Identification of gyrB and rpoB gene mutations and differentially expressed proteins between a novobiocin-resistant *Aeromonas hydrophila* catfish vaccine strain and its virulent parent strain

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**ABSTRACT**

A total of 10 and 13 missense mutations were found in the deduced gyrB and rpoB proteins, respectively, between avirulent AH11NOVO vaccine strain and its virulent parent strain AH11P. SDS-PAGE revealed that six proteins bands were significantly over-expressed in AH11NOVO whereas five bands were significantly over-expressed in AH11P. Mass spectrometry identified seven proteins from the over-expressed AH11NOVO gel bands and five proteins from the over-expressed AH11P gel bands. QPCR confirmed that all 12 genes corresponding to the proteins identified by mass spectrometry were significantly over-expressed in AH11NOVO or AH11P. When AH11NOVO proteins were subjected to Western blot analysis, 13 protein bands exhibited significantly stronger reactivity with hyper-immune catfish sera. Fifteen proteins were identified from immunogenic protein bands, including six (formate acetyltransferase, chaperone HtpG, transketolase, ATP synthase subunit alpha, asparagine-tRNA ligase, and serine hydroxymethyltransferase) that were over-expressed in AH11NOVO proteins and three (elongation factor G, class II fructose-bisphosphate aldolase, and a putative uncharacterized 23 kDa protein) that were over-expressed in AH11P. In addition, the following six proteins were also identified from the immunogenic protein bands: pyruvate dehydrogenase E1 component, ATP synthase subunit beta, ribose-phosphate pyrophosphokinase, glyceraldehyde-3-phosphate dehydrogenase, 50S ribosomal L10, and 50S ribosomal L15. Our results might provide insights on how to develop novel efficacious vaccine against *Aeromonas hydrophila* infection.

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1. Introduction

*Aeromonas hydrophila*, a gram-negative, motile, rod-shaped bacterium commonly found in aquatic environments throughout the world, is the causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al., 2003). Feeding infected fish with antibiotic-mediated feed has been a general practice to control MAS (DePaola et al., 1995). However, this practice is expensive and usually ineffective as sick fish tend to remain off feed. In addition, the widespread use of antibiotics for treating bacterial diseases in aquaculture has led to the development of antibiotic resistance in many fish pathogens worldwide. Therefore, alternative control methods are urgently needed for the aquaculture industry.
To prevent future disease outbreaks caused by a virulent Alabama isolate of *A. hydrophila* AH11P, an attenuated vaccine AH11NOVO was developed from AH11P through selection for resistance to novobiocin (Pridgeon et al., 2012). Novobiocin works as an inhibitor of DNA gyrase beta subunit (gyrB) (Gellert et al., 1976; Holmes and Dyall-Smith, 1991). Mutations in *gyrB* gene have been reported in novobiocin-resistant *Staphylococcus aureus* (Stieger et al., 1996; Fujimoto-Nakamura et al., 2005). In addition, mutations in RNA polymerase beta subunit (*rpoB*) have also been reported in novobiocin-resistant *Salmonella typhimurium* (Blanc-Potard et al., 1995). However, it is currently unknown whether the selection of resistance to novobiocin in AH11NOVO might also have induced mutation at target sites. In addition, it was unknown whether the in vitro protein expression levels of the two strains were different. If so, what were the identities of those proteins? Therefore, the objectives of this study were to: (1) compare the DNA sequences of *gyrB* and *rpoB* of AH11NOVO and AH11P; and (2) identify differentially expressed proteins between AH11NOVO and AH11P.

2. Materials and methods

2.1. Bacterial growth conditions, genomic DNA extraction, and PCR amplification

Bacterial strains used in this study included a virulent strain of *A. hydrophila* AH11P and an avirulent strain AH11NOVO (Pridgeon et al., 2012). The AH11P strain was isolated from diseased channel catfish from Alabama in 2005. The AH11NOVO strain was a vaccine strain that was highly resistant to novobiocin (~960-fold of resistance, Pridgeon et al., 2012). Both bacteria were cultured in tryptic soy broth (TSB) or agar plates at 28 °C for 24 h. Single colonies from agar plates were transferred to TSB in the absence of novobiocin at 28 °C for 24 h. Bacteria were harvested from broth cultures by centrifugation for 15 min at 5000 rpm and washed twice in phosphate buffered saline (PBS, pH 7.2). Cell pellets were stored at −80 °C. Genomic DNA was extracted from *A. hydrophila* AH11P or AH11NOVO cell pellets (Pridgeon et al., 2012) using DNeasy kit (Qiagen, Valencia, CA, USA). To amplify *gyrB* and *rpoB* genes, *A. hydrophila* ATCC7966 genome sequence (GenBank accession no. CP000462) and Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were used to design primers. PCR products were sequenced at least three times.

2.2. Protein extraction, SDS-PAGE, in-gel trypsin digestion, and LC–MS/MS

Protein was extracted from bacteria pellets using B-PER protein extraction reagents (Thermo Fisher Scientific, Rockford, IL.). Protein concentrations were determined using a micro BCA protein assay (Pierce, Rockford, IL, USA). Proteins (10 μg) were separated by SDS-PAGE using method of Laemmli (1970). Both soluble and insoluble proteins were stained with SimpleBlue SafeStain (Life Technologies, San Francisco, CA). Band intensities were analyzed using NIH Imagej analysis software (http://rsbweb.nih.gov/ij/). Significantly (*P* < 0.05) differential expressed protein bands between AH11NOVO and AH11P were excised from gel. Gel pieces were trypsin digested and subjected to LC–MS/MS using published procedures (Kojima et al., 2012).

2.3. RNA extraction, cDNA synthesis, primer design, and quantitative PCR

Total RNA was isolated from AH11NOVO or AH11P using RNAprotect Bacteria Reagent and RNeasy Mini Kit (Qiagen, Germantown, MD). First strand cDNA was synthesized using AMV reverse transcriptase and oligo-dT primer (Invitrogen, Carlsbad, CA). Sequencing results of different proteins were used to search for their corresponding genes in GenBank through NCBI BlastP program. Gene-specific primers were designed using Primer3 program. For each cDNA sample, *A. hydrophila* 16S ribosomal RNA primers were included as an internal control. All qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (ABI, Foster City, CA) using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA). The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (*Ct*) of the gene by that of the 16S rRNA, the calibrator or internal control, as per the formula: Δ*Ct* = *Ct* (gene) – *Ct* (16S). The relative expression level of the specific gene in AH11NOVO compared to that in AH11P was then calculated by the formula 2^ΔΔCt where ΔΔ*Ct* = Δ*Ct* (AH11P) – Δ*Ct* (AH11NOVO) as described previously (Pridgeon et al., 2010).

2.4. Western blot analysis, LC–MS/MS, and statistical analysis

Immunogenic antigens of AH11NOVO were identified by Western blot. At 90 days post-immunization, pre-immune or hyper-immune sera were obtained from ten channel catfish immunized without or with AH11NOVO vaccine (injected on day 0, 30, and 60). Soluble and insoluble proteins of AH11NOVO were separated by SDS-PAGE transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blotted with the pre-immune or hyper-immune catfish sera overnight at 4 °C. After washing the membranes three times, the membranes were then blotted with a goat anti-channel catfish Ig heavy chain (Klesius and Horst, 1991) at a 1:2000 dilution. The tertiary antibody was alkaline-phosphatase conjugated anti-goat IgG (Sigma, St. Louis, MO, USA) at a 1:2000 dilution. The antibody-bound proteins were then visualized using 5-bromo-4-chloro-3-indolylphosphate toluidine salt and 4-nitro blue tetrazolium chloride (Bio-Rad). Band intensities were analyzed using UN-SCAN-IT gel analysis software (Silk Scientific, Orem, Utah). Significantly (*P* < 0.05) over-reactive protein bands were then excised from the PVDF membrane and subjected to proteolysis using published procedures (Methego et al., 2005). Digested peptides were then subjected to LC–MS/MS. All statistical analyses were performed using SigmaStat 3.5 software (Systat Software, Inc, Point Richmond, CA). Correlation between protein expression levels and
transcriptional levels were analyzed with Mann–Whitney U significance test. Differences at transcriptional level or translational levels were analyzed with Student’s t-test. Significance level was defined as $P < 0.05$.

3. Results

3.1. Sequencing results of gyrB and rpoB

Primers used to obtain the full length gyrB and rpoB are listed in Supplementary Table 1. The gyrB sequences of AH11P and AH11NOVO were both 2412 bp in length, coding for 803 amino acids (Supplementary Figure 1). Both sequences were deposited at GenBank with accession numbers KC133524 and KC133525, respectively. Sequence alignment between gyrB of AH11P and that of AH11NOVO revealed the ten missense mutations (Table 1). The rpoB sequences of AH11P and AH11NOVO were both 4092 bp in length, coding for 1363 amino acids (Supplementary Figure 2). Both sequences were deposited at GenBank with accession numbers KC133526 and KC133527, respectively. Sequence alignment between rpoB of AH11P and that of AH11NOVO revealed thirteen missense mutations (Table 1).

3.2. SDS-PAGE protein profile of AH11NOVO compared to AH11P and LC–MS/MS results

SDS-PAGE revealed that the protein expression levels of AH11NOVO insoluble proteins were similar to that of AH11P insoluble protein (Fig. 1). However, 11 protein bands were found to possess significantly ($P < 0.05$) different expression levels in the two bacterial strains, including six bands (band 1, 3, 5, 6, 7, 10) that were significantly ($P < 0.05$) over-expressed in the avirulent strain AH11NOVO and five bands (band 2, 8, 11, 12, 13) that were significantly ($P < 0.05$) over-expressed in the virulent AH11P strain. Mass spectrometry results of the 11 bands were listed in Supplementary Figures 4 and 5, respectively. Seven proteins identified from the over-expressed AH11NOVO protein bands were: formate acetyltransferase, chaperone htpG, transketolase, ATP synthase subunit alpha, asparagine-tRNA ligase, thymidine phosphorylase, and phosphoglycerate kinase (Table 2). Mass spectrometry also identified the following five proteins from the over-expressed AH11P protein bands: elongation factor G, enolase, class II fructose-bisphosphate aldolase, outer membrane autotransporter protein, and elongation factor P (Table 2).

3.3. Transcriptional levels of the 12 genes in AH11NOVO compared to that in AH11P

The genes corresponding to the 12 proteins identified by LC–MS/MS and gene-specific primers used in quantitative PCR (QPCR) are listed in Supplementary Table 2. QPCR results (Supplementary Table 3) revealed that the mean transcriptional levels of 7 genes in AH11NOVO were at least 10-fold higher than that in AH11P. The transcriptional levels of the following five genes in AH11NOVO were significantly ($P < 0.05$) lower than that in AH11P: serine hydroxymethyltransferase, acetate kinase, periplasmic nitrate reductase, and thymidine phosphorylase.

<table>
<thead>
<tr>
<th>Codon in AH11P</th>
<th>Codon in AH11NOVO</th>
<th>Nucleotide (bp)</th>
<th>Deduced amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>gryB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAC</td>
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<td>136</td>
<td>138</td>
</tr>
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<tr>
<td>AGC</td>
<td>GGC</td>
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<td>2181</td>
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</table>

| rpOB           |                  |                |                          |
| GAG            | AAG              | 211            | 213                      | Glutamic acid/E                           |
| GCT            | GGT              | 1291           | 1293                     | Alanine/A                                 |
| ACT            | GTC              | 1318           | 1320                     | Threonine/T                               |
| GAT            | GAA              | 1342           | 1344                     | Aspartic acid/D                           |
| GTC            | GGC              | 1843           | 1845                     | Valine/V                                  |
| GTT            | ATC              | 3118           | 3120                     | Valine/V                                  |
| GAG            | GAC              | 3133           | 3135                     | Glutamic acid/E                           |
| TAC            | TAT              | 3331           | 3333                     | Tyrosine/Y                                |
| GTC            | ATC              | 3457           | 3459                     | Valine/V                                  |
| GAA            | GAT              | 3463           | 3465                     | Glutamic acid/E                           |
| GTG            | ATC              | 3544           | 3546                     | Valine/V                                  |
| GTC            | GGC              | 3580           | 3582                     | Valine/V                                  |
| GCC            | TCC              | 3694           | 3696                     | Alanine/A                                 |

Table 1: List of mutations between gyrB or rpoB of AH11P and that of AH11NOVO.
3.4. Correlation between transcriptional levels and translational levels

Mass spectrometry identified 12 proteins from the 11 over-expressed protein bands, with two proteins identified from band 3: chaperone htpG and transketolase. When the transcriptional level of chaperone htpG was used in the analysis, significant correlation ($P = 0.013$) was found between the protein expression level and the transcriptional level of the 11 genes. Similarly, when the transcriptional level of transketolase was used in the analysis, significant correlation ($P = 0.015$) between the two expression levels was found.

3.5. Western blot of AH11NOVO proteins and mass spectrometry

When subjected to Western blot, AH11NOVO proteins exhibited significantly ($P < 0.05$) stronger reactivity with hyper-immune catfish sera vaccinated with AH11NOVO vaccine compared to that with pre-immune sera (Fig. 2). A total of 13 bands were excised from the PVDF membrane and subjected to LC–MS/MS. Results of the mass spectrometry of immunogenic AH11NOVO proteins were summarized in Supplementary Table 4. A total of 15 proteins were identified, including: (a) all seven but one (phosphoglycerate kinase) that were over-expressed in
AH11NOVO proteins; (b) majority (3 out of 5: elongation factor G, class II fructose-bisphosphate aldolase, and a putative uncharacterized 23 kDa protein) of the proteins that were over-expressed in AH11P. In addition, the following six proteins were identified from the protein bands excised from the PVDF membrane: pyruvate dehydrogenase E1 component, ATP synthase subunit beta, ribose-phosphate pyrophosphokinase, glyceraldehyde-3-phosphate dehydrogenase, 50S ribosomal L10, and 50S ribosomal L15 (Supplementary Table 4).

4. Discussion

Sequencing results revealed that mutations were induced by the selection of resistance to novobiocin, resulting in 10 missense mutations in deduced gyrB protein and 13 missense mutations in deduced rpoB protein. Mutations in DNA gyrase B have been reported to be associated with novobiocin-resistant archaeabacteria (Holmes and Dyall-Smith, 1991) and S. aureus (Fujimoto-Nakamura et al., 2005). In addition, mutations in RNA polymerase beta subunit (rpoB) have also been reported in novobiocin-resistant S. typhimurium (Blanc-Potard et al., 1995). Taken together, these results suggested the mutations at gyrB as well as rpoB might be associated with the high resistance of AH11NOVO to novobiocin.

Of the seven proteins that were over-expressed in AH11NOVO, six of them were also found in the immunogenic bands, of which five have been reported as immunogens. For example, serum antibodies to chaperone htpG protein of Porphyromonas gingivalis was reported to be protective against periodontitis (Shelburne et al., 2008). Transketolase was reported to be one of the major antigens of Leishmania infantum promastigotes (Dea-Ayuela et al., 2006). ATP synthase subunit alpha was found to be an immunoreactive protein in Campylobacter concisus, an emergent intestinal pathogen (Kovach et al., 2011). Asparagine-tRNA ligase was reported to be an immunodominant antigen of Brugia malayi, a filarial nematode (Kron et al., 1995). Serine hydroxymethyltransferase was one of the immunogenic proteins identified in Mycobacterium immunogenum (Roussel et al., 2011). These results suggest that over-expression of some immunogens might have contributed to the efficacy of AH11NOVO vaccine.

Of the three proteins that were found to be over-expressed in the virulent AH11P, the following two were also found to be immunogenic: elongation factor G and class II fructose-bisphosphate aldolase. Elongation factor G was found to be an immunogen of secreted proteins of Streptococcus suis (Wu et al., 2011). Fructose-bisphosphate aldolase was reported to be an immunogenic protein of Streptococcus iniae recognized by immune sera of Nile tilapia (LaFrentz et al., 2011). However, elongation factor G was also reported to be over-expressed in a virulent strain of Clostridium sordelli, a toxin-producing anaerobic bacillus that causes severe infections in humans and livestock (Kachman et al., 2010). In addition, mutagenesis in class II fructose-bisphosphate aldolase gene of Xanthomonas oryzae pv. oryzae reduced its extracellular polysaccharide production and impaired bacterial virulence (Guo et al., 2012). These results suggest that some virulence factors could also act as immunogens. This finding has been reported by other researchers. For example, cholera toxin is the primary virulence factor responsible for severe cholera because Vibrio cholerae strains unable to produce CT are severe attenuated in animals and humans (Price et al., 2013). The pentameric B subunit of cholera toxin is also a potent immunogen and a promising protective vaccine antigen in animal models (Price et al., 2013).

In addition to the nine proteins identified by both mass spectrometry (over-expression and Western blot), the following six proteins were also identified from the protein bands excised from the PVDF membrane: pyruvate dehydrogenase E1 component, ATP synthase subunit beta, ribose-phosphate pyrophosphokinase, glyceraldehyde-3-phosphate dehydrogenase, 50S ribosomal L10, and 50S ribosomal L15. It was reported that the sera from patients with tuberculosis could recognize pyruvate dehydrogenase specific for primary biliary cirrhosis (Klein et al., 1993). Pyruvate dehydrogenase was also reported to be one of the immunogenic proteins of Bacillus cereus recognized by human sera (DelVecchio et al., 2006). ATP synthase subunit beta was reported to be an immunogenic protein of Brucella abortus (Ko et al., 2012). Glyceraldehyde-3-phosphate dehydrogenase and ribosomal proteins have
been identified as major antigens of *Edwardsiella tarda* recognized by Japanese flounder antibody (Sakai et al., 2009). These results might provide insights on how to develop novel efficacious vaccine against *A. hydrophila* infection.

5. Conclusions

In summary, a total of 10 and 13 missense mutations were found in the deduced gyrB and rpoB proteins, respectively, between the avirulent AH11NOVO vaccine strain and its virulent parent strain AH11P. SDS-PAGE revealed that six proteins bands were significantly over-expressed in the avirulent vaccine strain AH11NOVO whereas five bands were significantly over-expressed in the virulent parent strain AH11P. Mass spectrometry identified seven proteins from the over-expressed AH11NOVO gel bands and five proteins from the over-expressed AH11P gel bands. QPCR confirmed that all 12 genes corresponding to the proteins identified by mass spectrometry were significantly over-expressed in AH11NOVO or AH11P. When AH11NOVO proteins were subjected to Western blot analysis, 13 protein bands exhibited significantly stronger reactivity with hyper-immune catfish sera. Fifteen proteins were identified from immunogenic protein bands, including six (formate acetyltransferase, chaperone htpG, transketolase, ATP synthase subunit alpha, asparagine-tRNA ligase, and serine hydroxymethyltransferase) that were over-expressed in AH11NOVO proteins and three (elongation factor G, class II...
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Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.07.025.

References


