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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 *B. 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 *B. abortus*, *B. melitensis*, and *B. suis*, respectively) is also characterized

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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].

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 *Photobacterium luminescens* was used for mouse and cell infections [11]. *E. coli* strain DH5 α 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 μ g/ml) and ampicillin (100 μ g/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% CO₂.

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, RAW 264.7 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 hour at 37C, cells were washed twice with phosphate-buffered saline (PBS), and external bacteria were killed using RPMI containing 30 μ g/ml gentamycin at 37C for 30 min. Media was replaced with RPMI containing 5 μ g/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 hrs 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. RAW 264.7 macrophages were infected with *B. melitensis* 16M as described above. After addition of 30 μ g/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 hrs 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 extracellular to intracellular bacteria in each condition was determined at each time point.

2.5 Activation of erythritol catabolism promoter in intracellular bacteria

Erythritol sensing by intracellular bacteria was analyzed by determining the level of promoter activity of the erythritol catabolism operon. Erythritol catabolism is controlled by a four gene operon encoding three catabolic proteins (*BMEII0430–428*) and a regulatory protein (*BMEII0427*). The promoter region (P_{ery}) consisting of approximately 500bp upstream of the first gene in the operon (*BMEII0430*) was cloned into the luminescent reporter plasmid pEP3 [14, 15]. The resulting vector pEP4 (P_{ery} -*lux*) was transformed into *B. melitensis* strain 16M.

The *P_{ery}-lux* strain and the virulent, luminescent GR023 strain were separately used to infect RAW macrophages as described above. RAW cells were incubated with RPMI media either with or without 1% erythritol and 5ug/ml gentamycin to kill any extracellular bacteria. Luminescent measurements were taken at 0, 24, 48, and 72 hrs. The GR023 luminescent measurements were used to approximate the bacterial level under each condition. Luminescent levels of the *ery* promoter were standardized to the luminescent level of GR023 under RPMI and RPMI+erythritol conditions. All cellular assays were conducted in triplicate and analyzed using Student's *t* test.

2.6 Microarray analysis of *B. melitensis* subjected to growth in erythritol

Microarray analysis was conducted on *B. melitensis* strain 16M to determine transcriptional changes during growth in erythritol. Cultures of *B. melitensis* strain 16M were inoculated into YEMM containing either 1% glucose or 1% erythritol and grown to an OD of 1.0. Total RNA was extracted using the Masterpure RNA extraction kit (Epicentre). Generation of cDNA, labeling, hybridization to microarray chips, and imaging of hybridized chips were conducted as performed previously [16]. Microarray scanned images were analyzed with Nimblescan software (Nimblegen) using robust multichip analysis (RMA). Gamma-gamma (GG) analysis was conducted on expression data using the R statistical program and EBarry package [17, 18]. Expression data for which the GG value was >0.5 were considered significant. Microarray data was submitted to the NCBI Gene Expression Omnibus (accession number GSE37819).

2.7 Analysis of promoter activity of *B. melitensis* during growth in erythritol

A subset of genes seen up-regulated in the microarray under growth in erythritol were selected for promoter analysis. The genes *BMEI0095*, *BMEI0102*, *BMEI0347*, *BMEII0025*, *BMEII0159*, *BMEII1009*, *BMEII1089*, *BMEII1108*, and *BMEII1116* were selected to examine the effect of erythritol on promoter activity. A negative control *infA* (*BMEI1671*) and positive control *ery* (*BMEII0430*) were also used. Approximately 500bp upstream of each gene of interest was cloned into the luminescent reporter vector pEP3 as described above. Each reporter vector was transformed into wild type *B. melitensis* strain 16M. These strains were grown overnight in YEMM containing 1% glucose as a carbon source. Each strain was then diluted to 0.1 OD in YEMM containing either 1% glucose or 0.5% glucose and 0.5% erythritol. These cultures were grown at 37C in a 96-well plate for 14 hrs to an OD of 0.5. Luminescent values and the OD of each strain in both media were measured in a DTX 880 multimode plate reader (Beckman Coulter). Relative light units (RLU) were determined by the ratio of photons/OD₆₀₀. Student's *t* test was used to compare the levels of promoter activity in erythritol medium versus the glucose control. Fold change in promoter activity was determined by taking the RLU of the erythritol experimental culture over the control glucose culture.

3. Results

3.1 *B. melitensis* preferentially localized to erythritol sites during mouse infection and induced cell infiltration

The presence of erythritol in the placenta of cows, sows, and goats has been used to explain both the tropism of *Brucella* for these sites and the ability of the bacteria to replicate to very high numbers and contribute to abortion [3, 4, 19]. However, erythritol has not been found in any measurable quantities in the placentas of mice that are commonly used as model systems for *Brucella* spp. infection [19]. To determine if we could replicate the ability of *B. melitensis* to preferentially localize to sites of erythritol in mice simulating a placenta, we utilized the cellular substrate matrigel (BD Biosciences).

A sample of matrigel containing 10% glucose and one containing 10% erythritol were injected into the lower back of a BALB/c mouse (n=4) where it solidified into a solid gel. The glucose sample was injected into the left side of the mouse and the erythritol sample was injected into the right. The mice were then infected intraperitoneally with the luminescent, virulent *B. melitensis* strain GR023 and bacteria were localized using the *in vivo* imaging system (Caliper). At three days post infection, mice were imaged for the presence of bacteria in each gel site, and in all 4 mice the level of bacteria at the site of the erythritol injection was greater than the glucose site (Fig. 1A). After imaging, mice were euthanized and gels were removed for further imaging (Fig. 1B).

3.2 Both cell-associated and free *B. melitensis* were found within the erythritol matrigel and resulted in large cellular infiltration

To further analyze the effect of *B. melitensis* in the erythritol matrigel, gels were analyzed by histological staining. While glucose gels showed very little inflammatory reaction, erythritol gels showed a high level of cellular infiltration (Fig. 2A-B). In some cases, large masses of cells appeared to be forming adjacent to erythritol gels. These masses suggest that the higher level of bacteria in these sites may have stimulated a potent immune reaction and caused this cellular recruitment. These results are in contrast to erythritol gels isolated from uninfected mice, indicating that bacterial localization at the site of the erythritol gel was required (Fig. S2).

The presence of bacteria in the erythritol gels was also analyzed using immunocytochemistry with a polyclonal antibody directed against *B. melitensis* (Fig. 2C). Bacteria in several locations can be seen localized to cells within the erythritol matrigel, indicating a possible intracellular infection. In addition to potential intracellular bacteria, there is also the presence of considerable extracellular bacteria within the gel. Large numbers of extracellular bacteria have also been observed within the placentas of *Brucella*-infected cows [20]. To determine if erythritol was playing any role in the presence large numbers of extracellular bacteria, the effect of erythritol on intracellular bacteria was examined.

3.3 Addition of erythritol to growth medium encourages extracellular growth of *B. melitensis*

The effect of erythritol on intracellular *B. melitensis* was examined by infecting RAW macrophages and monitoring infection by determining colony forming units (CFUs) present at 0, 24, and 48 hrs. Extracellular erythritol (1% w/v) was added to the growth medium and compared to RPMI growth medium without erythritol (Fig. 3A). In contrast to the control where the level of bacteria increased steadily through 48 hrs, in the erythritol-treated cells the same level of bacteria was observed throughout the entire infection. To confirm that erythritol-toxicity to the macrophages was not responsible for the observed phenotype, necrotic and apoptotic levels within macrophage cultures were tested, and erythritol was found to be non-toxic (Fig. S2).

Further analysis of the growth of intracellular *B. melitensis* under the presence of erythritol was conducted to determine if erythritol encouraged extracellular growth over intracellular growth. RAW macrophages were infected with *B. melitensis* strain 16M for one hour, and then cells were treated with gentamycin to kill all extracellular bacteria. Gentamycin was washed from the cells, and cell culture media without gentamycin and either with or without 1% erythritol was added to allow the growth of extracellular bacteria. Supernatant samples were taken at 0, 24, and 48 hrs and the number of bacteria present was determined. These values were compared to the level of intracellular bacteria to determine the extracellular-to-intracellular ratio of bacteria (Fig. 3B). At 48 hrs, the ratio of extracellular bacteria to

intracellular bacteria was greater than 10-fold higher under the presence of erythritol, indicating that erythritol encourages extracellular growth over intracellular growth.

3.4 Extracellular erythritol reaches the intracellular niche of *B. melitensis*

Any effect of erythritol is dependent on erythritol being available to intracellular *B. melitensis*. To determine whether this occurs, expression of the erythritol-regulated *ery* promoter was examined under these conditions. The four gene *ery* operon (*eryABCD*) consists of three proteins required for breakdown of erythritol and a repressor of the operon (*eryD*) whose binding to erythritol allows for transcription of the promoter (P_{ery}). The P_{ery} promoter was cloned into a promoterless reporter vector containing the *luxCDABE* operon used previously to monitor promoter activity levels in *B. melitensis* [14, 15].

B. melitensis containing the P_{ery} -*lux* reporter vector was used to infect RAW macrophages and cells were grown in either RPMI or RPMI containing 1% erythritol. Parallel to this infection was a set of RAW macrophages that were infected with the luminescent *B. melitensis* GR023 strain. Resulting levels of luminescent output from each condition were measured. Promoter activity levels were taken relative to the level of GR023 to compensate for the significantly higher level of bacteria found in the control (no-erythritol) condition. Levels of the P_{ery} promoter activity were found to be 2–2.5 fold higher upon addition of erythritol to the growth medium at both 24 and 48 hrs, indicating that erythritol is able to reach the intracellular niche of *B. melitensis* (Fig. 3C).

3.5 Microarray analysis of *B. melitensis* exposed to erythritol indicated that the bacterial response to erythritol goes beyond simple metabolism

With the ability of *B. melitensis* to preferentially localize to sites with erythritol and the possibility that erythritol plays a strong role in the pathogenesis of *Brucella* spp., the transcriptional response to erythritol was determined using microarray analysis. Cultures of *B. melitensis* strain 16M grown in minimal medium containing either 1% glucose or 1% erythritol as a carbon source were compared to determine what effect erythritol had on transcription. Microarray analysis found that 95 genes were significantly up-regulated and 40 were significantly down-regulated in response to growth in erythritol (Table 1; full results in Table S3). Of these 135 genes, 30% are categorized in the cluster of orthologous groups (COGs) as general function prediction, unknown function, and hypothetical proteins. The remaining 70% of the genes represent 16 other COGs with a wide range of potential importance discussed below (Fig. 4).

A subset of up-regulated genes identified by the microarray analysis was selected to confirm the microarray results. Nine experimental genes (*BMEI0095*, *I0102*, *I0347*, *II0025*, *II0161*, *II1009*, *II1086*, *II1108*, and *II1116*), a positive control *ery* promoter (*BMEII0430*), and the constitutive integration host factor 1 (*infA*) promoter (*BMEII671*) negative control were examined via promoter-reporter assay. All nine tested promoters and the positive control *ery* promoter were up-regulated upon addition of erythritol to the culture (Fig. 5).

4. Discussion

The connection between *Brucella* spp. and erythritol has been evident for some time [4, 21]. Initial studies identified a correlation between the ability of *B. abortus* strains to utilize erythritol and the virulence of the strain with lower utilization rates leading to lower virulence [5]. Subsequent studies with the same strains suggested that the decrease in erythritol utilization and virulence were both side effects of long passages during laboratory culture, but were not directly linked [6, 22]. This position was further strengthened with work on the primary example of the erythritol-virulence link, the *B. abortus* vaccine strain S19. Strain S19 possessed a well-documented defect in the utilization of erythritol that was

considered a significant reason why it was successful in vaccination of animals without causing abortions [5, 23]. However, upon identification of the genes responsible for erythritol utilization, complementation of strain S19 with those genes did not restore virulence [7].

The only link between erythritol and virulence in *B. abortus* appeared to be the decrease in both erythritol utilization and virulence resulting from long periods spent passaged in laboratory culture, not a direct causation between erythritol utilization and virulence. However, recent work in *B. suis* found that deletion of one of the genes responsible for erythritol utilization (*eryC*) resulted in a strain attenuated during both macrophage and mouse infections [8]. Either this observation represents a significant difference between the species, or the link between virulence and erythritol is more significant than previously determined. Erythritol injection of guinea pigs infected with *B. melitensis* showed a dramatic increase in bacterial counts within the spleen, indicating that perhaps *B. melitensis* also showed a strong connection between virulence and erythritol [19].

Previously, the presence of erythritol was suggested as the primary cause for localization of *Brucella* spp. in the placenta [19, 24]. To determine if this effect could be replicated in mice with *B. melitensis*, a localized site of erythritol was introduced into mice. Using a luminescent strain of *B. melitensis*, we found that *B. melitensis* preferentially localized to the artificial site of erythritol (Fig. 1). Whether this is due to a preferential active process of the bacterium (i.e. a chemotactic system) or an increased level of replication upon reaching the site of erythritol is currently unknown. While *B. melitensis* is known to produce a sheathed flagellum, the lack of an identified chemotactic system and the presence of only a small initial placental infection suggests that it is the latter, but further work will be required to identify the precise mechanism [25, 26]. Not only does this result replicate what has been seen in natural infections, it presents a potential model system for the study of the role of erythritol in *Brucella* spp. during placental pathogenesis.

Histological analysis of the erythritol-containing gel indicated a high level of cellular infiltration around the infected gel (Fig. 2). Further analysis of the gel with an anti-*Brucella* antibody identified numerous intracellular bacteria within the erythritol-containing gel matrix. In addition to the intracellular bacteria, there were a large number of extracellular bacteria. A large colony of extracellular bacteria has also been observed in the placentas of *Brucella*-infected cows, suggesting a potential role for erythritol in this preference for extracellular over intracellular growth [20].

To identify a potential cause for this abundance of extracellular bacteria, the effect of erythritol on intracellular *B. melitensis* was examined. The addition of erythritol to cell culture medium of infected RAW macrophages significantly decreased the level of bacteria present during intracellular growth (Fig. 3A). Erythritol was also found to be able to reach the intracellular location of *B. melitensis* for use by the bacterium (Fig. 3C). This resulted in *B. melitensis* preferentially replicating extracellularly rather than intracellularly, consistent with the results of the histological analysis of the matrigel plug showing a high number of extracellular bacteria when erythritol was present. Extracellular growth in response to erythritol may be playing an important role in the pathogenesis of the bacterium, but further efforts to elucidate this role are needed.

If erythritol is able to stimulate the growth of *B. melitensis* in broth culture, it was interesting that it did not also stimulate bacterial growth inside of macrophages [27]. Previous work examining the effect of erythritol additions to the cell culture medium on intracellular *B. abortus* within bovine trophoblasts also found that erythritol was inhibitory to intracellular growth, although growth with erythritol in the culture medium was never

compared to growth without erythritol [28]. In *B. abortus*, it was hypothesized that this intracellular growth defect in the presence of erythritol was due to the fact that iron played an important role in erythritol utilization, and iron may be limited in availability within the *Brucella* intracellular compartment [29, 30]. Further, it appears that several iron utilization genes are up-regulated within *B. melitensis* during intracellular growth, perhaps illustrating the lack of intracellular iron [31]. However, supplementation of iron (in the form of ferric citrate) to intracellular bacteria grown in the presence of erythritol was unable to restore replication to normal levels (data not shown). Due to difficulties in determining the level of available iron at the intracellular location of *B. melitensis* we are unable to exclude iron availability as the cause of this phenotype, but it is possible that erythritol is having an iron-independent effect upon intracellular replication.

In an effort to assess the impact of erythritol on *B. melitensis*, microarray analysis was conducted on bacteria grown with or without erythritol added to the media. Ninety-five genes were up-regulated during growth in erythritol while forty genes were down-regulated, constituting nineteen different clusters of orthologous groups (COGs) (Fig. 5). Of those genes down-regulated, the four most significant constitute a four-gene operon that is potentially responsible for glucose transport and therefore down-regulated in the condition of growth in erythritol without glucose present.

Genes up-regulated in the presence of erythritol can be separated into six primary groups – sugar metabolism, iron utilization, nitrogen fixation, cytochrome synthesis, type IV secretion components, and flagellar proteins (Table 1). Sugar metabolism, iron utilization, nitrogen fixation, and cytochrome synthesis are all connected to the pathways used to convert erythritol into energy. Iron has been found to be an important component of erythritol utilization, and *B. abortus* mutants deficient in two iron siderophores have been found to utilize erythritol at much lower rates than wild type *B. abortus* [29, 30]. Similarly, the cytochrome electron transport system has been shown to be a crucial component of erythritol metabolism and nitrogen is coupled to the cytochrome system in *Brucella* spp. [32, 33]. The three genes encoding erythritol utilization proteins (*eryABC*) showed an increase of approximately 1.5-fold in the microarray but higher levels upon promoter testing (Fig. 5). This difference is likely due to experimental variation between the microarray conditions and promoter-testing conditions, such as growth time and erythritol:glucose ratio.

Of particular interest for this study were those groups of genes up-regulated during growth in erythritol that did not appear to play any role in the growth or energy production of the bacterium. These up-regulated genes can be separated into two groups, one consisting of genes encoding the type IV secretion system VirB and the other consisting of genes encoding flagellar proteins. Genes associated with VirB up-regulated during erythritol exposure were two structural proteins (VirB1 and VirB7), a protein shown to be secreted by the VirB secretion system (VceA), and a quorum sensing regulator that has been shown to regulate expression of the *virB* operon (VjbR) [34, 35]. Of the 12 structural proteins that make up the VirB type IV secretion system, VirB1 and VirB7 are two of the three that are required for *in vitro* infection of cell models but expendable in mouse models of infection [36, 37]. That both of these proteins are required for bacterial intracellular growth in cell culture but are not required for persistence in a mouse model suggests: 1) an additional protein not produced during cell culture conditions can replace their function, 2) the altered regulation of other VirB components *in vivo* makes VirB1 and VirB7 less important, or 3) that the secretion of VirB effector proteins required for cell culture infection is altered [36]. One such identified secreted effector protein (VceA) is also up-regulated in response to growth in erythritol [35]. To date, no function for VceA has been identified, but it is co-regulated along with the *virB* locus by the quorum sensing regulator VjbR, also up-regulated

in response to erythritol [35]. It is possible that the up-regulation of VjbR via erythritol is responsible for the subsequent up-regulation of the VirB proteins and VceA.

Flagellar proteins are the third largest COG up-regulated by erythritol behind “energy production and conversion” and “hypothetical proteins” and constitute 13% of the up-regulated genes. Although *B. melitensis* has been shown to produce a sheathed flagellum, the precise role for the flagellum is currently unknown [25]. Historically classified as non-motile, the identification of flagellar genes in the sequenced genome of *Brucella* spp. was presumed to be cryptic artifacts of previous motility or a type III secretion system [38]. However, subsequent analysis of *B. melitensis* flagellar mutants showed them to be attenuated during mouse and goat infection (although not during *in vitro* cellular infection) indicating a potential role in virulence [25, 39]. While it is still unlikely that *B. melitensis* uses flagella for motility, the flagella may instead be used for secretion or attachment. Another possible role of flagellin is through the intentional activation of various immune system components through the host TLR5 and IpaF flagellin-sensing proteins [40]. In addition to the 17 flagellar proteins up-regulated, the diguanylate cyclase BMEII1009 is also up-regulated in response to erythritol. Diguanylate cyclases produce the secondary messenger cyclic-di-GMP, a signaling molecule responsible for flagellar regulation in several bacterial species and recently implicated in *B. melitensis* [14, reviewed in 41]. In addition to flagellar regulation, cyclic-di-GMP may also be playing a role in a host of other regulatory capacities or even acting as an immunostimulatory agent [42, reviewed in 43, 44].

The effect of erythritol on the expression of factors of the VirB type IV secretion and flagellar systems suggest a potential role in signaling for this carbon source, potentially providing a cause for the increase in virulence seen with the concomitant infection with *Brucella* and injection of erythritol into hosts [19, 24]. As erythritol has been connected repeatedly with virulence in *Brucella* spp. pathogenesis, this work may provide a potential explanation for these phenotypes. This link between carbon metabolism and virulence is currently a rapidly expanding field [reviewed in 45]. Of particular interest is the recent study indicating that *Brucella* may use the cellular autophagy pathway as a mechanism to induce release from an infected cell [46]. While the *Brucella* factors responsible for this effect have not been identified, it is possible that erythritol is activating these factors intracellularly, promoting the rapid release from the infected cell.

In a natural infection, the presence of erythritol may encourage *Brucella* to leave the intracellular niche of the trophoblasts in the placental region and replicate extracellularly, potentially through the activation of the cellular autophagy pathways by erythritol-stimulated bacterial factors. Once extracellular, the bacteria can rapidly grow to huge levels while simultaneously activating several virulence pathways that will be necessary upon transmission to a new host. These high levels of bacteria cause the inflammation that will eventually lead to abortion of the fetus and ejection of the placenta into the environment. Now within the environment, *Brucella* are already expressing virulence factors required to establish an infection due to their activation by erythritol. Upon uptake by a new host, these primed bacteria are able to swiftly and efficiently generate a successful infection. Each of these potential crucial steps signifies a new potential target in the search to unlock the secrets of *Brucella* pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *B. melitensis* preferentially localizes to sites of erythritol during mouse infection

A) Gel solutions containing either 10% glucose (left) or 10% erythritol (right) was injected into the backs of BALB/c mice (n=4) where the gel solidified. The mice were then infected intraperitoneally with 10^6 CFU of virulent, luminescent *B. melitensis* GR023 and imaged three days post infection. In all four mice, the luminescent bacteria preferentially localizes at the site of the erythritol gel.

B) Gels from infected mice were extracted at day 6 and imaged for levels of luminescence. Gels containing erythritol (right gel) contained much higher levels of luminescent bacteria than gels containing glucose (left gel).

Fig. 2. *B. melitensis* at sites of erythritol induce a strong immune response

Mice were prepared as for Fig. 1. Gels were removed at day 6 for histological analysis.

A) Glucose-containing gel from an infected mouse shows little to no immune cell recruitment.

B) Erythritol-containing gel from an infected mouse shows a high level of cellular recruitment (lower right), consistent with an increase in bacterial levels within the erythritol gel.

C) Immunocytochemistry of the erythritol-containing gel with HRP-conjugated anti-*B. melitensis* polyclonal antibody and reaction with diaminobenzidine tetrahydrochloride identifies high levels of bacteria within the gel. Arrows mark bacteria-associated cells.

Fig. 3. *B. melitensis* preferentially replicates extracellularly after incubation with erythritol in cell culture medium

A) *B. melitensis* strain 16M was used to infect RAW 264.7 macrophages at an MOI of 100:1. Macrophage growth culture either consisted of RPMI or RPMI+1% erythritol. Bacteria grown in the presence of erythritol fail to replicate to wild type levels. Data presented is the mean of three individual experiments and analyzed via Student's *t* test. B) RAW macrophages were infected as described above. Extracellular bacteria were initially killed with gentamycin immediately after infection, and then gentamycin media was removed, allowing extracellular growth. The ratio of extracellular bacteria to intracellular bacteria in the presence of RPMI or RPMI+erythritol was determined. The addition of erythritol to the cell media causes *B. melitensis* to preferentially replicate extracellularly. Data presented is the mean of three individual experiments and analyzed via Student's *t* test.

C) To measure the level of erythritol gaining access to intracellular bacteria, *B. melitensis* containing a luminescent reporter construct measuring activity of the erythritol-responsive *ery* promoter was used to infect RAW264.7 macrophages. Intracellular bacteria were incubated in either RPMI or RPMI containing 1% erythritol and luminescent output of the P_{ery} reporter was measured. To correct for lower numbers of bacteria in erythritol-incubated cells, the virulent luminescent strain GR023 was infected alongside under identical conditions and promoter values were taken relative to GR023 values to correct for cell numbers. After incubation for 24 and 48 hours, levels of the *ery* promoter are higher in the erythritol-incubated cells, indicating that erythritol is able to access the intracellular bacteria in sufficient quantities to elicit a transcriptional response from *B. melitensis*. Data presented is the mean of three separate experiments and analyzed via Student's *t* test.

Fig. 4. Microarray analysis of *B. melitensis* grown in the presence of erythritol identified a high number of regulated genes, including virulence factors

Microarray analysis was conducted on *B. melitensis* strain 16M grown in either 1% glucose or 1% erythritol. A total of 135 genes were found to change in levels after growth in erythritol, and these genes fall within 19 clusters of orthologous groups (COGs). In addition to genes for erythritol metabolism, several virulence factors (flagellar and type IV secretion proteins) were found to be up-regulated.

Fig. 5. Erythritol up-regulates transcription of several virulence factors

To confirm the results of the microarray on increased transcription, several promoters were assessed for their activity using a luminescent reporter assay. Cultures were grown in either 1% glucose or 0.5% erythritol to an OD₅₉₅ of about 0.5 and photon output and OD₅₉₅ were measured. Relative light units (RLUs) were calculated as the number of photons/OD₅₉₅, and change in response to erythritol was calculated as the ratio to glucose-grown levels. The negative control initiation factor 1 (*infA*) and the positive control erythritol-responsive *ery* promoter were included to confirm the success of the experiment. All nine promoters tested were significantly up-regulated in response to growth in erythritol, including several promoters of genes encoding virulence factors (flagella, type IV secretion system VirB, cyclic-di-GMP regulation, quorum sensing). Data presented is the mean of three separate experiments each measured in triplicate and analyzed between erythritol and glucose RLUs for each promoter by Student's *t* test.

Table 1

Selected Genes Showing a Significant Change After Growth in Erythritol

Gene Name	Product	Fold change	GG Probability ^a .
<u>Type IV Secretion System VirB</u>			
BMEI0025	Attachment mediating protein VirB1 homolog	2.12	0.79
BMEI0031	Channel protein VirB7 homolog	2.61	0.98
BMEI0390	Putative cytoplasmic protein – (VceA)	2.40	0.92
BMEI1116	Transcriptional activator, LuxR family – (VjbR)	2.14	0.66
<u>Flagella</u>			
BMEI0150	Flagellin	2.47	0.97
BMEI0159	Flagellar hook protein FlgE	2.37	0.94
BMEI0161	Flagellar hook-associated protein FlgL	3.72	1.00
BMEI0162	FlaF protein	3.50	1.00
BMEI0164	Basal-body rod modification protein FlgD	4.88	1.00
BMEI0170	Flagellar protein FlgJ	2.97	1.00
BMEI0171	“FlgN protein”	5.49	1.00
BMEI0172	“FlhL protein”	2.70	0.99
BMEI1085	Flagella basal body P ring formation protein FlgA	2.16	0.83
BMEI1086	Flagellar basal-body rod protein FlgG	5.82	1.00
BMEI1087	Flagellar hook-basal body complex protein FlhE	5.45	1.00
BMEI1088	Flagellar basal-body rod protein FlgC	5.70	1.00
BMEI1089	Flagellar basal-body rod protein FlgB	2.76	0.99
BMEI1108	Flagellar basal-body rod protein FlgF	2.52	0.98
BMEI1109	Chemotaxis MotA protein	6.65	1.00
BMEI1110	Flagellar motor switch protein FlhM	1.99	0.63
BMEI1113	Flagellar motor switch protein FlhG	2.09	0.75
BMEI1009	C-di-GMP phosphodiesterase A	2.43	0.94
<u>Cytochrome Synthesis</u>			
BMEI0593	SCO2 Protein	4.03	1.00
BMEI1463	Cytochrome C oxidase assembly protein CtaG	–3.22	1.00
BMEI1564	Cytochrome C oxidase polypeptide I homolog, bacteroid	3.59	1.00
BMEI1565	Cytochrome C oxidase, monoheme subunit, membrane-bound	3.87	1.00
BMEI1566	Cytochrome C oxidase, diheme subunit, membrane-bound	2.29	0.90
BMEI1898	Cytochrome O ubiquinol oxidase operon protein CyoD	2.50	0.97
BMEI1900	Cytochrome O ubiquinol oxidase subunit I	2.14	0.82
BMEI1901	Cytochrome O ubiquinol oxidase subunit II	3.07	1.00
BMEI0759	Cytochrome D ubiquinol oxidase subunit II	1.95	0.60
BMEI1068	Cytochrome C2 precursor	2.99	1.00
<u>Nitrogen Fixation</u>			
BMEI1294	Nitrogen fixation regulation protein FixK	3.09	1.00
BMEI1567	Nitrogen fixation protein FixG	2.64	0.99
BMEI1967	N utilization substance protein A	2.02	0.68

Gene Name	Product	Fold change	GG Probability ^a .
BMEII0246	Nitroreductase	2.31	0.88
BMEII0948	Nitrite extrusion protein	2.35	0.93
BMEII0975	Regulatory protein NosR	2.60	0.97
<u>Iron Metabolism</u>			
BMEI0673	Thiosulfate-binding protein precursor	2.73	0.99
BMEI1591	Ferredoxin-NADP reductase	2.38	0.89
BMEII0105	Iron-regulated outer membrane protein FrpB	2.02	0.54
BMEII1002	Calcium or iron-binding protein	1.98	0.60
<u>Carbohydrate Metabolism</u>			
BMEII0424	Ribose 5-phosphate isomerase	2.51	0.95
BMEII0425	Triosephosphate isomerase	1.97	0.61
BMEII0590	Sugar-binding protein	-20.38	1.00
BMEII0591	Sugar transport system permease protein	-6.93	1.00
BMEII0592	Sugar transport system permease protein	-7.56	1.00
BMEII0593	Glucose ABC transporter ATPase	-4.11	1.00
<u>Other</u>			
BMEI0095	Succinoglycan biosynthesis regulator	2.39	0.95
BMEI0102	Universal stress protein family	4.16	1.00
BMEI0347	Phosphoserine aminotransferase	2.27	0.90
BMEI0454	Outer membrane protein W precursor	25.38	1.00

^a A GG probability of >0.5 was considered significant