Altered Transcriptome of the *B. melitensis* Vaccine Candidate 16MΔ*vjbR*, Implications for Development of Genetically Marked Live Vaccine

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Abstract The VjbR protein induced antibody responses in both human and animal brucellosis, and the *vjbR* mutant 16MΔ*vjbR* is an ideal vaccine candidate because of the feasibility of using the VjbR as diagnostic antigen. To further characterize this vaccine candidate and provide information for vaccine development, in the present study, a whole genome DNA microarray of 16M were used to compare the transcriptome of the *vjbR* mutant to that of the wild type strains. A total of 126 genes were greatly differentially expressed in the *vjbR* mutant. A great proportion

of *virB* and flagellar genes were differentially expressed in the *vjbR* mutant, implying that the *vjbR* regulate expression of virulence genes by sensing intracellular environments. Interestingly, the *virB* genes are regulated by the *vjbR* in independent manners as shown by their different fold changes and transcription abundances. A number of genes involved in translation, stress response, amino acid transport and metabolism, cell wall/membrane biogenesis, energy production and conversion, translation were differentially expressed. The *vjbR* mutant showed increased sensitivity to stresses of nutrition limitation, oxidative stress and acidification, and decreased survival in macrophage and mice, being consistent with its transcription profiles. These results indicated that the quorum sensing regulator *vjbR* could sense intracellular environments and response to them by regulate expression of virulence genes and other intracellular survival related genes, and therefore contribute to *Brucella* survival in host cells. This also provided direct evidence for the rational vaccine design by using antigenic global regulator for future development of genetically marked vaccine for brucellosis.

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Introduction

Brucella species are gram-negative intracellular pathogens, which can survive and replicate in macrophages and non-professional phagocytes. These bacteria are responsible for Brucellosis, a worldwide zoonotic disease causing abortion in domestic animals and Malta fever in humans [1]. Recent years saw a great increase in brucellosis incidence

worldwide, and therefore, *Brucellosis* is defined as re-emergent infectious disease worldwide [2].

Due to serious economic loss and public health risk, great efforts have been conducted to prevent the disease in animals through vaccination programs. Live attenuated vaccines have been proven to be the best vaccines and are used worldwide for prevention against animal brucellosis [3, 4]. However, its use is known to induce antibody responses indistinguishable by the current conventional serological tests from those observed in infected animals [5–7]. This fact limits the extended use of these vaccines in countries applying eradication programs based on serological testing and slaughtering of sero-positive animals. Therefore, numerous efforts have been made to develop new vaccines by targeted deletion of virulence genes or antigenic ones [8–12]. Recent research from other and our laboratory showed that the *vjbR* mutant is a good vaccine candidate for brucellosis, with the advantage of differentiation of immunization and infections [13].

To further understand the virulence regulation mechanism of *VjbR* and characterize the vaccine candidate 16MΔ*vjbR*, in the present study, transcription of *vjbR* under several in vitro conditions simulating the intracellular conditions was analyzed to define the one that *vjbR* was highly activated. Then, the transcriptome of the *vjbR* mutant was compared to that of the wild type strain under this condition to determine the target genes regulated by *vjbR*. The results showed that, besides the known target genes, a number of genes involved in several categories were differentially expressed in the *vjbR* mutant. The decreased expression of intracellular survival related genes in the *vjbR* mutant is consistent with its reduced virulence and survival under stresses conditions, indicating that *vjbR* may regulate *Brucella* intracellular survival and virulence by affecting the expression of target genes.

Materials and Methods

Ethics Statement

Mice were obtained from Experimental Animal Center of Academy Military Medical Science. All animals were handled in strict accordance with good animal practice as defined by the relevant national animal welfare bodies, and the animal work was approved by Beijing Institute of Disease Control and Prevention Animal Ethics Committee (Ethical Approval BIDCP012-2009).

Bacterial Strains

B. melitensis strain 16M was obtained from the Center of Chinese Disease Prevention and Control. Strain 16MΔ*vjbR*

was constructed previously. 16M-*vjbR* is the complementary strain of 16MΔ*vjbR*. All the *Brucella* was cultured in tryptic soy broth (TSB) or tryptic soy agar (TSA).

Determination of In Vitro Transcription Induction Conditions for *vjbR*

B. melitensis strain 16M, 16MΔ*vjbR* and 16M-*vjbR* were cultured and prepared as described previously [13]. The strain 16M was cultivated in TSB to the logarithmic phase ($OD_{600} = 1.3$) at 37 °C and then transferred to different stress conditions as described before [14]. The bacteria were subjected to TSB4.0 (acid shock), TSB5.5 (acid shock), GEM7.0 ($MgSO_4 \cdot 7H_2O$ 0.2 g/L, citric acid, H_2O 2.0 g/L, K_2HPO_4 10.0 g/L, $NaNH_4HPO_4 \cdot 4H_2O$ 3.5 g/L, glucose 20 g/L, pH 7.0, limited nutrition), GEM4.0 (pH 4.0, limited nutrition and acid shock), TSB7.0 with 1.5 mM H_2O_2 (oxidative stress), TSB7.0 with 50 mM H_2O_2 (oxidative stress), 42 °C (heat shock), TSB7.0 (control) for 30 min. Then, the transcription of *vjbR* under these stresses was quantified and compared by quantitative real time RT-PCR.

16M Whole Genome Microarray Construction, Hybridization and Data Analysis

The whole-genome DNA microarrays of 16M were developed based on the genomic sequences of *B. melitensis* strain 16M. PCR products of a total of 3,192 genes were obtained and used for microarray fabrication. The whole genome microarray hybridization was performed essentially as described previously [15]. Briefly, 16MΔ*vjbR* and 16M were grown in TSB to mid log phase and then subjected to the stress conditions. Total cellular RNA was isolated and cDNA were synthesized. The cDNA were labeled by Cy5 or Cy3, which were then hybridized to the slides. The scanning images were processed and the data were further analyzed by using GenePix Pro 4.1 software (Axon Instruments) combined with Microsoft Excel software. Significant changes of gene expression were identified with Significance Analysis of Microarrays (SAM) software. All microarray data is MIAME compliant and has been deposited in GEO.

Sensitivity to Nutrition Limitation, Heat Shock, Low pH and Oxidative Stresses

The sensitivity of *Brucella* strains to nutrition limitation, heat shock, low pH and oxidative stress was determined as follows: *B. melitensis* strains inoculated into TSB medium were grown to the early logarithmic phase ($OD_{600} = 0.6$) at 37 °C, and then subjected to different stresses. The bacteria was transferred to GEM 7.0 for 6 h, TSB 7.0 at

50 °C for 1 h, TSB 2.5 for 5 min, and TSB containing 440 mM H₂O₂ for 30 min for nutrition limitation, heat shock, low pH, and oxidative stress respectively. After the treatment, the survival percent was calculated by plating serial dilutions on TSA plates. All the results represent the averages from at least three separate experiments.

Statistical Analysis

Bacteria survivals under stress conditions were expressed as the mean log CFU $\pm$ the standard deviation (SD) for each group. The differences between groups were analyzed by ANOVA followed by Tukey's honestly significant difference post test comparing all groups to one another. For ANOVA, *P* values of <0.05 were considered statistically significant.

Results and Discussions

The *vjbR* is Highly Induced Under Stresses Simulating Intracellular Environments

For successful intracellular survival, bacteria must resistant or adapt to the intracellular environments [16]. To identify such conditions, 16M was subjected to several in vitro stress treatments. As shown in Fig. 1, compared with transcription in TSB 7.0, *vjbR* is highly activated under the stress conditions, implying that all tested stress conditions simulating intracellular environments could be sensed by *Brucella* and activate the expression of the *vjbR*. GEM 4.0, a condition simulating combination of acidification and nutrition limitation, is the strongest stimulus of the *vjbR* expression. Therefore, this condition is chosen for comparative transcriptome analysis for 16M and 16MΔ*vjbR*.

Fig. 1 Transcription of the *vjbR* under stress conditions. 16M was firstly cultured in TSB 7.0 to logarithmic phase and then subjected to different stresses. RNA was isolated and transcription of *vjbR* was quantified by qRT-PCR. The *vjbR* was greatly activated under GEM4.0

A Large Number of Genes of Different Functional Categories are Differentially Expressed in 16MΔ*vjbR*

Weeks et al. [17] compared transcriptome of a *vjbR* Tn5 mutant to wild type strain in the presence or absence of autoinducer C₁₂-HSL and found a number of genes were differentially expressed, and the *vjbR* regulons were putatively defined. Uzureau et al. [18] analyzed the quorum sensing targets of *B. melitensis* in 2YT medium by both proteomic and microarray analysis. However, *Brucella* is an intracellular bacterial and normal laboratory conditions, such as 2YT and TSB medium, could not correctly reflect the survival condition of *Brucella*. In this study, we chose an alternative means to analyze the regulation mechanism of the *vjbR* by comparing transcriptome of *vjbR* mutant to that of the wild type strain under a condition simulating intracellular environment. A total of 3,192 16M genes were successfully obtained and included in the microarray. This microarray was used for comparative transcriptome analysis. A total of 126 genes were identified to be differentially expressed in 16MΔ*vjbR* (Table S1, Fig. 2). Among these genes, 82 were up-regulated and 44 were down-regulated. These genes are distributed in 19 COG categories (Fig. 2). Genes involved in amino acid transport and metabolism, cell wall membrane biogenesis, energy production and conversion, translation accounted great proportion of these genes. Although some of the differentially expressed genes identified in the present study is consistent with those identified by Weeks and Uzureau, a great proportion of them is different from the gene set in their experiments, possibly due to different assay conditions and experimental system. This also implied the complex regulation mechanism of *vjbR* in *Brucella*.

The *virB* Genes are Regulated by *vjbR* in 16MΔ*vjbR*

The *virB* operon encodes a type IV secretion system, one of the most important virulence structures essential for *Brucella* intracellular survival and chronic infection of mice [19]. The regulation of *virB* by the *vjbR* is shown by results from lacZ reporter system [20]. Interestingly, in the present study, of the 11 *virB* genes, 10 (without *virB7*) were found to be differentially transcribed in 16MΔ*vjbR*. As shown in Fig. 3a, all the *virB* genes except of *virB7* are down-regulated at least twofolds. The unchanged expression of *virB7* implied that this protein might be tightly regulated at some special time points. Being consistent with our results, Weeks and Uzureau did not observe expression alteration of *virB7* [17]. The *virB4* is an ATPase, which provide energy for substrate export and pilus biogenesis. This gene is down-regulated in the present study, but remains unchanged in Jenni's experimental systems. Very interestingly, the two known effector proteins, VceA and VceC,

Fig. 2 Functional category distributions of differentially transcribed genes. The differentially transcribed genes identified by SAM are summarized according to their functional categories. A number of genes involved several functional categories were differentially transcribed in the *vjbR* mutant compared to parental strain. Genes involved in intracellular trafficking and secretion were down-regulated, and those involved in translation were up-regulated

were not differentially expressed in the *vjbR* mutant, being contrast to what observed by Weeks et al. [17], where the two proteins are differentially expressed, although the fold changes are lower than two. This implied that the *vjbR* might regulate the *virB* genes under intracellular environment in a manner different from that under standard culture condition.

The Different *virB* genes are Transcribed in Different Abundances and Regulated by *vjbR* in Different Manners

The *virB* encode a type IV secretion structure, and the *virB* operon is shown to be transcribed in a single transcript. Results from other secretion system showed that the structure component might be transcribed in different abundance [19, 21]. Therefore, we also examined the transcription abundance of the *virB* genes. As expected, the *virB* genes are transcribed in different abundance as shown by their different fluorescence intensity (FI). Both the gene length (2.4 kb) and probe length (2 kb) of the *virB4* is the longest ones, but its FI is not the highest one, indicating that the FI is related with transcription abundance, but not with its gene length (Fig. 3b, c). To further analyze the FI of each gene, the FI is divided by the gene probe length, as shown in Fig. 3c, all these genes showed different FI per bp. As shown in Fig. 3b, the *virB* genes are transcribed in different abundance. The different fold changes and abundances of the *virB* genes implied that they are independently regulated by *vjbR*, but not as a full transcript of the operon. Actually, the structure components of a secretion system are tightly regulated in a very complicated

manner [19]. Possible reasons for this include the different degradation rates of these transcripts, or they are transcribed from different start sites. As for the *virB* genes regulation, both of the two mechanisms are possible. For example, recent data indicated that there are initiation sites in the *virB* operon. The complicated regulation of *virB* genes by *vjbR* further confirms that the intracellular survival of *Brucella* is a tightly regulated process.

The Altered Transcriptome Profiles of 16MΔ*vjbR* is Consistent with its Decreased Growth Rate In vitro and Increased Sensitivity to Stresses

The increased expression of translation genes in 16MΔ*vjbR* prompts us to test whether 16MΔ*vjbR* will show altered growth rate. The growth curves of 16MΔ*vjbR*, 16M and 16M-*vjbR* were evaluated. As shown in Fig. 4a, 16MΔ*vjbR* showed reduced growth rate than 16M and 16M-*vjbR*. Resistance to intracellular stresses is considered to be one of the important virulence mechanisms for intracellular bacteria. Compared with 16M and 16M-*vjbR*, 16MΔ*vjbR* showed increased sensitivity to these stresses, confirming that *vjbR* promotes *Brucella* adaptation to these stresses (Fig. 4b). The increased expression of genes involved in energy production and conversion, amino acid transport and metabolism, and cell wall membrane biogenesis is consistent with the adaptation of *Brucella* to the acidified nutrition limitation. As observed in our previous study, 16MΔ*vjbR*, 16M-*vjbR* and 16M showed similar bacterial number at early infection phase [13]. However, at later infection phase, great declines was observed in 16MΔ*vjbR*, but not in 16M-*vjbR* and 16M, indicating that *vjbR* is involved in

Fig. 3 Fold changes and fluorescence intensity of the virB genes. **a** All the virB genes except virB7 were down-regulated in 16MΔvjbR compared with 16M. Compared with previous results, more virB genes are differentially expressed when bacteria was cultured under stress condition and high cell density. **b** Different fluorescence intensities of virB genes in 16M and 16MΔvjbR. **c** Fluorescence intensity per base pair of virB genes of 16M

Brucella survival and replication in macrophage. The reduced survival of 16MΔvjbR is consistent with previous results of vjbR deletion mutants, this further substantiates that vjbR is a key virulence regulator factor of *Brucella*. The virB is involved in *Brucella* intracellular survival, especially late stage of the survival. Therefore, vjbR might affect *Brucella* intracellular survival by regulating expression of virB and other related genes. This decreased expression of virB is different from the complete deletion of virB. Because virB is key virulence factor, deletion of which will make the mutant could not survive in macrophage, therefore, could not induce efficient protective immune responses. The efficient protection against virulence strain challenges indicated that, although with the decreased expression of related

Fig. 4 Phenotype characteristics of 16M, 16MΔvjbR and 16M-vjbR. **a** Growth curves of 16M, 16MΔvjbR and 16M-vjbR in TSB, 16MΔvjbR showed reduced growth rate than the other two strains. **b** Sensitivity of 16M, 16MΔvjbR and 16M-vjbR to nutrition deficiency, heat shock, low pH and oxidative stress. 16MΔvjbR showed reduced survival percent under these stress conditions

genes and intracellular survival capability, 16MΔvjbR could still survive in host and induce protective immune response.

Conclusions

Genetically marked live vaccine is an ideal vaccine form for brucellosis, with the advantages of reduced virulence and the feasibility of differentiation of immunization and infection. The choice of genes for screening and evaluation of this type of vaccine candidate is very important. As exemplified by the 16MΔvjbR, deletion of vjbR leads to reduced but not completely loss of survival both in vitro, macrophage and mice, making it possible to induce sufficient immune response in the hosts. Comparative transcriptome analysis of the 16MΔvjbR indicated the complicated regulation of other genes involved in environment sensing, intracellular survival. From this point of view, it could be conclude that for choice of genes for genetically marked live vaccine, genes involved in virulence regulation seemed to be good candidate, because the deletion will not lead to completely loss of virulence which provides the space to induce effective protective immune responses.

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