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 CTCTAA-N6-CTCTAA, where the CTCTAA repeats flank the −35 element of the promoter. The *ygiW–qseBC* expression could not be detected in *A. actinomycetemcomitans ΔqseB* 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*<sup>Δpho</sup>−, 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*hA* and Δ*hB* strains of *A. actinomycetemcomitans*, suggesting that IHF is a negative regulator of the *ygiW–qseBC* operon.

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 cytotoxic 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

**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.

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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 fhlDC 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 enterohemorrhagic *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 & Bearer 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* (Khanjanich 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 YAANNNTTGATW, 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* (affiliated, 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* D115S-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 RNXaprotect 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 synthesized 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 Select master mix (Applied Biosystems by Life Technologies), as specified by the 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 18S RNA expression level as the internal reference for normalization, and expressed as fold-differences by calculating 2^{-DDCT} using the 16S rRNA expression level as the internal reference for each time point of 1 cycle of 95°C for 1 min and extension at 60°C for 1 min 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 RNA expression level as the internal reference for normalization, and expressed as fold-differences by calculating 2^{-DDCT} using the 16S rRNA expression level as the internal reference for each time point.

**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-124F/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 *KpnI*–*BamHI* and cloned independently into *KpnI*–*BamHI*-digested pJT3 (Cruéjuel-Navarro et al., 2013b) to create pDJR55, pDJR56, pDJR57, pDJR58 and pDJR63, respectively.

**Site-directed mutagenesis.** Mutagenesis of the putative −10 element (TATAAG to GCGGCG) and −35 element (CAACAAA to GCAGAA) of ygiW–qseBC-P1 was carried out by annealing primers MDJR-158F/MDJR-159R and cloning the resulting double-stranded fragment into *KpnI*–*BamHI*-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 (CTTATACCTTTCTTAAC to GCGGACTACTTTCTTAAC) by PCR. The resulting product was then digested with *KpnI*–*BamHI* and cloned into pJT3 to generate pDJR86. Mutations in the ygiW–qseBC-P2 −10 element (TAATAG to GCGGCG) 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 *KpnI*–*BamHI* 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 GCC) encoding the conserved active site aspartate residue in QseB (DS1) 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* ΔqseBC.** Integration of genes in single copy into the *A. actinomycetemcomitans* ΔqseBC genome was carried out as previously described (Torres-Escobar et al., 2014). To integrate a single copy of qseB, the qseB mutant allele (qseB^{dmuts}) encoding the QseB_{DMJR} mutant protein, qseBC or qseB^{	ext{pho}}–qseC independently into the chromosone of *A. actinomycetemcomitans* ΔqseBC 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/MDJR126R (for qseB constructs). The 3135 and 1645 bp PCR fragments were digested with NotI–PstI and cloned into NotI–PstI-digested suicide vector pT11 to generate the plasmids pDJR45, pDJR46, pDJR47 and pDJR48. Each recombinant suicide plasmid (20 μg) was introduced individually into *A. actinomycetemcomitans* isogenic ΔqseBC mutant by electroporation. Ten Sp’ 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’ colonies of *A. actinomycetemcomitans* ΔqseBC::qseB^{pho}–qseC, ΔqseBC::qseBC, ΔqseBC::qseB^{pho} and ΔqseBC::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 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 Ncol–Apal and cloned into Ncol–Apal-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 harboured pDJR36 was used for the synthesis of the hexahistidine containing 50 mM Tris/HCl (pH 8.5), 4 M urea, 0.5 M L-arginine, for 1 h with shaking at 4°C.

Electrophoretic mobility shift assay (EMSA).

**Expression and purification of QseB protein.**

**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 (Juarez-Rodriguez 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 (TSS1) 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 (Juarez-Rodriguez 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, TSS2 was again identified, but two additional putative start sites at nucleotides −21 and −53 (TSS2 and TSS3, respectively) were also identified. TSS4 and TSS5 represented 10 and 20% of the positive clones identified by 5’-RACE, respectively. The main start site, TSS3, mapped to nucleotide −53 and represented 70% of the positive clones identified by 5’-RACE. Associated with TSS5 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 GCGCGG) 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.
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

<table>
<thead>
<tr>
<th>ygiW transcriptional start site</th>
<th>Position (nucleotide)*</th>
<th>No. (%) of 5′-RACE clones identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS₁</td>
<td>−15 (T)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>TSS₂</td>
<td>−21 (A)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>TSS₃</td>
<td>−53 (A)</td>
<td>14 (70)</td>
</tr>
</tbody>
</table>

*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 ΔqseB or ΔqseBC strains. However, transcription still occurred in the ΔqseC strain but was reduced by 58% relative to WT. This suggests that QseB may be activated in the absence of its cognate sensor kinase. This was further demonstrated by complementing the ΔqseBC mutant with single copy chromosomal insertions of either qseB or qseBC. As shown in Fig. 2(b), introducing qseBC in single copy into the ΔqseBC strain restored β-Gal activity to near WT levels. Complementation of the ΔqseBC 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 ΔqseBC strain was complemented with a mutated copy of qseB (qseB<sup>pho<sup>−</sup></sup>) encoding an inactive protein in which the functional aspartate residue was substituted with alanine (QseB<sub>DS1A</sub>). As shown in Fig. 2(b), β-Gal activity produced by strains complemented with qseB<sup>pho<sup>−</sup></sup> alone or with qseB<sup>pho<sup>−</sup></sup>–qseC was similar to the parent ΔqseBC 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, ΔqseB mutant (652-JR37), ΔqseC mutant (652-JR38) and ΔqseBC 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, ΔqseBC mutant and ΔqseBC mutant strains harbouring pDJR29 (Juárez-Rodríguez et al., 2013a) and complemented with a single copy chromosomal insertion of qseBC, qseB, qseB<sup>pho<sup>−</sup></sup>–qseC or qseB<sup>pho<sup>−</sup></sup>–qseC. 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–N₆–CTTAA) at nucleotides −78 to −93 (see Fig. S1) and this sequence resembles the DNA-binding site [CTTAA(G or T)–N₅–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 CGCGA 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 decrease 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.](http://mic.sgmjournals.org)
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 (YAANNNTTGATW, where Y = T or C, N = 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 IHFs1, IHFs2 and IHFs3, 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 control.

**Table 2. Mutagenesis of the putative QseB binding site**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Promoter fragment</th>
<th>Promoter fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>652/pDJR57</td>
<td>−1 to −122</td>
<td>584.6 ± 77.9</td>
</tr>
<tr>
<td>652/pDJR86</td>
<td>−1 to −122 (mutated CTTAA)</td>
<td>13.0 ± 0.7</td>
</tr>
<tr>
<td>652/pDJR58</td>
<td>−1 to −84 (deleted CTTAA)</td>
<td>10.5 ± 1.1</td>
</tr>
</tbody>
</table>
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 (ΔihfA) or IHFβ (ΔihfB) subunit. Total RNA was analysed by qRT-PCR from cultures of WT 652, ΔihfA mutant (652-TE78) and ΔihfB mutant (652-TE79), and as shown in Fig. 6, the transcription of ygiW–qseBC was significantly increased (P<0.05) in both the ΔihfA and ΔihfB 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

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**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.
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 CTAA 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; Ritzfeld et al., 2013; Tung & McMahon, 2012), consistent with our finding that CTAA 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 phospho-donor 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 QseB 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 ΔihfA and ΔihfB 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.

Fig. 6. qseB and ltxC transcriptional levels in IHF mutant strains. Cultures of WT 652, ΔihfA mutant (652-TE78) and ΔihfB mutant (652-TE79) were grown in BH 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. *, $P<0.05$. 

...
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 CTTTA direct repeat sequences that flank the −35 element, and IHF interacts with three distinct binding sites in the promoter and functions as a 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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