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Original article

Erythritol triggers expression of virulence traits in *Brucella melitensis*

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Abstract

Erythritol is a four-carbon sugar preferentially utilized by *Brucella* spp. The presence of erythritol in the placentas of goats, cows, and pigs has been used to explain the localization of *Brucella* to these sites and the subsequent accumulation of large amounts of bacteria, eventually leading to abortion. Here we show that *Brucella melitensis* will also localize to an artificial site of erythritol within a mouse, providing a potential model system to study the pathogenesis of *Brucella* abortion. Immunohistological staining of the sites of erythritol within infected mice indicated a higher than expected proportion of extracellular bacteria. Ensuing experiments suggested intracellular *B. melitensis* was unable to replicate within macrophages in the presence of erythritol and that erythritol was able to reach the site of intracellular bacteria. The intracellular inhibition of growth was found to encourage the bacteria to replicate extracellularly rather than intracellularly, a particularly interesting development in *Brucella* pathogenesis. To determine the effect of erythritol on expression of *B. melitensis* genes, bacteria grown either with or without erythritol were analyzed by microarray. Two major virulence pathways were up-regulated in response to exposure to erythritol (the type IV secretion system VirB and flagellar proteins), suggesting a role for erythritol in virulence.

Keywords: *Brucella*; Erythritol; Flagella; Virulence

1. Introduction

Brucella spp. are facultative intracellular α-proteobacteria and the causative agent of the zoonotic disease brucellosis. Human infection with *Brucella* spp. results in an acute undulating fever before developing into a chronic disease potentially displaying endocarditis, meningitis, and osteomyelitis [1]. Brucellosis of domesticated animals (cows, goats, and pigs with *Brucella abortus*, *Brucella melitensis*, and *Brucella suis*, respectively) is also characterized by a chronic infection resulting in orchitis in infected males and spontaneous abortions in infected females [2]. Despite the endemic nature of *Brucella* spp. in many areas of the world, no suitable human vaccine is available and all current animal vaccines present a variety of potential downsides. A further knowledge of *Brucella*-host interactions is necessary to improve our understanding of the role of the host in *Brucella* infections.

A key site of this *Brucella*-host interface is the infected placenta. The spontaneous abortions that occur as a hallmark of animal brucellosis were thought to be due to the amount of *Brucella* "endotoxin" produced in the placenta as the bacteria replicate to levels as high as 10^{13} bacteria/gram of tissue [3]. This high level of bacteria was found to result from the presence of a growth-stimulatory factor in the fetal tissues that was later identified as the 4-carbon sugar erythritol [4]. Initially the ability of *B. abortus* to utilize erythritol was considered directly related to virulence, evidenced by the fact that the *B. abortus* vaccine strain S19 was known to be deficient in erythritol utilization [5]. While subsequent analysis found that the link between virulence and erythritol utilization was not as clear as it may have appeared, an erythritol-virulence connection is suggested by recent analysis [6–8].

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Growth analysis of *Brucella* spp. in erythritol-containing media found that *Brucella* spp. will utilize erythritol as a carbon source preferentially over the presence of glucose in the media [9]. The genes responsible for erythritol utilization were found to be organized in a four-gene operon encoding three catabolic proteins and an erythritol-responsive repressor that controlled transcription of the operon [10]. While the catabolism of erythritol and its potential connection to virulence have been well studied, there are few studies on how the bacteria respond to the presence of erythritol. In the present study, we examined several aspects of how bacteria interact with erythritol. *B. melitensis* localized to artificial sites of erythritol in a mouse model of infection, resulting in a significant immune response. Further, the growth of intracellular *B. melitensis* is inhibited by the addition of erythritol to the cell culture medium. To determine a potential cause of this inhibition, microarray analysis of *B. melitensis* grown in erythritol was conducted. In addition to the expected activation of energy production and metabolism pathways, several virulence pathways (flagella, type IV secretion system VirB, quorum sensing) were also up-regulated in response to erythritol. From these data, erythritol may play a significant role in the regulation of virulence in *B. melitensis*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in Table S1. *B. melitensis* strain 16M (ATCC23456) was used as the wild type strain. A virulent strain of *B. melitensis* generated previously that contains the *luxCDABE* operon from *Photorhabdus luminescens* was used for mouse and cell infections [11]. *Escherichia coli* strain DH5α was used for cloning and propagation. All *B. melitensis* strains were maintained on brucella agar and grown in brucella broth (Becton Dickinson) at 37 °C. Microarray analysis and promoter activity levels of *B. melitensis* were conducted in yeast extract minimal medium (YEMM) [12]. *E. coli* was grown in Luria-Bertani broth at 37 °C. Kanamycin (50 μg/ml) and ampicillin (100 μg/ml) were added to the medium as needed. RAW264.7 macrophages were grown in RPMI media containing 10% fetal bovine serum at 37 °C with 5% CO_2.

2.2. Infection of mice containing sugar–matrigel mixtures

Analysis of the localization of *B. melitensis* to sites of erythritol in mice was examined using the non-immunogenic matrix substrate Matrigel (BD Biosciences). A 10% solution of glucose or erythritol was mixed with the high-concentration Matrigel substrate. Half a milliliter of glucose gel was injected into the lower left back of 4 BALB/c mice and the erythritol gel was injected into the lower right back. All 4 mice were then immediately infected intraperitoneally with 1×10^6 CFU of the virulent, luminescent *B. melitensis* strain GR023 in PBS [11]. Mice were imaged three days after infection using the *in vivo* imaging system and Living Image software (Caliper). This work was carried out in accordance with the protocol approved by the Animal Care and Use Committee at the University of Wisconsin–Madison (protocol #V554). All imaging was conducted under isoflurane anesthesia.

2.3. Histology and immunocytochemistry of sugar–matrigel mixtures from infected mice

After six days mice containing the sugar–matrigel mixtures and intraperitoneally infected with GR023 were euthanized and the gels removed. After luminescent imaging of both the glucose and erythritol gels, they were paraffin embedded and six micron sections were subjected to hematoxylin and eosin staining (University of Wisconsin–Madison School of Veterinary Medicine Histology Center). Additional sections were stained for *B. melitensis* by incubating with polyclonal antibody directed against *B. melitensis* (Tetracore, Inc.). Incubation of horse-radish peroxidase (HRP) conjugated secondary antibody and resulting treatment with 3,3′-Diaminobenzidine tetrahydrochloride produced characteristic brown precipitates at sites of antibody binding within gels.

2.4. B. melitensis infected RAW macrophages cultured with erythritol-containing media

To determine whether addition of erythritol to intracellular bacteria resulted in changes in intracellular growth, RAW264.7 macrophages (ATCC) were infected with *B. melitensis* strain 16M. Bacterial cultures were grown overnight in brucella broth, diluted in RPMI, and used to infect RAW macrophages at a multiplicity of infection (MOI) of 100:1. Bacteria were allowed to infect for 1 h at 37 °C, cells were washed twice with phosphate-buffered saline (PBS), and external bacteria were killed using RPMI containing 30 μg/ml gentamycin at 37 °C for 30 min. Media was replaced with RPMI containing 5 μg/ml gentamycin to ensure that any bacteria released from macrophages would be killed. Selected wells contained RPMI with an addition of 1% erythritol (w/v). Enumeration of bacteria within the cells was determined at 0, 24, and 48 h post infection by lysing cells with 0.1% Triton X-100. Colony forming units (CFUs) within each well were counted by diluting ten-fold within 96-well plates and plating on brucella agar as previously performed [13].

In addition to testing intracellular infection, the number of extracellular bacteria present during cell infections with erythritol was also tested. RAW264.7 macrophages were infected with *B. melitensis* 16M as described above. After addition of 30 μg/ml gentamycin-containing RPMI to kill extracellular bacteria, cells were washed and media was replaced with RPMI without gentamycin and either with or without 1% erythritol. Supernatant samples were taken at 0, 24, and 48 h to determine the number of extracellular bacteria present in each sample. After supernatant samples were taken, cells were lysed and plated as described above to determine the number of intracellular bacteria present. The ratio of