delta G = -60.7$ KJ/mol) and *ltxA* and *ltxB* ($\Delta G = -47.7$ KJ/mol) in *A. actinomycetemcomitans*. Thus, as shown in Fig. 5, the overall structure of the *ygiW-qseBC* operon is similar to the recently reported *ygiW-firRS* operon in *H. influenzae* (Steele *et al.*, 2012), but differs from the *qseBC* locus in *E. coli* and *S. Typhimurium*. *E. coli* EHEC, UPEC and *S. Typhimurium* possess *ygiW*, and it also resides immediately upstream from the *qseBC* operon, but the transcriptional orientation of *ygiW* is opposite to that of *qseBC* in these organisms.

Our results also indicated that expression of the *ygiW-qseBC* operon was dependent on the QseB response regulator because transcriptional activity of the *ygiW* promoter was significantly reduced in a *qseB*-deficient background. This is consistent with Merighi *et al.* (2009), who showed that although *ygiW* is not co-transcribed with *qseBC* in *S. Typhimurium*, the *ygiW*-STM3175 operon is positively regulated by QseB. In addition, the transcription of *ygiW* was increased in Δ*qseC* mutants of *E. coli* UPEC (Hadjifrangiskou *et al.*, 2011) and EHEC (Njoroge & Sperandio, 2012), presumably due to dysregulation in QseB activation (Hadjifrangiskou *et al.*, 2011). In contrast, our results showed that deletion of the QseC periplasmic sensory domain resulted in a significant reduction of *ygiW* expression, suggesting that a functional QseC sensor kinase is required for *ygiW* expression in *A. actinomycetemcomitans*. Thus, although *ygiW* expression is related to *qseBC* gene expression in all of these organisms, the organization of the *qseBC* locus and the role of *qseC* in regulating *ygiW* appear to differ.

Fig. 4. Three-dimensional renditions of biofilms formed by wild-type *A. actinomycetemcomitans* 652, an isogenic mutant harbouring an in-frame deletion in *ygiW* (*ifΔygiW*), *ifΔygiW* complemented [*ifΔygiW*(pDJR28)], by deletion mutants lacking *qseC* ($\Delta qseC$) or the sequence encoding the periplasmic sensor domain of *qseC* (*qseC* Δpr). Biofilms were cultured in open-flow cells as described in Methods and analysed using confocal laser scanning microscopy. Image stacks were assembled and analysed with Volocity image analysis software. The yellow scale bars in the *x*-*y*, *x*-*z* and *y*-*z* sections represent 50 μm .

An in-frame deletion of *ygiW* that prevents the production of a functional YgiW protein resulted in a slight increase in biofilm biomass and a significant increase in surface coverage relative to wild-type. This is interesting because deletion of *qseC* or an in-frame deletion of the QseC periplasmic sensory domain resulted in a significant decrease in biofilm formation by *A. actinomycetemcomitans*. Thus, although *ygiW* and *qseBC* are co-expressed, they have opposing effects on biofilm formation. The *ygiW* gene codes for a putative periplasmic protein that is a member of the oligonucleotide/oligosaccharide binding (OB-fold) family of proteins (Ginalski *et al.*, 2004; Murzin, 1993). Although YgiW has been reported to confer resistance to

H₂O₂, cadmium and acid pH stresses in *E. coli* (Lee *et al.*, 2010) and polymyxin B exposure in *Salmonella* (Pilonieta *et al.*, 2009), its mechanism of action and the ligand bound by YgiW have not been identified in these organisms or in *A. actinomycetemcomitans*. Recently, YgiW was designated VisP (virulence- and stress-related periplasmic protein) in *Salmonella* and suggested to bind peptidoglycan and inhibit lipid A modifications mediated by LpxO (Moreira *et al.*, 2013). Our results showing that YgiW regulates biofilm growth and that the periplasmic sensory domain of QseC is required for optimal biofilm growth and expression of the *ygiW-qseBC* operon suggest that at least two independent signals recognized by YgiW and QseC

Table 3. Biofilm growth of *A. actinomycetemcomitans* strains containing in-frame deletions of *ygiW* (*ifΔygiW*) or the periplasmic domain of *qseC* (*qseC* Δpr)

Strain	Average biofilm depth (μm)	Maximum biofilm depth (μm)	Total biomass ($\mu m^3 \times 10^3$)	Total surface coverage ($\mu m^2 \times 10^3$)
652	10.4 $\pm$ 5.1	24.2 $\pm$ 15.1	665.2 $\pm$ 340.1	708.5 $\pm$ 260.1
<i>ifΔygiW</i>	8.4 $\pm$ 1.5	23.2 $\pm$ 5.5	784.9 $\pm$ 135.8	916.9 $\pm$ 84.3†
<i>ifΔygiW</i> /pDJR28	7.1 $\pm$ 1.4	20.0 $\pm$ 8.6	622.8 $\pm$ 49.8	702.9 $\pm$ 50.9
<i>qseC</i> Δpr	6.4 $\pm$ 1.3†	12.6 $\pm$ 4.1*	363.5 $\pm$ 31.4†	452.9 $\pm$ 41.0‡
$\Delta qseC$	7.3 $\pm$ 0.6	16.1 $\pm$ 3.4	236.9 $\pm$ 72.3‡	309.8 $\pm$ 72.6‡

**P*<0.05; †*P*<0.01; ‡*P*<0.001 in comparison with the 652 wild-type strain.

Fig. 5. Genomic organization of the *ygiW-qseBC* locus in *A. actinomycetemcomitans* and related loci in *Pasteurellaceae* species *A. pleuropneumoniae*, *H. influenzae* and *P. multocida* and *Enterobacteriaceae* species *E. coli* and *S. Typhimurium*. Gene transcription orientation is indicated by open arrows.

may control biofilm formation in *A. actinomycetemcomitans*. Clearly, identifying the ligands/signals for YgiW and QseC is important because both proteins may be viable targets for therapeutic intervention to limit biofilm formation by *A. actinomycetemcomitans*, and indeed Rasko *et al.* (2008) have already identified a compound that inhibits virulence by preventing QseC signalling in *E. coli*.

In *E. coli*, QseC responds to autoinducer-3 (AI-3) and adrenergic hormones (Clarke *et al.*, 2006) to regulate virulence factor expression levels and motility. However, the *A. actinomycetemcomitans* genome does not contain many of the *qseBC*-regulated virulence factors or motility components that are present in *E. coli*. Furthermore, we have found no evidence that *A. actinomycetemcomitans* produces AI-3, nor does exposure of the organism to epinephrin result in detectable growth or a biofilm phenotype under the conditions tested. In addition, using pull-down assays and surface plasmon resonance experiments, we found no evidence to suggest that YgiW functions through QseC by interacting with the periplasmic sensor domain (not shown). Recently, Steele *et al.* (2012) reported that the *firRS* two-component system of *H. influenzae*, which represents the *qseBC* homologue in this organism, responds to ferrous iron and zinc. Interestingly, *qseBC* regulates the expression of several ferric uptake transporters in *A. actinomycetemcomitans* (E. Novak & D. Demuth, unpublished data), suggesting that iron may also serve as a signal for *A. actinomycetemcomitans* *qseC*. We are currently determining the complete transcriptional profiles of the *qseC* and *ygiW* mutants to gain insight into their mechanism of action.

ACKNOWLEDGEMENTS

This research was supported by the National Institutes of Health (grant no. RO1DE14605).

REFERENCES

- Amarasinghe, J. J., Scannapieco, F. A. & Haase, E. M. (2009). Transcriptional and translational analysis of biofilm determinants of *Aggregatibacter actinomycetemcomitans* in response to environmental perturbation. *Infect Immun* **77**, 2896–2907.
- Bearson, B. L. & Bearson, S. M. (2008). The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of *Salmonella enterica* serovar Typhimurium. *Microb Pathog* **44**, 271–278.
- Brogan, J. M., Lally, E. T. & Demuth, D. R. (1996). Construction of pYGK, an *Actinobacillus actinomycetemcomitans*-*Escherichia coli* shuttle vector. *Gene* **169**, 141–142.
- Clarke, M. B. & Sperandio, V. (2005). Transcriptional regulation of *flhDC* by QseBC and sigma (FliA) in enterohaemorrhagic *Escherichia coli*. *Mol Microbiol* **57**, 1734–1749.
- Clarke, M. B., Hughes, D. T., Zhu, C., Boedeker, E. C. & Sperandio, V. (2006). The QseC sensor kinase: a bacterial adrenergic receptor. *Proc Natl Acad Sci U S A* **103**, 10420–10425.
- Demuth, D. R., Novak, E. A. & Shao, H. (2011). Alternative autoinducer-2 quorum sensing response circuits: impact on microbial community development. In *Oral Microbial Communities: Genomic Inquiry and Interspecies Communication*, pp. 263–282. Edited by P. E. Kolenbrander. Washington, DC: American Society for Microbiology.
- Fine, D. H., Kaplan, J. B., Furgang, D., Karched, M., Velliyagounder, K. & Yue, G. (2010). Mapping the epithelial-cell-binding domain of the *Aggregatibacter actinomycetemcomitans* autotransporter adhesin Aae. *Microbiology* **156**, 3412–3420.

- Fong, K. P., Chung, W. O., Lamont, R. J. & Demuth, D. R. (2001). Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. *Infect Immun* **69**, 7625–7634.
- Fong, K. P., Gao, L. & Demuth, D. R. (2003). *luxS* and *arcB* control aerobic growth of *Actinobacillus actinomycetemcomitans* under iron limitation. *Infect Immun* **71**, 298–308.
- Ginalski, K., Kinch, L., Rychlewski, L. & Grishin, N. V. (2004). BOF: a novel family of bacterial OB-fold proteins. *FEBS Lett* **567**, 297–301.
- Hadjifrangiskou, M., Kostakioti, M., Chen, S. L., Henderson, J. P., Greene, S. E. & Hultgren, S. J. (2011). A central metabolic circuit controlled by QseC in pathogenic *Escherichia coli*. *Mol Microbiol* **80**, 1516–1529.
- Haffajee, A. D. & Socransky, S. S. (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontol* **2000** **5**, 78–111.
- Henderson, B., Nair, S. P., Ward, J. M. & Wilson, M. (2003). Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. *Annu Rev Microbiol* **57**, 29–55.
- Hughes, D. T., Clarke, M. B., Yamamoto, K., Rasko, D. A. & Sperandio, V. (2009). The QseC adrenergic signaling cascade in enterohemorrhagic *E. coli* (EHEC). *PLoS Pathog* **5**, e1000553.
- Hyvärinen, K., Mäntylä, P., Buhlin, K., Paju, S., Nieminen, M. S., Sinisalo, J. & Pussinen, P. J. (2012). A common periodontal pathogen has an adverse association with both acute and stable coronary artery disease. *Atherosclerosis* **223**, 478–484.
- James, D., Shao, H., Lamont, R. J. & Demuth, D. R. (2006). The *Actinobacillus actinomycetemcomitans* ribose binding protein RbsB interacts with cognate and heterologous autoinducer 2 signals. *Infect Immun* **74**, 4021–4029.
- Jinadasa, R. N., Bloom, S. E., Weiss, R. S. & Duhamel, G. E. (2011). Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. *Microbiology* **157**, 1851–1875.
- Juárez-Rodríguez, M. D., Torres-Escobar, A. & Demuth, D. R. (2013). Construction of new cloning, *lacZ* reporter and scarless-markerless suicide vectors for genetic studies in *Aggregatibacter actinomycetemcomitans*. *Plasmid* **69**, 211–222.
- Kachlany, S. C. (2010). *Aggregatibacter actinomycetemcomitans* leukotoxin: from threat to therapy. *J Dent Res* **89**, 561–570.
- Khajanchi, B. K., Kozlova, E. V., Sha, J., Popov, V. L. & Chopra, A. K. (2012). The two-component QseBC signalling system regulates in vitro and in vivo virulence of *Aeromonas hydrophila*. *Microbiology* **158**, 259–271.
- Kostakioti, M., Hadjifrangiskou, M., Pinkner, J. S. & Hultgren, S. J. (2009). QseC-mediated dephosphorylation of QseB is required for expression of genes associated with virulence in uropathogenic *Escherichia coli*. *Mol Microbiol* **73**, 1020–1031.
- Lee, J., Hiibel, S. R., Reardon, K. F. & Wood, T. K. (2010). Identification of stress-related proteins in *Escherichia coli* using the pollutant cis-dichloroethylene. *J Appl Microbiol* **108**, 2088–2102.
- Li, J., Attila, C., Wang, L., Wood, T. K., Valdes, J. J. & Bentley, W. E. (2007). Quorum sensing in *Escherichia coli* is signaled by AI-2/LsrR: effects on small RNA and biofilm architecture. *J Bacteriol* **189**, 6011–6020.
- Merighi, M., Septer, A. N., Carroll-Portillo, A. M., Bhatiya, A., Porwollik, S., McClelland, M. & Gunn, J. S. (2009). Genome-wide analysis of the PreA/PreB (QseB/QseC) regulon of *Salmonella enterica* serovar Typhimurium. *BMC Microbiol* **9**, 42.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moreira, C. G., Weinshenker, D. & Sperandio, V. (2010). QseC mediates *Salmonella enterica* serovar typhimurium virulence in vitro and in vivo. *Infect Immun* **78**, 914–926.
- Moreira, C. G., Herrera, C. M., Needham, B. D., Parker, C. T., Libby, S. J., Fang, F. C., Trent, M. S. & Sperandio, V. (2013). Virulence and stress-related periplasmic protein (VisP) in bacterial/host associations. *Proc Natl Acad Sci U S A* **110**, 1470–1475.
- Murzin, A. G. (1993). OB(oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences. *EMBO J* **12**, 861–867.
- Njoroge, J. & Sperandio, V. (2012). Enterohemorrhagic *Escherichia coli* virulence regulation by two bacterial adrenergic kinases, QseC and QseE. *Infect Immun* **80**, 688–703.
- Nørskov-Lauritsen, N. & Kilian, M. (2006). Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int J Syst Evol Microbiol* **56**, 2135–2146.
- Novak, E. A., Shao, H., Daep, C. A. & Demuth, D. R. (2010). Autoinducer-2 and QseC control biofilm formation and in vivo virulence of *Aggregatibacter actinomycetemcomitans*. *Infect Immun* **78**, 2919–2926.
- Paturel, L., Casalta, J. P., Habib, G., Nezri, M. & Raoult, D. (2004). *Actinobacillus actinomycetemcomitans* endocarditis. *Clin Microbiol Infect* **10**, 98–118.
- Perez, B. A., Planet, P. J., Kachlany, S. C., Tomich, M., Fine, D. H. & Figurski, D. H. (2006). Genetic analysis of the requirement for *flp-2*, *tadV*, and *rcpB* in *Actinobacillus actinomycetemcomitans* biofilm formation. *J Bacteriol* **188**, 6361–6375.
- Pilonieta, M. C., Erickson, K. D., Ernst, R. K. & Detweiler, C. S. (2009). A protein important for antimicrobial peptide resistance, Ydel/OmdA, is in the periplasm and interacts with OmpD/NmpC. *J Bacteriol* **191**, 7243–7252.
- Rahamat-Langendoen, J. C., van Vonderen, M. G., Engström, L. J., Manson, W. L., van Winkelhoff, A. J. & Mooi-Kokenberg, E. A. (2011). Brain abscess associated with *Aggregatibacter actinomycetemcomitans*: case report and review of literature. *J Clin Periodontol* **38**, 702–706.
- Rasko, D. A., Moreira, C. G., Li, R., Reading, N. C., Ritchie, J. M., Waldor, M. K., Williams, N., Taussig, R., Wei, S. & other authors (2008). Targeting QseC signaling and virulence for antibiotic development. *Science* **321**, 1078–1080.
- Reading, N. C., Torres, A. G., Kendall, M. M., Hughes, D. T., Yamamoto, K. & Sperandio, V. (2007). A novel two-component signaling system that activates transcription of an enterohemorrhagic *Escherichia coli* effector involved in remodeling of host actin. *J Bacteriol* **189**, 2468–2476.
- Saito, T., Ishihara, K. M., Ryu, M., Okuda, K. & Sakurai, K. (2010). Fimbriae-associated genes are biofilm-forming factors in *Aggregatibacter actinomycetemcomitans* strains. *Bull Tokyo Dent Coll* **51**, 145–150.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Shao, H., Lamont, R. J. & Demuth, D. R. (2007a). Autoinducer 2 is required for biofilm growth of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. *Infect Immun* **75**, 4211–4218.
- Shao, H., James, D., Lamont, R. J. & Demuth, D. R. (2007b). Differential interaction of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* LsrB and RbsB proteins with autoinducer 2. *J Bacteriol* **189**, 5559–5565.
- Sperandio, V., Torres, A. G. & Kaper, J. B. (2002). Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component

regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. *Mol Microbiol* **43**, 809–821.

Steele, K. H., O'Connor, L. H., Burpo, N., Kohler, K. & Johnston, J. W. (2012). Characterization of a ferrous iron-responsive two-component system in nontypeable *Haemophilus influenzae*. *J Bacteriol* **194**, 6162–6173.

Torres-Escobar, A., Juárez-Rodríguez, M. D. & Curtiss, R., III (2010). Biogenesis of *Yersinia pestis* PsaA in recombinant attenuated *Salmonella* Typhimurium vaccine (RASV) strain. *FEMS Microbiol Lett* **302**, 106–113.

Torres-Escobar, A., Juárez-Rodríguez, M. D., Lamont, R. J. & Demuth, D. R. (2013). Transcriptional regulation of *Aggregatibacter actinomycetemcomitans* *lsrACDBFG* and *lsrRK* operons and their role in biofilm formation. *J Bacteriol* **195**, 56–65.

Wang, C. Y., Wang, H. C., Li, J. M., Wang, J. Y., Yang, K. C., Ho, Y. K., Lin, P. Y., Lee, L. N., Yu, C. J. & other authors (2010). Invasive infections of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. *J Microbiol Immunol Infect* **43**, 491–497.

Wang, X., Wang, Q., Yang, M., Xiao, J., Liu, Q., Wu, H. & Zhang, Y. (2011). QseBC controls flagellar motility, fimbrial hemagglutination and intracellular virulence in fish pathogen *Edwardsiella tarda*. *Fish Shellfish Immunol* **30**, 944–953.

Yue, G., Kaplan, J. B., Furgang, D., Mansfield, K. G. & Fine, D. H. (2007). A second *Aggregatibacter actinomycetemcomitans* autotransporter adhesin exhibits specificity for buccal epithelial cells in humans and Old World primates. *Infect Immun* **75**, 4440–4448.

Edited by: R. Palmer