Brucella abortus strain 2308 produces brucebactin, a highly efficient catecholic siderophore

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Brucella abortus is known to produce 2,3-dihydroxybenzoate (2,3-DHBA) and to use this catechol as a siderophore to grow under iron-limited conditions. In this study a mutant (BAM41) is described that is deficient in siderophore production by insertion of Tn5 in the virulent B. abortus strain 2308. This mutant was unable to grow on iron-deprived medium and its growth could not be restored by addition of 2,3-DHBA. Production of catecholic compounds by both the Brucella mutant and parental strains under iron-deprivation conditions was assayed by TLC. Two catecholic substances were identified in the supernatant of the parental strain 2308. The faster migrating spot showed the same retention factor (Rf) as that of purified 2,3-DHBA. The mutant BAM41 overproduced 2,3-DHBA, but failed to form the slower migrating catechol. This defect could only be complemented by the addition of the slow-migrating catechol from strain 2308. The genomic region containing Tn5 in BAM41 was cloned and the position of the transposon was determined by nucleotide sequencing. The sequence revealed that the insertion had occurred at a gene with homology to Escherichia coli entF, a locus involved in the late steps of the biosynthesis of the complex catecholic siderophore enterobactin. Intracellular survival and growth rates of the B. abortus wild-type and entF mutant strains in mouse-derived J774 macrophages were similar, indicating that production of this siderophore was not essential in this model of infection. It is concluded that B. abortus synthesizes a previously unknown and highly efficient catecholic siderophore, different from 2,3-DHBA, for which the name brucebactin is proposed.

Keywords: iron acquisition, catechol, macrophage, 2,3-DHBA

INTRODUCTION

Iron, the most abundant metal ion in biological systems, is involved in numerous metabolic processes and is essential for almost all living organisms examined to date. However, given the oxidative properties of free iron, the intracellular concentration of this metal needs to be tightly regulated, because otherwise it would react with reduced forms of oxygen, resulting in free radicals known to cause lipid peroxidation, protein modification, nucleic acid damage, etc. (Actis et al., 1986). Free iron in body fluids of animals is kept at very low levels by iron-sequestering proteins such as transferrin or lactoferrin; therefore, it is postulated that bacterial high-affinity iron acquisition systems are a prerequisite for most infections (Ratledge & Dover, 2000). Most iron inside host cells is in the cytoplasm, complexed with haem proteins or in the storage protein ferritin. However, little is known about iron content in the phagosomes where some intracellular parasites multiply. It is generally believed that phagosomal iron content is low and this limitation poses a restriction for bacterial growth. Stimulation of macrophages with interferon-γ and/or lipopolysaccharide decreased iron uptake from

Abbreviations: 2,3-DHBA, 2,3-dihydroxybenzoate; CAS, chrome-azurol-S; DIP, 2,2-dipyridyl; EDDA, diethylenediamine di(o-hydroxyphenylacetic acid); NTA Fe(III), ferric nitriletriacetate.

The GenBank accession number for the sequence reported in this paper is AF361942.
transferrin caused by down-regulation of transferrin receptor expression (Mulero & Brock, 1999). Studies carried out with Legionella pneumophila, an intracellular parasite localized in phagosomes, indicate that IFN-γ-activated monocytes inhibit intracellular multiplication of the parasite by limiting the availability of iron, showing that phagosomal iron is scarce and the pathogen needs it to survive (Byrd & Horwitz, 1989).

Moreover, it has been shown that the iron content of host-cell vesicles containing facultative intracellular parasites is low enough to enhance the production of iron-regulated virulence factors (Garcia-del Portillo et al., 1992; Pope et al., 1996).

Brucella abortus is an intracellular parasite able to proliferate within macrophages, thereby successfully avoiding the bactericidal effects of phagocytes. Our knowledge about the mechanisms used by B. abortus for its intracellular survival has improved considerably in the last few years. Recent findings indicate that B. abortus is able to survive and replicate in phagosomes of the phagocytic cells by preventing the fusion between phagosomes and lysosomes (Frenchick et al., 1985; Pizarro-Cerda et al., 1998).

When grown under iron-limited conditions, B. abortus secretes 2,3-dihydroxybenzoate (2,3-DHBA), a simple catecholic compound. 2,3-DHBA was considered the only B. abortus siderophore responsible for Fe^{3+} uptake and able to relieve the growth inhibition caused by the strong iron chelator EDDA (Lopez-Goni et al., 1992). Production of other catecholic or hydroxamate siderophores in Brucella has not yet been reported. The role of 2,3-DHBA in protecting Brucella from the antibacterial activity of macrophages has been studied by the addition of exogenous 2,3-DHBA to B. abortus-infected murine macrophages in culture. In these experiments the number of intracellular brucellae recovered from 2,3-DHBA supplemented cells was higher than that of bacteria recovered from untreated macrophages (Leonard et al., 1997). This observation suggests that production of this catechol could provide intracellular brucellae with an efficient mechanism for acquiring iron in an iron-restricted environment that exists within the phagosome, thereby contributing to bacterial proliferation in the host. However, a recent report indicated that production of 2,3-DHBA was not required for replication of Brucella in murine macrophages or for the establishment of a chronic infection in the BALB/c mouse model (Bellaire et al., 1999).

In this work we report the isolation and characterization of a mutant B. abortus strain which, unlike the parental strain, was siderophore-negative in chrome-azurul-S (CAS) plates and unable to grow in iron-restricted medium, although it secreted 2,3-DHBA. The growth restriction observed under these conditions was overcome by the addition of iron in a soluble form or of a catecholic extract from culture supernatants of the parental strain grown in the presence of subinhibitory concentrations of EDDA. The mutant strain altered in siderophore production was deficient in iron assimilation but did multiply in macrophages.

**METHODS**

**Bacterial strains, plasmids, culture media and chemical reagents.** Strains and plasmids used in this study are described in Table 1. Escherichia coli strains were grown either in LB broth or on LB agar plates (Pronadisa). Brucella strains were usually grown in Brucella Broth (BB), Brucella Agar (BA) or Trypticase Soy Broth (TSB) (Pronadisa). MMB, a semi-synthetic low-iron medium containing yeast extract (Lopez-Goni, 1989), was used for the iron assimilation assays. 2,2-Dipyridyl (DIP; 150 mM; Sigma) or 10 mM diethylene diamine di(o-hydroxyphenylacetic acid) (EDDA; Fluka), were added to the medium as iron chelators. EDDA was previously defferated with HCl and acetone as described by Rogers (1973). EDDA stock solutions were stored at –20 °C in the dark. Agarose (0.7%; Pronadisa) was used instead of bacteriological agar in iron-deprived media. Every new batch of culture medium with chelator was tested for minimal inhibitory concentration for the strains tested. In some experiments iron bioavailability was increased by the addition of ferric nitritetriacetate [NTA Fe(III)] to the medium. Ampicillin (100 µg ml⁻¹), nalidixic acid (15 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) were added as appropriate.

**Detection of siderophore in solid medium.** Siderophore production was detected using a modification of the CAS plating assay (Schwyn & Neilands, 1987). The modification consisted of using MMB/c medium instead of MM9 to prepare CAS plates. MMB/c medium has the same composition as MM9 except that the K₂HPO₄ final concentration was 0.3 g l⁻¹ and glycerol and lactic acid were omitted. The final pH was adjusted to 6.8 with PIPES (Sigma). The CAS assay is a universal assay for the detection of siderophores and operates independently of the siderophore structure. The presence of a siderophore is indicated by the decolorization of the blue-coloured ferric-CAS complex, resulting in a yellow-orange halo around colonies growing on CAS medium. Since B. abortus was unable to grow on this medium we centrifuged 1 ml of Brucella cultured in MMB to early stationary phase, washed the cell pellet and loaded it on top of the plate. Plates were incubated for 2–3 d at 37 °C and checked for colour change surrounding the zone where the samples had been deposited.

**Isolation of mutants defective in siderophore production.** pSUP2021, an ampicillin-resistant plasmid containing Tn5, and unable to replicate in Brucella, was introduced into B. abortus 2308 by conjugation with E. coli S17-1(pSUP2021) as described previously (Sangari & Aguero, 1991). Transconjugants containing Tn5 were initially selected on BA containing nalidixic acid and kanamycin and purified on MMB plates with the same antibiotics. Isolated colonies from MMB plates were transferred to the blue CAS medium to screen for siderophore-deficient mutants. Colonies that were negative in this assay were tested for susceptibility to ampicillin to confirm the loss of the plasmid and were further tested for their growth on MMB plates containing different concentrations of the iron chelator EDDA.

**Growth of B. abortus in iron-deprived medium.** B. abortus strains were cultured at 37 °C in liquid MMB medium containing different concentrations of EDDA. Samples were taken at different time intervals and growth was determined by measuring A₅₀₀. In all these experiments the glass material was previously washed overnight with a 1 M HCl solution followed by three washes with ultrapure water (18 MΩ) to remove traces of iron.

**Bioassays for siderophores.** The presence of siderophore was detected with a cross-feed assay, consisting of checking the
growth of siderophore-negative mutants in iron-deprived solid medium around filter disks containing different substances. When the strain to be cross-fed was E. coli AN193 entA, half strength TSB plates containing 150 µM DIP were used, and in the case of BAM41 the medium used was MMB agarose with 150 µM EDDA. In both cases cells from 1 ml early-stationary-phase low-iron cultures were collected by centrifugation, resuspended in 10 µl sterile saline and seeded onto the indicator plates. Five microliters of the supernatants were spotted onto a Millipore filter (0.22 µm) and allowed to dry before the disk was applied to the plate. The plates were then checked for the presence of a halo of growth after 48–72 h at 37 °C. To assay ethyl acetate extracts or pure dissolved substances, they were placed on sterile filter paper disks and dried for 10 min in the dark at room temperature to allow the solvent to evaporate. The size of the halo of growth represents the amount/strength of the siderophore present in the sample.

**Preparation of ethyl acetate extracts and analysis by TLC.** Early-stationary-phase B. abortus cultures in MMB were centrifuged (4000 r.p.m. for 10 min at 4 °C) and the supernatants were filter-sterilized through Millipore 0.22 µm filters. Quantification of catechols in these supernatants was carried out by the colorimetric test of Arnow (1937), using 2,3-DHBA as control. Catechol-containing supernatants were acidified to pH 2.0 by addition of 6 M HCl and catechols were extracted by addition of ethyl acetate (20 ml per 100 ml supernatant). The extracts were pooled and concentrated at low temperature in a vacuum system.

TLC of ethyl acetate extracts was carried out on silicagel G plates (Merck). Chromatograms were developed with benzen/acetate acid/water (125:72:3, by vol.). 2,3-DHBA and its derivatives were first detected by fluorescence under UV light. The presence of iron-reacting substances was revealed by spraying the plate with 0.12 M FeCl₃ in 0.1 M HCl. When required, the compounds were recovered from the chromatograms by elution in aqueous acetic acid (15%, v/v), the resulting solutions were made up to 0.5 M HCl and the compounds were extracted with ethyl acetate.

**Recombinant DNA techniques.** Chromosomal DNA from B. abortus strains was extracted by the guanidium thiocyanate method (Pitcher et al., 1989). Construction of plasmids, restriction enzyme analysis, agarose gel electrophoresis and Southern hybridizations were carried out by standard protocols (Sambrook et al., 1989). A 3.5 kb HindIII fragment from Tn5 was used as a hybridization probe and labelled with digoxigenin with the DIG-High Prime Kit (Roche Diagnostics). The DNA sequence flanking transposon mutants was cloned using arbitrary PCR (Caetano-Annoles, 1993), as described by O’Toole & Kolter (1998), and sequenced. Database searches and alignments were performed at the National Center for Biotechnology Information (NCBI), using the BLAST network service.

**Infection and intracellular viability assay of B. abortus in J774 cells.** J774 murine macrophages were cultured in RPMI medium with 2 mM L-glutamine, penicillin (100 U ml⁻¹), streptomycin (0.1 mg ml⁻¹) and 10% fetal calf serum (FCS) at 37 °C under 5% CO₂ and 100% humidity. Confluent monolayers were trypsinized and 2 × 10⁵ cells per well were incubated for 24 h before infection in 24-well tissue culture plates. B. abortus cells used for infection were opsonized with decomplemented antisera for 30 min at 37 °C. Infection of macrophages was carried out for 45 min at 37 °C in RPMI without serum and antibiotics at a multiplicity of infection of 100. After infection the wells were washed five times with sterile phosphate-buffered saline (PBS) and further incubated in RPMI with 2 mM L-glutamine, 10% FCS and 40 µg gentamicin ml⁻¹ to kill extracellular bacteria. The number of intracellular viable B. abortus was determined at different times post-infection. To this end, the monolayers were washed twice with sterile PBS and treated for 5 min with 1 ml 0.1% Triton X-100 (Sigma) in deionized water. Lysates were serially diluted and plated onto BA plates for determination of c.f.u.

**RESULTS**

**Isolation of B. abortus mutants unable to secrete siderophore**

A bank of B. abortus 2308 Tn5 insertion mutants was grown in MMB agar and tested for siderophore secretion. Isolated colonies were deposited on modified CAS plates. Although B. abortus was unable to grow on this medium, the siderophore present in the sample was able to decolorize CAS. The presence of a siderophore was considered positive when an orange halo was present under the mass of bacteria. Three hundred colonies were assayed by this method. The orange halo was absent in one isolate which was named BAM41 (Fig. 1).

**Growth of BAM41 in an iron-deprived medium**

To assess the ability of the CAS-negative mutant BAM41 to grow in media with low availability of iron we tested the parental and the mutant strains in liquid MMB containing different concentrations of EDDA. The results showed that at EDDA concentrations higher than 15 µM the biomass of the mutant strain was considerably lower than that of the parental strain.
Bacterial samples recovered from early-stationary-phase culture in MMB were deposited on pieces of 0.2–22 µm Millipore filter on CAS plates and incubated for 72–96 h. Siderophore-positive strains produce an orange halo. A, *E. coli* AN193; B, *B. abortus* BAM41; C, 2,3-DHBA (10 µl of a 10 mM stock solution); D, *B. abortus* 2308; E, blank (MMB medium).

This result suggested that the mutant BAM41 was unable to assimilate iron when the metal was chelated by EDDA. Addition of Fe(III) in the form of NTA Fe(III) reverted the inhibitory effect of EDDA on strain BAM41, demonstrating that the inhibition was caused by the absence of bioavailable iron in the medium (Fig. 2).

Isolation and analysis of catecholic compounds present in *B. abortus* cultures

Supernatants from *B. abortus* cultures grown in MMB medium containing a subinhibitory concentration of EDDA were analysed by the Arnow test for catechol content at different time points. Maximal production of catechols was observed at the beginning of the stationary phase of growth. The amount of catechol detected for a particular strain under the same culture conditions varied considerably between different assays. However, when catechol was detected, the amount found was always proportional to the biomass of bacterial cells present in iron-limited cultures.

Catechols were extracted from supernatants with ethyl acetate, concentrated and analysed by TLC. This analysis indicated the presence of a catechol molecule (Arnow-positive) with an *R*<sub>f</sub> identical to that of 2,3-DHBA both in the mutant BAM41 and in the parental strain, 2308. Strain 2308 presented, in addition, a second Arnow-positive spot that exhibited a lower *R*<sub>f</sub> and was less abundant than 2,3-DHBA (Fig. 3).

To confirm that the fast-migrating catechol described above was 2,3-DHBA we performed a cross-feed assay with the *E. coli* entA mutant AN193, unable to synthesize 2,3-DHBA, and hence enterochelin. This strain cannot grow in media with inhibitory concentrations of a strong iron chelator, such as DIP, unless 2,3-DHBA is present. The results showed that filtered supernatants from both *B. abortus* 2308 and mutant BAM41, were able to cross-feed the *E. coli* entA mutant, indicating that they contained 2,3-DHBA. This cross-feed assay was more efficient with strain BAM41 than with 2308, suggesting overproduction of 2,3-DHBA by the mutant. The fast-migrating catechol from BAM41 and 2308 supernatants was purified from the TLC plate, eluted and blotted on a filter. This purified substance restored the growth of AN193, confirming its identification as 2,3-DHBA.

Phenotypic complementation of mutant BAM41

To further characterize the *Brucella* siderophore-deficient mutant, we tried to restore its growth in EDDA-chelated MMB medium with different substances spot-
A new *Brucella abortus* siderophore

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**Fig. 3.** Analysis of ethyl acetate extracts from culture supernatants by TLC. Culture supernatants from early-stationary-phase cultures in MMB containing subinhibitory concentrations of EDDA were extracted with ethyl acetate and analysed on TLC plates containing 2,3-DHBA as standard. The line at the top indicates the solvent front. A, 2,3-DHBA (3 µl of a 10 mM stock solution); B, *B. abortus* 2308; C, ethyl acetate extract of fresh MMB medium; D, *B. abortus* BAM41.

**Table 2.** Phenotypic complementation of mutant BAM41

One hundred microlitres from a stationary phase BAM41 culture was spread on MMB plates containing an inhibitory concentration of EDDA. Compounds were deposited on sterile filter paper disks as indicated in Methods.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Growth*</th>
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<tbody>
<tr>
<td>2,3-DHBA (30 nmol)</td>
<td>—</td>
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<tr>
<td><em>E. coli</em> extract†</td>
<td>++</td>
</tr>
<tr>
<td>NTA Fe(III) (45 nmol)</td>
<td>+++</td>
</tr>
<tr>
<td>2308 extract†</td>
<td>+++</td>
</tr>
<tr>
<td>2308 low R₃ catechol</td>
<td>+++</td>
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<tr>
<td>2308 high R₃ catechol</td>
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* = No growth; + indicates the diameter of the halo of growth (1 cm per symbol).

†Ethyl acetate extracts from filtered supernatants containing CAS decoloration activity.

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**Fig. 4.** Intracellular survival of *B. abortus* BAM41 (open bars) and 2308 (filled bars) in J774 murine macrophages. Bacteria were recovered from the macrophages at different times after infection and intracellular live brucellae were determined by a c.f.u. count on BA plates.

**Survival of BAM41 in macrophages**

The role of siderophore in the ability of *B. abortus* to survive in macrophages was tested by using the *B. abortus* BAM41 mutant in an *in vitro* macrophage survival assay. The results of the experiment are shown in Fig. 4. Both *B. abortus* 2308 and BAM41 survived and replicated in the macrophage without significant differences.

**Genetic characterization of mutants**

Total DNA from BAM41 was digested with *EcoRI* and hybridized with an internal Tn5 probe as described in Methods. A single hybridization *EcoRI* band of 8 kb was observed. This band was excised from an agarose gel and cloned in the plasmid vector pUC18, resulting in pSUBA41. A physical map of this DNA fragment is shown in Fig. 5. The nucleotide sequence of both ends of this DNA fragment was determined by sequencing the recombinant plasmid pSUBA41 with universal primers. To analyse the transposon insertion point we used the arbitrary PCR method described by O’Toole & Kolter (1998). Using this method we obtained and sequenced a PCR amplification product corresponding to the right flank of the transposon (Fig. 5). To determine the sequence of the left flank we used a synthetic primer derived from the previously determined sequence. These sequences were assembled in a contig of 1 kb corresponding to the *B. abortus* chromosome (GenBank accession no. AF361942). This sequence was compared with the non-redundant GenBank nucleotide sequence database using the *BLASTN* program. The sequence showed strong homology with the gene *vibH* from *Vibrio cholerae*, which encodes an amidase involved in the condensation steps of vibriobactin synthesis (Keating *et al.*, 2000a, b). The sequence also showed homology with the amino-terminal part of *E. coli entF*, a gene encoding a subunit of the enterobactin biosynthesis multienzyme complex (Rusnak *et al.*, 1991). According
to these results we may conclude that the Tn5 insertion in BAM41 occurred at position 275, near the 5′ end of a B. abortus gene homologous to E. coli entF. This insertion resulted in the inactivation of the gene, which could be involved in the biosynthesis of the new catecholic siderophore described above.

The sequence of the right end of plasmid pSUBA41 was also compared with sequences in the GenBank database. The sequence showed strong homology with the E. coli entC and entE genes, involved in the first stage of enterobactin biosynthesis. These data fit well in the physical map reported for the region encoding the 2,3-DHBA biosynthesis genes of B. abortus (Bellaire et al., 1999).

**DISCUSSION**

2,3-DHBA has been reported to be the only catecholic siderophore present in the supernatant of Brucella cultures growing under iron-limiting conditions. 2,3-DHBA was able to supply iron to the bacterium and accordingly was considered as a true siderophore for Brucella (Lopez-Goiñi et al., 1992).

The appearance of large amounts of 2,3-DHBA or salicylic acid in culture supernatants is a phenomenon commonly observed for bacteria producing phenolate siderophores in response to iron deprivation. However, those compounds are considered to be low-affinity siderophores (Actis et al., 1986), unable to compete with stronger iron-chelating compounds. Moreover, recent theoretical studies do not consider 2,3-DHBA to be capable of acting as a bacterial siderophore (Chipperfield & Ratledge, 2000). The facts question the role of 2,3-DHBA as a siderophore in B. abortus and suggest that Brucella either produces a stronger siderophore, different from 2,3-DHBA, or it uses a different iron assimilation system.

We have observed that B. abortus 2308 was able to decolorize CAS plates, indicating that this strain secretes a siderophore. To discover the role of this siderophore in intracellular survival we have isolated a Tn5 mutant of B. abortus 2308, named BAM41, which was negative in the CAS assay and thus considered to be siderophore-deficient. The minimal inhibitory concentration of EDDA for this mutant was lower than that of the parental strain, 2308. The correlation between absence of siderophore activity in the CAS assay and inability to grow in an iron-deprived medium indicated that the transposon insertion in this mutant was probably affecting genes involved in siderophore biosynthesis.

The growth of BAM41 in a low-iron medium was stimulated by filtered supernatants from a 2308 culture grown in an iron-deprived medium, and also by ethyl acetate extracts from the same culture, indicating that an iron-related growth factor was present in both samples. Analysis of ethyl acetate extracts of B. abortus 2308 low-iron culture supernatants by TLC showed the presence of two different catechol species, one with an $R_f$ identical to that of 2,3-DHBA and the other with a lower $R_f$. The concentration of the low $R_f$ catechol in culture supernatants was always considerably lower than that of 2,3-DHBA. When isolated from TLC plates, only the compound with the low $R_f$, but not 2,3-DHBA, was able to complement the growth of the mutant in an iron-deprived medium and to decolorize CAS plates. These results indicated that B. abortus 2308 produced a second catechol substance with a higher affinity for iron than the formerly reported 2,3-DHBA. Since this new compound, capable of decolorizing CAS medium, could be an efficient siderophore for B. abortus, we propose to call it Brucebactin. The presence of catehol compounds in addition to 2,3-DHBA had been described by Lopez-Goiñi et al. (1992). Brucebactin could be identical to the catehol with an $R_f$ of 0.02 described in that report. However, this catechol was not further assayed and the possibility that it could be a siderophore was discarded by the authors.

An alternative explanation for the inability of BAM41 to grow in iron-deprived media is that the mutation in this strain affected siderophore transport instead of siderophore biosynthesis. However, siderophore transport mutants usually present a large halo in CAS plates (Schwyn & Neilands, 1987) and this was not the case for BAM41. Moreover, the fact that this strain could be cross-fed by B. abortus 2308 ethyl acetate supernatant extracts indicated that siderophore transport was not affected in this mutant.

The results obtained in a cross-feed assay with an E. coli entA mutant confirmed that the fast-moving catechol was 2,3-DHBA. Cross-feed experiments and TLC also demonstrated that BAM41 produced more 2,3-DHBA than the parental strain 2308. This would be the expected behaviour of a mutant blocked in the late steps of the biosynthesis of a complex catecholic siderophore structurally based on 2,3-DHBA. Genes encoding the enzymes for this process are usually organized in two
clusters. The first encodes the enzymes for the production of 2,3-DHBA from chorismic acid. The second cluster encodes a multienzyme complex responsible for the condensation of 2,3-DHBA with amino acids or other molecules to produce the final catecholic siderophore (Ratlledge & Dover, 2000). On these grounds, BAM41, which did not produce the final catechol, but produced 2,3-DHBA, might well be affected in the condensation step.

Genetic analysis revealed that BAM41 contained a single Tn5 copy inserted in a gene homologous to E. coli entF (Rusnak et al., 1991). The product of this gene (EntF) together with EntB and EntE constitute a multienzyme complex responsible for the biosynthesis of the catecholic siderophore enterobactin in E. coli (Gehring et al., 1998). The Brucella gene presumably should form part of an operon responsible for brucebactin biosynthesis.

Attempts to determine the conditions for optimal brucebactin production indicated that the better yields were always obtained in iron-limited media at the beginning of the stationary phase of growth. Attempts to isolate high amounts of brucebactin for structural and chemical analyses were unsuccessful because of the instability of the purified compound.

We conclude that 2,3-DHBA can be used by Brucella as a low-affinity siderophore when iron in the medium is readily soluble, but this substance cannot work as an efficient siderophore when iron is complexed by EDDA or high-affinity physiological chelators such as transferrin or lactoferrin, as theoretically predicted by Chipperfield & Ratledge (2000). In this case Brucella must compete for iron by secreting another compound with similar or stronger affinity for iron (responsible for CAS decoloration). Moreover, concentrations of 2,3-DHBA higher that those secreted by BAM41 in iron-deprived medium were unable to decolorize CAS plates, indicating the lack of siderophore activity of this catechol.

Results presented here and previous evidence (Bellaire et al., 1999) have shown that siderophores appear not to play a relevant role in the intracellular survival of B. abortus. This finding has also been reported for other intracellular pathogens such as Salmonella (Benjamin et al., 1985), probably because iron availability in the phagosome is high enough for this pathogen or because other effective iron assimilation mechanisms are used by Salmonella. On the other hand, it has been shown that 2,3-DHBA improves the survival of B. abortus in the macrophage (Leonard et al., 1997). Considering the poor ability of 2,3-DHBA as a siderophore, this effect could be due to another circumstance different from iron assimilation. Brucella needs an acidic phagosome in the first hours of infection to survive (Porte et al., 1999). In this low pH compartment, iron is readily soluble and thus highly bioavailable. Apart from this, catecholic siderophores present less affinity for iron at acid than at neutral pH (Emery, 1978). Therefore, given the pH of Brucella-containing phagosomes, and taking into account the available results, we may conclude that the role of catecholic compounds in the phagosomal survival of B. abortus, if any, should be different from iron sequestration.

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