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Autoinducer-2 and QseC Control Biofilm Formation and *In Vivo* Virulence of *Aggregatibacter actinomycetemcomitans*▽

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Biofilm formation by the periodontal pathogen *Aggregatibacter actinomycetemcomitans* is dependent upon autoinducer-2 (AI-2)-mediated quorum sensing. However, the components that link the detection of the AI-2 signal to downstream gene expression have not been determined. One potential regulator is the QseBC two-component system, which is part of the AI-2-dependent response pathway that controls biofilm formation in *Escherichia coli*. Here we show that the expression of QseBC in *A. actinomycetemcomitans* is induced by AI-2 and that induction requires the AI-2 receptors, LsrB and/or RbsB. Additionally, inactivation of *qseC* resulted in reduced biofilm growth. Since the ability to grow in biofilms is essential for *A. actinomycetemcomitans* virulence, strains that were deficient in QseC or the AI-2 receptors were examined in an *in vivo* mouse model of periodontitis. The Δ*qseC* mutant induced significantly less alveolar bone resorption than the wild-type strain ($P < 0.02$). Bone loss in animals infected with the Δ*qseC* strain was similar to that in sham-infected animals. The Δ*lsrB*, Δ*rbsB*, and Δ*lsrB* Δ*rbsB* strains also induced significantly less alveolar bone resorption than the wild type ($P < 0.03$, $P < 0.02$, and $P < 0.01$, respectively). However, bone loss induced by a Δ*luxS* strain was indistinguishable from that induced by the wild type, suggesting that AI-2 produced by indigenous microflora in the murine oral cavity may complement the Δ*luxS* mutation. Together, these results suggest that the QseBC two-component system is part of the AI-2 regulon and may link the detection of AI-2 to the regulation of downstream cellular processes that are involved in biofilm formation and virulence of *A. actinomycetemcomitans*.

Dental plaque is a complex and dynamic microbial community that forms as a biofilm on the surfaces of teeth and oral tissues (20, 22, 31, 50). It is comprised of over 700 species of bacteria (1, 20–22, 31, 50) and is the prime etiological agent of three common human oral diseases: dental caries, gingivitis, and periodontal disease (25, 26, 43). Major shifts in microbial populations within the oral biofilm have been associated with the progression of disease, as diseased sites often have increased populations of pathogenic species relative to healthy sites in the oral cavity (24–26). The host and/or microbial signals that contribute to the population shifts associated with disease are still unknown. The oral cavity is subject to continual environmental flux, including changes in pH, temperature, osmolarity, and nutrient supply, and it is possible that these stresses contribute in part to microbial population shifts in the biofilm. However, it is clear that oral bacteria rapidly detect environmental fluctuations and respond appropriately, allowing them to successfully coexist and thrive in the oral cavity (2, 18, 26).

Both intra- and interspecies communication is known to occur between bacteria, and these signaling processes potentially enable the organisms to coordinate their behavior and function by regulating gene expression as a community. One mechanism of communication, termed quorum sensing, is a cell density-dependent response (21, 28, 39, 55) which, in Gram-negative bacteria, is mediated by the production, release, and detection of soluble signal molecules called autoinducers. A variety of chemical species function as autoinducers, including acylated homoserine lactones (15, 30), quinolone derivatives (32), and furan derivatives (e.g., autoinducer-2 [AI-2]) (28, 39, 49, 52). As a population of bacteria expands, the external concentration of the autoinducer increases until a threshold is attained, at which point a signal transduction cascade is initiated that alters gene expression and behavior of the microbial community. Quorum sensing has been shown to control cell density-dependent behaviors, such as the expression of virulence factors, biofilm formation, and iron acquisition, in a variety of organisms (4, 15, 49, 51). Thus, it has been suggested that quorum sensing may allow bacteria in a biofilm to react coordinately as a multicellular organism to changes in the external environment.

The dental pathogen *Aggregatibacter actinomycetemcomitans*, a Gram-negative organism associated with aggressive forms of periodontitis and other systemic infections (6, 29, 42, 54), possesses an AI-2-dependent quorum-sensing system (12). AI-2 produced by *A. actinomycetemcomitans* regulates expression of virulence factors, biofilm formation, and iron uptake and also influences the planktonic growth of the organism under conditions of iron limitation (13, 38, 41). *A. actinomycetemcomitans* possesses two periplasmic AI-2 receptors, LsrB and RbsB, both of which are linked to ABC transporters (19, 40), suggesting that *A. actinomycetemcomitans* may import AI-2. However, exactly how the detection and/or importation of AI-2 is linked to downstream gene regulation remains to be determined. In *Escherichia coli*, the QseBC two-component signal transduction system has been suggested to regulate biofilm formation (14), and *A. actinomycetemcomitans* contains an

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Description	Source or reference
A. actinomycetemcomitans strains		
652	Wild type, serotype c, minimally leukotoxic strain	7
652 Δ*lsrB*	*lsrB*::spec single-receptor mutant	40
652 Δ*rbsB*	*rbsB*::*kan* single-receptor mutant	19
652 Δ*lsrB* Δ*rbsB*	*lsrB*::spec *rbsB*::*kan* double-receptor mutant	40
652 Δ*qseC*	*qseC*::spec mutant	This study
652 Δ*qseC* comp	*qseC* mutant complemented with pYGK*qseC*	This study
JP2	Wild type, serotype b, highly leukotoxic strain	7
JP2-12	*luxS*::*kan* mutant	12
JP2-12/750	*luxS* mutant complemented with pJRD215*luxS*	13
E. coli strains		
DH5α TqseC	DH5α carrying pGEMT*qseC*	This study
DH5α TqseCcomp	DH5α carrying pBSKQseC-spec	This study
pVT1461	Contains Spec^r cassette	K. Mintz
P. gingivalis strains		
33277	Wild type	ATCC
Δ*luxS*	33277 *luxS*::*ermF* mutant	10
V. harveyi BB170	Sensor 1$^-$ sensor 2$^+$	5

operon that displays 70 to 80% sequence similarity to the QseBC genes of *E. coli*. QseBC is also similar to the FeuPQ two-component system that regulates iron uptake in *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Brucella suis* (11), and iron acquisition is known to be regulated by AI-2 (13) and dramatically influences biofilm formation (35, 36). In this study, we show that *qseBC* is part of the AI-2 regulon and that its induction requires a functional AI-2 receptor. The *qseBC* operon also contributes to biofilm formation and virulence of *A. actinomycetemcomitans*, since inactivation of *qseC* results in reduced biofilm growth and attenuates virulence *in vivo*. These results suggest that QseBC may couple the detection and/or importation of AI-2 to the downstream regulation of gene expression that controls these processes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains employed in this study are listed in Table 1. *A. actinomycetemcomitans* JP2 and 652 are afimbriated, smooth-colony-morphotype strains and were grown at 37°C under microaerophilic conditions in brain heart infusion broth (BHI; Becton Dickinson and Company [BD], Sparks, MD) supplemented with 40 mg of $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO) per liter. The *luxS*-deficient strain of *A. actinomycetemcomitans* was grown as described above, but in medium supplemented with kanamycin (25 μg/ml; Sigma-Aldrich). The Δ*luxS* mutant complemented with a plasmid-borne copy of *luxS* was grown in BHI supplemented with kanamycin (25 μg/ml) and streptomycin (50 μg/ml; Sigma-Aldrich). The *A. actinomycetemcomitans* Δ*lsrB* and Δ*rbsB* mutant strains were cultured in BHI supplemented with spectinomycin (50 μg/ml; Sigma-Aldrich) and kanamycin (25 μg/ml), respectively. The *A. actinomycetemcomitans* Δ*lsrB* Δ*rbsB* double mutant was cultured in BHI supplemented with kanamycin (25 μg/ml) and spectinomycin (50 μg/ml). The Δ*qseC* mutant was grown in BHI supplemented with spectinomycin (50 μg/ml), and the *qseC*-complemented strain was cultured in BHI supplemented with kanamycin (25 μg/ml).

Porphyromonas gingivalis strains were grown in reduced Trypticase soy broth (TSB; BD) supplemented with yeast extract (1 g per liter; BD), menadione (1 μg per ml; Sigma-Aldrich), and hemin (5 μg per ml; Sigma-Aldrich). The medium was reduced for 24 h under anaerobic conditions by equilibration in an atmosphere consisting of 10% CO_2, 10% H_2, and 80% N_2. The *P. gingivalis* Δ*luxS* mutant (kindly supplied by R. Lamont, University of Florida, Gainesville, FL) was grown as described above, but the medium was supplemented with erythromycin (10 μg/ml; Sigma-Aldrich) immediately before inoculation.

Vibrio harveyi BB170 (sensor 1$^-$ sensor 2$^+$) was a gift from B. Bassler (Princeton University) and was grown overnight in AB medium with aeration at 30°C (5). AB medium consists of 0.3 M NaCl, 50 mM $MgSO_4$, 0.2% Casamino Acids, 10 mM potassium phosphate (pH 7.0), 1 mM L-arginine, 2% glycerol, 1 μg per ml thiamine, and 10 ng per ml riboflavin. *E. coli* strains were grown in Luria-Bertani (LB) medium (BD) with aeration at 37°C. *E. coli* strains containing plasmid pGEM-T or pYGK were cultured as described above, using LB supplemented with 100 μg per ml ampicillin or 25 μg per ml kanamycin, respectively.

Construction of mutant strains. The *A. actinomycetemcomitans qseBC* operon was identified from the genomic sequence of strain HK1651 (Los Alamos National Laboratory [http://www.oralgen.lanl.gov/]) and was annotated as the *ygiX* and *qseC* genes. To construct the fragment for inactivation of *qseC*, parts of the *ygiX* and *qseC* genes were amplified using genomic DNA of strain 652 as the template, with primers P1 and P2 (Table 2). The following PCR program was used: 94°C for 10 min for 1 cycle and then 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min for 30 cycles. The PCR products were then ligated with pGEM-T Easy (Promega, Madison, WI) and transformed into *E. coli* DH5α. The resulting plasmid, pGEMTQseC, was purified from *E. coli*, cleaved by digestion with KpnI/BamHI, and ligated into pBSK. The resulting plasmid, pBSKQseC, was then cleaved with BamHI and treated with alkaline phosphatase for insertion of a spectinomycin resistance cassette. The spectinomycin resistance cassette was obtained by PCR amplification using plasmid pVT1461 (kindly supplied by K. Mintz, University of Vermont) as the template, with primers P3 and P4, and then ligated into pBSKQseC to create pBSKQseC-spec. This plasmid was then transformed into *E. coli* DH5α, and recombinant clones were confirmed by PCR using primers P1 and P4. Purified pBSKQseC-spec plasmid was introduced into *A. actinomycetemcomitans* by electroporation, with ampicillin resistance and spectinomycin resistance selection. The lack of a QseC transcript in the mutant strain was confirmed by reverse transcription-PCR (RT-PCR) using primers P6 and P7.

To make the *qseC*-complemented strain, the entire *qseC* gene was amplified using 652 genomic DNA as the template, with primers 5′ qseC-comp-HindIII and 3′ qseC-comp-BamHI. The leukotoxin promoter (ltx-pro) was also amplified from 652 genomic DNA, using the 5′ ltx-pro-KpnI and 3′ ltx-pro-HindIII primers. The following PCR program was used: 94°C for 10 min for 1 cycle and then 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min for 30 cycles. Both the *qseC* and leukotoxin PCR products were then ligated into pGEM-T Easy (Promega, Madison, WI) and transformed into *E. coli* DH5α. The resulting plasmids, pGEMTQseCcomp and pGEMTltx-pro, were purified from *E. coli*. The pGEMTQseCcomp plasmid was cleaved by digestion with HindIII and BamHI, and the pGEMTltx-pro plasmid was cleaved by digestion with KpnI and HindIII. Both digested fragments—the *qseC* gene and ltx-pro—were then ligated into pYGK. The resulting plasmid, pYGKqseC, was transformed into *E. coli*. The plasmid was then purified from an overnight culture of *E. coli* and transformed into the Δ*qseC* mutant for complementation.

Partial purification of *A. actinomycetemcomitans* AI-2. An enriched fraction containing AI-2 from *A. actinomycetemcomitans* was produced as described by

TABLE 2. Primers used for PCR

Primer	Sequence (5′–3′)[a]	Target gene	Product size (kbp)
P1	**GGTACC**TCGCCGTGGATTGGTTTACCGAC	*ygix* (5′ primer)	1.030
P2	**GGATCC**GGCGTTTATGCGACGGTTTG	*qseC* (3′ primer)	1.030
P3	**GGATCC**ATCGATTTTCGTTCGTG	Spec^r cassette (5′ primer)	1.141
P4	**GGATCC**CATATGCAAGGGTTTAT	Spec^r cassette (3′ primer)	1.141
P6	TAAGTGGAATAATTACAGCCTGCG	*qseC* (5′ primer)	0.139
P7	TTGTTGTGCGTCAAACACTTGGTTC	*qseC* (3′ primer)	0.139
5′ qseC-comp-HindIII	**AAGCTT**ATGAAACTGAGTAAGTGG	*qseC*	1.374
3′ qseC-comp-BamHI	**GGATCC**CAACTGAATCTCTGCC	*qseC*	1.374
5′ ltx-pro-KpnI	**GGTACC**AATGAAAAAAAACAAAGCG	Leukotoxin promoter	0.308
3′ ltx-pro-HindIII	**AAGCTT**ACTCGTTTTCCTTTTTCATTAG	Leukotoxin promoter	0.308
5′ QseBC induction	TCGCCGTGGATTGGTTTACCGAC	*qseB*	0.159
3′ QseBC induction	GAATCAGCACCGGCACATCCTGC	*qseB*	0.159
5′ 5S rRNA	GCGGGGATCCTGGCGGTGACCTACT	5S-2 rRNA	0.089
3′ 5S rRNA	GCGATCTAGACCACCTGAAACCATACC	5S-2 rRNA	0.089

[a] Bold sequences indicate restriction enzyme sites used to facilitate cloning into the appropriate shuttle vectors.

Sperandio et al. (44). Briefly, an overnight culture of *A. actinomycetemcomitans* cells was diluted 1:20 in fresh medium, cultured to mid-exponential phase (optical density, 0.3) at 37°C, and then harvested by centrifugation. AI-2 was obtained from a 7.2-ml aliquot of the conditioned medium. The culture supernatant was first filtered through a 0.22-μm-pore-size filter and then through a Centricon YM-3 3-kDa exclusion filter (Millipore, Bedford, MA). The resulting filtrate was lyophilized, suspended in 1 ml of cold 5 mM sodium phosphate buffer, pH 6.2, and chromatographed on a C_{18} Sep-Pak reverse-phase column (Waters Company, Milford, MA) according to the manufacturer's instructions. Induction of bioluminescence of *Vibrio harveyi* BB170 was monitored in order to follow the AI-2 activity in the column fractions. Active fractions were lyophilized and stored at 4°C.

RNA isolation and real-time PCR. Overnight cultures of the appropriate *A. actinomycetemcomitans* strains were diluted 1:20 in fresh BHI medium, with or without partially purified AI-2, and were incubated at 37°C until the mid-exponential growth phase for RNA isolation. Total RNA was isolated from *A. actinomycetemcomitans* cells by use of a 5 Prime PerfectPure RNA Cell & Tissue kit (5 Prime Inc., Gaithersburg, MD) according to the manufacturer's instructions. To ensure that the samples were free of contaminating genomic DNA, the RNA preparation was digested with RQ RNase-free DNase I (Promega Corporation, Madison, WI). The concentration and purity of each RNA sample were measured via spectrophotometry (ND-1000 spectrophotometer; NanoDrop Technologies, Inc., Wilmington, DE) and were also assessed by gel electrophoresis. Samples were checked for contamination of genomic DNA by real-time PCR, using *A. actinomycetemcomitans* 5S rRNA primers (Table 2). RNA samples were considered free of significant genomic DNA if no amplification product was detected by real-time PCR after at least 30 cycles of amplification. RNA that was not immediately utilized for a reverse transcription reaction was aliquoted into different tubes and stored at −80°C until future use.

First-strand cDNA was prepared by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA was amplified using a Smart Cycler system (Cepheid, Sunnyvale, CA), with a final reaction volume of 25 μl that contained 100 ng of cDNA, primers for *qseC* (5′ QseBC induction and 3′ QseBC induction; ~71 μM [final concentration of each]), and 1× FastStart SYBR green master mix (Roche, Indianapolis, IN). The amplification conditions for real-time PCRs were as follows: 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. The threshold cycle for each real-time PCR was determined from a second derivative plot of total fluorescence as a function of cycle number by using the software package supplied with the Smart Cycler system. All gene-specific threshold values were normalized against threshold values for primers specific for the *A. actinomycetemcomitans* 5S rRNA gene (~60 μM [final concentration]). Real-time PCRs were carried out in triplicate, with consistent results. Each real-time PCR end-point amplification product was visualized by electrophoresis on 2% agarose gels.

Biofilm formation and analysis. *A. actinomycetemcomitans* biofilms were grown on a saliva-coated cover glass in a polycarbonate flow chamber (model FC81; Biosurface Technologies Corp, Bozeman, MT) (chamber dimensions are 50.5 mm by 12.7 mm by 2.54 mm) at a flow rate of 5.8 ml per hour at 25°C, essentially as described by Shao et al. (41). Briefly, saliva was self-collected, filter sterilized (pore size, 0.22 μm), and incubated on the cover glass (60 mm by 24 mm) for 30 min at 37°C. The saliva-coated cover glass was then fixed in the flow chamber and washed with phosphate-buffered saline (PBS; 100 mM NaH_2PO_4, 150 mM NaCl) for 10 min at a flow rate of 60 ml per hour by use of a peristaltic pump (Manostat Sarah cassette; Fisher Scientific, Pittsburgh, PA). Overnight cultures of *A. actinomycetemcomitans* were resuspended in PBS at an optical density at 600 nm of 0.5, inoculated for 1 h into a polycarbonate flow chamber, and then washed with PBS for 30 min. Bound cells were fed BHI medium and allowed to grow for 60 h at a flow rate of 5.8 ml per hour. The resulting biofilm was stained with 0.2 mg/ml fluorescein isothiocyanate (FITC; Sigma-Aldrich) 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, Pittsburgh, PA) at a magnification of ×600, using an argon laser. Confocal images were captured from 9 randomly chosen frames from each flow chamber. Biofilm depth was determined by performing *z*-plane scans from 0 to 100 μm above the cover glass surface. The total biofilm biomass was determined by integrating fluorescence intensity across the *z* stack, using the Fluoview FV500 software provided by Olympus. Biofilm depth, total biomass, and biofilm topology were also quantified utilizing the COMSTAT image-processing software package (16). Biomass data were analyzed using a pairwise *t* test (Graphpad Software, Inc.) and are expressed as means ± standard deviations calculated for all the frames obtained for a given biofilm. Experiments were carried out in triplicate.

Mouse periodontitis model. To assess *A. actinomycetemcomitans* virulence, the Baker mouse model of periodontitis was utilized (3). This model measures alveolar bone resorption, the clinical presentation of periodontitis in humans, as an outcome, and we used the model previously to assess the virulence of *P. gingivalis*, another periodontal pathogen (33, 48). All animal procedures received Institutional Animal Care and Use Committee approval and were in accordance with federal guidelines for the care and use of laboratory animals. Specific-pathogen-free female BALB/cByJ mice (10 weeks old; Jackson Laboratory, Bar Harbor, ME) were orally infected with wild-type or mutant strains of *A. actinomycetemcomitans* or, in some experiments, with wild-type or mutant strains of *P. gingivalis*. Briefly, prior to infection, each group of mice was given the antibiotics sulfamethoxazole (final concentration of 800 μg/ml; Sigma-Aldrich) and trimethoprim (final concentration of 400 μg/ml; Sigma-Aldrich) in their water bottles *ad libitum* for 10 days to suppress the normal flora of the mouse oral cavity. After the 10-day antibiotic period, the mice were given regular drinking water without antibiotics for 4 days and then were orally infected a total of five times at 2-day intervals with 1×10^9 CFU/ml of bacteria resuspended in 2% carboxymethylcellulose (CMC; MP Biomedicals, Solon, OH) via a blunt-tipped syringe (Fisher Scientific, Pittsburgh, PA). Animals that served as the negative-control group (sham-infected mice) received only 2% CMC without bacteria. Mice were euthanized by CO_2 inhalation and cervical dislocation 47 days after the last infection (for a total experiment time of 70 days).

The maxillae were collected at the end of the experiment and were immersed in 3% H_2O_2 overnight. Maxillae were then sonicated in 1% bleach, cleaned, rinsed with water, and then stained for 30 s with 1% methylene blue (Ricca Chemical Company, Arlington, TX). To measure the loss of alveolar bone, the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest

TABLE 3. QseBC two-component system is influenced by AI-2

Strain and signal	ΔT[a]	$\Delta\Delta T$	Fold induction
JP2-12	−3.1 ± 0.6	0.11 ± 0.04	1.0
JP2-12 + AI-2	−0.66 ± 0.2	1.52 ± 0.3	14.5 ± 4.3
JP2-12 + mock[b]	3.6 ± 0.05	0.08 ± 0.01	0.8 ± 0.3
Δ*lsrB* Δ*rbsB* mutant[c]	3.2 ± 0.35	0.09 ± 0.13	0.95 ± 0.2

[a] ΔT was calculated by subtracting the C_T determined for the *qseC* reaction from the C_T for the 5S rRNA control.

[b] The mock control represents culture fluid from the Δ*luxS* strain subjected to the purification scheme used to partially purify AI-2.

[c] This strain contains a functional copy of *luxS* and produces but cannot respond to AI-2.

(ABC) was measured at 7 sites on the buccal side of the right and left maxillary molars (for a total of 14 measurements per animal), utilizing a dissecting microscope fitted with a video image marker measurement system (model VIA-170K; Fryer, Huntley, IL) standardized to give measurements in millimeters. Alveolar bone loss was calculated by subtracting the 14-site mean sum total CEJ-ABC distance for each experimental group from the mean sum total CEJ-ABC distance for the sham-infected group. Results are expressed as changes in bone size, in millimeters, and negative values indicate bone loss compared with sham-infected controls.

Statistical analysis. Data were evaluated by analysis of variance (ANOVA) and the Dunnette multiple-comparison test, using the InStat program (Graphpad). Two-tailed *t* tests were also performed where appropriate (comparison of two groups only). Statistical differences were considered significant with *P* values of <0.05.

RESULTS

AI-2 is required for induction of QseBC in *A. actinomycetemcomitans*. In some *E. coli* strains, the QseBC two-component system is induced by AI-2 and, furthermore, is part of the AI-2-dependent response circuit that controls biofilm growth (14). The genome of *A. actinomycetemcomitans* encodes a two-component system that is related to QseBC in *E. coli*. The genes are listed as *ygiX* and *qseC* and are annotated as genes for a sensor histidine kinase (*qseC*) and a response regulator (*ygiX*) of a two-component signal transduction system (www.oralgen.lanl.gov). In strain 652, the *ygiX* and *qseC* genes overlap by 11 bp (data not shown), and RT-PCR indicates that they are coexpressed in an operon (data not shown). The deduced amino acid sequences for *ygiX* and *qseC* from strain 652 exhibit significant sequence identity with QseB and QseC from *E. coli* (data not shown).

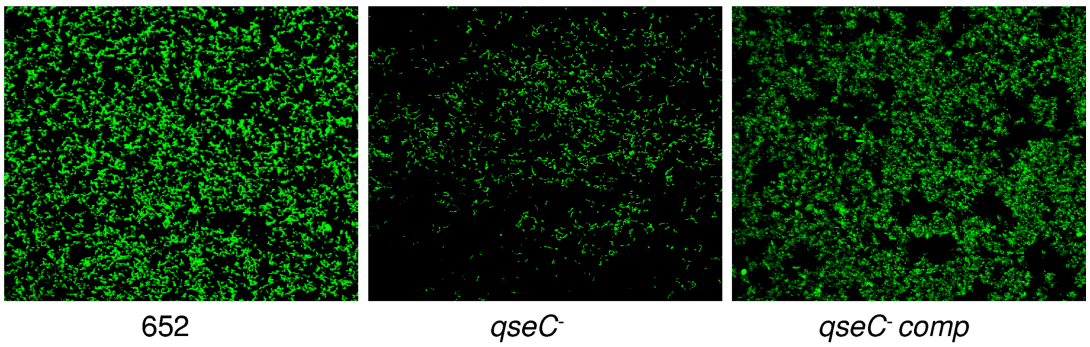
To determine if AI-2 regulates the putative QseBC two-component system in *A. actinomycetemcomitans*, the expression of *qseC* was examined using real-time PCR with RNA isolated from overnight cultures of the Δ*luxS* mutant grown in medium alone or in medium supplemented with partially purified AI-2. As shown in Table 3, the presence of AI-2 in the growth medium resulted in a 14.5-fold induction of *qseC*. As a control, cultures were also grown in medium supplemented with conditioned culture fluid obtained from a Δ*luxS* strain that was subjected to the same purification scheme used to obtain the AI-2 samples. No increase in *qseC* expression was observed under these conditions. In addition, the induction of *qseC* did not occur in the Δ*lsrB* Δ*rbsB* strain of *A. actinomycetemcomitans*, which lacks the AI-2 receptors but can still produce AI-2. This suggests that the QseBC two-component system in *A. actinomycetemcomitans* is regulated by AI-2 and may be linked to the detection and/or importation of AI-2 by the AI-2 receptors.

Inactivation of *qseC* influences biofilm formation in *A. actinomycetemcomitans*. Previous studies demonstrated that biofilm formation by *A. actinomycetemcomitans* was dependent on AI-2-mediated quorum sensing (41), but how the initial detection of the AI-2 signal leads to downstream regulation of gene expression that influences biofilm growth was not determined. To determine if QseBC plays a role in regulating biofilm growth, wild-type, Δ*qseC*, and *qseC*-complemented strains of *A. actinomycetemcomitans* were cultured in flow cells as described in Materials and Methods. Representative images of the biofilms formed by each of these strains are shown in Fig. 1A and illustrate that inactivation of *qseC* resulted in a significant reduction in biofilm growth. Complementation of the mutant with a plasmid-borne copy of *qseC* restored biofilm growth to wild-type levels. Analysis of the biofilms by use of COMSTAT is shown in Fig. 1B. The total biomass ($P < 0.03$), average depth ($P < 0.02$), and maximum depth ($P < 0.004$) of biofilms formed by the *qseC* mutant were significantly reduced relative to those of the wild type (Fig. 1B). All biofilm growth parameters were restored to wild-type levels in the complemented strain. Indeed, biomass and average biofilm depth for the complemented strain were greater than those for the wild type, which may reflect gene dosage, as *qseC* is expressed from a multicopy plasmid in the complemented organism. These results suggest that QseC controls biofilm formation by *A. actinomycetemcomitans*.

The *A. actinomycetemcomitans* Δ*qseC* mutant and AI-2 receptor mutants are less virulent in a murine model of periodontitis. The ability of *A. actinomycetemcomitans* to grow in a biofilm is essential for its survival in the oral cavity and is associated with virulence. Our previous studies showed that AI-2-dependent quorum sensing (41) and *qseC* (described above) are essential components that regulate biofilm formation. To determine if AI-2 and *qseC* influence *A. actinomycetemcomitans* virulence, we utilized a murine model of periodontitis (3) in which mice were orally infected with 1×10^9 CFU/ml of the wild-type strain or strains lacking either QseC or the AI-2 receptors. As a control, sham-infected mice were infected only with CMC, the vehicle used to deliver the bacterial suspensions. Alveolar bone loss, one of the clinical outcomes of periodontal disease in humans, was then measured 47 days after the last infection. As shown in Fig. 2A, mice that were infected with the wild type ($P = 0.0002$), Δ*lsrB* ($P = 0.0261$), or Δ*rbsB* ($P = 0.0036$) strain of *A. actinomycetemcomitans* exhibited significantly more alveolar bone resorption than did sham-infected mice. However, animals infected with either the Δ*lsrB* or Δ*rbsB* mutant strain exhibited a significant reduction in bone resorption relative to that of the wild type (for the Δ*lsrB* strain, $P = 0.0285$; for the Δ*rbsB* strain, $P = 0.0134$), suggesting that the detection of AI-2 by either receptor contributes to virulence. Consistent with this, mice that were infected with the Δ*lsrB* Δ*rbsB* double-receptor mutant did not display significant levels of bone resorption compared to the sham-infected controls.

As shown in Fig. 2B, animals that were infected with the Δ*qseC* mutant exhibited significantly less bone resorption than those infected with the wild type ($P < 0.02$) and were indistinguishable from the sham-infected controls. The virulence of

A.



B.

Strain	Biomass (μm³/μm²)	Average biofilm depth (μm)	Maximum biofilm depth (μm)
652	3.86 ± 0.83	5.77 ± 0.92	37 ± 1.41
qseC⁻	0.32 ± 0.057 [1]	0.4 ± 0.16 [3]	14 ± 1.41 [5]
qseC⁻ comp	8.61 ± 0.23 [2]	13.35 ± 0.72 [4]	38.2 ± 0.71

Biomass was determined using the COMSTAT package of biofilm analysis software.

[1] $p < 0.0265$ [2] $p < 0.0160$ [3] $p < 0.0147$ [4] $p < 0.0116$ [5] $p < 0.0038$

FIG. 1. *qseC* is necessary for biofilm growth in *A. actinomycetemcomitans*. (A) Representative confocal images (in the *x-y* plane) of 60-hour biofilms formed by wild-type *A. actinomycetemcomitans* (strain 652) and Δ*qseC* and *qseC*-complemented strains. The resulting biofilms were quantified using the COMSTAT program (16). (B) Values for microbial biomass, average biofilm depth, and maximum biofilm depth were determined using COMSTAT image-processing software as described in Materials and Methods. At least 9 individual microscopic frames were analyzed for each biofilm experiment, and three independent biofilm experiments were carried out for each strain.

the Δ*qseC* mutant was restored to wild-type levels when it was genetically complemented with a plasmid-borne copy of *qseC*. These results suggest that both QseC and the ability to detect AI-2 contribute to *A. actinomycetemcomitans* virulence and that a defective AI-2-dependent quorum-sensing pathway negatively impacts the ability of *A. actinomycetemcomitans* to induce alveolar bone resorption.

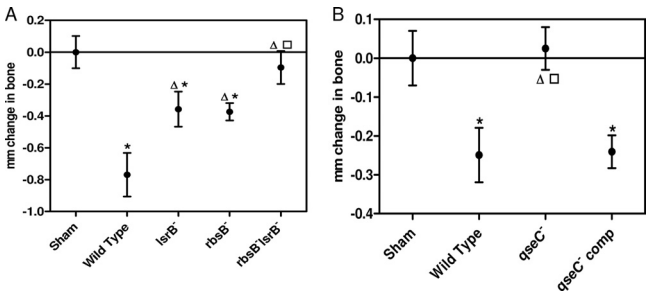


FIG. 2. *qseC* is necessary for *in vivo* virulence of *A. actinomycetemcomitans*. (A) AI-2 receptor mutants caused significantly less alveolar bone resorption than the wild type did. Infection with either the Δ*lsrB* or Δ*rbsB* mutant induced more alveolar bone loss than that in sham-infected mice but significantly less than that induced by the wild-type strain. The Δ*lsrB* Δ*rbsB* mutant induced bone loss comparable to that observed in sham-infected animals. The asterisks indicate statistically significant differences relative to sham-infected animals, triangles indicate significant differences relative to the wild type, and the square indicates a significant difference relative to the Δ*rbsB* strain. Values are means ± standard errors of the means (SEM). (B) Infection of mice with the Δ*qseC* mutant resulted in significantly less bone loss than that induced by the wild-type and complemented strains. The virulence of the mutant was restored to wild-type levels when the *qseC* mutation was complemented with a plasmid-borne copy of the *qseC* gene. Asterisks indicate statistical significance relative to sham-infected animals, the triangle indicates statistical significance relative to the wild type, and the square indicates statistical significance relative to the complemented strain. Values are means ± SEM.

Inactivation of *luxS* does not influence *A. actinomycetemcomitans* virulence. Previous studies showed that a Δ*luxS* mutant of *A. actinomycetemcomitans* formed biofilms that exhibited reduced total biomass and biofilm depth relative to those of wild-type biofilms (41). Since the *luxS* gene is responsible for the production of AI-2 and AI-2 is essential for the formation of biofilms, we next examined the importance of *luxS* in the *in vivo* virulence of *A. actinomycetemcomitans*. As shown in Fig. 3A, the extent of bone resorption induced by the *A. actinomycetemcomitans* Δ*luxS* mutant was indistinguishable from that induced by the wild-type or *luxS*-complemented strain, but all strains induced alveolar bone resorption over that in the sham-

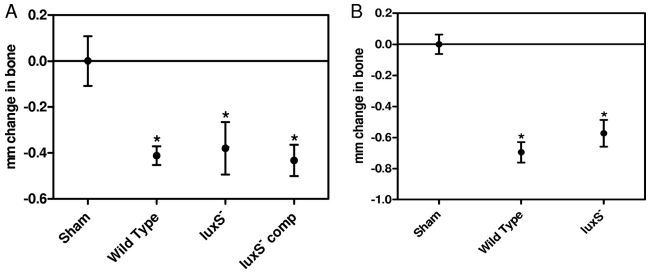


FIG. 3. The Δ*luxS* mutation is complemented *in vivo*. The Δ*luxS* mutants of *A. actinomycetemcomitans* (A) and *P. gingivalis* (B) induced alveolar bone resorption at levels that were comparable to those with the wild type or the *luxS*-complemented strain. Asterisks indicate statistically significant differences relative to sham-infected animals. Values are means ± SEM.

infected controls (for the wild type, $P = 0.0052$; for the Δ*luxS* mutant, $P = 0.0365$; and for the *luxS*-complemented strain, $P = 0.007$). Similar results were obtained when animals were infected with wild-type and Δ*luxS* mutant strains of another periodontal pathogen, *P. gingivalis* (Fig. 3B). Thus, inactivation of the AI-2 receptors, but not the AI-2 synthase, affected *A. actinomycetemcomitans* virulence. This suggests that AI-2 produced by indigenous bacteria in the mouse oral cavity may complement the *luxS* mutation in *A. actinomycetemcomitans*.

DISCUSSION

Dental plaque is a complex oral biofilm comprised of a diverse microbial community consisting of more than 700 different bacterial species (1, 20–22, 31, 50) that must coexist and adapt to the fluctuating environment of the oral cavity. It is likely that both intra- and interspecies communication occurs between these organisms and that these processes may enable oral organisms to coordinate their behavior and function based on their local environment and the other organisms that occupy the same niche. For *A. actinomycetemcomitans*, this intimate cell-to-cell communication potentially occurs via the quorum-sensing circuit dependent upon the soluble signaling molecule AI-2 (12). Indeed, previous studies showed that AI-2 regulates both biofilm formation and various iron acquisition pathways in *A. actinomycetemcomitans* (13, 41). However, it is not known how the initial detection of AI-2 is linked to downstream regulation of gene expression that controls these complex phenotypes. *A. actinomycetemcomitans* does not possess the dedicated two-component system that controls the cell density-dependent response of *Vibrio* spp. to AI-2 (i.e., the LuxQ sensor kinase, LuxU phosphorelay protein, LuxO response regulator, and LuxR master regulator encoded by the *lux* operon). Instead, the genome of *A. actinomycetemcomitans* encodes two periplasmic proteins, LsrB and RbsB, which function as receptors for AI-2, and each is linked to a putative ABC transporter that may import the signal (19, 40). Thus, the AI-2 response circuit in *A. actinomycetemcomitans* may be similar to that described by Li et al. for *E. coli*, in which the LsrR regulator controls the expression of genes involved in biofilm growth as well as regulating the expression of the *lsr* operon bound to AI-2 that has been phosphorylated by the LsrK kinase (23). AI-2 has also been suggested to regulate motility and biofilm formation in *E. coli* through the QseBC two-component system (14, 45). The *A. actinomycetemcomitans* genome also encodes the QseBC two-component system, and our results here show that *qseBC* is part of the AI-2 regulon in *A. actinomycetemcomitans*, since *qseBC* is induced by the AI-2 signal itself and its induction requires a functional AI-2 receptor. Furthermore, AI-2-dependent induction of QseBC in some *E. coli* strains requires the MqsR regulator (14). MqsR has been shown to be part of a toxin/antitoxin system and adopts an α/β fold similar to that of the RelE family of bacterial RNase toxins (8). Consistent with this, Yamaguchi et al. have shown that MqsR functions as a GCU-specific mRNA interferase (53). MqsR exhibits sequence similarity to open reading frame AA00673 in the *A. actinomycetemcomitans* genome (www.oralgen.lanl.gov), which is coexpressed with AA00672. AA00673 and AA00672 also exhibit approximately 65% sequence identity to the HigBA toxin/antitoxin system of *Vibrio cholerae*, and the HigB toxin is known to function as an mRNase (9). This suggests that QseBC may participate in the AI-2 response circuit in *A. actinomycetemcomitans* and that AA00673 may represent a paralog of MqsR and contribute to *qseBC* regulation.

Consistent with this, inactivation of the QseC sensor influenced the formation of biofilms by *A. actinomycetemcomitans* in that the Δ*qseC* mutant formed biofilms that were reduced in total biomass, average biofilm depth, and maximum biofilm depth relative to those for biofilms formed by the wild-type strain. The biofilm growth phenotype of the Δ*qseC* strain closely resembled the growth phenotype of the *A. actinomycetemcomitans* Δ*luxS* mutant previously reported by Shao et al. (41). Complementation of the Δ*qseC* mutation restored biofilm formation, and indeed, the complemented strain formed biofilms that exhibited greater average depth and biomass than those of the wild type. This may have been due to the gene dosage of *qseC* in the complemented strain, since *qseC* was expressed from a multicopy plasmid in this organism. These data suggest that the QseC sensor, and presumably the QseB response regulator, may be part of the quorum-sensing circuit mediated by AI-2 in *A. actinomycetemcomitans*.

However, although the QseC sensor may be common to the AI-2 signal transduction pathways of both *A. actinomycetemcomitans* and some *E. coli* strains, other aspects of these AI-2 response circuits appear to differ. Sperandio et al. have shown that *E. coli* O157:H7 QseC responds to stress hormones (epinephrine/norepinephrine) as well as the microbial signal AI-3 and that QseC may function in a one-to-many-branched signal transduction pathway that activates the QseB, QseF, and KdpE response regulators (17, 34). In addition, the QseE sensor kinase was shown to reside downstream of QseC in the adrenergic signal transduction cascade (17). At present, the signal that the *A. actinomycetemcomitans* QseC kinase senses is not known, and we have been unsuccessful in identifying AI-3 in *A. actinomycetemcomitans* extracts (D. R. Demuth, unpublished data). Furthermore, paralogs of the QseE, QseF, and KdpE polypeptides could not be identified in the *A. actinomycetemcomitans* genome at a search stringency that readily detected QseC with the *E. coli* QseC sequence as the query. This suggests that the components that reside downstream of QseC in *A. actinomycetemcomitans* may differ from those in some *E. coli* strains. Interestingly, *E. coli* O157:H7 lacks MqsR, and transcription of *qseBC* is independent of AI-2 in this organism (44).

Biofilm growth in *A. actinomycetemcomitans* is dependent upon AI-2-mediated quorum sensing (41), and formation of biofilms is essential to the *in vivo* survival and virulence of dental pathogens in the oral cavity. Our results show that AI-2-mediated quorum sensing is important for *A. actinomycetemcomitans* virulence *in vivo*, since mutants that were deficient in either of the AI-2 receptors (LsrB or RbsB) induced lower levels of alveolar bone resorption than the wild-type strain and since a double-receptor mutant was essentially avirulent. These phenotypes are also consistent with the biofilm phenotypes exhibited by these mutant strains (41). The Δ*qseC* mutant was also avirulent and did not induce bone loss over that in the sham-infected control group. This is consistent with QseC participating in and residing downstream of the AI-2 receptors in the AI-2 response circuit, as shown in the model

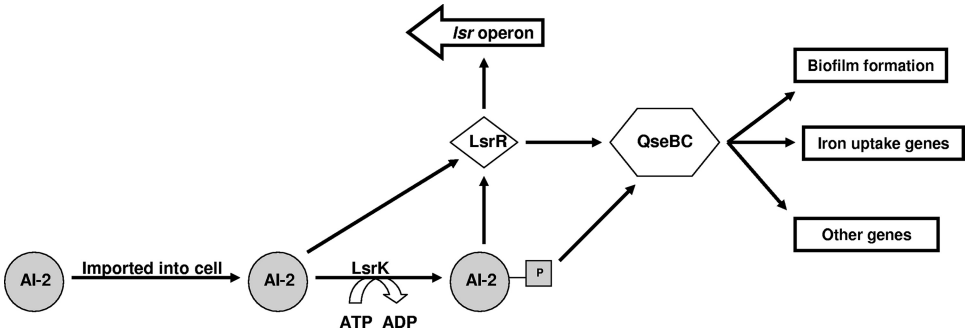


FIG. 4. AI-2 quorum-sensing circuit in *A. actinomycetemcomitans*. For *A. actinomycetemcomitans*, we hypothesize that AI-2-dependent biofilm formation and other potential AI-2-mediated cellular processes may result from one or two possible pathways. AI-2 is initially bound by its periplasmic receptors and imported into the cell by ABC-type transporters associated with the *lsr* and *rbs* operons. Once inside the cell, AI-2 is presumed to be phosphorylated by LsrK, and phosphorylated AI-2 is thought to interact directly with LsrR, resulting in derepression of the *lsr* operon. However, LsrR may also interact with unphosphorylated AI-2, as suggested by Li et al. (21). Regulation of the QseBC two-component system may occur through direct interaction with AI-2, or its expression may be induced by LsrR. The QseBC two-component system in turn alters the expression of genes that mediate biofilm formation, and potentially other genes, including those important for iron acquisition.

presented in Fig. 4. Nonetheless, the exact mechanism(s) through which QseBC contributes to the intracellular response of AI-2 as well as to biofilm growth in *A. actinomycetemcomitans* is not known. *A. actinomycetemcomitans* possesses the protein LsrR, which is the master regulator of the *lsr* operon in *Salmonella enterica* (46, 47), and Gonzalez-Barrios et al. suggested that AI-2-dependent biofilm growth of *E. coli* requires internalization of AI-2 (14). In *A. actinomycetemcomitans*, the QseBC two-component system may couple the detection/importation of AI-2, either alone or in cooperation with LsrR, to downstream regulation of biofilm formation and other cellular processes.

At present, the sets of genes that are regulated by the QseBC two-component system in *A. actinomycetemcomitans* are not known, but it is interesting that *qseBC* also exhibits sequence similarity to the *feuPQ* two-component system that regulates iron acquisition in several other bacterial species. Several operons that encode iron acquisition pathways have been shown to be regulated by AI-2 (13), and iron availability is also known to dramatically influence biofilm formation by *A. actinomycetemcomitans* (35, 36). Thus, it is possible that the biofilm defect that arises from inactivation of *qseC* occurs in part from the dysregulation of iron acquisition mechanisms. We are currently determining if the QseBC two-component system coregulates iron acquisition genes, and RT-PCR experiments suggest that at least one ferric uptake transporter is under QseBC control (Demuth, unpublished data).

Given that *luxS* was previously shown to influence biofilm growth, it was surprising that neither *A. actinomycetemcomitans* nor *P. gingivalis* Δ*luxS* mutants exhibited attenuated virulence in the murine model. A possible explanation for this result is that the Δ*luxS* mutation was complemented by AI-2 produced by organisms that are indigenous to the murine oral cavity. Consistent with this, McNab et al. have shown that AI-2 cross talk occurs in dual-species biofilms of *Streptococcus gordonii* and *P. gingivalis* (27). In this system, dual-species biofilms formed efficiently even if one of the strains harbored a *luxS* mutation, but no biofilms formed if both strains were LuxS deficient. Our previous studies also showed that AI-2-mediated cross talk is possible between *A. actinomycetemcomitans* and *P. gingivalis* and that the AI-2 signal of *A. actinomycetemcomitans* is capable of modulating the expression of *luxS*-regulated genes in *P. gingivalis* (12). In addition, Rickard et al. showed that the ability of the oral microbes *Actinomyces naeslundii* and *Streptococcus oralis* to form dual-species biofilms in saliva was dependent on AI-2 produced by *S. oralis* (37). The inability to detect or respond to AI-2 prevents the stimulation of the AI-2 response circuit and results in reduced biofilm growth and attenuated virulence, whereas the inability to produce AI-2 is likely overcome by the presence of exogenous signals produced by other bacteria in the murine oral cavity.

In summary, we have shown that the QseBC two-component system is induced by AI-2 and that QseC is important for biofilm formation and virulence of *A. actinomycetemcomitans*. Our results suggest that QseC is part of the AI-2 response circuit and resides downstream of the AI-2 receptor proteins LsrB and RbsB. Further definition of the genes regulated by the QseBC two-component system may identify new targets for therapeutic intervention of aggressive periodontitis and other systemic infections associated with *A. actinomycetemcomitans*.

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