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Transcriptional regulation of the *Aggregatibacter actinomycetemcomitans ygiW–qseBC* operon by QseB and integration host factor proteins

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The QseBC two-component system plays a pivotal role in regulating virulence and biofilm growth of the oral pathogen *Aggregatibacter actinomycetemcomitans*. We previously showed that QseBC autoregulates the *ygiW–qseBC* operon. In this study, we characterized the promoter that drives *ygiW–qseBC* expression. Using *lacZ* transcriptional fusion constructs and 5′-rapid amplification of cDNA ends, we showed that *ygiW–qseBC* expression is driven by a promoter that initiates transcription 53 bases upstream of *ygiW* and identified putative *cis*-acting promoter elements, whose function was confirmed using site-specific mutagenesis. Using electrophoretic mobility shift assays, two *trans*-acting proteins were shown to interact with the *ygiW–qseBC* promoter. The QseB response regulator bound to probes containing the direct repeat sequence CTTAA-N6-CTTAA, where the CTTAA repeats flank the −35 element of the promoter. The *ygiW–qseBC* expression could not be detected in *A. actinomycetemcomitans* Δ*qseB* or Δ*qseBC* strains, but was restored to WT levels in the Δ*qseBC* mutant when complemented by single copy chromosomal insertion of *qseBC*. Interestingly, *qseB* partially complemented the Δ*qseBC* strain, suggesting that QseB could be activated in the absence of QseC. QseB activation required its phosphorylation since complementation did not occur using $qseB^{pho-}$, encoding a protein with the active site aspartate substituted with alanine. These results suggest that QseB is a strong positive regulator of *ygiW–qseBC* expression. In addition, integration host factor (IHF) bound to two sites in the promoter region and an additional site near the 5′ end of the *ygiW* ORF. The expression of *ygiW–qseBC* was increased by twofold in Δ*ihfA* and Δ*ihfB* strains of *A. actinomycetemcomitans*, suggesting that IHF is a negative regulator of the *ygiW–qseBC* operon.

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INTRODUCTION

Periodontitis is a disease produced by a dental biofilm in association with anaerobic pathogenic bacteria. This severe infection causes a chronic inflammation of the gingival tissues, which leads to progressive loss of the alveolar bone that supports the teeth. Untreated periodontitis will eventually result in tooth loss. *Aggregatibacter actinomycetemcomitans* is a Gram-negative, facultative anaerobe that is associated with aggressive forms of periodontitis and other human infections including endocarditis and systemic infections (Haffajee & Socransky, 1994; Paturel *et al.*, 2004; Wang *et al.*, 2010; Murakami *et al.*, 2013; Brady *et al.*, 2014). *A. actinomycetemcomitans* expresses a variety of virulence factors that mediate colonization of oral tissues and biofilm formation, such as the autotransporter adhesins Aae and Api, outer-membrane protein Omp29, and the *tad* fimbriae (Fine *et al.*, 2010; Yue *et al.*, 2007; Kajiya *et al.*, 2011; Saito *et al.*, 2010). In addition, expression of the leukotoxin (LtxA) and cytolethal distending toxin (CdtABC) contribute to inflammation, tissue destruction and inactivation of the host immune response (Dietmann *et al.*, 2013; Fernandes *et al.*, 2008; Jinadasa *et al.*, 2011). *A. actinomycetemcomitans* biofilm formation, iron acquisition and virulence are also regulated by a quorum-sensing system that responds to autoinducer AI-2 (Demuth *et al.*, 2011; Fong *et al.*, 2001, 2003). The QseBC two-component system is part of this quorum-sensing system and is regulated by AI-2 (Novak *et al.*, 2010). In *A. actinomycetemcomitans*, the *qseBC* genes are co-expressed with *ygiW*, which codes for a polypeptide of unknown function in the OB-fold family of proteins and the

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Abbreviations: 5′-RACE, 5′-rapid amplification of cDNA ends; β-Gal, β-galactosidase; EMSA, electrophoretic mobility shift assay; Km, kanamycin; qRT-PCR, quantitative real-time PCR; UPEC, uropathogenic *Escherichia coli*.

Two supplementary tables and two supplementary figures are available with the online Supplementary Material.

operon is transcribed from a promoter that resides upstream of *ygiW* (Juárez-Rodríguez *et al.*, 2013a).

The QseBC two-component system is present in several Gram-negative pathogens, and in *Escherichia coli*; it is a key component for intercellular and interkingdom communication by sensing both autoinducer-3 (AI-3) and host catecholamine hormones to regulate virulence gene expression (Clarke *et al.*, 2006). Activation of QseC initiates the transcription of *flhDC* genes that encode the master regulator for the flagella and motility genes (Sperandio *et al.*, 2002; Clarke & Sperandio, 2005), and genes encoding the FucRS two-component system for sensing of fucose in the host intestine (Pacheco *et al.*, 2012). Furthermore, QseC can activate non-cognate response regulators KdpE and QseF that are involved in the formation of the attaching and effacing lesions by enterohaemorrhagic *E. coli* on epithelial cells (Hughes *et al.*, 2009). QseBC also has an important role in the pathogenesis of uropathogenic *E. coli* (UPEC) (Kostakioti *et al.*, 2009). In UPEC, QseC has been reported to function as both a kinase and phosphatase activity that modulates QseB phosphorylation. This is important since QseB is also phosphorylated by the PmrB histidine kinase (Kostakioti *et al.*, 2009; Guckes *et al.*, 2013). Deletion of *qseC* in UPEC causes an aberrant upregulation and overactivation of QseB by PmrB, since QseB cannot be dephosphorylated in the mutant. This leads to pleiotropic effects, such as the misregulation of nucleotide, amino acid and carbon metabolism, suggesting QseC controls the bacterial central metabolic circuit (Hadjifrangiskou *et al.*, 2011).

In *Salmonella*, the QseC and QseE sensors are part of the AI-3/adrenaline/noradrenaline signalling system, which influences *Salmonella* serovar Typhimurium pathogenesis (Moreira *et al.*, 2010) by regulating pathogenicity island 1 (SPI-1) genes responsible for the invasion of epithelial cells (Moreira & Sperandio, 2012). QseC has also been implicated in the regulation of flagellar genes in *Salmonella* (Bearson & Bearson 2008; Moreira *et al.*, 2010), but this was not observed by Merighi *et al.* (2009). This discrepancy has been suggested to arise from the labile nature of the adrenaline/noradrenaline signals *in vitro* (Moreira *et al.*, 2010; Moreira & Sperandio, 2012).

The QseBC system has also been found in other pathogens such as *Edwardsiella tarda* (Wang *et al.*, 2011), *Aeromonas hydrophila* (Khajanchi *et al.*, 2012) and *Haemophilus influenzae* (Steele *et al.*, 2012), and it plays an important role in the pathogenic processes of these bacteria. However, the *H. influenzae* QseBC system is induced by ferrous iron, but differs from *E. coli* since it is unresponsive to adrenaline or noradrenaline (Steele *et al.*, 2012). *A. actinomycetemcomitans* is a non-motile organism and lacks many of the genes that are regulated by QseBC in *E. coli* and *Salmonella*. Yet *A. actinomycetemcomitans* QseBC clearly plays a role in virulence, since deletion of *qseC* dramatically reduces biofilm growth and leads to a virulence attenuated phenotype (Novak *et al.*, 2010; Juárez-Rodríguez *et al.*, 2013a). This suggests that the function of QseBC in *A. actinomycetemcomitans* may differ from that in *E. coli* and other enteric organisms. The QseBC regulon in *A. actinomycetemcomitans* is currently unknown.

In addition, the expression of several genes encoding major virulence factors of *A. actinomycetemcomitans*, e.g. the leukotoxin, is also controlled by integration host factor (IHF) (Kolodrubetz *et al.*, 2010). IHF is a DNA-binding and -bending protein that recognizes the asymmetrical consensus sequence YAANNNNTTGATW, where Y=T or C, *N*=any base and W=A or T (Craig & Nash, 1984; Friedman, 1988). IHF concentration increases throughout growth and becomes the second most abundant nucleoid protein during the stationary phase (Azam *et al.*, 1999; Ishihama, 1999). Functionally, it plays many roles and is involved in the organization of the chromosomal DNA, DNA replication, DNA recombination and transcriptional regulation in Gram-negative bacteria. IHF is also implicated in the transcriptional regulation of genetic loci associated with virulence in many pathogenic bacteria (Mangan *et al.*, 2006; Blomfield *et al.*, 1997).

In this paper, we have characterized the promoter that drives the expression of *qseBC* in *A. actinomycetemcomitans*, and show that it is regulated by both QseB and IHF. The QseB response regulator is required for transcription of the operon and it is primarily activated by QseC sensor kinase. However, QseB may also be partially activated by other mechanism(s) in the absence of its cognate sensor kinase. QseB binds to a direct repeat sequence in the *ygiW–qseBC* promoter that resembles the PmrA DNA-binding site in *E. coli* and *Salmonella*. Three IHF binding sites were identified, two of them located in the *ygiW–qseBC* promoter region and the third motif in the *ygiW* ORF. In contrast to QseB, IHF binding negatively regulates the *ygiW–qseBC* operon.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table S1 (available in the online Supplementary Material). Luria–Bertani (LB) broth, LB agar [LB broth plus 1.5 % (w/v) agar], TYE broth [1 % (w/v) tryptone, 0.5 % (w/v) yeast extract (DIFCO)], brain heart infusion (BHI) broth, and BHI agar (all from DIFCO) were routinely used for the propagation and plating of bacteria. *A. actinomycetemcomitans* (afimbriated, smooth-colony-morphotype strain 652) was grown at 37 °C under microaerophilic conditions in a candle jar. When required, the medium was supplemented with 25 μg kanamycin (Km) ml^{-1}, 12.5 μg tetracycline (Tc) ml^{-1}, 50 μg spectinomycin ml^{-1} or 100 μg ampicillin ml^{-1}.

DNA procedures. DNA manipulations were carried out as described elsewhere (Sambrook & Russell, 2001). Transformation of *E. coli* and *A. actinomycetemcomitans* was carried out by electroporation (Bio-Rad). Transformants containing plasmids were selected on LB 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 (Integrated DNA Technology) are shown in Table S2 and restriction enzyme sites

are underlined in the primer sequences. Primer sequences were designed based on the genome information of *A. actinomycetemcomitans* D11S-1 strain available from the PathoSystems Resource Integration Center (www.patricbrc.org). All constructs were verified by DNA sequencing (University of Louisville Core Sequencing Facilities).

Identification of *ygiW–qseBC* transcriptional start sites. The transcriptional start sites of *ygiW–qseBC* were determined using a GeneRacer kit (Invitrogen). *A. actinomycetemcomitans* 652 was grown in BHI broth to exponential phase (OD_{600} 0.3). This culture was treated with Qiagen RNAprotect bacterial reagent and total RNA was extracted using RNeasy Lipid Tissue Mini kit (Qiagen) as specified by the manufacturer. RNA was treated with RNase-free DNase I (New England Biolabs) to eliminate contaminating DNA. RNA was quantified by spectrophotometry using a Nanodrop ND-1000 (Thermo Fisher Scientific). A 44 base 5′ RNA adaptor oligonucleotide was ligated to the 5′ ends of the total RNA (5 μg) using T4 RNA ligase. Reverse transcription was performed with Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) and random hexamers that were included in the GeneRacer kit. PCR was performed using the GeneRacer 5′ primer and *ygiW*-specific primers MDJR-57R and MDJR-94R. The PCR products were subsequently cloned into a pCR4-TOPO vector and used to transform *E. coli* Top10 (Invitrogen). Twenty independent positive clones were sequenced and analysed for the *ygiW–qseBC* promoter(s).

Quantitative real-time PCR (qRT-PCR). For qRT-PCR, total RNA was obtained as described above, and DNA was synthetized by using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems by Life Technologies) according to the manufacturer's instructions. Real-time PCR amplification of the cDNA was performed with SYBR Selected master mix (Applied Biosystems by Life Technologies), as specified by manufacturer, and primer pairs (Table S2) in a total volume of 20 μl. Reactions were performed in triplicate for each primer pair in an Applied Biosystems 7500 real-time PCR system, using the default thermal cycle conditions as follows: 1 cycle of 50 °C for 2 min for uracil-DNA glycosylase activation; then 1 cycle of 95 °C for 10 min for hot-start AmpliTaq Gold polymerase activation; followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s; and including at the end a dissociation stage of 1 cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Relative expression levels of the specific transcripts were calculated using the 16S rRNA expression level as the internal reference for normalization, and expressed as fold-differences by calculating $2^{-\Delta\Delta Ct}$ (Winer *et al.*, 1999). Differences in the expression levels of the target genes between the WT strain and deletion mutant strains were analysed by using ANOVA followed by Tukey's post test.

Construction of *ygiW–qseBC* promoter/*lacZ* fusion plasmids. Various fragments containing portions of or the entire 372 bp promoter region of *ygiW–qseBC* were amplified by PCR 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 20 s, 60 °C for 30 s and 72 °C for 2 min for 25 cycles. For the *ygiW–qseBC* promoter fusion constructs (pDJR55, pDJR56, pDJR57, pDJR63 and pDJR58), portions of the 372 bp regulatory region were amplified using the primer sets MDJR-142F/MDJR-70R, MDJR-143F/MDJR-70R, MDJR-154F/MDJR-70R, MDJR-157F/MDJR-70R and MDJR-176F/MDJR-70R, respectively (see Table S2). The 338, 172, 157, 119 and 128 bp PCR products were then digested with *Kpn*I–*Bam*HI and cloned independently into *Kpn*I–*Bam*HI-digested pJT3 (Juárez-Rodríguez *et al.*, 2013b) to create pDJR55, pDJR56, pDJR57, pDJR58 and pDJR63, respectively.

Site-directed mutagenesis. Mutagenesis of the putative −10 element (TATAAG to **GCCGC**G) and −35 element (CAACAAA to **GCAG**AA) of *ygiW–qseBC*-P1 was carried out by annealing primers MDJR-158F/MDJR-159R and cloning the resulting double-stranded fragment into *Kpn*I–*Bam*HI-digested pJT3 to generate pDJR60. For mutation in *ygiW–qseBC*-P2, primer pair MDJR-274F/MDJR-70R was used to introduce mutations into the QseB binding site (CTTAATACTTTCTTAAC to C**GCG**ATACTTTCTTAAC) by PCR. The resulting product was then digested with *Kpn*I–*Bam*HI and cloned into pJT3 to generate pDJR86. Mutations in the *ygiW–qseBC*-P2 −10 element (TAATAG to **GCGCG**G) were introduced using primer pair MDJR-175F/MDJR-70, and after verifying the correct base changes by DNA sequencing, the resulting amplicon was used as a template with primer pair MDJR-273F/MDJR-70. The resulting product was digested with *Kpn*I–*Bam*HI and cloned into pJT3 to create pDJR85. Similarly, primer pair MDJR-178/MDJR-70 was used to introduce mutations in both the −10 element and the QseB binding site of *ygiW–qseBC*-P2 by PCR to produce pDJR76. All mutations were confirmed by DNA sequencing.

The QuikChange site-directed mutagenesis kit (Stratagene) was used to alter the codon (GAT to GCG) encoding the conserved active site aspartate residue in QseB (D51) to an alanine residue using primers MDJR-99F/MDJR-100R. The mutations introduced into *qseB* were verified by sequencing in the resulting plasmid pDJR28-M1.

Single copy chromosomal complementation of *A. actinomycetemcomitans* Δ*qseBC*. Integration of genes in single copy into the *A. actinomycetemcomitans* Δ*qseBC* genome was carried out as previously described (Torres-Escobar *et al.*, 2014). To integrate a single copy of *qseB*, the *qseB* mutant allele (*qseB*$^{pho-}$) encoding the $QseB_{D51A}$ mutant protein, *qseBC* or *qseB*$^{pho-}$*qseC* independently into the chromosome of *A. actinomycetemcomitans* Δ*qseBC* strain by homologous recombination, the corresponding *qseB* or *qseBC* fragments together with *ygiW* were amplified by PCR from pDJR28 and pDJR28-M1, respectively, with the primer sets MDJR-124F/MDJR-125R (for *qseBC* constructs) or MDJR124F/MDJR/126R (for *qseB* constructs). The 3135 and 1645 bp PCR fragments were digested with *Not*I–*Pst*I and cloned into *Not*I–*Pst*I-digested suicide vector pJT1 to generate the plasmids pDJR45, pDJR46, pDJR47 and pDJR48. Each recombinant suicide plasmid (20 μg) was introduced individually into *A. actinomycetemcomitans* isogenic Δ*qseBC* mutant by electroporation. Ten Sp^r colonies were selected to verify by PCR the single-copy chromosomal insertion of *qseB* or *qseBC*, or *qseB*$^{pho-}$ or *qseB*$^{pho-}$*qseC*. Verification was carried out using primer sets MDJR-63F/MDJR-61R and MDJR-54F/MDJR-77R. The Sp^r colonies of *A. actinomycetemcomitans* Δ*qseBC*::*qseB*$^{pho-}$*qseC*, Δ*qseBC*::*qseBC*, Δ*qseBC*::*qseB*$^{pho-}$ and Δ*qseBC*::*qseB* that were PCR positive were selected for electroporation with the pDJR29 promoter/*lacZ* fusion plasmid.

Growth kinetics. A single colony of *A. actinomycetemcomitans* harbouring each recombinant plasmid was independently inoculated into 10 ml BHI broth supplemented with 25 μg Km ml^{-1} and was grown standing for 24 h at 37 °C. The next day, the overnight culture (OD_{600} 0.6) was diluted at a 1 : 30 ratio and used to inoculate 10 ml BHI broth with 25 μg Km ml^{-1} and grown standing at 37 °C. For the first 12 h of growth, an aliquot was removed each hour and culture density was determined by measuring the OD_{600}. Additional aliquots were taken from each culture for analysis at different time points. β-Galactosidase (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 X-Gal ml^{-1}. Quantitative evaluation of β-Gal activity was carried out using permeabilized cells incubated with ONPG substrate (Sigma) as described by Miller (1972). Mean value (±SDs) for activity units were routinely calculated from three independent assays.

Construction of *qseB* expression plasmid. The structural *qseB* gene was amplified from *A. actinomycetemcomitans* genomic DNA

using primer sets MDJR-80F and MDJR-81R, and the resulting 710 bp fragment was digested with *Nco*I–*Apa*I and cloned into *Nco*I–*Apa*I-digested pYA3883 (Torres-Escobar *et al.*, 2010) expression vector to create pDJR36.

Expression and purification of QseB protein. *E. coli* LMG194 harbouring pDJR36 was used for the synthesis of the hexahistidine fusion QseB protein. The expression and detection of the recombinant protein was performed essentially as described previously (Torres-Escobar *et al.*, 2010). Purification was carried out by cobalt-based immobilized metal affinity chromatography under denaturing conditions. Briefly, cells from a 200 ml culture were harvested and frozen at −70 °C overnight. The cell pellet was thawed and suspended in lysis buffer (6M guanidine hydrochloride, 100 mM NaH_2PO_4, 10 mM Tris/HCl, pH 8), and lysed by homogenization. Lysed cells were centrifuged and the lysate was loaded onto HisPur cobalt resin (Thermo Scientific) for 1 h with shaking at 4 °C. The resin was washed with lysis buffer and sequentially washed with wash buffer containing 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris/HCl pH 8, 6.3 and 5.9. Protein was then eluted with elution buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris/HCl, pH 4.5). Eluted fractions containing the purified QseB were selected based on SDS-PAGE analysis. The QseB selected pools were dialysed in a Slyde-A-Lyser 3K cassette (Pierce) at 4 °C against refolding buffer containing 50 mM Tris/HCl (pH 8.5), 4 M urea, 0.5 M L-arginine, 264 mM NaCl, 11 mM KCl, 8 mM $MgCl_2$, 0.1 % (v/v) Triton X-100, 20 % (v/v) glycerol for 24 h. Subsequently, samples were dialysed for 24 h against the buffer described above but containing 2 M urea, and then sequentially dialysed for 12 h each in buffer consisting of 50 mM Tris/HCl (pH 8.5), 100 mM NaCl, 0.1 mM KCl, 4 mM $MgCl_2$, 2 mM $Mg(CH_3COO)_2$, 0.1 mM EDTA, 0.1 mM DTT, 30 % (v/v) glycerol, and 2, 1 or 0.5 M urea. A final dialysis was then carried out for 6 h against the buffer described above without urea. Aliquots of the QseB purified protein were stored at −70 °C. Protein concentration was determined by the Bradford assay, using BSA as a standard.

Electrophoretic mobility shift assay (EMSA). The DNA fragments used for nonradioactive EMSA were obtained by PCR using the sets of primers described in Table S2. A biotin 3′ end labelling kit (Thermo Scientific) was used for labelling of DNA fragments according to the manufacturer's instructions. Binding reactions were performed with a total of 50 fmol each probe mixed with 4 μM or various amounts (2, 4, 6 and 8 μM) QseB protein, or 4 μM IHF protein purified previously (Torres-Escobar *et al.*, 2014), in 20 μl binding buffer consisting of 10 mM Tris/HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 μg poly (dI–dC) and 100 μg BSA ml^{-1}. The reactions were incubated for 20 min at room temperature. Afterward, 5 μl gel loading buffer [0.25 × Tris/borate-EDTA (TBE), 60 % (v/v); glycerol, 40 % (v/v); bromophenol, 0.2 % (w/v)] was added and mixtures were electrophoresed in a 6 % native polyacrylamide gel in 0.5 × TBE buffer (45 mM Tris/borate, 1 mM EDTA, pH 8.0) and immunoblotted. DNA bands were detected using the LightShift chemiluminescent EMSA kit according to the manufacturer's instructions. For competitive EMSA, the specific competitor (unlabelled fragment III) was added in increasing amounts (0.5-, 5-, 10- and 20-fold molar excesses) to the EMSA reaction containing the biotin-labelled fragment III and 4 μM QseB as specified above.

RESULTS

Identification of the *ygiW–qseBC* operon promoter

Our previous work showed that *qseBC* is co-expressed with the upstream *ygiW* gene in *A. actinomycetemcomitans* and that the promoter that drives expression of the operon resides in the 372 bp *fdhE–ygiW* intergenic region (Juárez-Rodríguez *et al.*, 2013a). To localize the putative *ygiW–qseBC* promoter, DNA fragments encompassing portions of the intergenic region were introduced into a promoterless *lacZ* reporter plasmid pJT3 (see Fig. 1a) and β-Gal activity was determined. As shown in Fig. 1(c), pDJR55 and pDJR56, in which nucleotides −307 to −372 and −139 to −372 were deleted, respectively, exhibited similar β-Gal activity as pDJR29, indicating that no regulatory elements exist upstream from nucleotide −138. However, β-Gal activity was reduced by approximately 3-fold in pDJR63 when nucleotides −94 to −372 were deleted and a 78-fold reduction in activity occurred upon deletion of nucleotides −85 to −372. This suggests that regulatory elements responsible for *ygiW–qseBC* expression reside between −84 and −138.

Previously, a transcriptional start site (TSS_1) was mapped to nucleotide −15 upstream of the *ygiW* start codon and putative −10 and −35 elements were identified at nucleotides −26 to −31 and −48 to −54, respectively (Juárez-Rodríguez *et al.*, 2013a). For this study, this putative promoter was designated *ygiW–qseBC*-P1. However, the results in Fig. 1 clearly show that an additional promoter or regulatory element(s) exists between nucleotides −85 to −138, upstream from the putative −10 and −35 elements that were previously identified. To further characterize these regulatory element(s), 5′-rapid amplification of cDNA ends (5′-RACE) was repeated and a 10-fold higher number of clones was analysed. As shown in Table 1, TSS_1 was again identified, but two additional putative start sites at nucleotides −21 and −53 (TSS_2 and TSS_3, respectively) were also identified. TSS_1 and TSS_2 represented 10 and 20 % of the positive clones identified by 5′-RACE, respectively. The main start site, TSS_3, mapped to nucleotide −53 and represented 70 % of the positive clones identified by 5′-RACE. Associated with TSS_3 are putative −10 and −35 elements located at nucleotides −59 to −64 and −83 to −88, respectively, and this putative promoter was designated *ygiW–qseBC*-P2. Consistent with this, a significant decrease in β-Gal activity was observed when nucleotides −85 to −93 were deleted (compare pDJR63 and pDJR58 in Fig. 1c) which disrupts the −35 element of *ygiW–qseBC*-P2 (nucleotides −83 to −88).

To further confirm that the regulatory elements in *ygiW–qseBC*-P2 are functional, the putative −10 element was altered by site-specific mutagenesis (TAATAG changed to **GCGCG**G) to generate pDJR85 (Fig. 1b). As shown in Fig. 1(c), this construct exhibited a 57-fold reduction in β-Gal activity. Finally, to determine whether *ygiW–qseBC*-P1 is functional, mutations were introduced into the putative −10 and −35 elements (see pDJR60 in Fig. 1b). As shown in Fig. 1(c), these mutations did not significantly reduce β-Gal activity (compare pDJR60 with pDJR58 and pDJR85), suggesting that *ygiW–qseBC*-P1 is non-functional. Together, these results suggest that *ygiW–qseBC*-P2 is the primary promoter that transcribes the *ygiW–qseBC* operon under the growth conditions that were used.

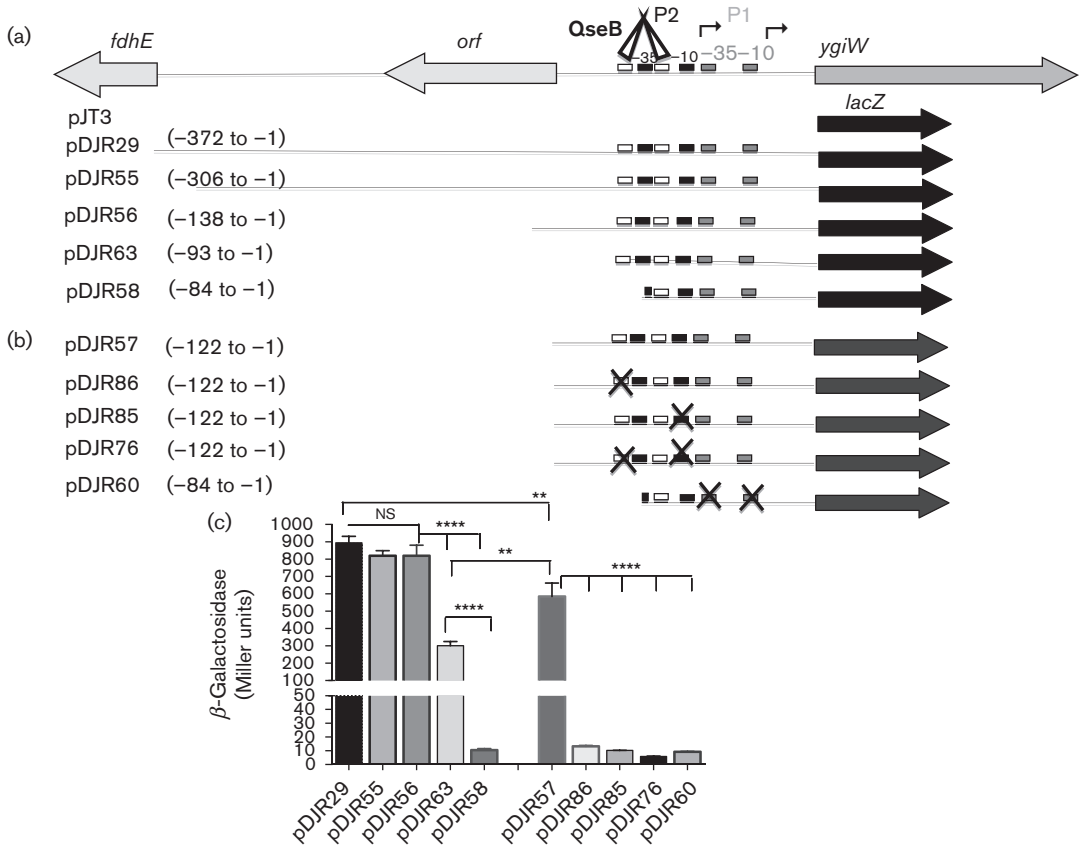

Fig. 1. Schematic diagrams of *ygiW* transcriptional fusion constructs showing the putative binding regions for QseB (white boxes), *ygiW–qseBC*-P1 promoter (grey boxes), *ygiW–qseBC*-P2 promoter (black boxes), QseB protein (white triangles) and transcriptional start sites (bent arrows). (a) Fragments derived from the 372 bp *fdhE–ygiW* intergenic region are represented by lines, and coding sequences are shown by large arrows. (b) *ygiW* transcriptional fusions derived from the *orf–ygiW* intergenic region. The putative promoter elements and QseB binding sites that were altered by site-specific mutagenesis are indicated with an X symbol. In both (a) and (b) the numbering of the nucleotides is relative to the *ygiW* start site and the *lacZ* gene is indicated by a solid black or grey arrow. (c) Graph depicting β-Gal activity in bacterial cells harbouring the transcriptional fusion constructs. β-Gal activity for each construct is expressed in Miller units and was measured in *A. actinomycetemcomitans* 652 strain transformed individually with each plasmid and grown in BHI medium as described in Methods. Measurements were made at the mid-exponential phase of growth, and values are means of results from three independent experiments ± SDs. Statistical analysis was performed using a two-tailed unpaired *t*-test. NS, Not significant; **, $P<0.01$; ****, $P<0.0001$.

QseC is the primary but not the only phosphodonor for QseB

In our previous work, we showed that the QseC sensor and QseB response regulator were required for expression of the *ygiW–qseBC* operon, since deletion of each gene or a *qseBC* double deletion resulted in significant downregulation of the operon (Juárez-Rodríguez *et al.*, 2013a). To determine whether QseC is required for activation of QseB,

Table 1. Transcriptional start sites identified by 5′-RACE

ygiW transcriptional start site	Position (nucleotide)*	No. (%) of 5′-RACE clones identified
TSS_1	−15 (T)	2 (10)
TSS_2	−21 (A)	4 (20)
TSS_3	−53 (A)	14 (70)

*The letters in brackets indicate the nucleotide determined for that 5′ end.

we first examined the effect of *qseC* deletion on the transcription of the *ygiW–qseBC* operon in *A. actinomycetemcomitans* by qRT-PCR using primers for the *qseB* gene. As shown in Fig. 2(a), transcription of the operon was not detected in the *A. actinomycetemcomitans* Δ*qseB* or Δ*qseBC* strains. However, transcription still occurred in the Δ*qseC* strain but was reduced by 58 % relative to WT. This suggests that QseB may be activated even in the absence of its cognate sensor kinase. This was further demonstrated by complementing the Δ*qseBC* mutant with single copy chromosomal insertions of either *qseB* or *qseBC*. As shown in Fig. 2(b), introducing *qseBC* in single copy into the Δ*qseBC* strain restored β-Gal activity to near WT levels. Complementation of the Δ*qseBC* mutant with *qseB* alone also significantly increased β-Gal activity, but the overall levels were still approximately 7.5-fold lower than the *qseBC* complemented strain. Together, these results indicate that QseB may be partially activated in the absence of QseC. Finally, to determine whether QseB activation in the absence of its cognate sensor requires phosphorylation of the response regulator, the Δ*qseBC* strain was complemented with a mutated copy of *qseB* (*qseB*$^{pho-}$) encoding an inactive protein in which the functional aspartate residue was substituted with alanine ($QseB_{D51A}$). As shown in Fig. 2(b), β-Gal activity produced by strains complemented with *qseB*$^{pho-}$ alone or with *qseB*$^{pho-}$*qseC* was similar to the parent Δ*qseBC* strain. Thus, *lacZ* transcription in the absence of QseC requires phosphorylation of QseB. Together, these results suggest that QseC is the primary phosphodonor for QseB but that phosphorylation of QseB can also occur via another mechanism in the absence of the cognate kinase resulting in partial activation of the response regulator.

Identification of the QseB binding site in the *ygiW–qseBC* promoter

To determine whether QseB interacts directly with the *ygiW–qseBC* promoter region, a QseB-hexahistidine fusion protein was purified (Fig. 3a) and used in mobility shift reactions with a family of DNA fragments spanning the 372 bp regulatory region between *ygiW–qseBC* and the upstream *fdhE* gene (Fig. 3b). Preliminary experiments showed that the protein and probe concentrations required for optimal formation of DNA–protein complexes were

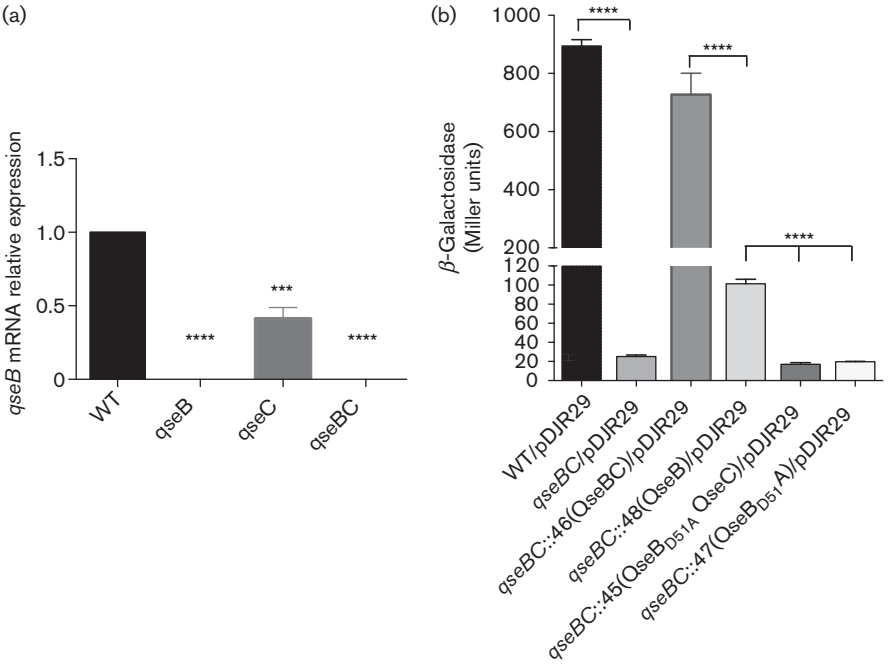

Fig. 2. Role of QseC and QseB in the expression of the *ygiW–qseBC* operon. (a) Expression of the *ygiW–qseBC* operon in gene deletion strains of *A. actinomycetemcomitans*. Cultures of WT 652, Δ*qseB* mutant (652-JR37), Δ*qseC* mutant (652-JR38) and Δ*qseBC* mutant (652-JR39) were grown in BHI medium to mid-exponential phase, and expression of the operon was measured by qRT-PCR and normalized to the expression determined in the WT strain. The data represent the means and SDs of three independent experiments. Significant differences in expression between WT and mutant strains were determined using one-way ANOVA. ***, $P<0.001$; ****, $P<0.0001$. (b) Graph depicting β-Gal activity in WT 652 strain, Δ*qseBC* mutant and Δ*qseBC* mutant strains harbouring pDJR29 (Juárez-Rodríguez *et al.*, 2013a) and complemented with a single copy chromosomal insertion of *qseBC*, *qseB*, *qseB*$^{pho-}$*qseC* or *qseB*$^{pho-}$. β-Gal activity is expressed in Miller units and was measured at the mid-exponential phase of growth in BHI medium. Values are means of results from three independent experiments ± SDs. Statistical analysis was performed using a two-tailed unpaired *t*-test. ****, $P<0.0001$.

4 μM and 50 fmol, respectively (data not shown). As shown in Fig. 3(c), QseB bound only to probes I and III within the regulatory region, suggesting that the QseB binding site resides between nucleotides −13 and −156. Increasing the concentration of QseB in reactions with probe III converted all of the free probe into DNA–protein complexes (Fig. 3d), and QseB binding was inhibited by the addition of increasing amounts of unlabelled probe III DNA (Fig. 3e). QseB did not produce DNA–protein complexes with a 200 bp DNA fragment derived from the *Yersinia pseudotuberculosis psaA* gene, which represents a negative control (Fig. 3f).

To more precisely localize the QseB binding site(s) within probe III, DNA fragments spanning nucleotides −186 to +70 were tested (Fig. 4a). As shown in Fig. 4(b), only probes VI and VIII formed DNA–QseB complexes, indicating that QseB interacts with a DNA sequence between nucleotides −122 to −59 relative to the *ygiW* start codon. The inability of probes XII and XIII to form DNA–QseB complexes suggests that the binding site spans or resides close to nucleotides −92 and −93. Interestingly, the sequence of this region of probe VIII contains two direct repeats separated by six nucleotides (CTTAA-N_6-CTTAA) at nucleotides −78 to −93 (see Fig. S1) and this sequence resembles the DNA-binding site [CTTAA(G or T)-N_5-CTTAA(G or T)] for the *E. coli* transcriptional activator PmrA, which is related to QseB (Chen & Groisman, 2013). To confirm that this putative QseB binding site regulates *ygiW–qseBC* expression, pDJR57 containing nucleotides −1 to −122 (see Fig. 1b) was altered by site-directed mutagenesis from CTTAA to C**GCG**A to generate pDJR86. In addition, a construct in which the upstream direct repeat was deleted (pDJR58 in Fig. 1a) was tested for β-Gal activity. As shown in Table 2, mutagenesis or deletion of the upstream direct repeat resulted in a 45-fold decease in β-Gal activity, suggesting that the QseB binding site is essential for *ygiW–qseBC* transcription.

Identification and function of IHF binding sites in the *ygiW–qseBC* operon

The results shown in Fig. 1(c) also suggest that additional regulatory elements exist upstream of the *ygiW–qseBC*–P2

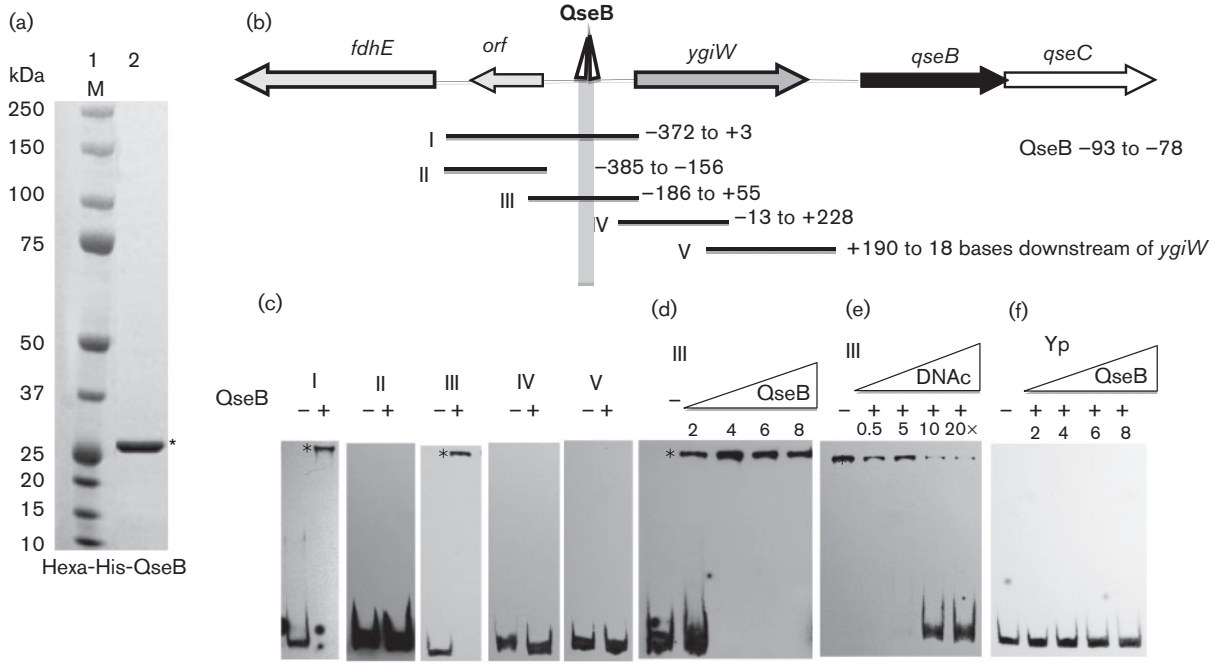

Fig. 3. Binding of purified QseB to the *ygiW–qseBC* promoter region. (a) SDS-PAGE of purified QseB protein. Lane 1, molecular-mass marker (M); lane 2, hexa-histidine QseB purified by cobalt-based immobilized metal affinity chromatography (indicated by the asterisk). (b) Schematic representation of the *ygiW–qseBC* promoter region showing the putative binding region for QseB. PCR fragments used for EMSA reactions are numbered from I to V and the nucleotides contained in each fragment are indicated to the right, numbered relative to the *ygiW* start codon. (c) PCR probes I–V were incubated in the absence (−) or presence (+) of 4 μM QseB protein. (d) Probe III was incubated in the absence (−) or in the presence of the indicated concentrations (μM) of QseB. (e) Probe III was incubated with 4 μM QseB in the absence (−) or presence of 0.5-, 5-, 10- and 20-fold molar excesses of competitive specific unlabelled fragment III (DNAc). (f) The non-specific Yp probe was incubated in the absence (−) or in the presence of the indicated concentrations (μM) of QseB. All binding reactions were performed for 30 min at room temperature and the DNA–protein complexes (indicated by an asterisk) were resolved in 6 % polyacrylamide gels. A 200 bp DNA fragment from the *psa* gene of *Y. pseudotuberculosis* (probe Yp) was used as a negative control.

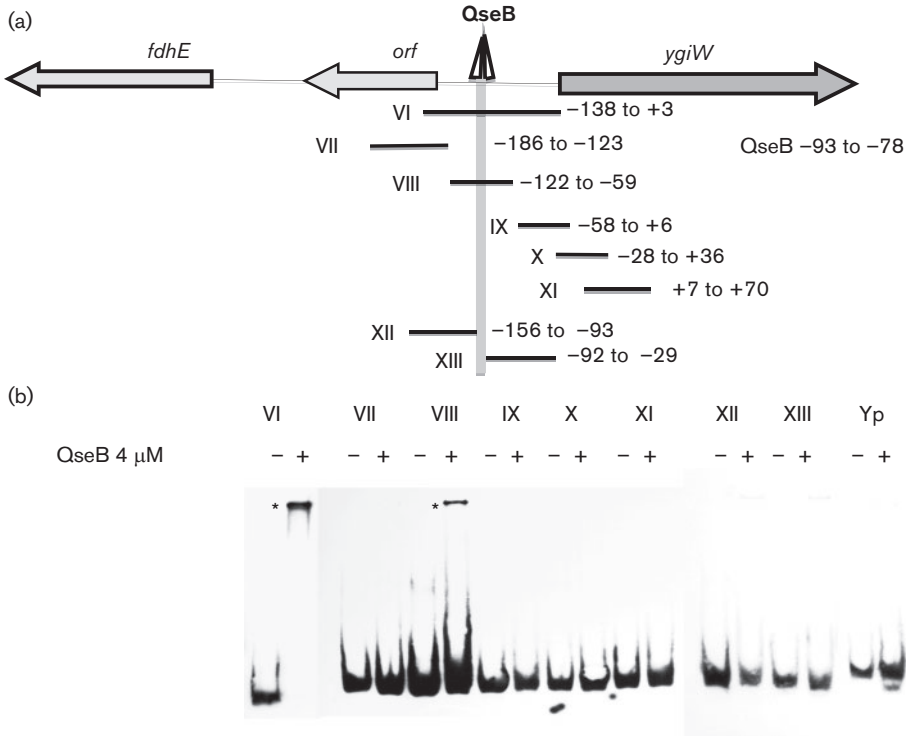

Fig. 4. Localization of the QseB binding site. (a) Schematic representation of the *fdhE–ygiW* intergenic region showing the putative binding region for QseB. PCR fragments used for EMSA reactions are numbered from VI to XIII and the nucleotides contained by each fragment are indicated to the right, numbered relative to the *ygiW* start codon. (b) PCR probes were incubated in the absence (−) and presence (+) of 4 μM QseB protein for 30 min at room temperature, and the DNA–protein complexes (indicated by an asterisk) were resolved in 6 % polyacrylamide gels. A 200 bp DNA fragment from *Y. pseudotuberculosis psa* (probe Yp) was used as a negative control.

promoter and QseB binding site (compare pDJR63 with pDJR56), between nucleotides −93 and −138. Further examination of the 372 bp intergenic region and the *ygiW* coding sequence identified three putative sequences that closely resemble the *E. coli* consensus recognition sequence of the IHF protein (YAANNNNTTGATW, where Y=T or C, N=is any base, W=A or T) (Mangan *et al.*, 2006). The first putative IHF binding sequence resides in the *ygiW* ORF at nucleotides +17 to +32. The second and third sequences reside at nucleotides −31 to –49 and –96 to −111, respectively, relative to the *ygiW* start codon. These sequences were designated $IHFs_1$, $IHFs_2$ and $IHFs_3$, respectively (see Fig. S1).

To determine whether the *A. actinomycetemcomitans* IHF protein interacts with these putative binding sites, *A. actinomycetemcomitans* IHF heterodimer was produced as previously described (Torres-Escobar *et al.*, 2014) and used in EMSA reactions with a family of DNA fragments spanning nucleotides −372 to +70 (see Fig. 6). As shown in Fig. 5, IHF bound only to probes that contained one or more of the putative binding sites described above (i.e. probes III, VI, VIII, IX, X, XI, XII and XIII), whereas probes comprising other regions did not form DNA–IHF complexes (i.e. II and VII). IHF binding was not detected using a 200 bp DNA fragment derived from the *Y. pseudotuberculosis psaA* gene, which represented a negative

Table 2. Mutagenesis of the putative QseB binding site

Strain/plasmid	Promoter fragment	Promoter fragment
652/pDJR57	−1 to −122	584.6 ± 77.9
652/pJDR86	−1 to −122 (mutated CTTAA)	13.0 ± 0.7
652/pDJR58	−1 to −84 (deleted CTTAA)	10.5 ± 1.1

control. To determine whether IHF regulates the expression of *ygiW–qseBC*, the transcriptional levels of *qseB* were determined in *A. actinomycetemcomitans* strains that lacked either the IHFα (Δ*ihfA*) or IHFβ (Δ*ihfB*) subunit. Total RNA was analysed by qRT-PCR from cultures of WT 652, Δ*ihfA* mutant (652-TE78) and Δ*ihfB* mutant (652-TE79), and as shown in Fig. 6, the transcription of *ygiW–qseBC* was significantly increased ($P<0.05$) in both the Δ*ihfA* and Δ*ihfB* strains compared with the WT strain. The *ltxC* gene was used as a positive control in these experiments, since *ltxC* was previously shown to be negatively regulated by IHF in *A. actinomycetemcomitans* (Kolodrubetz *et al.*, 2010). As expected, *ltxC* expression increased in 652-TE78 and 652-TE79 (Fig. 6). Thus, IHF binds to sites within the *ygiW–qseBC* promoter and within the coding sequence *ygiW*, and results in a net reduction of *ygiW–qseBC* expression.

DISCUSSION

In this study, we characterized the regulatory region of the *ygiW–qseBC* operon that encodes a two-component system that is essential for biofilm growth and virulence of *A. actinomycetemcomitans*. Transcription of *ygiW–qseBC* was directed by sequences between nucleotides −1 to −138 relative to the *ygiW* start codon, suggesting that the promoter resides within the intergenic region of *ygiW* and the small upstream ORF(D11S_1134) encoding a hypothetical protein. Two putative promoters, *ygiW–qseBC*-P1 and *ygiW–qseBC*-P2, were initially identified in this region, but our results strongly suggest that expression of the operon is primarily driven by *ygiW–qseBC*-P2 under the growth conditions used. This promoter comprises −10 and −35 elements as well as CTTAA direct repeat elements that flank the −35 sequence (between nucleotides −78 and −93) and function to interact with the QseB response regulator, a strong positive regulator of operon expression. Site-specific mutation of the P2 −10 sequence and the distal CTAA repeat reduced β-Gal activity almost to the level of the promoterless *lacZ* control construct, suggesting that *ygiW–qseBC*-P1 is not functional. Consistent with this, mutation of the P1 −10 and −35 elements had little effect on promoter activity. However, we cannot exclude the possibility that this promoter may be active under growth conditions different from those used in this study. In

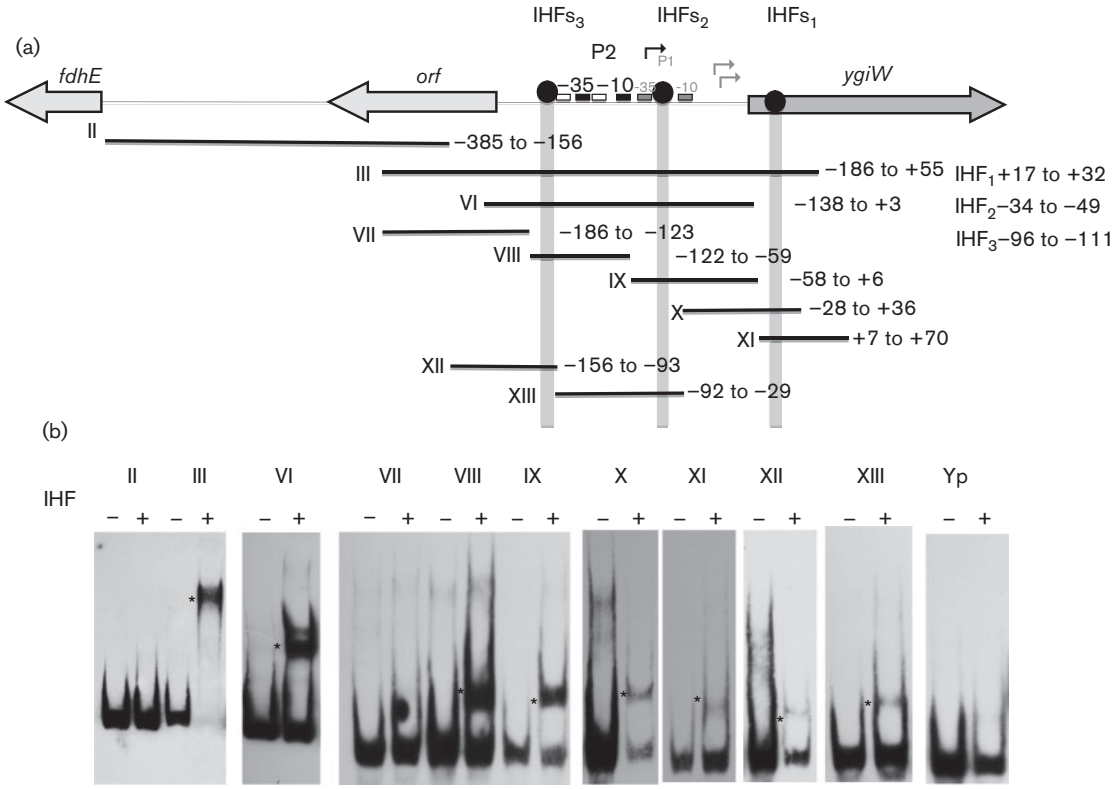

Fig. 5. Binding of purified IHF heterodimer to the *ygiW–qseBC* promoter. (a) Schematic representation of the *fdhE–ygiW* intergenic region showing the putative binding sites for IHF. PCR fragments used for EMSA reactions are numbered from II to XIII, and the nucleotides contained by each fragment are indicated to the right, and numbered relative to the *ygiW* start codon. (b) PCR probes were incubated in the absence (−) and presence (+) of 4 μM IHF protein for 30 min at room temperature, and the DNA–protein complexes (indicated by an asterisk) were resolved in 6 % polyacrylamide gels. A 200 bp DNA fragment from *Y. pseudotuberculosis psa* (probe Yp) was used as a negative control.

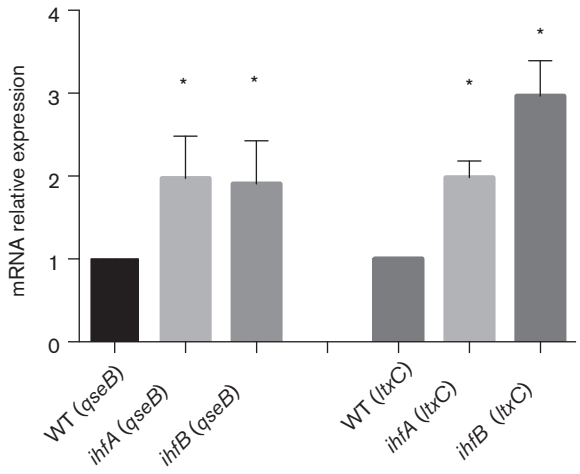

Fig. 6. *qseB* and *ltxC* transcriptional levels in IHF mutant strains. Cultures of WT 652, Δ*ihfA* mutant (652-TE78) and Δ*ihfB* mutant (652-TE79) were grown in BHI medium to mid-exponential phase, and expression of *qseB* and *ltxC* (positive control) was measured by qRT-PCR and normalized to the WT strain. The data represent the means and SDs of three independent experiments. Significant differences in transcription between WT and mutant strains were determined using one-way ANOVA. *, $P<0.05$.

addition, deletion of nucleotides −94 to −138, upstream from the QseB binding CTAA repeats in *ygiW–qseBC*-P2, resulted in a threefold reduction in expression, suggesting that additional regulatory elements exist upstream from the −35 sequence and the direct repeat sequences. One possibility is that IHF interaction with its binding site that is immediately upstream from the distal CTTAA repeat (nucleotides −96 to −111) induces a DNA conformation that facilitates QseB binding and promotes *ygiW–qseBC* expression.

QseB belongs to the OmpR/PhoB subfamily of response regulator transcriptional factors that are distinguished by a C-terminal winged helix–turn–helix DNA binding motif (Mizuno, 1997; Martínez-Hackert & Stock, 1997). As shown in Fig. S2, the *A. actinomycetemcomitans* QseB is structurally highly similar to other members of the OmpR/PhoB response regulators, and shares the phosphorylation domain, dimer interface and DNA contact motifs that are present in these transcriptional regulators. The DNA sequences recognized by the OmpR/PhoB subfamily have been determined for many members and all bind to specific DNA direct repeat sequences (Chen & Groisman, 2013; Liu & De Wulf, 2004; Yamamoto & Ishihama, 2006; Zwir *et al.*, 2012; Rhee *et al.*, 2008; Ritzefeld *et al.*, 2013; Tung & McMahon, 2012), consistent with our finding that CTTAA direct repeats represent the QseB binding site of *A. actinomycetemcomitans*. Phosphorylation of this subfamily of response regulators induces the formation of dimers, which increase the binding affinity of the C-terminal DNA-binding domain with the tandem DNA direct repeat sequences on the promoters of target genes (Toro-Roman *et al.*, 2005; Creager-Allen *et al.*, 2013; Barbieri *et al.*, 2013). As expected, the primary phosphodonor for QseB is its cognate sensor, QseC, but interestingly QseB could be partially activated and induce transcription of *ygiW–qseBC* in the absence of QseC. The absence of QseC in *E. coli* results in constitutively high *qseB* transcription that arises from bidirectional cross-regulation between the structurally related QseBC and PmrAB two-component systems (Guckes *et al.*, 2013). Without QseC, the non-cognate PmrB kinase phosphorylated QseB and transcription of *qseBC* was also activated by PmrA resulting in constitutive expression of *qseB*. However, the *A. actinomycetemcomitans* genome does not encode the PmrAB two-component system, which may explain why transcription of *ygiW–qseBC* is not constitutively high in the absence of QseC. Partial activation of QseB in the absence of QseC in *A. actinomycetemcomitans* may instead result from inefficient phosphorylation by other non-cognate kinases, or alternatively from the transfer of a phosphate from the small phosphate donor acetylphosphate (Wolfe, 2005). Consistent with latter possibility, the genes that encode enzymes required for the production of acetylphosphate are present in the *A. actinomycetemcomitans* genome [D11S-1227 (*pta*) and D11S-1228 (*ackA*)]. Interestingly, the PmrAB two-component system of *E. coli* senses ferric iron and induces the expression of *qseBC* in response to this signal (Chen & Groisman, 2013; Guckes *et al.*, 2013). In contrast, in the absence of PmrAB in *A. actinomycetemcomitans,* the induction of *ygiW–qseBC* expression in response to ferrous and ferric iron is dependent on QseBC itself, and appears to be similar to ferrous iron induction of *H. influenzae ygiW–fisRS* expression (Steele *et al.*, 2012). Indeed, QseC of *A. actinomycetemcomitans* may sense both iron and catecholamine hormones since the QseBC operon is synergistically induced when both signals are present (unpublished data).

Our data also show that IHF binds to two sites in the *ygiW–qseBC*-P2 promoter and a third site near the 5′ end of the *ygiW* ORF. Overall, IHF functions as a net negative regulator of *ygiW–qseBC* expression, since transcription of *ygiW–qseBC* increased approximately twofold in Δ*ihfA* and Δ*ihfB* strains of *A. actinomycetemcomitans*. Consistent with this, IHF binding to the IHF_{S1} site appears to downregulate operon expression since the β-Gal activity of the 372 bp *ygiW–qseBC* promoter–*lacZ* fusion in pDJR29 was previously shown to be higher in an *A. actinomycetemcomitans* strain containing an in-frame deletion in *ygiW* (in which IHF_{S1} was lost) than in the WT (Juárez-Rodríguez *et al.*, 2013a). However, our previous work characterizing the *lsr* locus of *A. actinomycetemcomitans* showed that *lsr* expression is differentially regulated by IHF interaction at each of the two binding sites that are present in this promoter (Torres-Escobar *et al.*, 2014). At present, the outcomes of IHF interaction at IHF_{S2} and IHF_{S3} are not known, although, as suggested above, it is possible that the interaction with IHF_{S3} may facilitate QseB binding to *ygiW–qseBC*-P2.

In summary, we have shown that transcription of the *ygiW–qseBC* operon of *A. actinomycetemcomitans* is primarily driven by the QseB-dependent *ygiW–qseBC*-P2 promoter. This promoter interacts with two *trans*-acting proteins; the QseB response regulator activates transcription of *ygiW–qseBC* by binding to CTTAA direct repeat sequences that flank the −35 element, and IHF interacts with three distinct binding sites in the promoter and functions as a net negative regulator of transcription. Finally, although QseB is mainly activated by the cognate QseC sensor, it can be partially activated by other mechanisms in the absence of QseC activity.

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