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Over-expression, purification, and confirmation of *Bacillus anthracis* transcriptional regulator NprR

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Abstract

Quorum sensing (QS) has been recognized as an important biological phenomenon in which bacterial cells communicate and coordinate their gene expression and cellular processes with respect to population density. *Bacillus anthracis* is the etiological agent of fatal pulmonary anthrax infections, and the NprR/NprX QS system may be involved in its pathogenesis. NprR, renamed as *aqsR* for anthrax quorum sensing Regulator, is a transcriptional regulator that may control the expression of genes required for proliferation and survival. Currently, there is no protocol reported to over-express and purify *B. anthracis* AqsR. In this study, we describe cloning, purification, and confirmation of functional full-length *B. anthracis* AqsR protein. The AqsR gene was cloned into the pQE-30 vector with an HRV 3C protease recognition site between AqsR and the N-terminal His₆-tag in order to yield near native AqsR after the His-tag cleavage, leaving only two additional amino acid residues at the N-terminus.

Keywords

Quorum sensing; the neutral protease regulator (NprR); *Bacillus anthracis*; purification

Introduction

Quorum sensing (QS) is a cell-cell communication system by which bacterial cells coordinate their gene expression and consequently, cellular processes in response to the cell density of the population [1]. A typical QS system in Gram-positive bacteria consists of a signaling peptide and a cognate transcriptional regulator. In general, a signaling peptide is

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Supplementary Content

Supplementary content includes the identified peptide sequences by Mass spectrometry. (Table S1).

produced as a pre-propeptide, which is processed into a propeptide during secretion into extracellular milieu and further cleaved proteolytically to become a mature signaling peptide (Fig. 1). Either the signaling peptide is sensed, which leads to the activation of a cognate regulator, or it is re-imported into the cell, in which it binds to and activates a cognate regulator [2]. In Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, and *Enterococcus faecalis*, signaling molecules are predominantly small peptides whose length ranges from 7 to 22 amino acids [2, 3].

Two QS systems, PlcR/PapR and NprR/NprX, are known in the *B. cereus* group of bacteria that includes the anthrax-causing *B. anthracis*, *B. cereus*, *B. thuringiensis*, and other *Bacillus* species to date [4]. Both PapR and NprX are produced as precursor peptides of 48 and 43 amino acids, respectively, and they are predicted to be proteolytically processed into a small mature signaling peptides. PlcR and NprR belong to the family of RNPP (Rap/NprR/PlcR/PrgX) transcriptional regulators, which are characterized by having multiple tetratricopeptide repeat domains (TPR domains) [5, 6]. The TPR domain is a motif of a degenerate 34 amino acids known to be involved in protein-protein interactions [7], and its role in the RNPP family regulators appears to be recognition of cognate signaling peptides [5, 6]. The PlcR/PapR system is shown to regulate the expression of several secreted virulence factors that are chromosomally encoded in both *B. cereus* and *B. thuringiensis* [8–11]. However, in *B. anthracis*, it is non-functional due to the nonsense mutation in the *plcR* gene [9]. The NprR/NprX system was recently identified and shown to be regulated by the PlcR/PapR system in both *B. cereus* and *B. thuringiensis* [12, 13]. Unlike the PlcR/PapR system, the NprR/NprX system appears to be functional in all three bacterial species, *B. anthracis*, *B. cereus*, and *B. thuringiensis*.

The NprR/NprX system was shown to control the expression of the neutral protease NprA (also known as NprB or Npr599) in *B. cereus* and *B. thuringiensis* [12, 13]. In *B. anthracis*, the NprR/NprX orthologous system also appears to regulate the expression of the NprA orthologue based on two separate observations. First, NprA has been shown to be part of the secretome of *B. anthracis* [14, 15]. Second, the disruption of the *nprR* orthologue resulted in mutants defective in extracellular protease production [16]. The NprR/NprX orthologous system of *B. anthracis* is predicted to control the expression of a set of genes distinct from those controlled by NprR/NprX of *B. cereus* and *B. thuringiensis*. *nprR* was highly expressed during the outgrowth of *B. anthracis* spores within murine macrophages [17], suggesting the NprR/NprX system may be required for survival and/or proliferation of *B. anthracis* during anthrax pathogenesis. In addition, our unpublished data suggest that the NprR/NprX regulon of *B. anthracis* is distinct from that of *B. cereus*. The *nprR* orthologue is BAS0566 and *nprX* is unannotated in the genome of *B. anthracis* Sterne. To recognize the difference mentioned above, we named BAS0566 and its unannotated cognate peptide as *aqsR* and *aqsP* for anthrax quorum sensing Regulator and Peptide, respectively.

Although the functionality of the AqsR/AqsP system remains to be determined, the nucleotide sequences of both *aqsR* and *aqsP* do not contain a nonsense mutation and appear to encode a functional regulator and a pre-propeptide, respectively. Zouhir et al. reported a purification procedure to obtain the NprR protein from *B. thuringiensis* [18]. However, only the construct with the helix-turn-helix (HTH) domain truncated from its N-terminus was

successfully crystallized. The NprR proteins of *B. cereus* and *B. thuringiensis* and AqsR of *B. anthracis* share high sequence identity including their N-terminal HTH domain (Fig. 2). No protocol has been reported to purify either full-length AqsR or HTH-truncated AqsR protein from *B. anthracis*. Herein, we have described the procedure for the cloning, over-expression, and purification of the full-length AqsR protein. The purification of this protein will facilitate the biochemical characterization of AqsR, which will include electro-mobility shift assay and DNase I foot-printing to elucidate the DNA sequence motif recognized by the AqsR/AqsP complex, and the generation of an AqsR-specific antibody to be used to identify genes directly regulated by the AqsR/AqsP system in *B. anthracis*.

Materials and methods

Reagents, chemicals, biological, and equipment

The primers used for polymerase chain reactions (PCR) were purchased from Integrated DNA Technologies (Coralville, IA). Enzymes required for cloning, such as the Phusion Taq polymerase was purchased from New England Biolab (Beverly, MA), whereas the FastDigest restriction enzyme and T4 DNA ligase were purchased from Fischer-Scientific (Pittsburg, PA). The GenElute Genomic DNA purification kit and plasmid miniprep kit were purchased from Sigma (St. Louis, MO). The PCR product was purified using the Wizard SV Gel and the cleanup purification kit purchased from Promega (Madison, WI). The pQE vector series, as well as *Escherichia coli* M15/pREP4 were purchased from Qiagen (Valencia, CA). The materials for expression and purification, Luria-Bertani (LB) medium, ampicillin, Tris base, NaCl, imidazole, TCEP, and GelCode Blue Safe Protein Stain were purchased from Fisher Scientific (Pittsburgh, PA). Isopropyl β -D-thiogalactopyranoside (IPTG), beta-mercaptoethanol (BME), DNase I, Lysozyme, and Triton X-100 were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Recombinant type 14 3C protease from human rhinovirus (HRV 3C) was purchased from Novagen (San Diego, CA). The equipment used for purification, the Sonic Dismembrator Model 500, was obtained from Fisher Scientific (Pittsburgh, PA). The HisTrap HP column (5 mL, 16 $\times$ 25 mm), AKTApurifier FPLC system, HiLoad 16/60 Superdex 75 prep grade (120 mL, 16/600 mm) gel filtration column, and HiLoad 16/60 Superdex 200 prep grade (120 mL, 16/600 mm) gel filtration column, were obtained from GE Healthcare (Piscataway, NJ). NuPage 4–12% Bis-Tris Protein Gel was purchased from Invitrogen (Grand Island, NY). The surface plasmon resonance (SPR) was performed on the Biacore T200 instrument using a CM5 chip, both were purchased from GE Healthcare (Piscataway, NJ), as well as the HBS-P running buffer, EDC, NHS, and ethanolamine.

Construction of AqsR overexpression plasmid

Genomic DNA from the *B. anthracis* Sterne 34F2 (pXO1⁺, pXO2⁻) strain was purified according to company provided protocol using the GenElute Genomic DNA purification kit (Sigma). The *aqsR* (*BAS0566*) gene was PCR amplified with a pair of primers HP0859 (5'-CTAGGGATCCCTCGAAGTTCTGTTCCAGGGCCCGATGCAACAAACATTAGAAAAA A TAG-3') and HP0860 (5' CTAGGAGCTCACTATTATTCCTCCTT ATCATTCAATTAATC-3') using purified genomic DNA as a template. An HRV 3C protease recognition site in the N-terminus is underlined in the forward primer, HP0859. The

PCR reaction contained: 20 ng of template, 4 µl of 10 mM dNTPs, 4 µl each of forward and reverse primers (10 mM), 20 µl of 5X PCR buffer, 2 U of Phusion High-Fidelity DNA polymerase, and sterile water adjusted to 100 µl total volume. Amplified PCR products were purified using the Wizard SV Gel and a PCR cleanup purification kit. Enzyme double digestion was then performed with *Bam*HI and *Sac*I for both purified PCR products and pQE-30 vector. Double digested PCR products were ligated into linearized pQE-30. The resulting plasmid with correct *aqsR* sequence, pQE-30-*aqsR*, was transformed into *E. coli* M15/pREP4 cells via heat shock at 42 °C for 30s, followed by plating onto LB agar supplemented with ampicillin (100 µg/mL) and kanamycin (25 µg/mL). Cells containing pQE-30-*aqsR* were stored at –80 °C with 20% glycerol for long term storage.

Expression and purification of recombinant His-HRV-AqsR

The M15/pREP4 cells containing the recombinant His-HRV3C-AqsR plasmid were grown in 1 mL LB media supplemented with kanamycin (50 µg/mL) and ampicillin (100 µg/mL) while shaking at 220 rpm for 8 hours at 37 °C. The seed culture was diluted 1:500 with fresh LB media with the same antibiotics and grown for 16 hours at 37 °C. The overnight culture was diluted to OD₆₀₀ ~ 0.1 in 1 L LB medium (50 µg/mL kanamycin and 100 µg/mL ampicillin). The culture was grown, with shaking at 220 rpm at 37 °C, to OD₆₀₀ ~ 0.4, at which temperature was lowered down to 25 °C. Cells were further grown until the OD₆₀₀ reached 0.75 and were induced with 0.5 mM IPTG for 15 additional hours at 25 °C. The induced cells were harvested by centrifugation at 8000 rpm for 20 minutes (Beckman, JA-10 rotor), weighed, and stored at –80 °C.

For purification, the thawed cell pellet was resuspended in 20 mL per 1 g of lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1.5% Triton X-100, 0.025 mg/mL DNase I, 1 mg/mL lysozyme) and sonicated (Sonic Dismembrator Model 500, Fisher Scientific) at 65% amplitude for 5 minutes (3 seconds on, 6 seconds off). The crude extract was separated by centrifuging the sample at 16000 rpm for 15 minutes (Beckman, JA-20 rotor), and then filtered through a 0.45-µm membrane filter prior to being loaded onto a 5-mL HisTrap HP affinity column which was equilibrated with binding buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, and 5 mM BME). Ten column volumes of binding buffer were applied to wash unbound impurities followed by a step-wise gradient elution with elution buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 500 mM imidazole, and 5 mM BME). The elution gradient used was 50 mM, 200 mM, and 500 mM of imidazole in elution buffer at a flow rate 1.5 mL/min flow rate. The sharpest and highest peak was pooled and loaded onto either a HiLoad 16/60 Superdex 200 PG or HiLoad 16/60 Superdex 75 PG gel filtration column that was equilibrated with gel filtration buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% hydrogenated Triton X-100, and 1 mM TCEP). The protein was loaded at a flow rate of 1.2 mL/minute and eluted with 1.5 column volumes and collected in 2-mL fractions. The eluate was monitored by UV₂₈₀, and the protein peak samples were analyzed by SDS-PAGE.

Removal of His-tag

HRV 3C protease (1000 U, 500 µL) was added to the purified His₆-HRV3C-AqsR according to the Novagen recommendations in order to cleave the His₆-tag and linker amino acid

residues. The protein sample was dialyzed in 4 L of cleavage buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 0.5 mM TCEP) for 16 hours at 4 °C, while cleaving the tag at the same time. The mixture was then loaded onto a 5-mL HisTrap HP column equilibrated with binding buffer without imidazole (50 mM Tris, pH 8.0, 500 mM NaCl, and 5 mM BME), and the flow-through containing cleaved AqsR was collected. The collected protein was monitored by UV₂₈₀, and the protein peak samples were analyzed by SDS-PAGE.

Protein identification by Mass Spectroscopy

The His-tag cleaved AqsR protein was purified as described above. Purified AqsR was then applied to 4 12% NuPAGE Bis-Tris gels (Novex). A protein band with approximately 44 kDa was cut and sent for protein identification using Mass Spectrometry. Trypsin digestion was performed to cleave the proteins into peptides, and the peptide mixture was subjected to LC-MS/MS analysis using a Linear Trap Quadrupole (LTQ) ion-trap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Electron), high-performance liquid chromatography pump, and an auto-sampler (LC Packings). A full-scan MS (m/z 320 1800) experiment was performed, followed by MS/MS experiments on abundant ions detected in the full-MS scan. The collection of MS and tandem MS data were analyzed using the SEQUEST data analysis program. The *E. coli* protein database with the addition of *B. anthracis* AqsR was searched using SEQUEST to identify the protein.

Binding analysis by Surface Plasmon Resonance (SPR)

His-tagged AqsR was purified with IMAC in the manner described above prior to injection into a HiLoad 16/60 Superdex 200 PG equilibrated with 50 mM Sodium Phosphate, pH 7.5, 500 mM NaCl, 0.2% Triton-X 100, and 0.5 mM TCEP. The binding affinity (K_D) was determined by surface plasmon resonance (SPR) using the Biacore T200 instrument and a CM5 chip. Blank immobilization was performed by activating the sensor surface with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) followed by ethanolamine blocking on the activated surface area on flow channel 1 as a control. The purified AqsR was diluted with 10 mM sodium acetate (pH 4.0) and immobilized on flow channel 2 at 25 °C with running buffer PBS-P (10 mM phosphate pH 7.4, 2.7 mM KCl, 137 mM NaCl, and 0.05% surfactant P-20). The AqsR immobilization level was ~4500 response units (RU). A serial dilution of the peptide AqsP7 (SKPDIVG) ranging from 1.95 nM to 250 nM was prepared on a 96-well plate and injected through both flow channels 1 and 2 at a flow rate of 25 uL/min for 60 seconds association and 60 seconds dissociation time at 25 °C with binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% surfactant P-20, and 0.5 mM TCEP). The resulting sensorgrams were analyzed using Biacore T200 Evaluation Software v2.0. Data were referenced with RU values of blank channel and assay buffer alone before fitting the data with 1 to 1 steady-state affinity equation embedded in the Biacore T200 Evaluation Software.

Results and discussion

Cloning, expression and purification of His-tagged AqsR

The *aqsR* gene from *B. anthracis* Sterne 34F2 (pXO1⁺, pXO2⁻) strain encodes a 50.3 kDa protein with 422 amino acid residues. The desired gene was amplified using the purified *B.*

anthracis genomic DNA as a template with a cleavage site for 3C human rhinovirus (HRV 3C) protease inserted between the N-terminal His₆-tag and AqsR, and cloned into the pQE-30 vector. The HRV 3C cleavage site was inserted to allow for greater His₆-tag cleavage efficiency compared to the traditional thrombin digestion. In addition, there are only two amino acid residues left at the N-terminus of AqsR after His₆-tag cleavage by HRV 3C protease, producing a near native full-length AqsR protein. The pQE-30 vector contains a T5lac promoter allowing protein expression to be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). After the AqsR DNA sequence was confirmed, the plasmid was transformed into *E. coli* M15/pREP4 competent cells for overexpression.

The expression of His₆-HRV3C tagged full-length AqsR protein was induced by addition of 0.5 mM of IPTG at 25 °C, and was monitored by a SDS-PAGE analysis (Fig. 3A). The temperature used for induction was lower than that used in the *B. thuringiensis* NprR overexpression protocol [18] in order to yield increased amounts of soluble AqsR. After the addition of IPTG, a high level of protein expression was observed, as shown by an intense protein band in lane 3 compared to lane 2 (Fig. 3A). The absence of a protein band in lane 2 indicates that very little to no leaky expression occurs without IPTG induction.

The purification protocol for the full-length AqsR required several different chromatographic steps. First, the His₆-HRV3C tagged protein was purified from the crude extract by immobilized metal ion affinity column (IMAC) chromatography. Next, the tagged protein was purified from any contaminants by size exclusion chromatography. Finally, the cleaved protein was separated from the His₆-HRV3C tag by another IMAC chromatography step. AqsR is a hydrophobic protein, so a higher concentration of Triton X-100 and a higher volume (20 mL/g cell pellet) was used in the lysis buffer to increase the solubility. After cell lysis by sonication, the protein is reasonably soluble as shown in lanes 2 and 3 (Fig. 3B) with the clarified extract (lane 3) retaining similar amounts of the target protein as the crude extract (lane 2). The first IMAC chromatography step used a step gradient of elution buffer to isolate His₆-HRV3C tagged AqsR from the other *E. coli* soluble cellular components. At a 40% concentration of elution buffer, or approximately 300 mM imidazole, His₆-HRV3C-AqsR was eluted as determined by the distinct peak in the UV₂₈₀ absorbance measurement on the chromatograph. The corresponding 3-mL fractions were collected, and a portion of each fraction was run on SDS-PAGE gel to confirm the presence of expressed protein (lane 5 in Fig. 3B).

Table 1 shows the resulting yield and purity of a typical AqsR purification. In order to determine the protein concentration at the various stages of purification, the UV absorbance at 280 nm was measured. The yield after the first IMAC step is ~83% (Table 1) compared to the clarified extract. The lowered percent yield can be due to the high level of impurities that interfere with the protein measurements in the clarified extract, leading to a false sense of significant protein loss. Actual loss of protein is most likely due to a lowered binding affinity of the protein to the Ni-IMAC column, resulting in the protein being washed out. This is confirmed by the presence of the protein in the flow-through during the unbound wash step (lane 4 in Fig. 3B). Despite the loss in the flow-through, however, the SDS-PAGE shows high retention of the purified protein (lane 5 in Fig. 3B). To remove any additional impurities and provide preliminary structural information, the purified His₆-HRV3C tagged

AqsR was subjected to size exclusion chromatography equipped with the HiLoad 16/60 Superdex 200 PG gel filtration column. The His₆-HRV3C-AqsR (lane 6 in Fig. 3B) eluted at a volume corresponding to ~45–50 kDa, indicating it may exist as a monomer. (Fig. 4A) It is important to note that although the molecular weight of the His₆-HRV3C tagged AqsR is approximately 52 kDa, the protein band is apparent at approximately 44 kDa in the gel (Figs. 3 and 4B). A follow-up confirmation with Mass Spectrometry is described below.

His-tag removal and purification of native AqsR

The AqsR protein is a transcriptional regulator, and the introduction of a tag (His₆-HRV3C) of 17 amino acid residues at the N-terminus may abrogate or reduce its transcriptional activity, consequently affecting results of experiments (such as electrophoretic mobility shift assay) involving the purified AqsR. Although functions/activities of some proteins are not affected by the presence of a tag and/or by the tag size [19], others, do show complete/partial loss of their activities even with a tag consisting of a few residues [20]. Therefore, to avoid any potential complication for downstream studies with the purified AqsR, we tried to prepare as-native-as-possible AqsR. His₆-HRV3C tag cleavage was performed using HRV 3C protease, and removed by reloading the protease-treated sample into the Ni-IMAC column as follows. The collected fraction from size excluded AqsR was pooled and incubated with 1.5 units of HRV 3C protease per mg of protein. For maximal cleavage, the solution was dialyzed in dialysis buffer for 16 hours at 4 °C. Native AqsR was isolated from the cleaved His₆-HRV3C tag by injecting the dialyzed sample into the Ni-IMAC column pre-equilibrated with binding buffer without imidazole. Only the His₆-HRV3C tag and the HRV 3C protease, which is also a His-tag fusion, will bind to the Ni-IMAC column. As a result of tag removal, native AqsR will not be able to bind to the Ni column and will be collected in the flow through. The presence of cleaved AqsR was confirmed with SDS-PAGE gel (lane 7 in Fig. 3B), and the final yield was ~58% compared to the clarified extract (Table 1) at over 95% purity.

A typical AqsR purification from 1 L of LB culture results in ~5.2 grams of total cell weight which yields approximately 7 mg of pure native protein. There is minor loss in the initial chromatography step most likely due to the hydrophobic nature of AqsR, but the following steps show minimal loss in amount of protein.

Confirmation of AqsR

The size of the purified AqsR protein seemed to be approximately 44 kDa according to the SDS-PAGE, which is ~6–7 kDa smaller than the full-length AqsR size (50.3 kDa) (Fig. 3 and 4B). AqsR should be slightly above the 49 kDa protein marker in the SDS-PAGE gel, but it was shown below that marker closer to a 44 kDa area. This could indicate three very different possibilities. First, the purified protein is not AqsR, but some other *E. coli* protein that is 44 kDa in size. Second, the purified AqsR was truncated by an *E. coli* protease and became smaller. Fig. 5A illustrates where a truncation could have occurred in order to produce ~44 kDa AqsR. If this is the case, about 51 amino acid residues at the C-terminus would not be identified by Mass Spectrometry. Third, it is the correct AqsR of 50.3 kDa, but its migration is slightly faster than what it is supposed to be, causing it to show up as a smaller protein in the SDS-PAGE gel.

In order to clarify which possibility is occurring, the gel band containing the final purified protein of ~44 kDa was sliced and the identity of the protein was determined by mass spectroscopy. While protein identification by mass spectrometry can clearly indicate whether the purified protein is AqsR or not, it can also reveal if the purified AqsR is truncated. Truncation can only happen at the C-terminus because IMAC purification was done by capturing N-terminal His-tags, and hence the N-terminus was intact. Gel slices were treated with trypsin to generate small peptides before being subjected to LC-MS/MS analysis. Identified peptides were searched in the *E. coli* protein database with the addition of the *B. anthracis* AqsR protein sequence. Search results clearly indicated that our purified protein was indeed *B. anthracis* AqsR with > 80% coverage of total AqsR protein sequence (Fig. 5B). Identified peptides are shown in pink squares in Fig. 5B. As can be seen, more than 130 amino acid residues at the C-terminus are present in the purified AqsR, clearly indicating the purified protein is the full-length AqsR. All identified peptide sequences are listed in Supplementary Table S1). Therefore, we concluded that despite the apparent smaller size on the SDS-PAGE gel, the purified protein is the full-length AqsR.

Binding affinity of Peptide to AqsR

In order to confirm the purified AqsR was properly folded and functional, binding affinity analysis was performed between AqsR and its cognate signaling peptide AqsP. Previously, a heptapeptide (SKPDIVG), NprX7, was determined to bind to NprR at an estimated binding affinity (K_D) of 50 nM in *B. thuringiensis* [21]. Hence, the same heptapeptide orthologue in *B. anthracis*, AqsP7 (SKPDIVG), was tested with AqsR using the surface plasmon resonance (SPR) technique for K_D determination. Peptide concentrations ranging from 1.95 nM to 250 nM were passed through immobilized AqsR, and real time response units at each concentration were monitored. The 1 to 1 Steady-State Affinity model was used to analyze the data, and the resulting K_D was 57.3 nM, which is similar to that of NprR/NprX7 (Fig. 6). A shorter peptide, AqsP5 (SKPDI), was also investigated in the same manner, and its K_D was 2161 nM, ~38-fold weaker than that of AqsP7 (data not shown), indicating that at least seven residues might be required for signaling in *B. anthracis*, as was the case for *B. thuringiensis* [21]. Therefore, our purified full-length AqsR was able to recognize its signaling peptide AqsP7 at a comparable binding affinity to the NprR/NprX7 interaction, suggesting that it is functional.

Conclusions

Quorum sensing (QS) is a crucial cell-cell communication process that, in some bacterial pathogens, regulates expression of virulence factors. The AqsR/AqsP QS system may be one of the QS systems that regulate certain aspects of pathogenesis of *B. anthracis*. In this study, we have cloned the full-length *B. anthracis* AqsR gene into a pQE-30 vector with an HRV 3C protease cleavage site, optimized expression and purification conditions, and generated a protocol to yield the native AqsR protein. Lower temperature induction compared to the *B. thuringiensis* NprR over-expression protocol resulted in a reasonable yield of *B. anthracis* AqsR. Native full-length AqsR has been purified using a simple three step purification protocol with only two types of columns. Finally, the purified AqsR was able to recognize and bind to its signaling peptide AqsP7, confirming that it is well-folded and functional.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

QS	Quorum sensing
<i>B. anthracis</i>	<i>Bacillus anthracis</i>
PlcR	phospholipase C regulator
NprR	neutral protease regulator
RNPP	Rap/NprR/PlcR/PrgX
TPR domains	tetratricopeptide repeat domains
AqsR	anthrax quorum sensing Regulator
SPR	Surface plasmon resonance

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Figure 1. A schematic diagram of a potential quorum sensing regulatory system consisting of AqsR and its cognate signaling peptide AqsP

This diagram shows a possible pathway for the activation of anthrax quorum sensing Regulator (AqsR) controlling the expression of a putative target gene *nprA* in *B. anthracis*. Opp stands for oligopeptide permease (Opp) system, and the expression of *nprA* encoding a neutral protease A is known to be regulated by the NprR/NprX quorum sensing system (orthologous to AqsR/AqsP) in *B. cereus*.

Figure 3. AqsR expression and purification gel documentation and analysis

(A) SDS-PAGE of AqsR expression. Lane 1: SeeBluePlus2 pre-stained standard. Lane 2: After 16 hour cell culture without IPTG induction. Lane 3: After 16 hour cell culture with 0.5 mM IPTG induction. (B) SDS-PAGE of a typical purification of AqsR. Lane 1: SeeBluePlus2 pre-stained standard. Lane 2: Crude extract. Lane 3: Soluble portion of the clarified extract. Lane 4: Flow-through from first Ni-IMAC chromatography step. Lane 5: Pooled 40% elution fractions from the first Ni-IMAC. Lane 6: Pooled elution fractions from size exclusion chromatography step. Lane 7: Pooled His₆-HRV3C tag cleaved AqsR from the third IMAC step.

Figure 4. AqsR size exclusion chromatography analysis

(**A**) Gel filtration UV profile of His₆-HRV-AqsR (Monomer, ~52 kDa). Column volume is 120 mL. Void volume is ~45 mL. (**B**) SDS-PAGE of size exclusion chromatography. Lane M: SeeBluePlus2 pre-stained standard. Lanes 1 to 13 are each 2-mL fractions of size exclusion chromatography step starting from 74 mL to 100 mL shown in dashed lines in Figure 4A (A total of 13 fractions).

Figure 5. Validation of the purified AqsR protein by mass spectrometry

(A) Schematic of the His₆-HRV3C-tagged AqsR protein. The His₆-tag and HRV 3C protease cleavage sites are shown in black and cyan, respectively. (B) Peptide sequences identified by mass spectrometry. Highlighted in pink indicates ~80% of the total AqsR sequences covered by peptide sequences detected by mass spectrometry. Fifty one residues from the C-terminus shown in red indicate a potentially cleaved region to produce a truncated AqsR of 44 kDa in size.

Figure 6. Binding affinity analysis by surface plasmon resonance (SPR)
(A) Sensorgrams of AqsP7 binding to AqsR at varying concentrations. (B) Steady-State Affinity fitting curve using Biacore T200 evaluation software v2.0.

Summary of purification of recombinant AqsR.

Table 1

Step	Volume (mL)	Concentration (mg/mL) ^d	Total Protein (mg) ^e	Target Protein (mg) ^f	Yield (%)	Purity (%)
Crude Lysate ^a	56	4.14	232	19	100	8
Clarified Extract	53	2.78	146	15	79	10
Tagged AqsR HisTrap ^b	12	0.834	10.0	9.0	47	90
Tagged AqsR size exclusion	27	0.442	11.9	11	58	95
Tag-cleaved AqsR	40	0.174	6.96	6.6	35	>95

^aFrom 5.23 g of wet weight *E. coli* cell pellet (from 1 L culture).

^bFirst IMAC purification step (pooled fractions).

^cSecond IMAC purification step, after tag cleavage and dialysis (pooled fractions).

^dThe protein concentration determined by UV measurement at A280.

^eTotal protein is calculated based on concentration and volume.

^fTarget protein is calculated based on purity. Purity is estimated from the gel.