Functional analysis of the ClpATPase ClpA of *Brucella suis*, and persistence of a knockout mutant in BALB/c mice

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The protein ClpA belongs to a diverse group of polypeptides named ClpATPases, which are highly conserved, and which include several molecular chaperones. In this study the gene encoding the 91 kDa protein b-ClpA of the facultative intracellular pathogen *Brucella suis*, which showed 70% identity to ClpA of *Rhodobacter blasticus*, was identified and sequenced. Following heterologous expression in *Escherichia coli* strains SG1126 ($\Delta clpA$) and SG1127 ($\Delta lon \Delta clpA$), b-ClpA replaced the function of *E. coli* ClpA, participating in the degradation of abnormal proteins. A b-clpA null mutant of *B. suis* was constructed, and growth experiments at 37 and 42 °C showed reduced growth rates for the null mutant, especially at the elevated temperature. The mutant complemented by b-clpA and overexpressing the gene was even more impaired at 37 and 42 °C. In intracellular infection of human THP-1 or murine J774 macrophage-like cells, the clpA null mutant and, to a lesser extent, the strain of *B. suis* overexpressing b-clpA behaved similarly to the wild-type strain. In a murine model of infection, however, the absence of ClpA significantly increased persistence of *B. suis*. These results showed that in *B. suis* the highly conserved protein ClpA by itself was dispensable for intramacrophagic growth, but was involved in temperature-dependent growth regulation, and in bacterial clearance from infected BALB/c mice.

Keywords: *Brucella*, ClpA, ClpATPase, chaperone

INTRODUCTION

Brucella spp. are facultative intracellular bacteria and the causative agents of brucellosis, an anthroponosis. The disease can be spread by aerosolization of the pathogen, by contact with infected animal tissues such as placenta or by ingestion of contaminated food, mainly dairy products (Young, 1983). It has been shown that survival and multiplication of bacteria in host phagocytic cells are crucial to *Brucella* infection (Young, 1983; Price *et al.*, 1990; Liautard *et al.*, 1996). Inside the host cell, brucellae remain enclosed in phagocytic compartments (Harmon *et al.*, 1988), and macrophage activation and cell-mediated immunity are prerequisites for the elimination of the bacteria (Smith, 1990). To date, little is known about the mechanisms and the factors that allow brucellae to survive within macro-

phages. The genes encoding HtrA (Elzer *et al.*, 1994), PurE (Drazek *et al.*, 1995), a two-component system (Sola-Landa *et al.*, 1998), and a homologue of the VirB type IV secretion system (O'Callaghan *et al.*, 1999) have been described as being involved in virulence.

Under environmental stress conditions such as elevated temperatures, variation in pH or starvation, a certain number of proteins are induced or repressed in *Brucella* spp., among which are the GroE and DnaK heat-shock proteins, and other, not yet identified, proteins (Lin *et al.*, 1992; Köhler *et al.*, 1996; Rafie-Kolpin *et al.*, 1996). The hostile conditions such as acidic pH encountered by brucellae inside phagocytic cells (Porte *et al.*, 1999) mimic certain environmental stress signals. In intracellular brucellae, the known stress proteins GroEL and DnaK are induced during infection, and DnaK is essential for multiplication of *Brucella suis* in macrophage-like cells (Lin & Ficht, 1995; Köhler *et al.*, 1996). Both DnaK and GroEL/GroES belong to the class of molecular chaperones (Hendrick & Hartl, 1993). The

The GenBank accession number for the sequence reported in this paper is AJ224881.

ClpATPases (HSP100 proteins) are a family of proteins represented in prokaryotes and eukaryotes with a high degree of conservation, and of which several members have been described as molecular chaperones. These proteins, which are characterized by the presence of consensus ATP-binding sites, can be divided into two major classes with eight subfamilies, including ClpA, ClpB, ClpC and ClpX (Schirmer *et al.*, 1996). The ClpA (Hwang *et al.*, 1988; Katayama *et al.*, 1988) and ClpX (Gottesman *et al.*, 1993) proteins of *Escherichia coli* were first described as regulatory ATPases forming a complex with the unrelated, ATP-dependent, proteolytic component ClpP (Maurizi *et al.*, 1990). This complex, called Clp protease or protease Ti, removes toxic denatured proteins by degradation. In addition, ClpA can act as a molecular chaperone *in vitro* in the absence of ClpP, as has been shown for the *in vitro* activation of plasmid P1 RepA replication initiator protein, where purified ClpA performs the chaperone function of DnaK and DnaJ, and for the protection of luciferase from irreversible heat inactivation (Wickner *et al.*, 1994). A chaperone function of ClpA has not been described *in vivo* yet and the natural substrates have not been identified.

It became evident that ClpATPases are implicated in the stress tolerance of many organisms and protect vital cellular components during stress conditions (Squires & Squires, 1992; Schirmer *et al.*, 1996; Wawrzynow *et al.*, 1996). Recent work on ClpC and ClpE of the pathogen *Listeria monocytogenes* has demonstrated that these chaperones are required for the virulence of the bacterium (Rouquette *et al.*, 1996, 1998; Nair *et al.*, 1999). To our knowledge, these are the first reports on the study of members of the ClpATPase family in an intracellular pathogen. In contrast, all functional studies reported on ClpA have been performed exclusively on *E. coli* ClpA. Other bacterial ClpA homologues have been identified in systematic genome sequencing programs without any further characterization.

Additional knowledge of ClpATPases of pathogenic bacteria contributes to a more profound understanding of the role of these proteins in a large spectrum of bacteria, and may shed light on the complex mechanisms of bacterial virulence. In the present work, we have isolated and characterized the gene encoding the ClpA homologue of the facultative intracellular pathogen *B. suis* and undertaken a first functional analysis of the protein. We constructed a $\Delta clpA$ mutant, which showed restricted growth at elevated temperature, but behaved normally during intracellular survival within phagocytic cells. Overexpression of *clpA* in *B. suis* led to increased growth restriction, as compared to the *clpA* null mutant and the wild-type strain. In a murine model of infection, the *clpA* null mutant of *B. suis* persisted significantly longer in the animal, as compared to the wild-type.

METHODS

Bacterial strains, plasmids and growth conditions. Characteristics of bacterial strains and plasmids used are described in Table 1. *E. coli* strains were routinely grown in Luria-Bertani

(LB) medium. Strains of *B. suis* were grown in Tryptic Soy (TS) medium. Solid media were supplemented with 1.5% agar (Life Technologies). The standard growth temperature for all strains was 37 °C, in the absence of supplementary CO₂. The appropriate antibiotics were added at the following concentrations when needed: ampicillin, 50 µg ml⁻¹; chloramphenicol, 25 µg ml⁻¹; kanamycin, 50 µg ml⁻¹.

Reagents. Unless otherwise stated, all chemical reagents were purchased from Sigma. Molecular biology reagents were purchased from Eurogentec. 1 α ,25-Dihydroxy-vitamin D₃ (1 α ,25-dihydroxycholecalciferol) was a kind gift of C. Damais, INSERM U-313, Paris, France.

Bacterial growth experiments. *Brucella* strains were grown in the presence of antibiotics until stationary phase at 37 °C and diluted 1:20 in preheated TS medium without antibiotics to an OD₆₀₀ of 0.05–0.07. Cultures of each strain were incubated with shaking at 37 °C or 42 °C, respectively. At the time points indicated, the OD₆₀₀ was measured in a spectrophotometer. The stable maintenance of plasmid pBBR1MCS or its derivative containing *b-clpA* was verified at the end of the experiment by plating serial dilutions of the cultures on TS agar with and without chloramphenicol, followed by colony enumeration. Growth curve measurements were performed twice.

DNA isolation, PCR amplification and Southern blots. Plasmid DNA preparations, DNA treatments with restriction and modification enzymes, and cloning techniques were performed according to manufacturer's instructions or standard protocols (Sambrook *et al.*, 1989). Extraction of genomic DNA of *E. coli* and *B. suis* strains was performed as described by Ausubel *et al.* (1989). For DNA amplification by PCR, two 20-mer oligonucleotides (5'-TGTGGCGGATATTGAGTC-CG-3' and 5'-TGGTGGTCATCACCAGCACC-3'; Eurogentec), annealing to the second ATP-binding domain of *clpA* from *E. coli*, were used. Amplification conditions were as follows: 30 cycles of a denaturation step at 95 °C for 45 s, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min, followed by final elongation step at 72 °C for 5 min.

For labelling of DNA probes, PCR products or DNA fragments obtained by restriction enzyme digestion were purified from agarose gels by electroelution and labelled using a random prime kit (Boehringer Mannheim) with digoxigenin for nonradioactive labelling or with [³²P]dCTP (NEN; 600 Ci mmol⁻¹) for radiolabelling. Southern blots were performed as described by Sambrook *et al.* (1989) on to Biotodyne B nylon membranes (Pall). Under low-stringency conditions, the membrane was washed twice at 45 °C for 15 min in 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS. For highly homologous probes, two 20 min washes under stringent conditions of 65 °C in 0.1 × SSC and 0.1% SDS were applied.

Cloning of the *b-clpA* gene. A 550 bp DNA fragment was amplified by PCR from the second ATP-binding domain of the *E. coli clpA* gene (see above), radiolabelled and hybridized to a genomic Southern blot of *B. suis* DNA. Under low-stringency conditions, the probe bound to a 4.6 kb *EcoRI* fragment, which was cloned into pUC18. To that purpose, the screening of recombinant *E. coli* clones was performed on pools of plasmid DNA preparations to avoid background hybridization with the chromosomal *clpA* gene of *E. coli*. The 3' end of the gene was, however, missing on this *EcoRI* insert, and a second, overlapping cloning step was performed. The 3' part of the gene of interest was isolated as a 2.3 kb *HincII*–*HindIII* fragment from *B. suis* genomic DNA, overlapping by 640 bp with the previously identified *EcoRI* fragment. Both inserts were fused in the proper orientation via the single

Table 1. Bacterial strains and plasmids used in this study

Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; r, resistance.

Strain/plasmid	Genotype and/or description	Source
<i>E. coli</i> strains		
DH5 α F'	F' <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 ϕ80lacZΔM15 Δ(lacZYA-argF)U169</i>	Life Technologies
SG1101	Δ arg	Katayama <i>et al.</i> (1988)
SG1102	Δ arg lon-146:: Δ Tn10	Katayama <i>et al.</i> (1988)
SG1126	Δ arg clpA319:: Δ kan	Katayama <i>et al.</i> (1988)
SG1127	Δ arg clpA319:: Δ kan lon-146::Tn10	Katayama <i>et al.</i> (1988)
<i>B. suis</i> strains		
<i>B. suis</i> 1330	Biovar 1; ATCC 23444	ATCC
<i>B. suis</i> 1330 Δ clpA	<i>B. suis</i> 1330 Δ b-clpA::kan (<i>b-clpA</i> null mutant)	This work
Plasmids		
pUC18	Amp ^r	Pharmacia
pUC4K	Contains a Kan ^r cassette flanked by polylinker	Pharmacia
pCVD442	Vector with <i>sacB</i> encoding sucrose sensitivity, Amp ^r	Donnenberg & Kaper (1991)
pBBR1MCS	Broad-host-range cloning vector, Cm ^r	Kovach <i>et al.</i> (1994)
pUC18-clpA	pUC18 derivative with the <i>b-clpA</i> gene in the <i>Sma</i> I/ <i>Hind</i> III site	This work
pUC18 Δ clpA::kan	pUC18 derivative with <i>b-clpA</i> null allele in <i>Sma</i> I site	This work
pBBR1-clpA	pBBR1MCS derivative with the <i>b-clpA</i> gene in the <i>Bam</i> HI/ <i>Sac</i> I site	This work
pCVD Δ clpA::kan	pCVD442 with <i>b-clpA</i> null allele in <i>Sac</i> I/ <i>Sph</i> I site	This work

common site *Apa*I, and the site of fusion was verified by DNA sequencing. The *clpA* homologue in *B. suis* was further subcloned into pUC18 as a 3.9 kb *Eco*RV-*Hind*III fragment, yielding clone pUC18-clpA.

The *b-clpA* gene was recloned into the broad-host-range vector pBBR1MCS (Kovach *et al.*, 1994) by excision from pUC18-clpA as a 4 kb *Bam*HI-*Sac*I fragment and insertion into the corresponding restriction sites of pBBR1MCS, resulting in construct pBBR1-clpA.

Sequencing. DNA sequencing with pUC18-based templates was done by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using universal, reverse and specific internal primers synthesized by Genome Express. Each base was sequenced 3–5 times.

Computer-based sequence analysis. The DNA sequence obtained was translated into the six reading frames and compared to the polypeptides in the SWISS-PROT database by using the programs FASTA (Pearson & Lipman, 1988) or BLAST (Altschul *et al.*, 1990) to identify similar sequences. Multiple sequence alignment of ClpA amino acid sequences was carried out with CLUSTAL W 1.60 (Thompson *et al.*, 1994). The Prosite database (Bairoch *et al.*, 1997) allowed the identification of ClpA/B signature sequences. Secondary structure prediction of ClpA was performed using Predict-Protein (Rost *et al.*, 1994).

Electroporation of *B. suis*, and inactivation of *b-clpA* by homologous recombination. A fragment of 550 bp, flanked by two *Cla*I sites and internal to *b-clpA*, was deleted and replaced

by the 1.2 kb blunt-ended kanamycin resistance gene from plasmid pUC4K (Pharmacia), leading to the vector pUC18 Δ clpA-kan. The *b-clpA*-kan insert was excised as a 4.6 kb *Sac*I-*Sph*I fragment, and recloned into pCVD442 (Donnenberg & Kaper, 1991), containing the gene *sacB* encoding sucrose sensitivity (Gay *et al.*, 1983, 1985). *B. suis* was transformed with this suicide vector, named pCVD Δ clpA::kan, by electroporation, as described by Köhler *et al.* (1996), and recombinants that integrated the construct into the chromosome were isolated on TS agar containing kanamycin. For selection of the second crossing-over event resulting in inactivation of the chromosomal *b-clpA* gene, an overnight culture of such a recombinant was plated on to TS agar containing 5% sucrose and kanamycin. Only clones that had lost the pCVD442 plasmid resistance marker *bla* encoding ampicillin resistance were taken into consideration for further analysis. The inactivation of chromosomal *b-clpA* was verified by Southern blot analysis and immunoblotting.

Production of polyclonal anti-ClpA antiserum. A rabbit antiserum was raised against a synthetic ClpA peptide of 14 aa coupled to keyhole limpet hemocyanin (Synt:em). Three subcutaneous booster injections followed at 14, 28 and 56 d after the first injection, and the rabbit was bled 4 weeks after the last injection. For each injection, 2 mg coupling product in 500 μ l phosphate buffer (20 mM, pH 7.4) were diluted with 1 vol. Freund's adjuvant.

Expression of *b-clpA* in *B. suis*.

At different pH. A stationary-phase culture of *B. suis* 1330 was

diluted 1:500 and grown to mid-exponential phase at 37 °C. Bacteria were harvested by centrifugation, washed once in 0.9% NaCl and resuspended in equal volumes of TS medium adjusted to pH 3.0, 4.0 and 5.0, and in regular TS. After incubation at 37 °C for 2 h, bacteria were centrifuged and total cell lysates were prepared for SDS-PAGE.

At different temperatures. A mid-log culture was obtained at 30 °C as described above. Aliquots of the culture were then incubated at 30, 37, 42 and 45 °C for 2 h, followed by centrifugation and preparation of cell lysates for SDS-PAGE analysis.

SDS-PAGE and immunoblotting. For total cell lysates, the OD₆₀₀ of the cultures was determined, and the concentration of bacteria was calculated from a previously established standard curve relating optical density to the concentration via serial plating and colony enumeration on agar plates. Equal numbers of bacteria were harvested from cultures, washed once in 0.9% NaCl, resuspended in Laemmli sample buffer and heated to 100 °C for 5 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels (Laemmli, 1970). Bands were visualized by Coomassie brilliant blue staining after transfer onto a PolyScreen membrane (NEN) by a semi-dry transfer procedure (Harlow & Lane, 1988). Transferred ClpA protein from *B. suis* and recombinant *E. coli* was detected using the 1:1000-fold diluted rabbit anti-b-ClpA polyclonal antiserum and anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma). Bound antibodies were detected using chemiluminescence (ECL; Amersham).

Canavanine disk assay inhibition test. *E. coli* strains were grown to stationary phase in TS medium, centrifuged and the pellet was resuspended at an OD₆₀₀ of 0.25 in 0.9% NaCl. Each suspension (100 µl) was plated on to agar plates containing RPMI 1640 medium supplemented with 1.5% agar (w/v). RPMI 1640 contains L-arg at 200 µg ml⁻¹. All strains used grew equally well in this medium. Sterile 6 mm filter disks were saturated with 20 µl of a solution of canavanine (100 mg ml⁻¹), and laid on to the inoculated culture plates, followed by incubation of the plates at 37 °C for 16 h. For each strain, 10 independent measurements of the diameter of the inhibition zone around the filter plates were performed.

Infection and intracellular survival assay of *B. suis* in human THP-1 and murine J774A.1 macrophage-like cells. Experiments were done as described earlier (Caron *et al.*, 1994). Briefly, THP-1 cells, differentiated by 1 α ,25-dihydroxy-vitamin D₃ at a concentration of 10⁻⁷ M for 72 h, were resuspended at 5 × 10⁵ cells ml⁻¹ in RPMI 1640 medium supplemented with 10% foetal calf serum. Alternatively, murine J774 cells were resuspended at 1.2 × 10⁵ cells ml⁻¹ in the same medium. Adherent cells were incubated for 24 h at 37 °C in 5% CO₂ prior to infection at a m.o.i. of 20 with stationary-phase *B. suis* grown in the presence of the corresponding antibiotics. After 30 min, cells were washed three times with PBS and reincubated in RPMI 1640/10% foetal calf serum with gentamicin (30 µg ml⁻¹) for at least 1 h. At 1.5, 7, 24 and 48 h post-infection, cells were washed twice with PBS and lysed in 0.2% Triton X-100. Numbers of c.f.u. were determined by plating serial dilutions on TS agar and incubation for 3 d at 37 °C. In parallel, plating on TS agar containing chloramphenicol allowed checking for the presence of pBBR1MCS or its derivative containing *b-clpA*. Viable counts were identical with and without chloramphenicol, showing great stability of the plasmids even in the absence of selective pressure. Viability of the cells was measured by trypan blue dye exclusion at different time points and was not affected throughout the experiment. Experiments were performed three times in duplicate.

Infection of mice, preparation of spleens and bacterial counts. Eight-week-old female BALB/c mice obtained from our animal facilities or from IFFA Credo were challenged intraperitoneally with 5 × 10⁴ c.f.u. of either wild-type *B. suis* 1330 or the *b-clpA* null mutant in 0.2 ml. Brucellae were grown on Trypticase Soy agar (Life Technologies) supplemented with 0.1% (w/v) yeast extract (Difco) (TSA-YE) for 24 h, harvested in buffered saline solution, adjusted spectrophotometrically at 600 nm and diluted to 2.5 × 10⁵ c.f.u. ml⁻¹. Viable counts were determined retrospectively by enumeration on TSA-YE plates. Five mice for each *B. suis* strain were killed at days 1, 4, 11, 18, 25 and 32 by cervical dislocation. Spleens and livers were harvested, weighed and frozen at -20 °C. After homogenization in buffered saline solution, bacterial counts were determined on TSA-YE plates. When no c.f.u. was detected in the plated volume, calculations were based on the presence of one c.f.u. per plate (= limit of detection).

To normalize the distribution of individual counts for statistical analysis, the number of c.f.u. per spleen was transformed as log (c.f.u./log c.f.u.), as previously published (Bosserey & Plommet, 1976). The normalized distribution was used to evaluate the significance of the results, and analysis of variance (ANOVA) was performed by comparison of the growth curves and the weight curves of liver and spleen for the two strains. The software used were Excel (Microsoft) and InStat (Graphpad Software) for Macintosh.

EMBL/GenBank nucleotide sequence accession numbers. The nucleotide sequence of the *b-clpA* gene of *B. suis* was submitted to GenBank and its database accession number is AJ224881. Formerly published accession numbers for *clpA* of *E. coli* and *Rhodobacter blasticus* are M31045 and P05444, respectively.

RESULTS

Cloning of the *b-clpA* gene of *B. suis*

All known homologues of ClpA and ClpB contain two large ATP-binding regions that are highly conserved among the members of the family, sharing at least 85% similarity within these regions (Gottesman *et al.*, 1990a). Based on the sequence alignments performed earlier (Gottesman *et al.*, 1990a), a pair of oligonucleotides was designed (see Methods) that amplify a 550 bp DNA fragment from the second ATP-binding region of *clpA* from *E. coli*. Oligonucleotide 1 was located at position 2265–2284 and the reverse oligonucleotide 2 was located at position 2815–2796 on the complementary strand of the published sequence (Gottesman *et al.*, 1990a). This PCR product, used as a probe, allowed the identification and isolation of the *clpA* homologue from the genome of *B. suis*. The establishment of a physical map of the gene we called *b-clpA*, localized the binding of the *E. coli* probe to a region adjacent to the 200 bp *Cla*I fragment of the insert (not shown). Sequence data analysis confirmed that the DNA region recognized by the heterologous *clpA* probe was located within an ORF highly homologous to *clpA*.

DNA and deduced amino acid sequences of *b-clpA*

The *b-clpA* ORF of 2475 bp encoded a protein of 824 aa. The *b-clpA* gene had a G + C content of 57.6 mol%, which is typical of *Brucella* spp. DNA (Corbel, 1990). It is flanked by a putative Shine–Dalgarno sequence at

	signature sequence N-terminus	A Box (N1)	B Box 1 (N1)	Prositesign. I	B Box 2 (N1)
<i>B. suis</i>	---LERALHQAALTIANERHHEYATLEHLLALLI---	GDPGVGKT---	KELEEFPGAVLFID---	DASNLLKPALSSG---	RKLDPKVIDID---
<i>R. blasticus</i>	---LEQAIHAALALANARRHELATLEHLLALLI---	GDPGVGKT---	KELEHDPDAILFID---	DASNLLKPALQGG---	RKLDPKVIDID---
<i>E. coli</i>	---LELSLNMAFARAREHRHEFTVEHLLALLI---	GESGVGKT---	KQLEQDTSILFID---	DAANLIKPLSSG---	RHLDPKVIDID---
	---**...*...*...*...*****---//--**...*****---//--**...*****---//--**...*****---//--				
	A Box (N2)	Prositesign. II	B Box (N2)	signature sequence C-terminus	SSD-motif
<i>B. suis</i>	---GPTGVGKT---	RFDMSYMERHTVSRLIGA---	VLLLD---	FTPEFRNRDLAIIPFGPL---	GARPLARVIQ---
<i>R. blasticus</i>	---GPTGVGKT---	RFDMSYMERHTVSRLIGA---	VLLLD---	FTPEFRNRDLAIVISFAPL---	GARPLGRVIQ---
<i>E. coli</i>	---GPTGVGKT---	RFDMSYMERHTVSRLIGA---	VLLLD---	FTPEFRNRDLNIWFDHL---	GARPMARVIQ---
	---*****---//--*****---//--*****---//--*****---//--*****---//--*****---//--*****---//--				

Fig. 1. Amino acid alignment of characteristic portions of the ClpA ATPases of *B. suis*, *R. blasticus* and *E. coli*. -- represents regions of the proteins not shown in the alignment.

position 668–671 and a potential rho-independent transcription terminator at position 3155–3184 of the sequence. The theoretical isoelectric point of the b-ClpA protein was 6.34 and its theoretical molecular mass 91.07 kDa. ClpA of *B. suis* was highly homologous over its entire amino acid sequence to two other bacterial ClpA proteins previously described, ClpA of *R. blasticus* (Tybulewicz *et al.*, 1984) and of *E. coli* (Gottesman *et al.*, 1990b), with identities of 71% and 59%, respectively. Of these three ClpAs, b-ClpA had the highest number of amino acid residues (824 aa), followed by *R. blasticus* (793 aa) and *E. coli* (758 aa). A multiple sequence alignment was performed with the three ClpA proteins (Fig. 1). Based on a previous alignment between members of the ClpATPase family (Gottesman *et al.*, 1990a) and on subfamily associations (Schirmer *et al.*, 1996), various well-conserved regions and consensus sequences were localized in b-ClpA. Two characteristic internal signature sequences for chaperonins ClpA and ClpB were found by Prosites database search: DASNLLKPALSSG and RFDMSYMERHTVSRLIGA, at positions 336–348 and 552–570, respectively (Fig. 1). N-terminal and C-terminal signature sequences present in b-ClpA are also well-conserved in the other ClpA molecules. Most characteristic of the ClpATPase family were the two nucleotide-binding domains, designated N1 and N2. They contained Walker-type consensus motifs A and B (Walker *et al.*, 1982) for ATP binding. The first nucleotide-binding domain is unique in having two B motifs, B Box 1 and B Box 2 (Fig. 1). Domains N1 and N2 were separated by a spacer of 5 aa, which is also present in ClpA of *E. coli* and *R. blasticus* (Squires & Squires, 1992). Another characteristic region of the protein was the sensor- and substrate-discrimination (SSD) domain, containing the well-conserved SSD motif (Fig. 1). This domain seems to play a critical role in the mechanism by which ClpA recognizes the correct substrates (Smith *et al.*, 1999). Secondary structure predictions (data not shown) for the regions around the ATP-binding domains revealed the presence of structures known to participate in the formation of a nucleotide-binding pocket or Rossmann fold (β -sheet-Gly-Lys-Thr-loop- α -helix around the A Boxes, and α -helix-loop- ϕ_4 - β -sheet around the B Boxes in Fig. 1; Senior, 1988), which is in agreement with previous results on ClpA from *E. coli* and *R. blasticus* (Gottesman *et al.*, 1990b).

Table 2. Sensitivity of *E. coli* strains to canavanine

Results represent the mean of ten measurements per strain.

Strain	Diameter zone of inhibition in mm (mean $\pm$ SD)
SG1101 (Lon ⁺ ClpA ⁺)	6.66 $\pm$ 0.28
SG1102 (Lon ⁺ ClpA ⁺)	7.75 $\pm$ 0.29
SG1126 (Lon ⁺ ClpA ⁺)	7.60 $\pm$ 0.25
SG1126 complemented with <i>b-clpA</i>	6.52 $\pm$ 0.39
SG1127 (Lon ⁺ ClpA ⁺)	12.50 $\pm$ 0.40
SG1127 complemented with <i>b-clpA</i>	7.41 $\pm$ 0.30

Functional complementation of a *clpA* mutant of *E. coli*

It has been reported previously that *E. coli* mutants defective in protease Lon show a reduced degradation rate of canavanine-containing proteins and that this rate is further reduced in a $\Delta lon \Delta clpA$ double mutant, as ClpA regulates the other major protease ClpP (Katayama *et al.*, 1988). These experiments were short-time radiolabelling experiments performed in liquid cultures. Here, we report the development of a disk inhibition assay where the arginine analogue canavanine diffused into solid medium of defined composition, thus allowing us to assess an effect on bacterial growth by measurement of the inhibition zone diameter around the different *E. coli* strains. The arginine auxotroph, Lon⁺ ClpA⁺ strain SG1101 showed very little sensitivity to canavanine, as the inhibition zone corresponded to the filter diameter (Table 2). In contrast, strains SG1102 (Lon⁺ ClpA⁺), SG1126 (Lon⁺ ClpA⁺), and SG1127 (Lon⁺ ClpA⁺) were significantly more sensitive to canavanine incorporation (Student's *t*-test; *P* < 0.005) than SG1101. Complementation of strains SG1126 and SG1127 with *b-clpA* reduced growth inhibition by canavanine to levels significantly different from those obtained with the parental strains SG1126 and SG1127 (*P* < 0.0001) and not significantly different from those obtained with SG1101 and SG1102, respectively, as shown in Table 2. ClpA of *B. suis* therefore appeared to functionally replace *E. coli* ClpA and to restore protease ClpP activity in an *E. coli* background.

Fig. 2. Expression of *b-clpA* in recombinant *E. coli* and in *B. suis*. Lysates equivalent to 3×10^8 bacteria were loaded per lane, and separated by SDS-PAGE prior to Western blot analysis with polyclonal anti-b-ClpA antibody. Lane 1, *E. coli* DH5α with pUC18-*clpA*; lane 2, *E. coli* DH5α with pUC18; lane 3, *B. suis* 1330; lane 4, *B. suis* $\Delta clpA$; lane 5, *B. suis* $\Delta clpA$ complemented with pBBR1-*b-clpA*.

b-clpA expression is not increased at low pH or at high temperatures

In our studies, *b-clpA* expression was followed with a polyclonal rabbit anti-b-ClpA antiserum. Antibodies were raised against the internal peptide SKRPQGSEPRTPRG of the protein (position 166–179). Sequence alignments with other ClpA proteins revealed very little conservation of the molecule in this region (not shown), and there was no homology to other ClpATPases, particularly in *E. coli*. Secondary structure prediction revealed that this peptide was part of a region rich in loop structures, flanked by α -helical domains (not shown). These features made the peptide a good candidate for a specific b-ClpA epitope. The anti-ClpA

antiserum specifically recognized recombinant b-ClpA in the host *E. coli* strain. As expected, *E. coli* ClpA did not cross-react with the antiserum (see Fig. 2, lanes 1 and 2). As in recombinant *E. coli*, a single protein band of a total *B. suis* 1330 wild-type lysate reacted with anti-b-ClpA (Fig. 2, lane 3). The molecular mass of the protein was calculated to be 90 kDa according to SDS-PAGE migration, which was in good agreement with the theoretical molecular mass of 91.07 kDa.

To analyse if ClpA behaved as a stress protein in *B. suis*, further experiments were conducted where the bacteria were exposed to the temperatures of 30, 37, 42, and 45 °C, or to pH values ranging from pH 3.0 to 7.2 for 2 h during exponential phase. Bacterial lysates were then subjected to Western blot analysis with anti-b-ClpA antiserum. No differences were visible in the amounts of ClpA produced under these various conditions (data not shown). This is consistent with results described for *E. coli* ClpA, which is not a classical heat-shock protein (Katayama *et al.*, 1988).

Insertional inactivation of *b-clpA* and complementation of a *clpA* null mutant of *B. suis*

A *b-clpA* deletion mutant was constructed by replacement of an internal fragment of the gene by a kanamycin-resistance cassette. This construct was introduced into the vector pCVD442, which is unable to replicate in *Brucella* spp. and contains the selection marker *sacB*, encoding sucrose sensitivity. Double recombination events between chromosomal *b-clpA* and the inactivated gene on the suicide plasmid led to kanamycin- and sucrose-resistant clones that had lost the plasmid marker encoding ampicillin resistance (Fig. 3a).

Fig. 3. Inactivation of *b-clpA* in *B. suis* by homologous recombination. (a) Schematic representation of double crossing-over between chromosomal *b-clpA* and the inactivated gene containing *Kan^r* cloned into suicide vector pCVD. The hatched bar indicates the *clpA* ORF, and the region labelled P corresponds to the *b-clpA* portion homologous to the *clpA* probe from *B. suis* used in (b). A, *ApaI*; C, *Clal*; Ev, *EcoRV*; Hc, *HincII*; Hd, *HindIII*; Sa, *SacI*; Sp, *SphI*. (b) Southern blot of genomic DNA from *B. suis* strains and hybridization to a 650 bp *HincII*-*ApaI* probe from *b-clpA*. Lane 1, *b-clpA* null mutant/*HindIII*; lane 2, *b-clpA* null mutant/*HindIII* + *HincII*; lane 3, wild-type/*HindIII*; lane 4, wild-type/*HindIII* + *HincII*.

Fig. 4. Effect of *b-clpA* inactivation and overexpression on growth at 37 °C (a) and 42 °C (b). The data shown are the means from duplicate cultures of *B. suis* 1330 wild-type (●), *b-clpA* null mutant (○) and complemented mutant overexpressing *b-clpA* (▲).

The correct disruption of chromosomal *b-clpA* was verified by Southern and Western blot analyses. For Southern blotting, an internal 650 bp *HincII*–*ApaI* fragment was used as a probe (Fig. 3a). The hybridization profiles obtained on *HindIII*- and *HindIII*/*HincII*-digested genomic DNAs from wild-type or mutant strains revealed fragments of the expected sizes: 7 kb and 2.5 kb for the wild-type, and 4.6 kb and 1.1 kb for the mutant (Fig. 3b). Western blot analysis using polyclonal anti-b-ClpA confirmed the absence of ClpA in the mutant of *B. suis* (Fig. 2, lane 4). No truncated or partially degraded ClpA products were detectable.

The *b-clpA* null mutant of *B. suis* was complemented *in trans* with the intact *b-clpA* gene cloned into pBBR1MCS. We noticed strong overexpression of *b-clpA* under these conditions, as shown in Fig. 2, lane 5. Western blot experiments also revealed that expression of *b-clpA* was strongly increased in the *B. suis* background as compared to heterologous expression using pUC18 in *E. coli*, despite the considerably lower copy number of the pBBR1MCS derivative in *Brucella* spp. (Elzer *et al.*, 1994). This indicated that *b-clpA* promoter recognition was of low efficiency in *E. coli*.

Temperature-dependent growth rates of the *b-clpA* null mutant and of the complemented mutant of *B. suis*

Previous work on ClpA of *E. coli* had shown that, as a chaperone, ClpA protected proteins from irreversible heat denaturation (Wickner *et al.*, 1994). We compared the growth of the wild-type strain containing pBBR1MCS to that of the isogenic *b-clpA* null mutant with pBBR1MCS and of the complemented strain with pBBR1–*clpA* at temperatures of 37 and 42 °C. At 37 °C, all strains reached the same optical density during stationary phase (OD₆₀₀, 1.8), but growth rates were significantly reduced for the *b-clpA* mutant, and especially for the complemented strain (Fig. 4a). In mid-exponential phase, at OD₆₀₀ between 0.2 and 0.4, the mean generation time was 2 h for the wild-type and approximately 4 h for the mutant and the complemented strain. The wild-type maintained its growth rate until an

OD₆₀₀ of about 1.2, whereas the other strains reduced their growth rates at an OD₆₀₀ of 0.4–0.55.

Following a temperature shift to 42 °C, the growth curve of the wild-type strain was, as expected, not affected. In contrast, growth rates of both the *b-clpA* mutant and the complemented strain were strongly reduced as soon as the OD of the culture reached 0.2. At this point, the generation time was 4.5 h for both strains and it further increased to 10 and 20 h at ODs of 0.4 and 0.25 for the mutant and the complemented strain, respectively. In stationary phase, the optical density of the *b-clpA* null mutant culture reached only 50% of wild-type culture density and that of the complemented mutant did not exceed 25% (Fig. 4b). The same results were obtained at 37 and 42 °C for a second, independently isolated *clpA* mutant of *B. suis* (data not shown).

Intracellular survival of the *b-clpA* null mutant is not affected in human macrophage-like THP-1 cells and in murine J774A.1 macrophages

We investigated the question of whether ClpA was involved in survival of *B. suis* within its host cell, the macrophage. Infection of differentiated, human macrophage-like THP-1 cells by *B. suis* 1330 containing pBBR1MCS, the isogenic *b-clpA* null mutant containing pBBR1MCS, and the complemented mutant with pBBR1–*clpA* leading to overexpression of *b-clpA*, yielded similar results for intracellular multiplication of the three strains (Fig. 5a). After an initial drop in survival during the first 7 h post-infection, all strains used showed a 30–100-fold increase in viable intracellular bacteria over 48 h. Differences between strains were not significant, except at 48 h for the complemented strain (Student's *t*-test; *P* < 0.05). The slower intracellular multiplication of this strain may, to some extent, reflect the reduced growth rate in late exponential phase at 37 °C described above. Similar intracellular growth curves were obtained upon infection of murine J774 macrophagic cells over a period of 48 h (Fig. 5b), but the differences in the rate of multiplication between the wild-type and the *clpA* null mutant versus the overexpressing strain were further enhanced.

Fig. 5. Growth of *B. suis* in (a) THP-1 human macrophage-like cells, or in (b) J774 murine macrophages. Adherent cells were infected with the wild-type strain (●), the *b-clpA* null mutant (○) or the complemented mutant overexpressing *b-clpA* (▲). Error bars indicate standard deviations.

Fig. 6. Course of infection of *B. suis* 1330 wild-type (●) and the *b-clpA* null mutant (○) in spleen (a) and liver (c) of BALB/c mice, and corresponding spleen (b) and liver (d) weights. Growth of the bacteria in the spleen and in the liver were determined at several time points for 32 d. Results represent means $\pm$ SD.

The *b-clpA* null mutant of *B. suis* is more persistent in BALB/c mice

The well-established murine model of infection was used for the study of *Brucella* spp. *in vivo* (Bosserey & Plommet, 1990; Allen *et al.*, 1998; Godfroid *et al.*, 1998).

The fate of the bacteria was monitored by the enumeration of the organisms in spleen and liver of BALB/c mice at different times post-infection.

Colonization of the spleen by *Brucella* was significantly different between the wild-type strain and the *b-clpA*

null mutant ($P < 0.0001$), and at different time points after infection ($P < 0.0001$). With the wild-type strain, the number of *Brucella* in the spleen peaked at day 4, followed by a 10^4 -fold decrease until day 18. The *b-clpA* mutant colonized the spleen significantly less than the wild-type on day 4 ($P = 0.002$), but infection was then persistent with only a 10^2 -fold decrease over a period of 28 d (Fig. 6a). The number of *b-clpA* null mutant brucellae in the spleen was significantly higher than the number of wild-type bacteria between days 18 and 32 ($P < 0.0001$). Recent experiments with the complemented mutant overexpressing *b-clpA*, however, did not allow us to study intramurine behaviour of this strain, due to loss of the pBBR1-*clpA* during the course of infection, as determined by serial platings in the presence and absence of chloramphenicol, and by plasmid preparations (not shown). The control plasmid pBBR1MCS was, in contrast, stably maintained in the wild-type and the *b-clpA* null mutant.

The weight of spleens increased after infection until day 11 ($P < 0.0001$) independently of the strains, i. e. 7 d after the peak of intrasplenic survival. This splenomegaly was followed by rapid reduction of weight for the wild-type strain (Fig. 6b), in contrast to splenomegaly induced by the *b-clpA* null mutant which persisted until day 32. Splenomegaly induced by *Brucella* strains was indeed significantly different between 18 and 32 d after infection ($P < 0.0001$), and weight of spleens infected by the *b-clpA* null mutant was higher than the weight of spleens infected by the wild-type strain ($P < 0.0001$).

Differences observed in the spleens were confirmed in the livers. Clearance of *Brucella* from livers was observed for three of five livers on days 18 and 25, and five of five livers on day 32 after infection with the wild-type strain. On day 18 after infection, colonization of livers by the wild-type strain was 10^3 times lower than that of the *b-clpA* null mutant ($P < 0.0001$), where the elimination rate was clearly reduced (Fig. 6c). In analogy to results described above, the increase in weight of livers infected by the *b-clpA* mutant was higher between 18 and 32 d after infection (Fig. 6d).

DISCUSSION

Conservation of *b-clpA* and evidence for its functional interaction with *E. coli* ClpP

In this study we described the isolation and characterization of the ClpATPase b-ClpA from *B. suis*. The protein showed extensive homology to ClpA of *R. blasticus*, which belongs, as do *Brucella* spp., to the α -*Proteobacteria*. Although several putative bacterial *clpA* genes have now been sequenced in genome-sequencing programs, *b-clpA* is, with *clpA* of *E. coli*, only the second bacterial gene encoding ClpA that was isolated and studied more extensively. The very high degree of conservation of the two ATP-binding sites in b-ClpA made activities identical to those observed in *E. coli* most likely in brucellae. Regions of very low homology

and alignment gaps between the three proteins were located immediately in front of and behind the nucleotide-binding domains, and at the C-terminus of the molecules. These regions may therefore not be important for the function(s) of the different ClpA proteins, or, in contrast, they could encode as yet unknown properties specific for the various bacteria.

ClpA has been identified in *E. coli* as the ATP-binding regulatory component of a two-component complex, in which the ATP-dependent protease ClpAP degrades polypeptide substrates (Katayama *et al.*, 1988). The proteolytic activity of ClpP in *E. coli* is therefore dependent on the presence of ClpA (Woo *et al.*, 1989). The *in vivo* function of ClpA has been studied by the comparison of the breakdown of abnormal canavanine-containing proteins in Δlon and $\Delta lon \Delta clpA$ mutants (Katayama *et al.*, 1988). Based on these experiments, we set up a modified canavanine-incorporation assay, in which we performed heterologous complementations of the existing $\Delta clpA$ and $\Delta lon \Delta clpA$ mutants of *E. coli* with *b-clpA*. Both single Δlon or $\Delta clpA$ mutations had comparable effects on growth inhibition, suggesting that the concerned proteases Lon and ClpAP both degrade a number of specific proteins, but are also both involved in general degradation of abnormal proteins. Compared to the single mutants, the double mutant $\Delta lon \Delta clpA$ was highly sensitive to canavanine. One possible explanation for this observation is that both proteases can substitute for each other to a large extent during bacterial growth. Hence, simultaneous inactivation or decrease in activation of both proteases, either by direct mutation or by elimination of the regulatory component ClpA, strongly increased growth inhibition. Our findings on canavanine-related growth inhibition partially reflect previously published results (Katayama *et al.*, 1988) and differences may be explained by the lack of direct correlation between the two experiments, as we were studying long-term effects of canavanine exposure on the mutant strains.

Complementation of the $\Delta clpA$ mutant and of the $\Delta lon \Delta clpA$ mutant of *E. coli* with the intact *b-clpA* gene of *B. suis* reduced canavanine-related growth inhibition to a level not significantly different from the inhibition observed in strain SG1101 and in the Δlon mutant alone. This is indirect evidence for the restoration of full activity of protease ClpP, mediated by a heterologous ClpA molecule. To this end, b-ClpA must functionally interact with ClpP from *E. coli* on a molecular level. Recently, ATP-dependent proteolysis combining the action of ClpA and ClpP in *E. coli* has been dissociated into discrete steps, and analysed in detail (Hoskins *et al.*, 1998). We suggest that in our heterologous complementation system using ClpA from *B. suis* and ClpP from *E. coli*, the reduction in growth inhibition caused by canavanine was due to an intimate interaction between both components. This leads to the assumption that the domains of ClpA responsible for the interaction with ClpP must be extremely well conserved in the different bacterial genera. The region of ClpA that interacts with ClpP has not been determined yet, although portions of

the C-terminal end may be involved (Gottesman *et al.*, 1993).

***b-clpA*: a component of Clp protease and a chaperone?**

In addition, ClpA and other ClpATPases can function alone as molecular chaperones preventing aggregation, and are involved in refolding and remodelling of proteins (Wickner *et al.*, 1994; Wawrzynow *et al.*, 1996; Pak & Wickner, 1997; Suzuki *et al.*, 1997). To date, the chaperone activities have only been observed *in vitro* for ClpATPases from *E. coli* and using defined model systems. ClpA of *E. coli*, in contrast to other ClpATPases, shows similar rates of synthesis at 32 and 43 °C, and thus is not a heat-shock protein (Katayama *et al.*, 1988). We confirmed these findings for *Brucella* ClpA, and, in addition, could not find any increase in ClpA synthesis under acid-shock conditions. The absence of a σ^{32} -consensus sequence 5' of *b-clpA* was in agreement with these observations. Nevertheless, b-ClpA was important for the normal growth of *B. suis* in liquid medium, as a $\Delta b-clpA$ mutant showed reduced growth rates in broth. This phenomenon was strongly accentuated at high temperatures, but already visible at 37 °C. In *E. coli*, inactivation of *clpA* does not affect growth on minimal and rich media at temperatures between 25 and 42 °C (Katayama *et al.*, 1988). However, detailed analysis of the behaviour of a $\Delta clpA$ mutant of *E. coli* at high temperature, including growth curve experiments, was reported only recently: at 46 °C, the growth rate of the mutant is reduced, despite the fact that ClpA is not a heat-shock protein (Thomas & Baneyx, 1998), hence strengthening our data obtained on *B. suis*. The reduced growth rate of the $\Delta b-clpA$ mutant at 42 °C suggested the participation of b-ClpA in preventing the accumulation of heat-inactivated and aggregated proteins in *B. suis*. Two different mechanisms of action may be considered for b-ClpA. (i) Our findings may be explained by a chaperone activity of b-ClpA in brucellae. A protective effect of ClpA from *E. coli* had been shown *in vitro* with purified luciferase, qualifying ClpA as a molecular chaperone (Wickner *et al.*, 1994). (ii) ClpA in brucellae most likely has, as in *E. coli*, the role of binding substrates and presenting them to ClpP for degradation. This protease function may be crucial in the elimination of inactivated or denatured proteins in *B. suis*, occurring for example at higher temperature. Whereas it appears easily conceivable that the absence of ClpA disturbs bacterial growth under certain conditions, we made the surprising observation that overexpression of *b-clpA* had a similar or even stronger effect. This result was confirmed lately for ClpA of *E. coli* (Thomas & Baneyx, 1998). A previously published report stated that overexpression of the gene encoding a different chaperone, DnaK, also has effects comparable to its inactivation (Blum *et al.*, 1992). We propose two possible explanations for the effects due to high concentrations of ClpA. (i) In the case of a possible chaperone activity of ClpA, we assume that the interactions between different chaperones in the cell represent

a well-balanced network, sensitive to disturbances that may arise from the absence or the overexpression of one of the components. High concentrations of ClpA may lead to high concentrations of disaggregated or unfolded substrate polypeptides which may reaggregate among themselves or may not be properly refolded by the chaperones of the Hsp60 and Hsp70 family. (ii) As an essential component of the protease ClpAP complex, ClpA at unphysiologically high concentrations may lead to deleterious protein degradation by the proteolytic complex activity, or may result in a shift of the balance between ClpP and the second ClpATPase presenting specific substrates to the protease, ClpX (Gottesman *et al.*, 1993), towards the interaction ClpA and ClpP. ClpX plays an essential role in *B. suis* and its gene cannot be inactivated (S. Köhler & E. Ekaza, unpublished). We consider that formation of ClpX–ClpP is crucial for viability in brucellae, and that disturbance of a balance ClpA–ClpP–ClpX may result in the phenotype described above. Recently, ClpX has been shown to be involved in the regulation of essential cell-cycle processes in *Caulobacter* (Jenal & Fuchs, 1998).

Chaperones play an important role in intramacrophagic survival, as has been described for DnaK in *B. suis* (Köhler *et al.*, 1996). Here, ClpA was chosen, as it has been shown to perform certain ATP-dependent chaperone functions of DnaK and DnaJ *in vitro* (Wickner *et al.*, 1994), and because it is the regulatory component of the protease ClpAP, hence playing an important role within the organism. In contrast to DnaK, ClpA of *B. suis* was by itself not essential for the multiplication of the bacteria within macrophages. We cannot exclude that one or several other proteins of the ClpATPase family such as ClpX can functionally replace the inactivated *b-clpA* gene in the intramacrophagic environment. To address the question, further insight into the *in vivo* substrates of these proteases has to be gained.

Knockout of *b-clpA* correlates with a higher persistence of the mutant in BALB/c mice

In a murine model of infection that has been well established in the study of various *Brucella* spp. strains, we observed that the rate of persistence of *B. suis* in the spleen and liver increased with the *b-clpA* null mutant strain. Analysis of the spleen and liver weights during *B. suis* infection suggests a delay in the establishment of an inflammatory response to infection by the *clpA* mutant strain, and a lower intensity of this response during 2 weeks post-infection. The better persistence of the mutant in mice thereafter correlated with slower reduction in spleen weight in comparison to the wild-type strain. One possible explanation of this persistence is the reduction of the growth rate at 37 °C observed for the *b-clpA* mutant in broth and in mice. Another explanation could be a direct effect of brucellae on proinflammatory cytokines and/or specific immune response induction. However, no direct experimental evidence concerning this matter has been obtained yet. In such a complex *in vivo* system, b-ClpA therefore rather seemed to be a

disadvantage for *B. suis* and could be a potential immunogen.

In contrast to what we could observe *in vitro* during macrophage infection or in broth culture in the absence of selective pressure, pBBR1-*clpA* was lost during murine infection experiments. We believe that this was due to overexpression of *b-clpA*, as pBBR1MCS *per se* is stably maintained at a level of about 10 copies per cell even in the absence of antibiotics (Elzer *et al.*, 1994), and we conclude that for a yet unknown reason overexpression of *b-clpA* is of disadvantage for *B. suis* during murine infection.

To summarize, our studies on b-ClpA are, to our knowledge, the first molecular genetic investigation of this specific ClpATPase in a facultative intracellular bacterium. We demonstrated the functional replacement of ClpA in *E. coli* by the heterologous protein of *B. suis*, and we described the participation of b-ClpA in bacterial growth at elevated temperatures. ClpA deficiency alone did not affect multiplication of *B. suis* in macrophages, but it reduced the rate of elimination in mice. The study of other ClpATPases in *B. suis* and their individual and simultaneous inactivations are currently in progress.

ACKNOWLEDGEMENTS

We thank S. Gottesman for her kind gift of *E. coli* strains and M. Kovach for providing us with pBBR1MCS. We wish to acknowledge P. Lechopier, H. Le Roux and staff for animal husbandry, and N. Bosseray for helpful comments on the statistical analysis. This work was supported by grant PL 980089 from the European Union.

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Received 22 November 1999; revised 25 February 2000;
accepted 24 March 2000.