The *Actinobacillus actinomycetemcomitans* Ribose Binding Protein RbsB Interacts with Cognate and Heterologous Autoinducer 2 Signals

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Autoinducer 2 (AI-2) produced by the oral pathogen *Actinobacillus actinomycetemcomitans* influences growth of the organism under iron limitation and regulates the expression of iron uptake genes. However, the cellular components that mediate the response of *A. actinomycetemcomitans* to AI-2 have not been fully characterized. Analysis of the complete genome sequence of *A. actinomycetemcomitans* (www.oralgen.lanl.gov) indicated that the RbsB protein was related to LuxP, the AI-2 receptor of *Vibrio harveyi*. To determine if RbsB interacts with AI-2, the bioluminescence of the reporter strain *V. harveyi* BB170 (sensor 1–, sensor 2+) was determined after stimulation with partially purified AI-2 from *A. actinomycetemcomitans* or conditioned medium from *V. harveyi* cultures in the presence and absence of purified six-His-tagged RbsB. RbsB efficiently inhibited *V. harveyi* bioluminescence induced by both *A. actinomycetemcomitans* AI-2 and *V. harveyi* AI-2 in a dose-dependent manner, suggesting that RbsB competes with LuxP for AI-2. Fifty percent inhibition occurred with approximately 0.3 nM RbsB for *A. actinomycetemcomitans* AI-2 and 15 nM RbsB for *V. harveyi* AI-2. RbsB-mediated inhibition of *V. harveyi* bioluminescence was reversed by the addition of 50 mM ribose, suggesting that *A. actinomycetemcomitans* AI-2 and ribose bind at the same site of RbsB. The RbsB/AI-2 complex was thermostable since *A. actinomycetemcomitans* AI-2 could not be recovered by heating. This was not due to heat inactivation of *A. actinomycetemcomitans* AI-2 since signal activity was unaffected by heating in the absence of RbsB. Furthermore, an isogenic *A. actinomycetemcomitans* mutant that was unable to express *rbsB* was deficient in depleting *A. actinomycetemcomitans* AI-2 from solution relative to the wild-type organism. Inactivation of *rbsB* also influenced the ability of the organism to grow under iron-limiting conditions. The mutant strain attained a cell density of approximately 30% that of the wild-type organism under iron limitation. In addition, real-time PCR showed that the expression of *afuABC*, encoding a major ferric ion transporter, was reduced by approximately eightfold in the *rbsB* mutant. This phenotype was similar to that of a LuxS-deficient mutant of *A. actinomycetemcomitans* that is unable to produce AI-2. Together, our results suggest that RbsB may play a role in the response of *A. actinomycetemcomitans* to AI-2.

Quorum sensing is a mechanism that allows a bacterial population to monitor its cell density through the action of soluble signal molecules called autoinducers, which may be acylated homoserine lactones (15, 24), peptides (8), quinolones (25), or furan derivatives (40). The concentration of autoinducer increases as the microbial population grows and eventually reaches a critical threshold that triggers changes in gene expression. It is thought that this process may allow organisms to synchronize the expression of genes that may be required for community survival (2, 13, 38).

Autoinducer 2 (AI-2) is a furan-like quorum-sensing signal that was initially identified in *Vibrio harveyi* (3) and is produced by the *luxS* gene (28). The enzyme encoded by *luxS* cleaves S-ribosylhomocysteine to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione (39), which in turn may undergo further rearrangement to produce AI-2. At least two structural forms of AI-2 have currently been identified: *Salmonella enterica* serovar Typhimurium produces 2R,4S-2,3,3,4-methyl-

tetrahydroxytetrahydrofuran (R-THMF), whereas *V. harveyi* produces the borate diester form of 2S,4S,-2,3,3,4-methyl-tetrahydroxytetrahydrofuran (S-THMF) (6, 22). In addition, Sperandio et al. suggest that LuxS may be required to produce another signal in *Escherichia coli*, designated AI-3 (32). However, the structure of AI-3 and its similarity to the known AI-2 isoforms are not currently known. AI-2 regulates the expression of a variety of genes in *Vibrio* species, including the *lux* operon of *V. harveyi*. Detection of AI-2 by *V. harveyi* is mediated by LuxP, a periplasmic AI-2 receptor (6) which activates the LuxQ sensor kinase/phosphatase and initiates a phosphotransfer cascade involving LuxU (12) and the response regulator LuxO (11). LuxO in turn influences the expression of small RNAs that are bound by Hfq, an RNA chaperone. This complex destabilizes the *luxR* transcript encoding the activator of the *lux* operon (17).

Interestingly, *luxS* is highly conserved in a wide range of gram-positive and gram-negative bacteria, and many, if not all, of these organisms produce an AI-2-like signal that is capable of inducing the expression of the *lux* operon of *V. harveyi*. As a result, AI-2 has been suggested to represent a universal signal that is recognized by many organisms (40). AI-2 has been reported to influence a wide variety of cellular processes, including type III secretion (31), cell motility (18, 30), the de-

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velopment of biofilms (4, 7, 14, 20, 21, 41), the expression of virulence factors (10, 16, 19), and iron uptake (9). However, in organisms outside of the genus *Vibrio*, the mechanism that governs the response to AI-2 is not well understood since some of the downstream proteins that are involved in AI-2-dependent signal transduction in *Vibrio* spp. are absent or not well conserved in these organisms.

Our studies have focused on the oral pathogen *Actinobacillus actinomycetemcomitans*, a gram-negative organism that is associated with aggressive forms of early-onset periodontitis and other systemic infections (5, 23, 29, 42). We previously showed that *A. actinomycetemcomitans* produces an AI-2-like signal that induces *Vibrio harveyi* bioluminescence (10) and regulates growth of the organism under iron limitation and expression of a variety of iron storage and uptake genes (9). However, *A. actinomycetemcomitans* appears to lack direct homologs of the AI-2 sensor kinase/phosphatase (LuxQ) and phosphotransfer protein (LuxU) that are dedicated to the *Vibrio harveyi* response to AI-2. However, this organism does possess two genes that encode polypeptides (RbsB and LsrB) related to LuxP, the receptor for AI-2 in *V. harveyi*. The *A. actinomycetemcomitans* RbsB protein is similar to the *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 periplasmic ribose binding proteins (76% sequence identity to each) encoded by the *rbs* operon (*rbsDABCK*), which functions to transport ribose into the cell. LsrB is homologous (80% sequence identity) to the LsrB polypeptide of *S. enterica* serovar Typhimurium (35), which is part of an ABC transporter involved in the uptake of AI-2. Presumably, LsrB functions in a similar manner in *A. actinomycetemcomitans* since the *lsr* operon is highly conserved with the *S. enterica* serovar Typhimurium gene cluster. Since AI-2 is derived from the ribose moiety of *S*-ribosylhomocysteine, we hypothesized that the *rbsDABCK* operon may also function in the transport of AI-2 in *A. actinomycetemcomitans*. The focus of this study was to determine if the periplasmic ribose binding protein RbsB interacts with AI-2 and represents a potential receptor for AI-2 in *A. actinomycetemcomitans*. We show that purified RbsB protein inhibits AI-2-mediated induction of *V. harveyi* bioluminescence. An *A. actinomycetemcomitans* mutant that does not express RbsB was deficient in depleting AI-2 from solution relative to the parent strain. The mutant also failed to attain high cell density under iron-limiting growth conditions and exhibited reduced expression of a ferric ABC transport system. Together, these results suggest that RbsB may play a role in the response of *A. actinomycetemcomitans* to AI-2.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. actinomycetemcomitans* strains were cultured on brain heart infusion medium (Difco, Detroit, MI) containing 0.5% yeast extract and supplemented with 40 mg of NaHCO₃ per liter. Cultures were maintained at 37°C in an atmosphere of 5% CO₂. Growth of the *rbsB* mutant strain of *A. actinomycetemcomitans* was carried out in the medium described above, which was further supplemented with 25 µg per ml kanamycin. *E. coli* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with aeration at 37°C. *E. coli* strains containing plasmids pQE60 and pRE4 were cultured as described above in LB supplemented with 100 µg per ml ampicillin and 25 µg per ml kanamycin. *V. harveyi* BB170 (sensor 1–, sensor 2+) was a gift from B. Bassler (Princeton University) and was grown overnight with aeration in AB medium (34) at 30°C. AB medium consists of 10 mM potassium phosphate, pH 7.0, 0.3 M NaCl, 0.05 M MgSO₄, 0.2% vitamin-

free Casamino Acids (Difco), 2% glycerol, 1 mM L-arginine, 1 µg per ml thiamine, and 0.01 µg per ml riboflavin.

Expression and purification of *A. actinomycetemcomitans* RbsB. *A. actinomycetemcomitans* *rbsB* was identified from the complete genome sequence of *A. actinomycetemcomitans* HK1651 (26; www.oralgen.lanl.gov) and amplified from *A. actinomycetemcomitans* genomic DNA using primers *rbsNS5* (5'-GGAGATATACCATGGAAAACTAACCACATTAGCC-3') and *rbs3B* (5'-CCGGATCCTTCGCTGATGACTTT-3'). The underlined sequences in the primers represent NcoI and BamHI restriction sites used to facilitate cloning into the expression plasmid pQE60. Amplification was carried out using the following profile: 95°C for 10 min followed by 30 cycles of 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min. For some reactions, an overnight soak at 4°C followed the profile described above. The resulting PCR product was ligated with pGEM-T Easy, and the ligation product was transformed into *E. coli* DH5α to produce strain pGEMrbsB. Recombinant clones were confirmed by restriction digestion with EcoRI to release the *rbsB* insert. Plasmid pGEMrbsB was subsequently digested with NcoI and BamHI (specified by the primers listed above), and the resulting fragment was isolated from a 1% agarose gel and ligated with pQE60 digested with NcoI and BamHI. After transformation into *E. coli* DH5α (which contains a mutation in *luxS* and does not produce AI-2), recombinant clones were confirmed by restriction analysis. The resulting *E. coli* strain, QE60rbsB, was used for expression of the RbsB polypeptide.

E. coli QE60rbsB was grown to early exponential phase in 100 ml LB supplemented with ampicillin (100 µg per ml) and kanamycin (25 µg/ml), and expression of *rbsB* was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. Growth was continued with aeration for an additional 3 to 4 h, after which cells were harvested, suspended in 4 ml of loading buffer (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 10 mM imidazole), lysed, and centrifuged at 10,000 × g to remove cellular debris. RbsB was purified from the resulting supernatant by affinity chromatography on nickel resin (HisTrap resin; Amersham Pharmacia Biotech). The column was first washed with distilled H₂O (10 ml), followed by equilibration with an equal volume of loading buffer. The protein extract was loaded by gravity, and the column was subsequently washed with 20 ml of loading buffer. Bound RbsB was eluted with 10 ml of elution buffer (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 0.5 M imidazole). The eluted protein was dialyzed overnight against 10 mM sodium phosphate, pH 7.5, and lyophilized. Prior to use, RbsB was suspended in distilled H₂O and protein concentration was determined using a Micro bicinchoninic acid assay kit from Pierce according to the manufacturer's instructions. Purity was assessed by visualizing the polypeptide by Coomassie blue staining after polyacrylamide gel electrophoresis (PAGE) on 4 to 15% gradient gels and by Western blotting using polyclonal anti-six-His antibodies.

Determination of *V. harveyi* bioluminescence. Induction of *V. harveyi* bioluminescence was carried out essentially as described by Surette and Bassler (34). Preparations of *V. harveyi* AI-2 were obtained from conditioned medium of overnight *V. harveyi* cultures grown in AI broth (34). After cells were harvested, the medium was filtered through a 0.22-µm-pore-size filter and used immediately or stored at –70°C. AI-2 from *A. actinomycetemcomitans* was partially purified by chromatography of conditioned medium from an exponential-phase culture on a Sep-Pak C₁₈ column (Waters) as described by Sperandio et al. (32). Briefly, cells were harvested from 4 ml of culture and the supernatant was filtered through a 0.22-µm-pore-size filter and subsequently through a Centricon YM-3 1-kDa exclusion filter (Millipore Co.). The filtered solution was lyophilized and concentrated by resuspension in 200 µl of cold 5 mM sodium phosphate, pH 6.2. Chromatography on a C₁₈ Sep-Pak column was carried out according to the instructions of the manufacturer. AI-2 activity was present in the column flowthrough fraction.

For the determination of *V. harveyi* bioluminescence, an aliquot of an overnight *V. harveyi* culture was diluted 1:5,000 into fresh sterile AB medium and 80 µl of cells was added to wells on a 96-well microtiter dish. Positive-control wells received cell-free conditioned medium from *V. harveyi* or partially purified *A. actinomycetemcomitans* AI-2 (20 µl). Negative-control wells received 20 µl sterile AB medium. Experimental wells received 20 µl of *V. harveyi* or *A. actinomycetemcomitans* AI-2 that was preincubated for 30 min at 30°C with various concentrations of purified RbsB protein. Samples were then added to wells containing the diluted *V. harveyi* cells to give a final concentration of 0.1 to 5,000 ng per ml RbsB. The microtiter plate was shaken on a rotary shaker at 500 rpm at 30°C, and bioluminescence was measured at half-hour or hourly intervals by use of a Wallac Victor³ 1420 MultiLabel counter (PerkinElmer). Bioluminescence of each well was initially calculated as induction (*n*-fold) of light relative to that for the negative-control reaction (*V. harveyi* BB170 cells incubated in sterile AI medium). Bioluminescence of the positive-control reaction was subsequently

normalized to a value of 1.0, and experimental data were calculated as percentages of the positive-control value.

Ribose-dependent reversal of RbsB inhibition of *V. harveyi* bioluminescence was determined as follows. *V. harveyi* or *A. actinomycetemcomitans* AI-2 was added to diluted *V. harveyi* BB170 cells (in the absence of RbsB), and the reaction mixtures were incubated as described above until the onset of the induction of bioluminescence in the positive control (3-h time point). At that time point, all reaction mixtures were removed from the shaker and experimental wells received purified RbsB protein (to a final concentration of 2.5 to 10 μ g per ml) or RbsB protein (2.5 μ g per ml final concentration) in the presence of ribose (0 to 500 mM final reaction concentration). After the addition of RbsB or RbsB-ribose, plates were incubated with shaking for an additional 1 to 2 h to allow the induction of *V. harveyi* bioluminescence to continue, and then light production was determined as described above. Data were analyzed as already described.

The heat stability of RbsB, AI-2, and the RbsB/AI-2 complex was determined by heating the reactants at the desired temperature (37°C to 100°C) for 15 min prior to addition to diluted *V. harveyi* BB170 cells. Light production by the reporter strain was monitored as described above.

Inactivation of *A. actinomycetemcomitans* *rbsB*. Inactivation of the *rbsB* gene was carried out with suicide plasmid pGEMrbsKan, which does not replicate in *A. actinomycetemcomitans*. A portion of the *A. actinomycetemcomitans* *rbsB* operon spanning the *rbsC-rbsB* genes was amplified from *A. actinomycetemcomitans* genomic DNA by using oligonucleotides R5 (5'-ATTCTCAACATTTTGC GCC-3') and R3 (5'-CGGCAACGTGTGCGGATGC-3'). R5 anneals in the *rbsC* gene upstream from the *rbsB* coding region in the *rbs* operon (*rbsDACBK*). The reverse primer R3 anneals within *rbsB*. The 1,250-bp fragment was ligated into pGEM-T and transformed into *E. coli* DH5 α . The resulting plasmid was isolated, digested with PstI, and subsequently modified by inserting the kanamycin resistance marker obtained by PstI digestion of pUC4K (Promega). Kan^r Amp^r clones were selected, and pGEMrbsKan was confirmed by restriction digestion.

pGEMrbsKan (2 μ g) was introduced into *A. actinomycetemcomitans* by electroporation essentially as described by Sreenivasan et al. (33), and Kan^r Amp^r clones were selected. To confirm Campbell-type integration of the plasmid into the *A. actinomycetemcomitans* genome, genomic DNA was isolated and used as a template for PCR amplification with primers rbsC5 (5'-TGCTTATTGGCGT CGTGTGCGG-3') and kan3 (5'-GTGCAATGTAACATCAGAG-3'). Primer rbsC5 anneals in *rbsC* immediately upstream from the annealing site of primer R5 and therefore does not anneal to the suicide plasmid. Primer kan3 anneals within the kanamycin resistance gene of pUC4K. Together, these primers will generate a 1.4-kbp product from *A. actinomycetemcomitans* genomic DNA only if pGEMrbsKan is integrated in the *rbs* operon. Clones exhibiting the desired 1.4-kbp product were chosen for further study. The resulting mutant contains intact *rbsDAC* genes but is unable to express *rbsB* and the downstream *rbsK* gene.

To complement the *rbsB* mutation, a plasmid-borne copy of *rbsB* under the control of the *A. actinomycetemcomitans* leukotoxin promoter was transformed by electroporation into the mutant strain. The *rbsB* gene was amplified from the *A. actinomycetemcomitans* genome by use of primers RC5 (5'-CCGGATCCAA AACTAACCACATTAGCC) and RC3 (5'-CCTCTAGATTATTCGTGAT GACTTTGAG-3'), which possess restriction sites for BamHI and XbaI, respectively. The resulting fragment contains the entire *rbsB* open reading frame except for the start codon and also contains the translational stop codon. After digestion with BamHI and XbaI, the fragment was ligated in frame into pBluescript-ltx, which contains a fragment encompassing the *A. actinomycetemcomitans* leukotoxin promoter, its ribosome binding site, and the start codon of the leukotoxin operon. The desired plasmid, designated pBSrbsB, was identified by restriction analysis after transformation of *E. coli* DH5 α . Plasmid pBSrbsB was subsequently cleaved with KpnI and XbaI, and the *ltx-rbsB* fragment was subsequently ligated into the *A. actinomycetemcomitans* shuttle vector pYGS (9). After transformation by electroporation into the *rbsB* knockout strain, clones exhibiting resistance to both kanamycin and streptomycin were selected. The presence of pYGSrbsB in these clones was confirmed by restriction analysis.

Depletion of AI-2 by wild-type and RbsB-deficient strains of *A. actinomycetemcomitans*. Overnight cultures of *A. actinomycetemcomitans* strains were harvested by centrifugation, washed twice with sterile AB medium, and suspended in 0.5 ml of partially purified *A. actinomycetemcomitans* AI-2 at a cell density of approximately 4×10^8 cells per ml. Cell suspensions were incubated at various times up to 30 min at 37°C. The bacteria were then removed by centrifugation, and the supernatant was filtered through a 0.22- μ m-pore-size filter. Filtered supernatants were subsequently analyzed in triplicate for AI-2 activity by using the *V. harveyi* BB170 reporter strain. Bioluminescence was measured and analyzed as described above.

Growth of *A. actinomycetemcomitans* strains under iron limitation. The growth of *A. actinomycetemcomitans* strains under iron-limiting conditions was determined by use of brain heart infusion medium supplemented with 100 μ M ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) essentially as previously described (9). EDDHA was incubated overnight with medium prior to inoculation with *A. actinomycetemcomitans*. All cultures were inoculated at an identical cell density (2×10^8 CFU/ml) and were incubated at 37°C for 6 h. The optical density at 600 nm of each culture was determined. Cell density, expressed in CFU per milliliter, was calculated as previously described (9) by using standard curves of the optical density at 600 nm versus CFU (per ml) for each strain.

RNA isolation and real-time PCR. *A. actinomycetemcomitans* total RNA was isolated from cells by using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The RNA preparations were subsequently digested with RQ RNase-free DNase I (Promega) to remove contaminating genomic DNA. Prior to conducting real-time reactions, samples were assayed for the presence of genomic DNA by standard PCR using primers afuA5 (5'-GGAAGCCTTTACTGCTGTGCG-3') and afuA3 (5'-TCCTGCCTGTTCA ATCAATT-3') and *A. actinomycetemcomitans* genomic DNA as a template. Samples that produced the 150-bp product were discarded or treated again with DNase until a negative result was obtained by normal PCR. For real-time reverse transcription-PCR, first-strand cDNA was prepared by using Ready-To-Go You-Prime first-strand beads (Amersham Pharmacia) as described by the manufacturer, with primer afuA3 and 2 μ g total RNA. The resulting cDNA was amplified using a SmartCycler system (Cepheid) with a final reaction volume of 25 μ l that contained 8 μ l of first-strand cDNA mix, primer mix (0.3 μ M each primer), 0.5 $\times$ SYBR green dye (Roche Applied Science), and 2.5 U of *Taq* polymerase (Roche Applied Science). Amplification conditions for real-time PCR were as follows: denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 30 s for 40 cycles. The cycle threshold was determined from a second derivative plot of total fluorescence as a function of cycle number by using the analysis software supplied with the SmartCycler system. Real-time PCRs were carried out at least twice using independently isolated RNA samples with consistent results. Control reactions were carried out as described above but using the primers rpoA5 (5'-TGAAGTAGAGATTGATGG-3') and rpoA3 (5'-GAC AGATTACATGCTCCGG-3').

RESULTS

***A. actinomycetemcomitans* RbsB inhibits AI-2-dependent induction of *V. harveyi* bioluminescence.** To determine if RbsB interacts with *A. actinomycetemcomitans* AI-2, purified protein was incubated with partially purified *A. actinomycetemcomitans* AI-2 for 30 min at 30°C and the mixture was subsequently added to the AI-2 indicator strain *V. harveyi* BB170. The RbsB polypeptide was expressed with a C-terminal six-His tag in *E. coli* DH5 α , which cannot produce AI-2 (34), and was purified by affinity chromatography on nickel resin (HisTrap resin). The purified protein migrated as a doublet with a molecular mass of approximately 34 kDa (consistent with the value of 32 kDa predicted from the deduced amino acid sequence of *rbsB*) (Fig. 1A) and reacted with polyclonal anti-six-His antibodies (Fig. 1B). The doublet likely arises from incomplete processing of the RbsB signal sequence in the *E. coli* host. Incubation of purified RbsB with *V. harveyi* BB170 and *A. actinomycetemcomitans* AI-2 potently inhibited the induction of *V. harveyi* bioluminescence in a dose-dependent manner (Fig. 2A). The level of *V. harveyi* bioluminescence that was induced in the positive-control reaction was approximately 1,300-fold greater than light production by *V. harveyi* BB170 incubated with sterile AB medium. The concentration of RbsB required to inhibit *A. actinomycetemcomitans* AI-2-mediated induction of *V. harveyi* bioluminescence by 50% was approximately 1 ng per ml (0.35 nM). Interestingly, RbsB was also an effective inhibitor of bioluminescence induced by AI-2 from *V. harveyi* (Fig. 2B), with 50% inhibition of light induction occurring in the presence of approximately 40 ng per ml of RbsB protein. Light

FIG. 1. Expression of *A. actinomycetemcomitans* RbsB polypeptide. The *rbsB* gene was amplified from *A. actinomycetemcomitans* genomic DNA by use of primers derived from the complete genome sequence of *A. actinomycetemcomitans* HK1651 (26). Cloning of the amplified product into the vector pQE60, expression, and affinity purification of RbsB protein were done as described in Materials and Methods. (A) Coomassie-stained gel subjected to PAGE. Lane 1, size markers; lane 2, affinity-purified RbsB. (B) Western blot of gel subjected to PAGE (from panel A). RbsB was visualized using anti-six-His monoclonal antibody. The doublet likely arises from incomplete processing of the RbsB signal sequence in the *E. coli* host. Molecular size markers are labeled on the left.

induction by the positive control in these reactions was approximately 1,100-fold greater than that achieved by the negative control. Addition of bovine serum albumin instead of RbsB to *V. harveyi* BB170 cells stimulated with *A. actinomycetemcomitans* AI-2 or *V. harveyi* AI-2 had no effect on the production of light (data not shown). These results suggest that exogenously added *A. actinomycetemcomitans* RbsB effectively competes with *V. harveyi* LuxP for AI-2 and that RbsB is capable of interacting not only with its cognate signal but also with a heterologous signal produced by an unrelated organism.

RbsB-mediated inhibition of *V. harveyi* bioluminescence is reversed by ribose. Since RbsB is a periplasmic ribose binding protein and is a component of an ABC-type transporter of ribose, we next determined if the addition of ribose could reverse RbsB-mediated inhibition of *V. harveyi* bioluminescence by competing with *A. actinomycetemcomitans* AI-2 or *V. harveyi* AI-2 for the RbsB binding site. These reactions were carried out as direct competitions, in which RbsB (or RbsB mixed with ribose) was added to *V. harveyi* BB170 cells that were already stimulated with AI-2 rather than preincubated with signal prior to addition to the reporter cells. As shown in Fig. 3A, the addition of RbsB at the onset of induction of *V. harveyi* AI-2-mediated bioluminescence (e.g., the 3-h time point in the experiment shown in Fig. 3A) resulted in a significant inhibition of bioluminescence of cells at later time points. The addition of 2.5 μg per ml RbsB (0.08 μM) inhibited light production by 87%, and complete inhibition of bioluminescence occurred using 10 μg per ml (0.3 μM) RbsB. Consistent with the results presented in Fig. 2, RbsB was a more potent inhibitor of *V. harveyi* bioluminescence induced by *A. actinomycetemcomitans* AI-2 than of that induced by *V. harveyi* AI-2.

FIG. 2. RbsB inhibits *V. harveyi* BB170 bioluminescence induced by *A. actinomycetemcomitans* AI-2 (A) and *V. harveyi* AI-2 (B) in a dose-dependent manner. Purified RbsB protein (0.3 to 5,000 ng per ml) was incubated with partially purified AI-2 from *A. actinomycetemcomitans* or with conditioned AI medium from an overnight *V. harveyi* culture for 30 min at 30°C. After subsequent addition of *V. harveyi* BB170 cells, bioluminescence was measured at hourly intervals by using a Wallac Victor³ microtiter plate reader. The data shown represent the 6-h time point after the addition of AI-2, at which time the positive control (*V. harveyi* BB170 cells exposed to conditioned AI medium from an overnight *V. harveyi* culture) exhibited approximately 1,100- to 1,300-fold induction of bioluminescence over that of the negative-control reaction mixture containing sterile AI medium. Reactions were performed in triplicate.

FIG. 3. (A) Direct competition of RbsB with *V. harveyi* BB170 cells induced with *V. harveyi* AI-2. RbsB protein (2.5 µg per ml, ●; 5 µg per ml, ▼; or 10 µg per ml, ■) was added to *V. harveyi* BB170 cells at the onset of induction of AI-2-mediated bioluminescence (e.g., the 3-h time point in the experiment shown), and light production was monitored at various time points after the addition of RbsB. The positive-control reaction mixture (◆) did not receive RbsB and exhibited ~1,200-fold induction of bioluminescence at the 5.5-h time point. (B) RbsB-mediated inhibition of *V. harveyi* BB170 bioluminescence induced by *A. actinomycetemcomitans* AI-2 is reversed by the addition of ribose. *V. harveyi* BB170 cells were mixed with partially purified AI-2 from *A. actinomycetemcomitans* and incubated at 30°C with shaking. At the onset of the induction of bioluminescence (3 h after the addition of *A. actinomycetemcomitans* AI-2), RbsB protein alone (2.5 µg per ml, left bar), RbsB (2.5 µg per ml) in the presence of 10 mM ribose (middle bar), or 50 mM ribose (right bar) was added. Light production was subsequently determined at the 5.5-h time point. Positive-control reaction mixtures did not receive RbsB and exhibited approximately 1,000-fold induction of light over levels for the negative control (*V. harveyi* BB170 induced with sterile AI broth). (C) Influence of ribose on induction of *V. harveyi* BB170 bioluminescence in the absence of RbsB. *V. harveyi* BB170 cells were induced with partially purified *A. actinomycetemcomitans* AI-2 or with *A. actinomycetemcomitans* AI-2 in the presence of ribose at a final concentration of 0 to 1,000 mM. The induction of bioluminescence was determined for each sample as described in Materials and Methods. All reactions were performed in triplicate.

Addition of 2.5 µg per ml RbsB inhibited light production induced by *A. actinomycetemcomitans* AI-2 by approximately 97% (Fig. 3B). However, inclusion of 50 mM ribose with RbsB (2.5 µg per ml) dramatically reversed RbsB-mediated inhibition and increased light production by *A. actinomycetemcomitans* AI-2-stimulated cells to 47% of the level for the positive control (Fig. 3B). This suggests that ribose competes with *A. actinomycetemcomitans* AI-2 for RbsB and that both AI-2 and ribose bind to the same site on the RbsB polypeptide. As shown in Fig. 3C, ribose itself had no effect on *A. actinomycetemcomitans* AI-2-mediated induction of *V. harveyi* bioluminescence at concentrations up to 50 mM.

***A. actinomycetemcomitans* AI-2 and the RbsB/*A. actinomycetemcomitans* AI-2 complex are heat stable.** Chen et al. (6) previously reported that thermal denaturation of the *V. harveyi* LuxP/AI-2 complex released active AI-2. To determine if bound AI-2 could be recovered from the RbsB/AI-2 complex, RbsB protein was preincubated with *A. actinomycetemcomitans* AI-2 for 30 min at 30°C and the complex was subsequently heated at 37°C, 55°C, and 65°C prior to addition to *V. harveyi* BB170 cells. Samples were then assayed for AI-2 activity by monitoring induction of *V. harveyi* BB170 bioluminescence. As shown in Fig. 4A, no *A. actinomycetemcomitans* AI-2 activity was recovered from these samples, suggesting that the interaction of *A. actinomycetemcomitans* AI-2 with RbsB may differ from the previously characterized interaction of *V. harveyi* AI-2 with LuxP. To exclude the possibility that *A. actinomycetemcomitans* AI-2 was heat labile, samples of *A. actinomycetemcomitans* AI-2 were pretreated at various temperatures prior to addition to BB170 cells. As shown in Fig. 4B, *A. actinomycetemcomitans* AI-2-mediated induction of *V. harveyi* biolumines-

cence was not affected by exposing the signal to temperatures up to 65°C, but *A. actinomycetemcomitans* AI-2 lost activity when heated at 100°C. Thus, our inability to recover *A. actinomycetemcomitans* AI-2 from the RbsB complex did not arise from heat inactivation of *A. actinomycetemcomitans* AI-2. We next examined the thermostability of the RbsB polypeptide. Samples of RbsB protein were subjected to increasing temperatures (37°C to 65°C) prior to incubation with *A. actinomycetemcomitans* AI-2 for 30 min at 30°C. The RbsB/*A. actinomycetemcomitans* AI-2 mixtures were then tested for induction of *V. harveyi* BB170 bioluminescence. As shown in Fig. 4C, exposure of RbsB to temperatures up to 65°C did not significantly alter its ability to compete with *V. harveyi* LuxP for *A. actinomycetemcomitans* AI-2. Together, these results suggest that the RbsB/*A. actinomycetemcomitans* AI-2 complex is thermostable or, alternatively, that RbsB may be capable of refolding to an active conformation upon cooling to physiologic temperatures after thermal denaturation.

Inactivation of *rbsB* influences AI-2 transport by *A. actinomycetemcomitans*. Taga et al. (36) showed that the *S. enterica* serovar Typhimurium *lsr* operon encoded a transporter that functioned to internalize AI-2. To determine if the *A. actinomycetemcomitans* *rbs* operon may function as a transporter of AI-2, we constructed an isogenic mutant deficient in RbsB and compared its ability to deplete signal from solutions of partially purified *A. actinomycetemcomitans* AI-2 with that of the wild-type strain. As shown in Fig. 5, wild-type *A. actinomycetemcomitans* depleted AI-2 activity by approximately 85% after incubation of cells with *A. actinomycetemcomitans* AI-2 for 10 min, and almost-complete depletion of AI-2 (>95%) was observed after 15 min. In contrast, incubation of the RbsB-defi-

FIG. 4. RbsB, AI-2, and the RbsB/AI-2 complex are heat stable. (A) *A. actinomycetemcomitans* AI-2 was incubated for 30 min at 30°C with purified RbsB (2.5 μ g) and then heated for 10 min at 37°C, 55°C, or 65°C. Samples were subsequently tested for induction of *V. harveyi* BB170 bioluminescence. (B) To assess the heat stability of AI-2, partially purified *A. actinomycetemcomitans* AI-2 was heated for 10 min at 37°C, 55°C, 65°C, or 100°C and then added to *V. harveyi* BB170 cells. Bioluminescence was determined as described above. (C) For the RbsB protein, purified RbsB (2.5 μ g) was heated for 10 min at 37°C, 50°C, or 65°C and then incubated with partially purified *A. actinomycetemcomitans* AI-2 for 30 min at 30°C. Induction of *V. harveyi* BB170 bioluminescence was determined as described in Materials and Methods.

cient mutant with *A. actinomycetemcomitans* AI-2 for 10 min resulted in only a 10% loss of AI-2 activity, and approximately 50% of the AI-2 activity still remained after 15 min. However, both the wild-type and mutant strains completely depleted

AI-2 activity after longer incubation with *A. actinomycetemcomitans* AI-2 (e.g., 30 min). Thus, inactivation of *rbsB* influences the rate of depletion of AI-2 but does not completely inhibit the ability of the mutant strain to deplete AI-2 from solution. These results suggest that the *rbs* operon may function to internalize AI-2 but also suggest that other redundant mechanisms may exist in *A. actinomycetemcomitans* to transport AI-2. This is consistent with the findings of Taga et al. (36), who reported that inactivation of the *S. enterica* serovar Typhimurium Lsr transporter did not completely inhibit AI-2 transport.

Inactivation of *rbsB* influences aerobic growth of *A. actinomycetemcomitans* under iron limitation. The interaction of RbsB with *A. actinomycetemcomitans* AI-2 suggests that the RbsB protein may play a role in the response of *A. actinomycetemcomitans* to AI-2. If so, the loss of RbsB function would be predicted to generate a phenotype similar to that of a LuxS-deficient *A. actinomycetemcomitans* strain that cannot produce AI-2. Fong et al. (9, 10) previously showed that inactivation of *luxS* affected aerobic growth of *A. actinomycetemcomitans* under conditions of iron limitation and influenced several genes involved in iron uptake, transport, and storage. To determine the potential role of RbsB in the response of *A. actinomycetemcomitans* to AI-2, we examined the growth of the RbsB-deficient organism under iron limitation. As shown in Fig. 6A, the levels of growth of wild-type and RbsB-deficient *A. actinomycetemcomitans* strains were similar under iron-replete conditions. As expected, iron limitation did not affect the growth of the wild-type strain, but growth of the RbsB mutant was only approximately 30% that of the wild-type strain under iron-limiting conditions. Complementation of the *rbsB* mutation with a plasmid-borne copy of *rbsB* restored growth to

FIG. 5. Inactivation of the *A. actinomycetemcomitans* *rbs* operon influences the kinetics of depletion of AI-2 from solution by bacterial cells. Wild-type (●—●) and Rbs-deficient (○—○) strains of *A. actinomycetemcomitans* were incubated at 37°C with a solution of partially purified *A. actinomycetemcomitans* AI-2. At various times up to 30 min, bacterial cells were removed by centrifugation and the supernatant was analyzed in triplicate for the induction of *V. harveyi* bioluminescence as described in Materials and Methods.

FIG. 6. Inactivation of *A. actinomycetemcomitans* *rbsB* influences aerobic growth under iron limitation and expression of *afuA*, encoding a ferric iron transporter. (A) Levels of growth of wild-type *A. actinomycetemcomitans* (wt), isogenic *rbsB* and *luxS* mutants (RbsB⁻ and LuxS⁻, respectively), and the *rbsB* mutant complemented with a plasmid-borne copy of *rbsB* (RC) were determined under iron-replete conditions (black bars) and in the presence of 100 μ M EDDHA (gray bars). Cultures were analyzed at mid-exponential to late exponential phases of growth by determining optical density at 600 nm (O.D._{600nm}). The results presented are averages from three independent experiments. (B) Expression of *afuA* was monitored by real-time PCR using gene-specific primers and the fluorophore SYBR green. Fluorescence as a function of cycle number was plotted for reactions using RNA from wild-type *A. actinomycetemcomitans* (solid line) and an isogenic *rbsB* mutant (dotted line). Results from a negative-control reaction using RNA from the mutant strain but without reverse transcriptase (RT) are shown by the dashed line. The cycle threshold was calculated from the raw fluorescence data by using the onboard analysis software supplied with the SmartCycler system.

near-wild-type levels under iron limitation. The LuxS-deficient *A. actinomycetemcomitans* mutant exhibited a cell density of only 8% that of the control.

To determine if inactivation of *rbsB* influenced the expres-

sion of iron uptake genes, real-time PCR was used to follow the expression of *afuA*, which encodes a major ferric iron binding protein of *A. actinomycetemcomitans* that was previously shown to be regulated by AI-2 (9). As shown in Fig. 6B, expression of *afuA* is reduced by approximately eightfold in the *rbsB* mutant relative to expression in the wild-type strain. This is consistent with the eightfold reduction in *afuA* expression that was previously observed for the LuxS-deficient strain of *A. actinomycetemcomitans* (9). Together, these results suggest that RbsB may play a role in the response of *A. actinomycetemcomitans* to AI-2 and may function as a receptor for AI-2 in *A. actinomycetemcomitans*.

DISCUSSION

Many gram-positive and gram-negative bacterial species express LuxS and secrete and respond in various ways to a signal that is related to autoinducer 2 of *V. harveyi* (4, 7, 9, 10, 14, 16, 18, 19, 20, 21, 30, 31, 41). However, few of these organisms possess the dedicated two-component circuit that mediates the cell density-dependent response of *V. harveyi* to AI-2. Our previous studies showed that AI-2 secreted by the oral pathogen *A. actinomycetemcomitans* was capable of inducing *V. harveyi* bioluminescence (10) and also influenced aerobic growth of *A. actinomycetemcomitans* under iron-limiting conditions (9). These studies also suggested that AI-2-mediated regulation of iron uptake and storage genes contributed to optimal growth of *A. actinomycetemcomitans* under iron limitation. However, the mechanism by which *A. actinomycetemcomitans* responds to AI-2 is not fully understood. Analysis of the complete genome sequence of *A. actinomycetemcomitans* HK1651 (26; www.oralgen.lanl.gov) indicates that this organism lacks direct homologs of the dedicated sensor kinase/phosphatase (LuxQ) and the phosphotransfer polypeptide (LuxU) that exist in *Vibrio* spp. However, *A. actinomycetemcomitans* does possess genes that exhibit significant similarity to the *V. harveyi* RNA chaperone Hfq and the receptor for AI-2, LuxP. Indeed, the *A. actinomycetemcomitans* genome contains two genes that are related to LuxP, one encoding a protein similar to the periplasmic ribose binding protein RbsB of *E. coli* and the second encoding a polypeptide that is homologous to the periplasmic LsrB protein of *S. enterica* serovar Typhimurium. The LsrB polypeptide of *S. enterica* serovar Typhimurium has recently been shown to bind to AI-2 and to function as part of a transport system encoded by the *lsrACDBFGE* operon, which internalizes AI-2 (35, 36). However, inactivation of the Lsr transporter did not completely inhibit the internalization of AI-2 by *S. enterica* serovar Typhimurium (36), suggesting that other AI-2 transport mechanisms may exist. Our current studies show that *A. actinomycetemcomitans* RbsB interacts with AI-2 and plays a role in the cellular response to AI-2, suggesting that RbsB may also function to transport AI-2.

RbsB protein that was purified from an AI-2-deficient background inhibited *V. harveyi* bioluminescence induced by both *A. actinomycetemcomitans* AI-2 and *V. harveyi* AI-2 in a dose-dependent manner, suggesting that RbsB competes with LuxP (the periplasmic receptor for AI-2 in *V. harveyi*) for AI-2. Furthermore, *A. actinomycetemcomitans* AI-2 appeared to interact with the ribose binding fold of the RbsB protein, since RbsB-mediated inhibition of *V. harveyi* bioluminescence was

reversed in the presence of ribose. This observation is consistent with the structural similarity that exists between ribose and the different known isoforms of AI-2. Furthermore, the precursor of AI-2 (4,5-dihydroxy-2,3-pentanedione) is generated by LuxS from the ribose moiety of *S*-ribosylhomocysteine (15). Interestingly, RbsB was a more effective competitive inhibitor of *V. harveyi* bioluminescence that was induced by *A. actinomycetemcomitans* AI-2 than of that induced by *V. harveyi* AI-2. Fifty percent inhibition of light production induced by *A. actinomycetemcomitans* AI-2 occurred with the addition of 1 ng per ml (0.03 nM) of RbsB, whereas approximately 40 ng per ml (1.3 nM) RbsB was required for 50% inhibition of bioluminescence stimulated by *V. harveyi* AI-2. This may reflect a difference in binding affinity for *A. actinomycetemcomitans* AI-2 and *V. harveyi* AI-2 by RbsB, possibly arising from structural variation of the *A. actinomycetemcomitans* and *V. harveyi* AI-2 signals. Indeed, multiple forms of AI-2 are known to exist. For example, the structure of the LsrB/AI-2 complex reported by Miller et al. (22) showed that *S. enterica* serovar Typhimurium produces a form of AI-2 that differs in stereochemistry from and lacks the borate diester that is present in AI-2 produced by *V. harveyi*. Since boron is present at much lower levels in terrestrial environments than in seawater, RbsB and LsrB may have evolved to interact at higher affinity with the AI-2 isoform that lacks boron, whereas marine organisms expressing LuxP bind to the borate diester form of AI-2 at higher affinity.

An alternative explanation for the different levels of RbsB-mediated inhibition observed is that the concentration of *A. actinomycetemcomitans* AI-2 in conditioned medium from *A. actinomycetemcomitans* is significantly lower than the level of AI-2 that is present in conditioned medium from *V. harveyi*. We feel that this explanation is unlikely since the *A. actinomycetemcomitans* AI-2 that was used in these experiments was partially purified and concentrated 20-fold from *A. actinomycetemcomitans* conditioned medium. However, a difference in AI-2 levels cannot be excluded because a direct biochemical assay to quantify the levels of AI-2 in conditioned medium is not currently available. Nonetheless, our results clearly show that *A. actinomycetemcomitans* RbsB interacts with both its cognate signal and AI-2 produced by *V. harveyi*. Thus, the activity of RbsB is consistent with the hypothesis that AI-2 functions as a universal signal that is recognized by a broad and diverse group of bacteria (1, 27). Furthermore, if structural variation of AI-2 extends beyond the two known isoforms (*S. enterica* serovar Typhimurium and *V. harveyi*), it is possible that the affinity of the receptor interaction with AI-2 may allow an organism to distinguish and respond to AI-2 signals produced by different organisms.

Our previous results showed that inactivation of *luxS* affected aerobic growth of *A. actinomycetemcomitans* under iron limitation and influenced the expression of iron uptake/transport genes, e.g., *afuA* (9). Interestingly, inactivation of *rbsB* resulted in a similar phenotype. Growth of the RbsB-deficient strain was inhibited under iron limitation, attaining only approximately 30% of the cell density exhibited by wild-type *A. actinomycetemcomitans* during late exponential growth. In addition, real-time PCR indicated that expression of *afuA* was reduced by eightfold in the mutant. Together, these results suggest that RbsB contributes to the cellular response of *A. actinomycetemcomitans* to AI-2. However, the molecular

mechanism whereby RbsB contributes to the response to AI-2 remains to be determined. In *E. coli*, RbsB is the periplasmic binding component of an ABC-type ribose transport complex that functions to internalize ribose and subsequently phosphorylate the internalized sugar via the kinase RbsK (ribokinase). Our findings that RbsB competes with LuxP (Fig. 2 and 3) and that inactivation of *rbsB* inhibits the ability of the mutant strain to deplete AI-2 from solution (Fig. 5) suggest that the RbsB protein may bind to and deliver AI-2 to the membrane permease (RbsC), facilitating internalization of AI-2 in *A. actinomycetemcomitans*. Thus, our hypothesis is that the RbsB protein represents a periplasmic receptor for AI-2 and that the *rbs* operon of *A. actinomycetemcomitans* may function to internalize AI-2, similarly to the activity of the *lsr* operon which internalizes AI-2 in *S. enterica* serovar Typhimurium (35).

Inactivation of *rbsB* did not completely inhibit the ability of the mutant to deplete AI-2 from solution (Fig. 5). This result is of interest since Taga et al. (36) showed that some internalization of AI-2 by *S. enterica* serovar Typhimurium still occurred in a strain that was unable to express a functional Lsr transporter. However, Taga et al. (36) also noted that the *S. enterica* serovar Typhimurium genome contains the *rbsDA CBKR* operon, and our analysis of the *A. actinomycetemcomitans* genome indicates the presence of an operon that encodes a potential Lsr-like transporter (open reading frames AA02215 to AA02226 in the *A. actinomycetemcomitans* HK1651 genome sequence). Indeed, the *lsr* operons of *A. actinomycetemcomitans* and *S. enterica* serovar Typhimurium are highly conserved, with the exception of *lsrE*, which does not appear to function in AI-2 transport in *S. enterica* serovar Typhimurium (36). This raises the possibility that multiple transport mechanisms exist to internalize AI-2. Our results suggest that the residual transport activity observed by Taga et al. (36) in the Lsr-deficient strain of *S. enterica* serovar Typhimurium may arise from internalization of AI-2 by the *rbsDACBKR* operon. Furthermore, the ability of the *A. actinomycetemcomitans* *rbsB* knockout strain to deplete AI-2 during longer incubations could occur via the Lsr transporter, which presumably is functional in the *rbsB* mutant. It is also interesting to note that the growth of the RbsB mutant under iron limitation was not stunted to the extent of the LuxS mutant of *A. actinomycetemcomitans* that we previously analyzed (9). This phenotype might arise if the putative *A. actinomycetemcomitans* Lsr transporter partially complements the loss of Rbs-mediated AI-2 transport. Studies to inactivate the *lsr* operon of *A. actinomycetemcomitans*, both alone and in combination with the *rbsB* mutation, and to compare the kinetics of the interaction of the LsrB and RbsB proteins with AI-2 are underway.

The physiologic role of AI-2 remains controversial since it has been shown to function as a quorum-sensing signal in *Vibrio* spp. (1, 2, 37) and yet is suggested to be a metabolic by-product that is possibly consumed by many other organisms (37). *A. actinomycetemcomitans* does not possess the two-component phosphorelay signal transduction pathway that is coupled to LuxP (or related proteins) of *Vibrio* spp., suggesting that it does not perform a quorum-sensing role at least in the context of the *Vibrio* paradigm. Yet, in the presence of a rich nutrient source, *A. actinomycetemcomitans* requires AI-2 for optimal growth under iron limitation and for the development of biofilms (D. R. Demuth, unpublished). This suggests that

AI-2 does not function solely as a consumable metabolite. To understand the physiologic role of AI-2, it will be necessary to determine the structure of the *A. actinomycetemcomitans* AI-2 and to clarify the molecular pathway that governs the response of the organism to AI-2. Our studies provide some of the first evidence that the *rbs* operon may be involved in the recognition of AI-2 by *A. actinomycetemcomitans*. However, additional studies are required to determine if redundant transporters of AI-2 exist in *A. actinomycetemcomitans* and to determine the downstream events that occur within the bacterium after internalization of signal.

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