Veterinary Microbiology 159 (2012) 90–98

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

OtpR regulated the growth, cell morphology of *B. melitensis* and tolerance to β-lactam agents

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ARTICLE INFO

Article history:
Received 18 December 2011
Received in revised form 10 February 2012
Accepted 20 March 2012

Keywords:
Brucella
otpR
Morphology
Real-time qPCR
β-Lactam agents

ABSTRACT

The intracellular pathogen, *Brucella melitensis*, possesses an operon with two components: *otpR* (BMEI0066), which encodes a response regulator, and BMEI0067, which encodes a putative *cAMP*-dependent protein kinase regulatory subunit. Previous studies have shown that a polar mutation in the BMEI0066 gene significantly decreased virulence and stress tolerance in *Brucella*. In this study, we constructed non-polar mutant with deletion of *otpR*, as well as its complementary strain to further investigate the function of *otpR*. The Δ*otpR* mutant produced smaller colonies on TSA plates, and grew slower in tryptic soy broth compared to 16M or the *otpR*-complemented strain C*otpR*. Electron microscopy revealed that Δ*otpR* displayed an unusual, irregular deformation of the cell surface in contrast to the native coccobacillus shape of 16M. These results showed that OtpR played a key role in the maintenance of cell shape. To determine the effect of the *otpR* mutant on antibiotic susceptibility, compared the parent strain, the mutant was two- to eight-fold more suscepcible to all the β-lactam antibiotics tested. Furthermore, comparative real-time qPCR of genes that related to penicillin binding proteins of cell wall synthesis and cell division showed that the *otpR* mutation resulted in reduced expression of *pbp1C*, *pbp6B*, *pbp6C* and *ftsQ*. Taken together, these data revealed that the OtpR activity is necessary for growth, and cell morphology and tolerance to β-lactam agents of *B. melitensis*.

1. Introduction

Brucella melitensis is a facultative, intracellular, and Gram-negative bacterial pathogen that induces abortion and infertility in domestic animals, and undulant fever in humans. After invasion of phagocytes such as macrophages, *Brucella* inhibits the fusion of phagosomes to lysosomes and replicate within a safe intracellular niche, which is essential to disease establishment by intracellular pathogens. It is known that *Brucella* is subjected to harsh conditions, such as low pH, hypotonic environments and exposure to oxygen intermediates in phagosomes (van Furth, 1994). In the recent years, many of the gene products or mechanisms *Brucella* employs to combat the harsh intracellular environment have been identified or elucidated (Roop et al., 2009; Seleem et al., 2008).

B. melitensis possesses a response regulator gene BMEI0066, which encodes one member of the OmpR protein family. In our previous study, using the marked knock-out technique, we showed that the BMEI0066 of *B. melitensis* plays an important role in resistance to stress factors such as low pH, high temperature and hyperosmotic conditions (Zhang et al., 2009). Hence, we referred to BMEI0066 as *otpR* gene. The amino acid sequence of OtpR displays 65% identity to that of CenR (cell envelope regulator) in *C. crescentus*. Despite the high homology of the amino acid sequences between OtpR and CenR, there are some different functions between *otpR* and *cenR*. Deleption of the *cenR* led to a severe membrane blebbing phenotype and cannot form colonies on plates and fail to

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http://dx.doi.org/10.1016/j.vetmic.2012.03.022

grow in liquid medium (Skerker et al., 2005). In *C. crescentus*, CenR and CenK (cell envelope kinase) compose a two-component regulatory system. However, in *B. melitensis* there is only gene BMEI1648 with 33% identity to CenK of *C. crescentus*, and no gene was predicted to encode the corresponding sensor component of OtpR in *Brucella* genome.

Up to now, the OtpR role in *B. melitensis* has been poorly documented. The only indirect data concerning BMEI0066 function in *B. melitensis* were obtained by Zhang et al. (2009), who showed the *B. melitensis* BMEI0066::km played an important role in virulence and resistance to stresses (Zhang et al., 2009). As BMEI0066 and BMEI0067 share the same promoter, the translation of BMEI0067 in ΔBMEI0066::km mutant might be influenced by the kanamycin gene, which was used to take the place of BMEI0066 in the marked knock-out. It is important to further explore the singular roles of BMEI0066 in *B. melitensis*. In this study, a non-polar mutant of the *otpR* gene and its complementation stain were constructed to investigate the functions of OtpR.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

B. melitensis were cultured on tryptic soy agar (TSA, Bacto) or in tryptic soy broth (TSB) at 37 °C on a rotary shaker. *Escherichia coli* cultures were routinely grown on Luria-Bertani (Oxoid) plates or broth overnight at 37 °C. Antibiotics, when required, were added at the following concentrations: Ampicillin, 100 μg/ml; kanamycin, 100 μg/ml. Bacterial inoculates for mouse infection were cultured on TSA, as growth on this medium has been shown to minimize the appearance of spontaneous rough mutants (Alton et al., 1975). All bacterial strains (Table 1) were stored frozen at −80 °C in medium supplemented with 25% (v/v) glycerol. Liquid cultures used for experiments were inoculated at an optical density of 10^6 CFU/ml. CFUs were determined at 0, 24, 48, 60, and 72 h. All work with live *B. melitensis* was performed at biosafety level 3 laboratory in the College of Veterinary Medicine, China Agricultural University.

2.2. Recombinant plasmid construction

Genomic DNA was isolated from *B. melitensis* strain 16M. Plasmid DNA was isolated using plasmid Mini- or Max-purification kits (Omega). In order to construct the plasmid for creation of unmarked mutant with *otpR* (BMEI0066) deletion, primers were designed to amplify sequences flanking the genes. These flanking regions, referred to as the 5′ fragment and the 3′ fragment, were amplified in separate reactions, gel purified, and used as templates for a second round of overlapping PCR (Murphy et al., 2000). The forward primer of the 5′ fragment and the reverse primer of the 3′ fragment were utilized in a second round of PCR to engineer a product that represented the ligation of the 5′ and 3′ fragments. The ends of this joined product were removed by restriction digestion at primer adapter sites. The final fragment was gel purified and ligated to the pEX18Ap plasmid, which contained *sacB* at the appropriate restriction sites (den Hartigh et al., 2004). This construct is referred to as the unmarked plasmid pEX-1.1.

2.3. Selection of Δ*otpR*

Deletion mutants Δ*otpR* was created in *B. melitensis* via allelic exchange, following electroporation of the pEX-1.1 plasmid into the 16M. Selection of Δ*otpR* was performed as previously described (Zhang et al., 2009). Verification of mutant genotypes was conducted via PCR and sequencing analysis.

2.4. Complementation of otpR in mutant 16M Δ*otpR*

The pMR*otpR* plasmid containing the *otpR* gene fragments from the *B. melitensis* 16M genome was used for generating a complementation strain of the Δ*otpR* mutant. For construction of the unmarked mutant complementation strain, pMR*otpR* was electroporated into Δ*otpR* and then cells were plate onto TSA containing

Table 1
Bacterial strains and plasmids.

Bacteria or plasmid	Relevant characteristic(s)	Source or reference
B. melitensis		
16M	Wild-type	Virulent laboratory
Δ*otpR*	16M unmarked mutant of BMEI0066	This work
C*otpR*	*B. melitensis* Δ*otpR* harboring the plasmid pMR*otpR*	This work
CBBR*otpR*	*B. melitensis* Δ*otpR* harboring the plasmid pBBR*otpR*	This work
E. coli		
DH10B	F⁻*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ 80*lacZ*Δ*M15* Δ*lacX74 recA1 endA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *nupG*	Invitrogen
Plasmids		
pEX18Ap	*sacB, bla*, Amp^r	den Hartigh et al. (2004)
pBBR1MCS	Broad-host-range plasmid, Cm^r	Kovach et al. (1994)
pEX-1.1	WU451F/WU454R cloned into pEX18Ap	This work
pMR10	RK2 derivative, Low-copy-no plasmid, Kan^r	R. Wright
pBBR*otpR*	WU281F-WU632R product (BMEI0066) cloned into pBBR1MCS for complementation assay	This work
pMR*otpR*	16M WU631F-WU632R PCR product cloned into pMR10A for complementation assay	This work