

## Analysis of the Behavior of *eryC* Mutants of *Brucella suis* Attenuated in Macrophages

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**The facultatively intracellular pathogen *Brucella*, characterized by its capacity to replicate in professional and non professional phagocytes, also causes abortion in ruminants. This property has been linked to the presence of erythritol in the placenta, as brucellae preferentially utilize erythritol. The *ery* operon encodes enzymes involved in erythritol metabolism, and a link with virulence has since been discussed. Allelic exchange mutants in *eryC* of *Brucella suis* were erythritol sensitive in vitro with a MIC of 1 to 5 mM of erythritol. Their multiplication in macrophage-like cells was 50- to 90-fold reduced, but complementation of the mutant restored wild-type levels of intracellular multiplication and the capacity to use erythritol as a sole carbon source. In vivo, the *eryC* mutant colonized the spleens of infected BALB/c mice to a significantly lower extent than the wild type and the complemented strain. Interestingly, *eryC* mutants that were in addition spontaneously erythritol tolerant nevertheless exhibited wild-type-like intramacrophagic and intramurine replication. We concluded from our results that erythritol was not an essential carbon source for the pathogen in the macrophage host cell but that the inactivation of the *eryC* gene significantly reduced the intramacrophagic and intramurine fitness of *B. suis*.**

Members of the genus *Brucella* are gram-negative intracellular pathogens that cause abortion and sterility in domestic animals and Malta fever in humans. These bacteria can survive and multiply inside trophoblasts and within a membrane-bound compartment in professional and nonprofessional phagocytic cells (4, 7, 11, 23, 24). In ruminants, *Brucella abortus* colonizes the placenta and fetus, causing abortion. This tropism for the reproductive organs correlates with the presence of erythritol in the bovine placenta, and it has been shown that, in the presence of erythritol and glucose, the genus *Brucella* preferentially metabolizes erythritol (31) and that erythritol promotes the growth of some *Brucella* strains (1, 21). A possible correlation between erythritol oxidation and virulence in animal models has been, however, a matter of controversy (15, 20). The catabolic pathway of erythritol has since been elucidated: the polyalcohol is phosphorylated, and three oxidation steps finally result in the formation of dihydroxyacetone phosphate, which can be metabolized to pyruvic acid (32). In the attenuated *B. abortus* B19 vaccine strain, the enzyme D-erythrulose-1-phosphate dehydrogenase, which plays an important role in the erythritol metabolism, has been shown to be absent (33). Genetic analysis of the chromosomal region containing the erythritol catabolic genes confirmed a deletion in this strain (29). In the presence of erythritol, ATP is consumed with the accumulation of D-erythrulose-1-phosphate. A general ATP depletion due to high-level erythritol kinase activity and interference with hexose kinase activity involved in glucose metab-

olism have been suggested as possible explanations for the sensitivity of *B. abortus* B19 to erythritol (33). However, the defect in erythritol metabolism of this vaccine strain is unrelated to its attenuated virulence in mice, as a strain obtained by gene replacement of the deleted *ery* region shows residual virulence similar to that of *B. abortus* B19 (30). A detailed analysis of the sequence and the organization of the *ery* genes was performed more recently (27). The four genes *eryA*, *-B*, *-C*, and *-D* form an operon of approximately 5 kb. The EryA protein is the erythritol kinase (19), and EryB and EryC are the two dehydrogenases in the catabolic chain. EryD is a transcriptional repressor, inactivated by erythritol binding (27). These sequences and the *ery* operon organization are highly conserved in the other two species whose genomes have been entirely sequenced, *Brucella melitensis* and *Brucella suis* (6, 22). Tn5 mutagenesis of the *B. abortus* 2308 genome allowed the isolation of a mutant where the transposon was inserted into *eryB* (26). As it has been observed for *B. abortus* B19, this Tn5 mutant is sensitive to erythritol. In addition, it is not significantly less virulent in a murine model of infection than the erythritol-tolerant *B. abortus* 2308 (30).

In contrast to these results, we have lately reported the isolation of two attenuated Tn5 mutants, obtained from a large-scale screening for genes of *B. suis* involved in intramacrophagic replication, where the transposon was shown to be integrated in *eryB* and *eryC* (16). It therefore appeared that the *ery* genes were somehow involved in the multiplication of brucellae in macrophages. The aim of this work was to analyze the possible role of erythritol in the virulence of *B. suis* in the macrophage and in the murine model of infection and to get insights into the possible causes of the attenuation of *ery* mutants.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *B. suis* 1330 (ATCC 23444) was grown to stationary phase in tryptic soy (TS) broth at 37°C. Kanamycin or chloramphenicol was added at concentrations of 50 or 25 µg/ml, respectively, when appropriate. The *Escherichia coli* strains CC118λpir (12) and DH5α (Invitrogen, Cergy Pontoise, France) were used as host strains for the cloning experiments with the erythritol gene of *B. suis*; they were grown in Luria-Bertani broth in the presence of kanamycin or ampicillin at a concentration of 50 µg/ml when appropriate. Constructions were performed using plasmids pUC4K, pUC18 (both Amersham Biosciences, Orsay France), pGEM-T Easy (Promega, Charbonnières, France), pBBR1MCS (18), and pCVD442 (8).

**Construction of *B. suis* ΔeryC deletion mutants.** For the cloning of the *eryC* gene from *B. suis* (locus BRA0866) (22), chromosomal DNA was prepared from a stationary-phase culture as previously described (2). The entire gene was then amplified by PCR as a 1,181-bp fragment using the primers 5'-CGCCGCACC AAGCATTACC-3' and 5'-TTGGCGATGAGACGATGCG-3'. For the construction of the ΔeryC::Kan<sup>r</sup> mutant, the PCR product was cloned into pGEM-T Easy. A 387-bp StuI-HindIII internal fragment was deleted from *eryC* and replaced by the 1.3-kb HincII Kan<sup>r</sup> cassette from pUC4K. This construct was amplified in *E. coli* and then electroporated as a suicide vector into *B. suis* as described earlier (17). After growth at 37°C for 3 days, colonies were streaked on kanamycin and on ampicillin, and only Kan<sup>r</sup>/Amp<sup>s</sup> clones were taken into consideration for further analysis. PCR analysis performed with the primers described above on chromosomal DNA isolated from these clones allowed the identification of mutants containing the correct allelic exchange within *eryC*. The ΔeryC deletion mutant, devoid of any resistance cassette to exclude any possible polar effects on genes located downstream, was constructed as follows. Instead of inserting the Kan<sup>r</sup> cassette to replace the StuI-HindIII fragment of *eryC* as described above, the deleted gene was religated and recloned as an SalI-SphI fragment into the vector pCVD442. This vector carries the *sacB* gene conferring sucrose sensitivity to gram-negative bacteria (8), hence allowing the application of selective pressure for allelic exchange, which would then yield sucrose-resistant clones. To this purpose, following electroporation, brucellae were first plated onto TS agar plates containing ampicillin to select for transformants. Positive clones were restreaked in the presence of 5% sucrose, and sucrose-resistant, isolated colonies were assayed by PCR using the *eryC* primers. The deleted gene yielded a PCR product of 800 bp. Standard DNA manipulations were performed according to established protocols (25).

**Complementation of the unmarked *B. suis* ΔeryC deletion mutant by *eryC*.** The gene was amplified by PCR as a 1-kb fragment using the primers 5'-TAACTG CAGTGGAAATGGTTTCGACAACGCC-3' and 5'-TAAGGATCTGAACGCG GTCCGGTCTGG-3' and cloned via BamHI/PstI restriction sites into pBBR1MCS in the orientation of the *lacZ* gene. The recombinant plasmid was isolated from *E. coli* and introduced into the ΔeryC mutant of *B. suis* by electroporation, and complemented mutants were selected on TS agar supplemented with chloramphenicol.

**Erythritol sensitivity assay with the ΔeryC deletion mutant of *B. suis* and growth curves in RPMI cell culture medium.** In experiments directly monitoring erythritol sensitivity, ΔeryC mutants grown to stationary phase were diluted in TS broth and incubated in the presence of erythritol at a concentration of 80 mM for 54 h. Growth curves in RPMI 1640 (Cambrex Bio Science, Paris, France) containing 10% fetal calf serum (FCS) were obtained following 100-fold dilution of a stationary-phase culture and incubation at 37°C for 5 days. In both types of experiments, growth was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>).

**Determination of the MIC of erythritol for the ΔeryC deletion mutant of *B. suis*.** Stationary-phase precultures of wild-type *B. suis* 1330 containing pBBR1MCS, the ΔeryC deletion mutant with plasmid pBBR1MCS, and ΔeryC complemented with pBBR1-eryC were diluted 1/1,000 in TS broth containing chloramphenicol and erythritol at concentrations of 0.1, 0.5, 1, 5, or 20 mM. At various times of incubation, the OD<sub>600</sub> was recorded.

**Growth curves of *B. suis* strains in TS broth or in minimal medium in the presence of erythritol as sole carbon source.** Stationary-phase precultures of wild-type *B. suis* 1330, the ΔeryC deletion mutant, ΔeryC complemented with pBBR1-eryC, and a spontaneously erythritol-tolerant mutant of *B. suis* ΔeryC were diluted either 1/1,000 in TS broth with or without erythritol (25 mM) or (following a washing step in saline) 1/100 in Gerhardt's modified minimal medium containing 25 mM erythritol as a sole carbon source (10). Growth was monitored at different time points by OD<sub>600</sub> measurements.

**Cell culture, infection, and intracellular survival assay of *B. suis* in macrophage-like cells and in murine bone marrow-derived macrophages.** Experiments with macrophage-like cells were performed as described previously (4). Briefly,

THP-1 cells, differentiated by 1,25-dihydroxyvitamin D<sub>3</sub> at a concentration of 10<sup>-7</sup> M for 72 h, were resuspended at 5 × 10<sup>5</sup> cells/ml in RPMI 1640 medium supplemented with 10% FCS. Alternatively, murine J774 cells were resuspended at 1.2 × 10<sup>5</sup> cells/ml in the same medium. Bone marrow-derived macrophages were obtained from BALB/c mice as described previously (5). Adherent cells were incubated for 24 h at 37°C in 5% CO<sub>2</sub> prior to infection at a multiplicity of infection of 20 with stationary-phase *B. suis* grown in the presence of the corresponding antibiotics. After 30 min, cells were washed twice with phosphate-buffered saline (PBS) and reincubated in RPMI 1640–10% FCS with gentamicin (30 µg/ml) for at least 1 h. At 1.5, 7, 24, and 48 h postinfection, cells were washed twice with PBS and lysed in 0.2% Triton X-100. CFU were determined by plating serial dilutions on TS agar and incubation for 3 days at 37°C.

When infection experiments were performed in the absence of FCS, adherent cells were washed once with phosphate-buffered saline and cultivated in RPMI 1640 medium devoid of FCS for the duration of the experiment. All experiments were performed twice in triplicate.

**Infection of BALB/c mice by *B. suis* strains.** *Brucella* suspensions were prepared as described elsewhere (14). Eight- to 9-week-old female BALB/c mice (University of Granada, Granada, Spain) were distributed in three groups and then injected intraperitoneally with 5 × 10<sup>4</sup> CFU (in a volume of 100 µl) of one of the following strains: wild-type *B. suis* 1330 containing pBBR1MCS, *B. suis* ΔeryC deletion mutant with pBBR1MCS, or ΔeryC complemented with pBBR1-eryC. The actual infectious dose was confirmed later by diluting and plating the inocula on TS agar (14). Five mice from each group were killed by CO<sub>2</sub> asphyxiation at 3 days, 7 days, 4 weeks, or 8 weeks postinoculation. Spleens were aseptically removed, weighed, and homogenized with 10 ml of PBS (Gibco BRL Life Technologies) with a homogenizer (Stomacher 80 lab blender; Seward, London, United Kingdom) for the determination of *Brucella* counts. The homogenates were serially 10-fold diluted in PBS and plated on TS agar containing chloramphenicol (13). To assess intramurine behavior of the spontaneously erythritol-tolerant ΔeryC mutant, an additional infection experiment was performed as described with the *B. suis* 1330 wild type, the ΔeryC deletion mutant, and the erythritol-tolerant ΔeryC strain. A total of 10<sup>5</sup> CFU were effectively injected intraperitoneally. Per strain, five mice were killed at 7 days postinoculation, and survival of brucellae in the spleens was determined as described above.

*Brucella* colonies were counted after incubation for 3 to 5 days. Data are presented as log<sub>10</sub> values of CFU (log CFU)/spleen ± standard deviation of the mean (SD). The limit of detection in these experiments was ≤10 CFU per spleen.

**Statistical analysis.** Analysis of variance using the Fisher protected least-significant-difference test and the Tukey-Kramer test was performed to determine the level of significance of differences in CFU and in spleen weights observed with the mouse infection experiments. In macrophage infection experiments, Student's *t* test was used for statistical analysis. In both cases, *P* values of ≤0.05 were considered significant.

## RESULTS

**The *eryC* knockout mutant of *B. suis* was attenuated in human and murine macrophage-like cells.** Our previously published results described the isolation of independent Tn5 mutants of *B. suis* where the transposon was shown to be localized in the *eryB* and *eryC* genes, and both mutants were attenuated in the human macrophage-like THP-1 cells (16). To know whether this observation was limited to the use of human cells or if was a general phenomenon, we performed infection experiments using the murine macrophage-like J774 cells. The results obtained with J774 cells confirmed those previously obtained with human THP-1 macrophages: the number of intracellular *B. suis* *eryC*::Tn5 mutants was significantly lower at 24 and 48 h postinfection than with the wild-type *B. suis* 1330 (*P* ≤ 0.001). At 48 h, the difference between both strains was 50 fold (Fig. 1). Thus, the lower replication rate observed for the mutant in macrophage-like cells was independent of the type of macrophagic cell line used. Similar results of reduced intracellular multiplication were obtained with both macro-

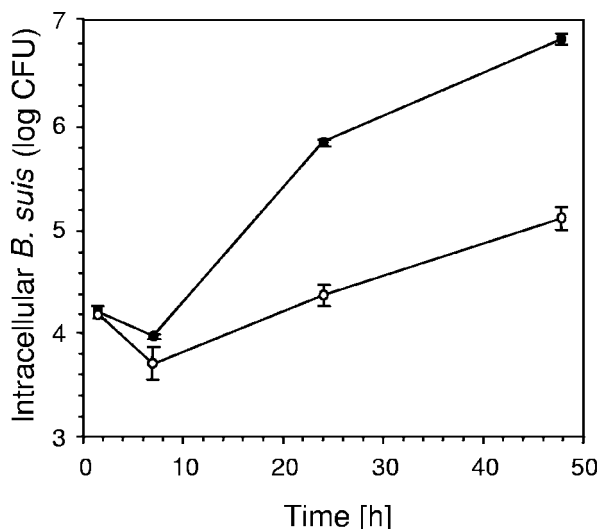


FIG. 1. Multiplication of *B. suis* 1330 wild-type (●) and *B. suis* *eryC*::Tn5 (○) in murine J774A.1 macrophage-like cells over a time course of 48 h. Values represent means of one out of two experiments performed in triplicate (each), and error bars indicate SD.

phage-like cell lines when *eryB* was inactivated (data not shown).

The construction of allelic exchange mutants was performed to verify that the transposon-based inactivation of genes of the *ery* operon was at the origin of the reduced intramacrophagic multiplication of *B. suis*. In the first allelic exchange mutant, a 387-bp internal fragment was deleted from *eryC* and replaced by a cassette carrying a kanamycin resistance gene. The gene *eryC* encodes the enzyme D-erythrose-1-phosphate dehydrogenase; it is the last of the three genes (*eryA*, *-B*, and *-C*) located within the *ery* operon and involved in erythritol degradation (27). It was conceivable, however, that the introduction of the cassette with the resistance marker would exert a polar

effect on the gene *eryD* located immediately downstream, which has been described as encoding a putative repressor of the *ery* operon (27), and therefore might affect the correct functioning of the enzymes encoded by the gene cluster. To verify this hypothesis, we constructed an additional, nonpolar deletion mutant of *eryC* in *B. suis*, which contained an internal deletion devoid of any selection marker. Infection experiments with THP-1 macrophage-like cells revealed that both mutants,  $\Delta$ *eryC*::Kan<sup>r</sup> and  $\Delta$ *eryC*, showed a very similar intracellular behavior, resulting in an approximately 90-fold-lower intracellular replication than the wild type at 48 h postinfection (Fig. 2A and data not shown). Differences between the wild type and the mutants were significant at 7, 24, and 48 h ( $P \leq 0.001$ ). Thus, the lower replication rate observed for the mutants in macrophage-like cells was clearly linked to *eryC* inactivation, and the attenuated phenotype of the  $\Delta$ *eryC*::Kan<sup>r</sup> mutant was not due to a polar effect on *eryD*.

In parallel, bone marrow-derived macrophages from BALB/c mice were infected by the wild type and the  $\Delta$ *eryC* deletion mutant under experimental conditions identical to those described above. At 48 h postinfection, the number of intramacrophagic bacteria was 90-fold higher with the wild type than with the  $\Delta$ *eryC* mutant (data not shown), confirming that this phenomenon was independent of the macrophage infection model used.

**Complementation in trans of  $\Delta$ *eryC* with the intact *eryC* gene restored wild-type levels of intramacrophagic replication.** The definite proof that *eryC* was responsible for the attenuated phenotype described above was obtained by complementation with an intact copy of the gene on plasmid pBBR1MCS. Following insertion of the gene in the proper orientation, the *lacZ* promoter of the plasmid was used to express *eryC*, as the gene is devoid of a promoter, because it is the third gene of the *ery* operon. In macrophage infection experiments with THP-1 cells, the complemented mutant of *B. suis* reached wild-type levels of intracellular multiplication, confirming that the erythritol metabolism pathway was in some way involved in optimal

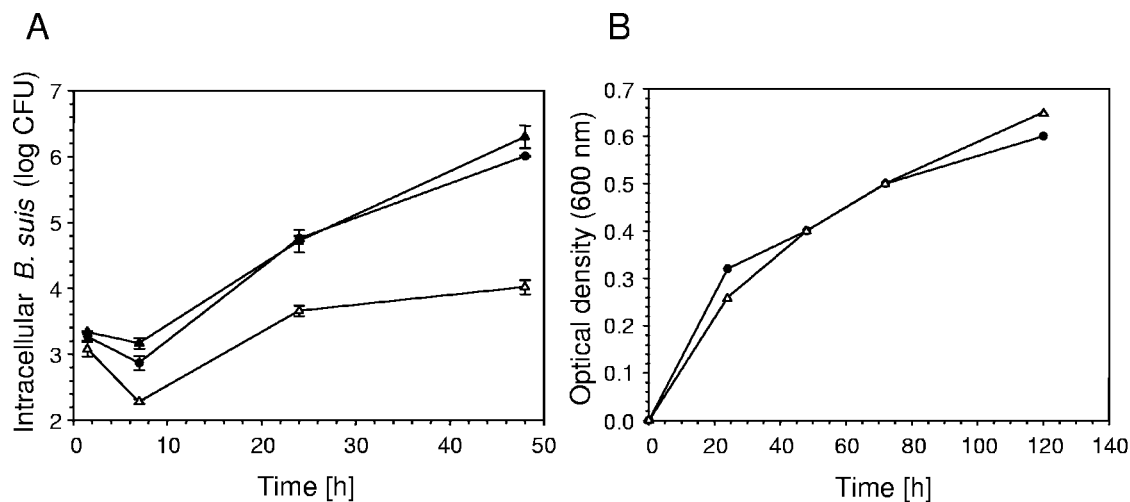


FIG. 2. (A) Comparison of the intracellular survival of *B. suis* 1330 wild-type containing plasmid pBBR1MCS (●), the  $\Delta$ *eryC* deletion mutant with plasmid pBBR1MCS (△), and  $\Delta$ *eryC* complemented with pBBR1-*eryC* (▲) in human THP-1 macrophage-like cells. Results are displayed as means  $\pm$  SD, and one out of two experiments performed in triplicate (each) is shown. (B) Growth curves of wild-type *B. suis* 1330 (●) and *B. suis*  $\Delta$ *eryC* (△) in RPMI 1640 medium with 10% FCS, where bacterial growth was measured by monitoring OD<sub>600</sub> increase.

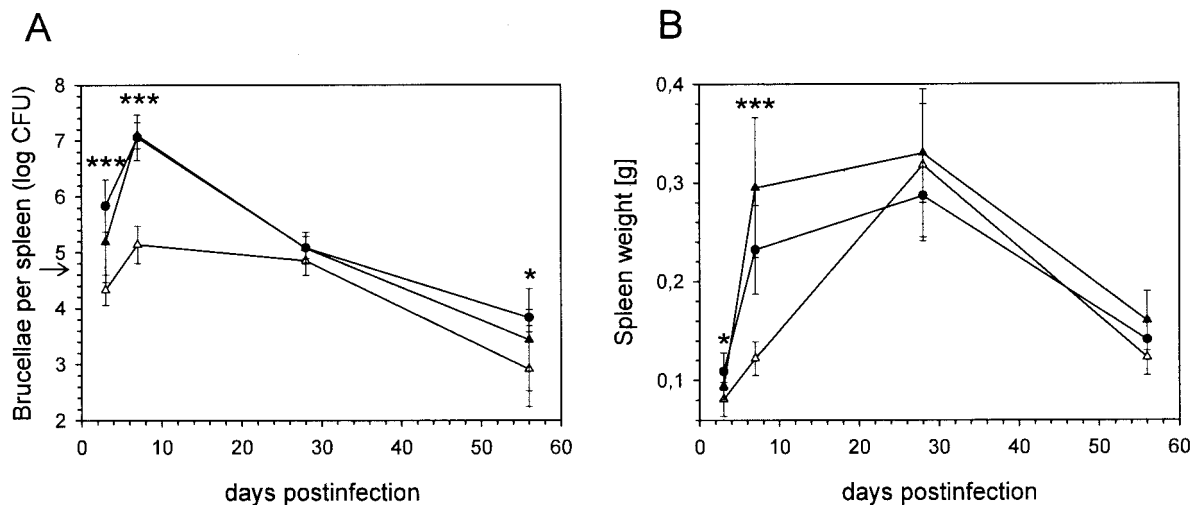


FIG. 3. Infection of BALB/c mice with the following *B. suis* strains over a period of 56 days. Wild-type *B. suis* 1330 containing plasmid pBBR1MCS (●), the  $\Delta$ eryC deletion mutant with plasmid pBBR1MCS (Δ), and  $\Delta$ eryC complemented with pBBR1-eryC (▲). (A) Survival of *B. suis* strains in the spleens. Viable bacteria were counted at different time points, and results are represented as means  $\pm$  SD. The infection dose of  $5 \times 10^4$  viable bacteria for each strain is indicated by an arrow. (B) Weights of the infected spleens removed for the determination of viable *Brucella* counts. In both panels, statistically significant differences between the wild-type and the  $\Delta$ eryC mutant were marked by asterisks (\*,  $P \leq 0.05$ ; \*\*\*,  $P \leq 0.001$ ).

adaptation of *Brucella* to the intramacrophagic environment (Fig. 2A). In bone marrow-derived macrophages, similar intracellular replication results were obtained with the complemented mutant (data not shown).

**Extracellular factors were not involved in reduced intramacrophagic survival of *B. suis*  $\Delta$ eryC.** It has been known for several decades that the absence of D-erythrulose-1-phosphate dehydrogenase in the live vaccine strain *B. abortus* B19 inhibits the growth of the strain in the presence of erythritol (33). The generation of a transposition mutant in *eryB* of the virulent *B. abortus* strain mimics the erythritol sensitive response of the B19 strain (26). We therefore had to consider the possibility that extramacrophagic factors present in the infection model, namely, in the FCS used in the cell culture medium, could be responsible for the attenuated phenotype observed with our mutant. To address this question, two complementary experiments were performed: (i) in vitro growth curves of the wild-type and the  $\Delta$ eryC deletion mutant in the cell culture medium used during infection, i.e., RPMI 1640 containing 10% FCS; and (ii) infection experiments with THP-1 cells in the absence of fetal calf serum.

In the cell culture medium, despite the fact that growth rates were considerably lower than in TS broth, where an optical density of 1.5 was reached in stationary phase after 20 h of culture, both the wild-type strain and the  $\Delta$ eryC mutant showed very similar growth rates over the entire duration of the experiment, which lasted for 5 days (Fig. 2B). The possibility that factors inhibiting growth of the mutant could be present in the cell culture medium used was therefore excluded. In the THP-1 infection model, the absence of fetal calf serum during the infection by *B. suis* strains did not alter the intracellular growth curves of both the wild type and the  $\Delta$ eryC mutant strain (data not shown). At 48 h, a 50-fold difference between both strains was measured, which was comparable to the results obtained in the presence of FCS (Fig. 1 and 2A). As the

RPMI 1640 medium and the FCS used cannot be implicated, it appeared evident that the intramacrophagic environment itself was responsible for the reduced replication observed with the *eryC* mutants.

**In vivo replication of the  $\Delta$ eryC mutant of *B. suis* in a murine model was affected in the early phase of infection.** The results presented above, describing the intramacrophagic attenuation of the *eryC* mutants, were obtained with cell culture infection models. It was therefore of interest to study the fate of an *eryC* mutant in vivo, and we chose the well-established BALB/c murine model of infection. At 3 and 7 days postinfection, the number of intrasplenic, viable  $\Delta$ eryC brucellae was significantly lower ( $P \leq 0.001$ ) than the number of wild-type bacteria, which increased rapidly during that period (Fig. 3A). The observed difference at 3 days (a factor of 33) was similar to the difference obtained in J774 macrophages at 48 h postinfection (Fig. 1). The strong and rapid replication of the wild-type strain during the first 7 days of infection was followed by a decline in the number of intrasplenic brucellae, confirming our previously published results with *B. suis* 1330 (9), whereas the level of the mutant strain was maintained during the following 3 weeks. Numbers of residual bacteria in the mice infected with *B. suis* 1330 or  $\Delta$ eryC were again significantly different ( $P \leq 0.05$ ) at 8 weeks postinfection (Fig. 3A). The  $\Delta$ eryC mutant was therefore characterized by its reduced capacity to set up an infection in the mouse, as opposed to the wild-type strain and the complemented mutant, which were not significantly different from each other during the course of the experiment. Evolution of the spleen weights of the infected animals confirmed these observations. The increase in weight, indicator of an inflammatory response of the organism, was significantly delayed in the mice infected by the mutant and hence corroborated the reduced capacity of infection of this strain (Fig. 3B).

With respect to the above-mentioned results, two major hypotheses had to be taken into consideration as possible ex-

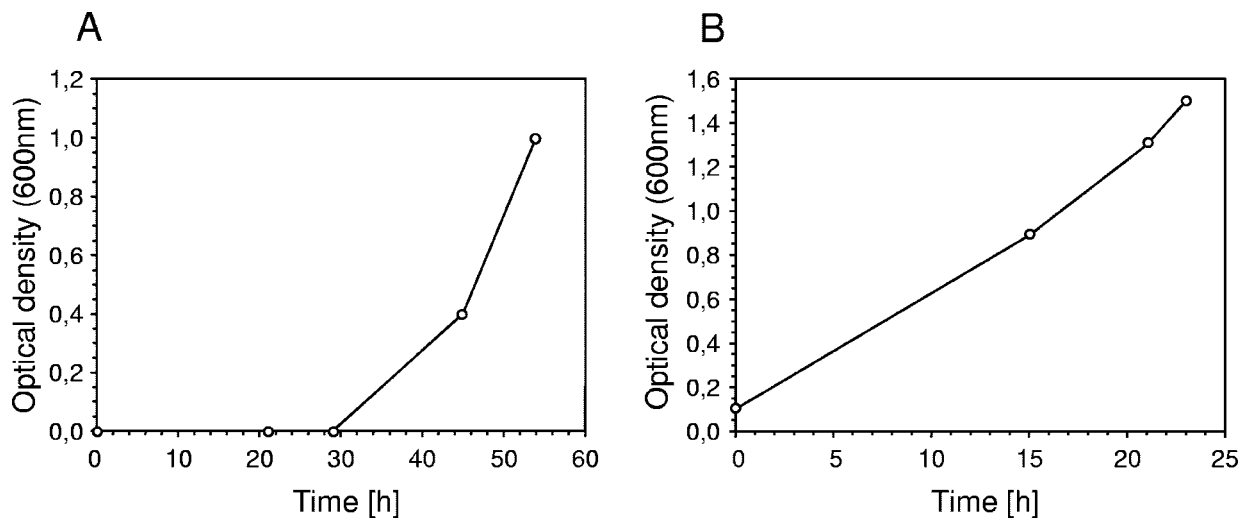


FIG. 4. Growth curves of *B. suis*  $\Delta eryC$  (A) and spontaneously erythritol-tolerant mutants of *B. suis*  $\Delta eryC$  (B) in TS broth containing 80 mM erythritol. The growth curve in panel B was obtained from a subculture of bacteria harvested at the end of the experiment shown in panel A. The  $OD_{600}$  value was measured in both sets of experiments, and one representative curve is shown for each.

planations for the observed phenomenon of reduced intramacrophagic replication of the *eryC* mutants of *B. suis*. (i) Erythritol contributed as a carbon or energy source to the capacity of multiplication within the macrophage host cell. (ii) The possible intracellular presence of erythritol at low concentrations partially inhibited the growth of  $\Delta eryC$ . To address these points, the properties of the  $\Delta eryC$  mutant of *B. suis* were further characterized.

**The  $\Delta eryC$  mutant of *B. suis* was sensitive to erythritol, and spontaneously resistant mutants occurred readily.** It has been previously described that *ery* mutants of *B. abortus* are sensitive to erythritol (26). However, erythritol-tolerant mutants unable to oxidize erythritol appear regularly in cultures of strain *B. abortus* B19 at a frequency of  $10^{-4}$  to  $10^{-6}$ , and the most likely explanation for this observation is a mutation in the erythritol uptake system (28). We confirmed in our assays that a  $\Delta eryC$  mutant of *B. suis* was also sensitive to erythritol, by incubation of the strain in broth in the presence of 1% erythritol (80 mM) for 54 h (Fig. 4A). No growth was measurable until 30 h of culture, when growth of resistant mutants started, as determined by an increase in optical density. The erythritol-tolerant character of the mutants was confirmed by the demonstration of rapid growth of a subculture in erythritol-containing broth (Fig. 4B). The experiments described below involving such an erythritol-tolerant  $\Delta eryC$  mutant were performed with a single, isolated mutant. In several cases, different individual erythritol-tolerant mutants were used in parallel experiments, and the results were always identical for all mutants used (not shown).

**The MIC of erythritol for the *B. suis*  $\Delta eryC$  mutant was in the millimolar range.** The erythritol-sensitive character of the  $\Delta eryC$  mutant of *B. suis* justified a thorough analysis of the conditions under which this sensitivity was observed. To this end, the approximate MIC for erythritol was determined during *Brucella* growth in TS broth in the presence of various concentrations of the compound. The wild-type strain and the complemented mutant grew well at concentrations of at least up to 25 mM, with a slightly higher growth rate of the com-

plemented strain during the log phase (Fig. 5 and 6B). In contrast, the growth rate of the  $\Delta eryC$  mutant was clearly affected at erythritol concentrations of 0.5 to 1 mM, and it was totally inhibited at concentrations of 5 mM and above. At the concentration of 0.1 mM, growth in the early log phase was slower than for the wild type (Fig. 5).

**Growth of a spontaneously erythritol-tolerant mutant of *B. suis*  $\Delta eryC$  was not inhibited by erythritol, but this polyalcohol could not be used as a carbon source.** To compare the properties of the  $\Delta eryC$  mutant and of a selected, spontaneously erythritol-tolerant  $\Delta eryC$  mutant with respect to tolerance and use of erythritol, growth experiments were performed with rich TS broth and in minimal medium in the absence or presence of erythritol, respectively. In rich medium and in the absence of

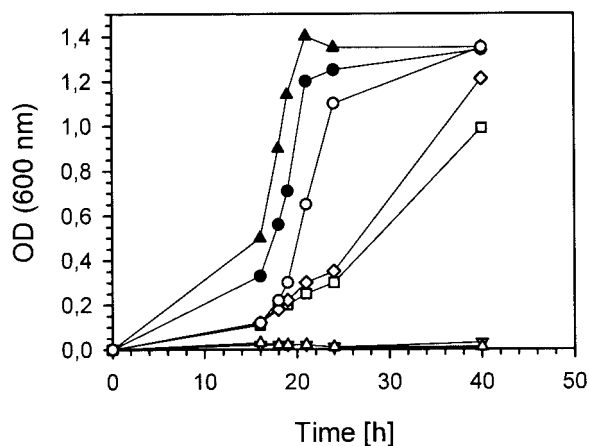


FIG. 5. Determination of the MIC of erythritol for the  $\Delta eryC$  mutant of *B. suis*. A growth curve measuring the  $OD_{600}$  value of the culture was performed at concentrations of 0.1 (○), 0.5 (◇), 1 (□), 5 (△), and 20 mM (▽) erythritol in TS broth. In addition, growth of the wild-type strain (●), and of the complemented *ery* mutant (▲) was followed in the presence of 20 mM erythritol.

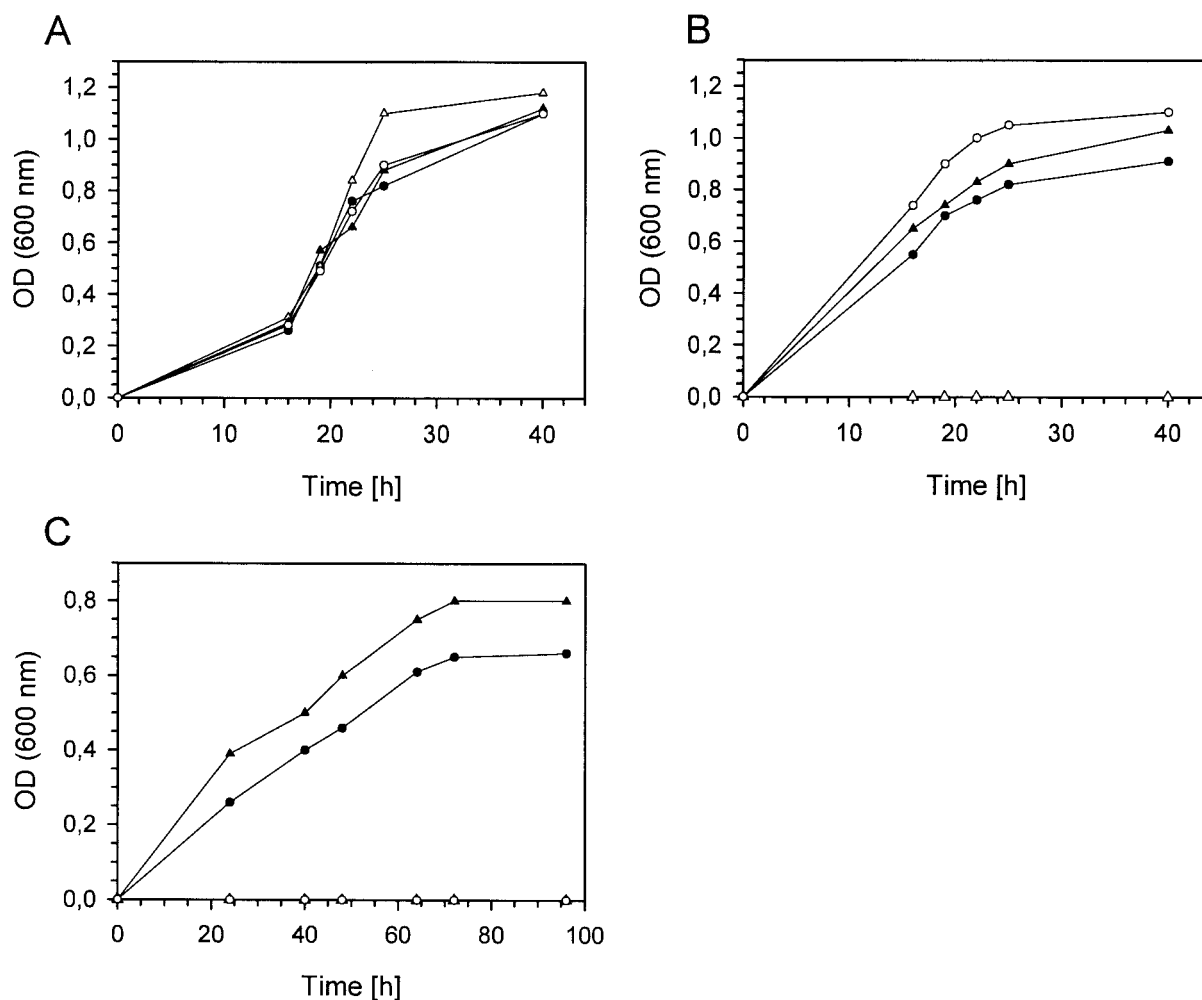


FIG. 6. Growth curves of *B. suis* strains in TS broth (A), in TS broth supplemented with 25 mM erythritol (B), and in minimal medium containing only erythritol (25 mM) as a carbon source (C). The strains used were the wild type (●), the  $\Delta$ eryC deletion mutant ( $\Delta$ ), the complemented  $\Delta$ eryC mutant containing pBBR1-eryC ( $\blacktriangle$ ), and an isolated, spontaneously erythritol-tolerant mutant of  $\Delta$ eryC ( $\circ$ ). One typical set of curves out of three independent experiments performed is shown for each.

erythritol, all strains grew comparably well, excluding any general growth defect of the mutants (Fig. 6A). In the same culture medium containing erythritol, the wild type, the complemented  $\Delta$ eryC mutant, and the spontaneously erythritol-tolerant  $\Delta$ eryC mutant grew well; only the  $\Delta$ eryC mutant was inhibited (Fig. 6B). Finally, although all strains grew in standard Gerhardt's minimal medium (not shown), the use of erythritol as a sole carbon source in this minimal medium prevented growth not only of the  $\Delta$ eryC mutant but also of the erythritol-tolerant  $\Delta$ eryC mutant (Fig. 6C). This finding was consistent with the assumption that the internal deletion of a 400-bp fragment of the *eryC* gene in the latter mutant, verified by PCR (not shown), could not spontaneously revert into a functional gene allowing complete erythritol metabolism. Altogether, these results made clear that the erythritol-tolerant  $\Delta$ eryC deletion mutant, as expected, did not recover the capacity to metabolize erythritol, suggesting therefore a defect in the uptake of the polyalcohol.

**A spontaneously erythritol-tolerant mutant of *B. suis*  $\Delta$ eryC exhibited wild-type-like replication in THP-1 cells and in BALB/c mice.** The spontaneously erythritol-tolerant mutant was then used to study its intramacrophagic behavior in THP-1 cells, compared to the wild-type strain and the original  $\Delta$ eryC mutant. In contrast to the original, erythritol-sensitive  $\Delta$ eryC mutant of *B. suis*, the tolerant mutant showed an intracellular replication rate over a period of 48 h that was identical to the growth rate observed with the wild-type strain (Fig. 7). Identical results were obtained with spontaneously erythritol-tolerant mutants of the  $\Delta$ eryC::Kan<sup>r</sup> deletion mutant and of the original *eryC*::Tn5 mutant (not shown). These results suggested (i) that although an intact erythritol degradation pathway was essential for normal intramacrophagic multiplication of the *B. suis* wild-type strain, erythritol was not an essential carbon source under these conditions; and (ii) that the reduced intracellular replication of the erythritol-sensitive  $\Delta$ eryC mutants might be due to the presence of erythritol in the host cell. In

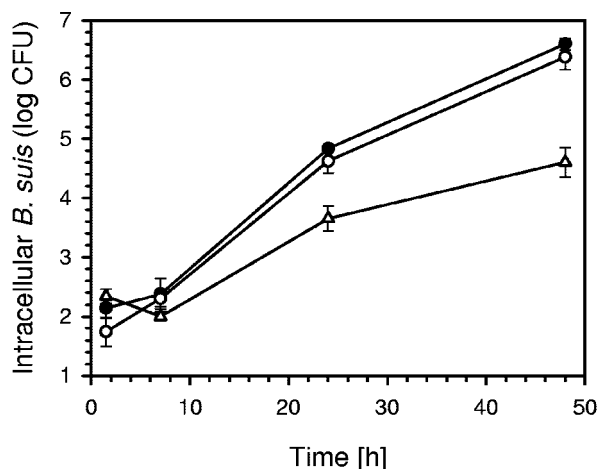


FIG. 7. Intracellular multiplication of *B. suis* 1330 wild-type (●), of an isolated, spontaneously erythritol-tolerant mutant of *B. suis*  $\Delta$ eryC (○), and of the  $\Delta$ eryC deletion mutant (△) in human THP-1 cells. The experiment was performed twice in triplicate (each), and results of one of the experiments are shown, presented as the means  $\pm$  SD.

this context, we verified that the reduced intracellular replication of the original *ery* mutants was not due to the occurrence of spontaneously erythritol-tolerant mutants that might allow a certain degree of multiplication of a mixed population in the macrophages: *ery* mutants reisolated from infected macrophages at 48 h postinfection were all sensitive to erythritol (data not shown).

An additional infection experiment using BALB/c mice was performed with the *Brucella* wild-type strain, the erythritol-sensitive  $\Delta$ eryC mutant, and the erythritol-tolerant  $\Delta$ eryC mutant to address the question of the behavior of the latter in the murine model of infection at 7 days postinfection, where differences between the wild type and the erythritol-sensitive  $\Delta$ eryC mutant were most significant (Fig. 3A). With effective infection doses of  $10^5$  bacteria, we obtained the following results for intramurine survival ( $\log_{10}$  total counts of *Brucella* CFU in the spleens):  $7.49 \pm 0.15$  for the wild type,  $6.0 \pm 0.39$  for the  $\Delta$ eryC mutant, and  $7.30 \pm 0.1$  for the erythritol-tolerant strain. Statistical analysis confirmed that the difference between wild-type and erythritol-tolerant bacteria was not significant ( $P = 0.096$ ), whereas it remained significant between the wild type or the erythritol-tolerant strain and the *ery* mutant ( $P < 0.0001$ ). The erythritol-tolerant strain therefore survived as well as the wild type in the mouse model of infection, confirming our results obtained with macrophages (Fig. 7).

## DISCUSSION

We have recently described for the first time the observation that *eryB* and *eryC* mutants of *B. suis* obtained in a Tn5 mutagenesis screen are characterized by reduced levels of intramacrophagic replication in human macrophage-like cells (16). This rather unexpected finding led to a more profound analysis of the interaction between an *eryC* mutant and the macrophage host cell. Allelic exchange mutants of *eryC* confirmed the results obtained with the original Tn5 mutants: the lack of an enzyme of the catabolic pathway of erythritol affects intracel-

lular *B. suis* multiplication, and the effect of the mutation was entirely reversible by complementation of the mutant. The observed attenuation could therefore be attributed to the inactivation of *eryC*. We extended the choice of in vitro infection models to a murine macrophage-like cell line and to bone marrow-derived macrophages, as attenuation could have been specific to human THP-1 cells. Nevertheless, a very similar degree of attenuation was recorded in all cell culture models used. However, if one considers that *eryB* and *eryC* are part of the same metabolic pathway (27, 32), there was contradiction with previously published results by Sangari et al., who reported the lack of attenuation of an *eryB* mutant of *B. abortus* in a murine model of infection (30); it was therefore possible that our results obtained in cell culture models of infection were not reproduced in vivo. Infection of BALB/c mice eliminated this concern, as we showed that an *eryC* mutant of *B. suis* was impaired in replication in this model. One possible explanation for this discrepancy may be differences in the strategy of infection for both species, as intramurine survival curves of the respective wild-type strains differed, notably the lack of an infection peak and the absence of decrease in the case of *B. abortus* (30). Results obtained with the complemented  $\Delta$ eryC mutant showed a good complementation in all infection models used, confirming that the observed attenuation was indeed linked exclusively to the inactivation of *eryC* by deletion of an internal fragment.

Previously published observations reported that the live vaccine strain *B. abortus* B19 and *ery* mutants of *B. abortus* are sensitive to erythritol (3, 26, 33) and that erythritol-tolerant mutants unable to oxidize erythritol appear regularly in cultures of strain *B. abortus* B19 (28). We confirmed erythritol sensitivity for the *eryC* mutant of *B. suis* and spontaneous occurrence of erythritol-tolerant phenotypes among these mutants. This tolerance was most likely due to a defect in the erythritol uptake system, as described and discussed previously (28). The possibility that traces of erythritol were present in fetal calf serum during infection experiments, leading to partial inhibition of mutant replication, could be excluded experimentally, and it was therefore straightforward to conclude that the intracellular environment of the host cell itself was the cause of the reduced intramacrophagic replication of the  $\Delta$ eryC mutant.

Two explanations were conceivable for the observed reduced intramacrophagic replication of the *eryC* mutants of *B. suis*. (i) The possible intracellular presence of erythritol at low concentrations partially inhibited the growth of  $\Delta$ eryC. (ii) Erythritol contributed as a carbon or energy source to the capacity of multiplication within the macrophage host cell. To further elucidate the possible reason(s) for intramacrophagic attenuation of the  $\Delta$ eryC deletion mutant, we made use of the availability of the spontaneously erythritol-tolerant mutants of  $\Delta$ eryC. In vitro growth experiments allowed us to determine the minimal erythritol concentrations that affected or inhibited growth of the erythritol-sensitive  $\Delta$ eryC deletion mutant and confirmed that the complemented mutant grew at least as well as the wild type and the erythritol-tolerant mutant at high concentrations of erythritol. Whereas a concentration of 5 mM was needed to completely inhibit growth of the  $\Delta$ eryC deletion mutant, concentrations as low as 100  $\mu$ M reduced the rate of bacterial multiplication in the logarithmic growth phase in rich medium. As suggested earlier, a general ATP depletion due to

high-level erythritol kinase activity and interference with hexose kinase activity may be explanations for the sensitivity of this mutant to erythritol (33). Additional experiments with minimal medium containing erythritol as a sole carbon source demonstrated the capacity of the *B. suis* wild-type strain and the complemented mutant to grow under these conditions and the absence of any growth for the erythritol-sensitive and -tolerant  $\Delta$ eryC deletion mutants. These results made clear that the capacity of *B. suis* to use erythritol as a C source depended on the presence of an intact *eryC* gene and that the disruption of this gene resulted in sensitivity of the mutant to erythritol, as described for *eryB* inactivation in *B. abortus* (26).

Phenotypically, the *eryC* deletion mutants had in common their incapacity to grow with erythritol as a C source, but in addition, the erythritol-tolerant mutants grew in rich medium in the presence of this polyalcohol. These properties were the starting point for macrophage infection and survival experiments with the  $\Delta$ eryC erythritol-tolerant mutant, experiments aimed at eventually explaining the attenuated intracellular phenotype linked to the *eryC* deletion. We reasoned that if erythritol was a molecule essential for intramacrophagic replication of brucellae, the reduced survival would persist. If, in contrast, the phenotype was only due to the erythritol sensitivity of the mutant, then wild-type-like multiplication would be observed with this spontaneously erythritol-tolerant mutant. The results presented in this work confirmed the latter hypothesis. We therefore speculated that brucellae were in contact with erythritol inside the macrophage, as it has never been reported that substrates other than erythritol were catabolized by these enzymes. The measurement of erythritol in tissue samples is, however, technically difficult and requires the application of very sensitive methods such as high-performance liquid chromatography coupled to gas chromatography-mass spectrometry (34). Although the spontaneously erythritol-tolerant mutants were not further characterized genetically, several independent isolates obtained from the three types of *eryC* mutants (*eryC*::Tn5,  $\Delta$ eryC::Kan<sup>r</sup>, and  $\Delta$ eryC) showed identical phenotypes in the described growth experiments in different media and in macrophage infection experiments, and it therefore appeared plausible that mutations in the as-yet-unknown erythritol uptake system were at the origin of the erythritol tolerance observed with the  $\Delta$ eryC mutants.

It has been previously described that brucellae utilize the carbon source erythritol in preference to glucose (1). In pregnant ungulates, *B. abortus*, *B. melitensis*, and *B. suis* have a marked tropism for the placenta where they preferentially replicate, causing acute placentitis, resulting in fetal death and abortion. The presence of erythritol in these tissues has therefore been linked to this tropism, and erythritol catabolism has been suggested to increase virulence in this host environment (15, 31). On the other hand, the erythritol-sensitive vaccine strain *B. abortus* B19 is attenuated and induces only few or no abortions. It carries a deletion in the *ery* operon (26, 27). These evidences suggested a role of erythritol catabolism in virulence of *B. abortus*, but studies of a murine model of infection using complementation experiments showed that the defect in erythritol metabolism of *B. abortus* B19 is not linked to its attenuated virulence in mice (30). Nevertheless, a stimulatory action of erythritol in vivo was described several decades ago for the infection of guinea pigs with *B. melitensis* or *B. suis* (15); in-

jected erythritol increased both the proportion of animals with detectable splenic infection and the level of the infection. Larger amounts of erythritol than those needed to produce enhancement of *B. suis* growth, however, tend to result in a smaller increase in the infection rate with this species. In addition, the authors mention that the erythritol content in tissue extracts of the placenta is three times higher in the cow than in the sow, and it is therefore conceivable that mutants of *B. suis* affected in the *ery* operon are generally more sensitive to the polyol than corresponding mutants of *B. abortus* or *B. melitensis*. As-yet-unknown differences in basic metabolic processes yielding, for example, various concentrations of ATP could be the cause of the observed discrepancies between *Brucella* strains and might explain why our results differed from those described earlier by Sangari et al. for *B. abortus* (30). Unfortunately, no data are yet available concerning the replication of a defined *ery* mutant of *Brucella* spp. in placenta trophoblasts and the rate of abortion it induces in ungulates or on the in vivo behavior of *B. abortus* B19 complemented with the *ery* operon. Future work in this direction may help to elucidate the questions remaining open today with respect to the role of erythritol in *Brucella* infection.

The general conclusion of our work was that the inactivation of the erythritol degradation pathway by mutation of *eryC* reduced the fitness of *B. suis* in the intracellular environment of the macrophage, although we could not show any direct benefit of its presence for the pathogen in this specific niche. The brucellae must be able to completely metabolize any erythritol they encounter in their environment to avoid toxicity and to maintain wild-type levels of survival in the host phagocytes. This constraint may be considered a safeguard, since maintaining constant pressure on the presence of a functional erythritol degradation pathway in *Brucella* appears to represent a selective advantage for rapid and intense colonization of erythritol-rich tissues such as the placenta of host animals, an origin for the further spread of disease and preservation of the pathogen.

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