Effect of entF deletion on iron acquisition and erythritol metabolism by Brucella abortus 2308

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Abstract

Brucella abortus has been shown to produce two siderophores: 2,3-dihydroxybenzoic acid (2,3-DHBA) and brucebactin. Previous studies on Brucella have shown that 2,3-DHBA is associated with erythritol utilization and virulence in pregnant ruminants. The biosynthetic pathway and role of brucebactin are not known and the only gene shown to be involved so far is entF. Using cre-lox methodology, an entF mutant was created in wild-type B. abortus 2308. Compared with the wild-type strain, the ΔentF strain showed significant growth inhibition in iron minimal media that became exacerbated in the presence of an iron chelator. For the first time, we have demonstrated the death of the ΔentF strain under iron-limiting conditions in the presence of erythritol. Addition of FeCl3 restored the growth of the ΔentF strain, suggesting a significant role in iron acquisition. Further, complementation of the ΔentF strain using a plasmid containing an entF gene suggested the absence of any polar effects. In contrast, there was no significant difference in survival and growth between the ΔentF and wild-type strains grown in the murine macrophage cell line J774A.1, suggesting that an alternate iron acquisition pathway is present in Brucella when grown intracellularly.

Introduction

Iron is the second most abundant metal on earth (Clarke et al., 2001), and throughout evolution, most organisms have evolved or acquired iron-dependent enzymes that are involved in the essential life processes including electron transport and glycolysis (Wandersman & Delepelaire, 2004). Although iron is abundant in the environment, it is not readily available inside the host (Payne, 1993). The host limits the availability of free iron to prevent either oxidative damage to itself or replication of pathogens. Most of the iron in the host is present in the form of hemoglobin in red blood cells and the rest in bound forms including proteins such as transferrin, lactoferrin and ferritin (Payne, 1993). To obtain iron from iron-binding proteins, pathogens have developed special mechanisms. A common mechanism is the production of strong iron chelators called siderophores (Ratledge, 2007). These are low-molecular-weight molecules with high affinity for Fe III (Neilands, 1995). Limitation of iron is more notable for intracellular pathogens such as Brucella spp. and Mycobacterium spp. because of the ability of macrophages to reduce cytoplasmic iron further through proteins such as natural resistance-associated macrophage protein (Nramp1 and Nramp2) (Gruenheid et al., 1999).

The role of siderophores is not well-understood in case of the intracellular pathogen Brucella. Unlike Mycobacterium (De Voss et al., 2000), siderophore mutants derived from virulent Brucella abortus 2308 do not lose their ability to survive and replicate inside macrophages (Gonzalez Carrero et al., 2002; Bellaire et al., 2003b). Brucella siderophore research began with the discovery of 2,3-dihydroxybenzoic acid (2,3 DHBA) in Brucella (Lopez-Goni et al., 1992). Through extensive studies it was found that Brucella does not need 2,3-DHBA for its survival in mice (Bellaire et al., 1999). Subsequently, it was found that a siderophore is extremely important for Brucella to acquire iron in the presence of erythritol (Bellaire et al., 2003a). Erythritol, a four-carbon sugar, is abundant in bovine placental trophoblasts and preferred by Brucella as the carbon and energy source (Smith et al., 1962). A defective ery operon (Fig. 1) in
the B. abortus S19 vaccine strain has been associated with its attenuation in pregnant cattle (Meyer, 1966; Sperry & Robertson, 1975a). The entC mutant lacking the ability to synthesize DHBA could not cause abortions in the pregnant ruminants because of its inability to metabolize erythritol (Bellaire et al., 2003b). Involvement of an iron-coupled enzyme in the erythritol catabolic pathway (Fig. 1) was considered as a possible reason for these observations.

Brucella also has the ability to produce another siderophore, named ‘brucebactin’, through an unknown pathway (Gonzalez Carrero et al., 2002). Similar to Escherichia coli and based on homology, Brucella also possesses entD, entE and entF genes, whose specific roles have still to be confirmed, but are likely to be involved in brucebactin synthesis. Using transposon mutagenesis, the entF gene upstream to the entCEBA operon in B. abortus was interrupted, leading to the inability to synthesize brucebactin (Gonzalez Carrero et al., 2002). As the mutant was unable to grow in iron-deprived media, a possible role for the entF gene in the biosynthesis of brucebactin was predicted. However, its role was disputed when Bellaire et al. (2003b) did not find any entF transcript under iron-limiting conditions; they therefore suggested that Brucella may synthesize brucebactin during late stages of growth and at much lower levels (Bellaire et al., 2003b). The entF gene in Brucella is homologous with the vibH gene of Vibrio cholera that is involved in the synthesis of the siderophore vibriobactin, but its role in Brucella is not clearly understood. The work presented here clearly suggests a role of the entF gene in iron acquisition and subsequently in erythritol metabolism by B. abortus 2308.

Materials and methods

Bacterial strains, culture media and chemicals

Brucella abortus 2308 was grown in tryptic soy broth or tryptic soy agar (TSA). Iron minimal media (IMM) was prepared as described previously (Lopez-Goni et al., 1992). The concentration of iron in minimal media was determined using atomic absorption spectrophotometry (flame method) and found to be $0.099\,\mu\text{g} \cdot \text{mL}^{-1}$. All other chemicals were bought from Sigma-Aldrich Inc. (St. Louis, MO) unless specifically stated.

ΔentF deletion (BAN1) and complemented mutant (BAN2)

An unmarked mutation was created in the entF gene of strain B. abortus 2308 using the cre-lox methodology as described previously (Rajasekaran et al., 2008). A segment containing 497 base pairs were deleted within the entF gene in the biosynthesis of brucebactin was predicted. However, its role was disputed when Bellaire et al. (2003b) did not find any entF transcript under iron-limiting conditions; they therefore suggested that Brucella may synthesize brucebactin during late stages of growth and at much lower levels (Bellaire et al., 2003b). The entF gene in Brucella is homologous with the vibH gene of Vibrio cholera that is involved in the synthesis of the siderophore vibriobactin, but its role in Brucella is not clearly understood. The work presented here clearly suggests a role of the entF gene in iron acquisition and subsequently in erythritol metabolism by B. abortus 2308.
RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was isolated from each of the three strains, *B. abortus* 2308 (wild type), ΔentF mutant (BAN1) and ΔentF complemented mutant (BAN2), at 72 h of growth in IMM using a Pure link kit (Invitrogen) and RNA Easy kit (Qiagen) as per the manufacturer’s protocol. Turbo DNase (Ambion) was used to treat RNA samples for DNA contamination according to the manufacturer’s protocol. The absence of contaminating chromosomal DNA was confirmed by failure of the detectable cDNA gene amplification reactions, in the absence of reverse transcriptase. To synthesize cDNA, iScript cDNA synthesis kit (Bio-Rad) was used as per the manufacturer’s instructions and finally PCR was performed with entF internal primers (forward primer: 5'-GGCGGAGGTTCTTTCCAT-3', reverse primer: 5'-CGTCCTCTCATGAATG-3') and entB-A intergenic primers (forward primer: 5'-CTACGGCCTCGATTCGCTA-3', reverse primer: 5'-GATGACGGTTGCGCCTTCGG-3') and products were checked on 1% agarose gel containing ethidium bromide under UV light.

In vitro growth

Cultures in IMM were started at 10^6 CFU mL^-1 from a frozen culture of *B. abortus* 2308. All the supplements including FeCl₃ (50 μM), erythritol (0.1% or 0.05%) and ethylenediamine-N,N,N',N'-tetracetic acid (EDDA, 15 μM) were added 48 h before start of the growth to allow the homogeneous distribution and binding of chemicals. EDDA was first deferrated using hydrochloric acid and acetone, as described previously (Rogers, 1973). To monitor growth, 200 μL of culture was sampled in triplicate and 10-fold serial dilutions were made in phosphate-buffered saline (PBS) and 50 μL of the dilutions were spread on TSA plates to determine the CFUs after incubation for 2–3 days at 37°C under 5% CO₂.

Growth and survival in macrophages

The J774A.1 murine macrophage-like cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum in a humidified 5% CO₂ atmosphere at 37°C. About 1 x 10⁶ cells were seeded per well in a 24-well plate (Corning Incorporated). After 18 h of the incubation, the cells were infected with a multiplicity of infection of 100:1 (100 Brucella per macrophage). After 1 h of incubation (which was considered as the 0-h time point for all the experiments), the cells were washed three times with DMEM media containing 50 μg mL⁻¹ gentamicin to wash off all the extracellular bacteria. Fresh DMEM containing 50 μg mL⁻¹ gentamicin and 10% fetal bovine serum (FBS) was added for further incubation. As needed, 30 μM deferoxamine mesylate (DFA) was added to the culture media 48 h before infecting the J774A.1 cells to allow the chemical binding between DFA and iron. At 0, 24 and 48 h postinfection, macrophages were lysed using 1 mL of 0.1% TritonX-100, and lysates were collected and serial dilutions prepared in PBS and spread on TSA plates to determine the CFUs of Brucella.

Statistical analysis

All statistical analyses were performed using the Student two-tailed t-test using Microsoft EXCEL. P-values  ≤ 0.005 were considered significant (*).

Results and discussion

Deletion and complementation of entF gene

Deletion of 497 base pairs from the entF gene (BAN1 strain) and complementation by pNSGro:entF plasmid (BAN2 strain) were confirmed by PCR using the entF forward and reverse primers (data not shown). Further, to confirm the expression of entF gene in the complemented strain, RT-PCR (Fig. 2) revealed that the entF gene was expressed in the BAN2 strain, but not in the ΔentF mutant (BAN1). Intergenic expression of entB-A or dhbB-A, shown (Bellaire et al., 2003b) to occur under iron-limiting conditions by *B. abortus* 2308, was used as the positive control.

Growth under iron-limiting conditions

Under iron limitation, the wild-type strain did not reach the same cell density and had a slower growth rate compared with growth in IMM supplemented with iron (Fig. 3). This confirms the importance of iron for the growth of *B. abortus*.
2308 as shown by others (Evenson & Gerhardt, 1955; Parent et al., 2002; Wandersman & Delepelaire, 2004). The ΔentF strain (BAN1) grew even more slowly compared with the wild-type strain in IMM, suggesting the importance of entF gene with respect to growth. The addition of 50 μM FeCl₃ restored the growth of both wild-type and mutant strains, suggesting that iron was the only limiting factor in the medium.

If a particular mutated gene affects the growth of a bacterium under iron-limiting conditions, the mutated gene might be involved in acquisition, transport or metabolism within the iron pathway. In our study, addition of EDDA to IMM considerably restricted the growth of the ΔentF strain (BAN1) compared with its growth in IMM (Fig. 4). The ΔentF strain was able to survive in the presence of EDDA in IMM, but could not multiply over a period of 10 days. Thus, the role of the entF gene depends on the degree of iron restriction in the growth medium. This suggests a significant role for entF gene in iron acquisition as compared with iron metabolism. There was no effect of the addition of EDDA on bacterial counts of wild-type Brucella in IMM until 192 h. This indicates a stronger iron acquisition system in the wild-type strain compared with the ΔentF strain (BAN1). Comparing the growth of the ΔentF strain in the IMM with and without EDDA, it appears that the role of entF gene is more important when iron is strongly bound to iron chelators. This finding agrees with the observation by Gonzalez Carrero et al. (2002), who suggested that brucebactin may be a stronger chelating agent than DHBA.

**Survival and growth in the presence of erythritol under iron-limiting conditions**

When grown in the presence of 0.1% erythritol in IMM, the ΔentF mutant was unable to grow and began to die after 48 h (Fig. 5). Wild-type Brucella also had a longer lag phase in the presence of erythritol and the CFUs in the stationary phase were less compared with that in minimal medium without erythritol. This clearly suggests that much more iron is needed for the efficient metabolism of erythritol. The only link that directly connects erythritol catabolism and iron is the enzyme 3-keto-L-erythrose 4-phosphate dehydrogenase, which is involved in the pathway leading to conversion of erythritol into dihydroxy acetone phosphate (Fig. 1). This enzyme is an iron-containing flavoprotein (Sperry & Robertson, 1975a). Much more iron is needed in the presence of erythritol because of the involvement of an iron-linked enzyme in erythritol metabolism; this observation also agrees with the results from others (Bellaire et al., 2003a). This need could also explain the rapid death of the ΔentF strain, which is deficient in the ability to acquire iron and is thus unable to catabolize erythritol efficiently. The lack of the entF gene restricts the ability of the mutant to acquire iron, thus resulting in a scarcity of iron that leads to inactivity of the enzyme that is required to carry on the erythritol catabolism. Figure 5 shows the rapid death of the mutant strain in the presence of 0.1% erythritol in IMM. To
rule out the possibility of any toxic effect of erythritol, supplementation with 50 μM FeCl₃ restored the growth of the mutant strain comparable to that of the wild type. The first step in erythritol catabolism by Brucella involves the phosphorylation of erythritol via an ATP-dependent kinase (Sperry & Robertson, 1975a). Thus, the pathogen needs to invest energy first before it can metabolize the substrate and generate ATP. Moreover, erythritol kinase is eight times stronger in its activity than glucose kinase in B. abortus (Sperry & Robertson, 1975b). The need for ATP in the first step and the higher activity of erythritol kinase could have resulted in slowing down the growth process and result in a longer lag phase of growth of the wild-type strain in the presence of erythritol in IMM. This may also explain the killing of the ΔentF strain that constantly utilizes ATP to charge erythritol, but could not further metabolize it to obtain energy. Further, complementation of the ΔentF strain successfully overcame the growth restriction in IMM supplemented with erythritol (Fig. 6) and argues against any possible polar effects relative to entF.

In contrast to the in vitro results, using the wild-type strain 2308 as a comparator, the ΔentF strain was not affected with respect to survival and growth inside murine macrophages (data not shown). This suggests either a lesser requirement or an alternate pathway for iron acquisition inside macrophages by Brucella spp. In addition, cell culture medium with 10% FBS contains many iron-containing proteins that may not be chelated by 30 μM DFA. Increasing the concentration of DFA to 60 μM inhibited the growth of macrophages (data not shown), and further DFA studies on the survival and growth of bacterial strains inside the macrophages was not pursued.

In conclusion, these results suggest a role of the entF gene in iron acquisition by B. abortus 2308 under iron-limiting conditions. Deletion of the entF gene also had a major effect on erythritol metabolism by the pathogen under iron-limiting conditions. However the exact role of EntF and its relation to erythritol metabolism is still open for further analysis.

References


### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Intracellular survival and growth of *Brucella abortus* 2308 and BAN1 in J774.A1 murine macrophages growth as a function of DFA.

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