Cloning of genes for proline and leucine biosynthesis from *Brucella abortus* by functional complementation in *Escherichia coli*

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By selecting for growth of *Escherichia coli* mutant strains in the absence of the required amino acid, clones were found in a *Brucella abortus* library carrying genes for glutamylphosphate reductase (*proA*) and β -isopropylmalate dehydrogenase (*leuB*). These clones hybridized to unique fragments in a genomic digest of *B. abortus* DNA. The *proA*-complementing DNA was found in a region of 1.3 kb, which directed the synthesis of a protein of 48000 Da with a pI of 6.3 in maxicells. The *leuB*-complementing activity was in a region of 1.4 kb and directed synthesis of a protein of 46000 Da with a pI of 5.9.

Introduction

Brucella abortus is usually considered a fastidious organism and is cultured on complex media, frequently including serum. Strains of *Brucella* have been found which will grow on defined media, in some cases using only ammonia as nitrogen source, but more often using a single amino acid (Gerhardt, 1958). That this can happen implies that *Brucella* must be able to synthesize virtually all amino acids. Species of *Brucella* are capable of surviving in macrophages and other cell types, and grow in enveloped vesicles in these cells. It is possible that some nutrients either in blood or the cytoplasm of the infected cells are unavailable in these vesicles, so in this state, the *Brucella* cells may have to use their ability to synthesize amino acids. Certain *Salmonella* auxotrophs, including ones for leucine, are less virulent in mice, suggesting these nutrients are not readily available to a pathogen living inside a phagosome (Bacon *et al.*, 1951). The ability of *B. abortus* strain 19 to synthesize amino acids was tested by attempting to complement defects in various *Escherichia coli* mutants unable to synthesize amino acids and other nutrients. We report here the isolation of *Brucella* genes for proline and leucine biosynthesis.

Methods

Bacterial strains, plasmids and media. The *E. coli* strains used in this work are shown in Table 1. *B. abortus* strain 19 was obtained from the Oklahoma Animal Disease Diagnostic Laboratory (Oklahoma State Univ.). For DNA isolation, *Brucella* cells were grown on plates of tryptose medium (Difco) solidified with agarose. Routine culture of *E. coli* used LB (Luria & Burrous, 1957). Transformants were selected on LM medium (Hanahan, 1983) containing 50 μ g ampicillin ml⁻¹. Amino acid requirements were tested using medium A (Miller, 1972) with 10 mM-glucose as carbon and energy source supplemented with the appropriate amino acids, bases and vitamins.

Strain RE484 was constructed from strain JM107 by introducing the *phoA8* deletion from strain JF4281 by cotransduction with the transposon from strain χ 2844. It was then made *thyA* by selection for resistance to trimethoprim (Miller, 1972). Using strain WL66 as donor, *recBC* was transduced in by selecting for Thy⁺. The strain was cured of Tn10 by selection for tetracycline sensitivity by the method of Maloy & Nunn (1981). *sbcB* was transduced from strain JC7623 by cotransduction with the *hisG*::Tn10 insertion from strain NK5526. Finally, the strain was transduced to His⁺, tetracycline sensitive from a wild-type donor.

Strains RE623, 625 and 799 were constructed from a derivative of χ 462 in which the *leuB2* lesion was replaced by wild-type by transduction, leaving the strain *ara-14*. The *leuA*, *C* and *D* lesions from strains CV512, 522 and 526, respectively, were transduced in by selecting for Ara⁺. Finally, the *recA* deletion was put in by mating with strain RE506 (RE799) or RE507 (RE623 and 625), selecting for tetracycline resistance.

General procedures. Cells were transformed by the PEG-DMSO method (Chung *et al.*, 1989) unless very high efficiency was needed, in which case the method of Hanahan (1983) was used. Plasmids were isolated by the alkaline-SDS method (Ish-Horowicz & Burke, 1981). Restriction enzymes were used under conditions specified by their manufacturers.

Construction of library. DNA was isolated from *Brucella abortus* strain 19 after growth on plates solidified with agarose to minimize

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Table 1. *E. coli* strains used

Strain	Genotype	Source/reference*
JM107	F ⁺ <i>traD36 proAB⁺lacP_ZΔM15/Δ(lac-pro) thi gyrA96 endA1 hsdR17 relA1 supE44</i>	C. O. Gardner, Jr ^a (Yanisch-Perron <i>et al.</i> , 1985)
JF4281	HfrC <i>ΔphoA8 fadL701 relA1 pit-10 spoT1 tonA22 ompF627 λ⁻</i>	J. D. Friesen ^b (Hayashi <i>et al.</i> , 1964)
χ2844	<i>tsx-462::Tn10, λ⁻</i>	GCSC 6683
WL66	<i>hsdR recB21C22 supE supF metB trpR</i>	Amersham
NK5526	<i>hisG213::Tn10 IN(rrnD-rrnE1)1</i>	CGSC 6416
RE484	F ⁺ <i>traD36 proAB⁺lacP_ZΔM15/Δ(lac-pro) thi gyrA96 endA1 hsdR17 relA1 supE44 ΔphoA8 recBC sbcB15</i>	See text
JC7623	<i>thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 rac sbcB15 hisG4, rfbD1 recB21 recC22 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1</i>	CGSC 5188 (Kushner <i>et al.</i> , 1971)
χ462	<i>thi-1 metE70 lysA23 trpE38 purE42 proA leuB6 mtl-1 xyl-5 ara-14 lacZ36 azi-6 rpsL109 tonA23 tsx-67 supE44</i>	M. C. Jones-Mortimer ^c (Berg & Curtiss, 1967)
ED1032	Hfr <i>Δ(gpt-lac)5 relA1 rpsE2123 thi-1 supE44 TP3</i>	CGSC 5395 (Broda <i>et al.</i> , 1972)
JC10289	<i>thr-1 leuB6 Δ(gpt-proA)62 hisG4 Δ(recA-srlR)306 srlR301::Tn10-84 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44</i>	R. D. Porter ^d (Csonka & Clark, 1979)
RE506	Hfr <i>Δ(gpt-lac)5 relA1 rpsE2123 thi-1 supE44 TP3 Δ(recA-srlR)306 srlR301::Tn10-84</i>	ED1032 transduced to Tc ^r with P1 grown on JC10289
RE521	as χ462, but <i>Δ(recA-srlR)306 srlR301::Tn10-84</i>	χ462 mated with RE506, selecting Tc ^r
χ474	as χ462, but <i>proB91</i>	CGSC 6452 (Berg & Curtiss, 1967)
RE603	as χ474, but <i>Δ(recA-srlR)306 srlR301::Tn10-84</i>	χ474 mated with RE506, selecting Tc ^r
χ478	as χ462, but <i>proC32</i>	J. D. Friesen ^b (Berg & Curtiss, 1967)
RE604	as χ478, but <i>Δ(recA-srlR)306 srlR301::Tn10-84</i>	χ478 mated with RE506, selecting Tc ^r
CV512	F ⁺ / <i>leuA371</i>	CGSC 5539 (Somers <i>et al.</i> , 1973)
RE623	as RE521, but <i>ara⁺ leuA371</i>	See text
CV522	F ⁺ / <i>leuC222</i>	CGSC 5543 (Somers <i>et al.</i> , 1973)
RE799	as RE521, but <i>ara⁺ leuC222</i>	See text
CV526	F ⁺ / <i>leuD101</i>	CGSC 5545 (Somers <i>et al.</i> , 1973)
RE625	as RE521, but <i>ara⁺ leuD101</i>	See text
CSR603	<i>thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44</i>	J. D. Friesen ^b (Sancar & Rupert, 1978)

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extraction of inhibitors of restriction enzymes. Cells were washed off plates in 0.03 M-Tris/HCl, 0.01 M-EDTA and 0.1 M-NaCl, pH 8.2, sedimented and resuspended in 9 ml of the same buffer per plate. Lysozyme was added to 280 µg ml⁻¹ and cells were digested at 37 °C for several hours. Pronase was added 1 mg ml⁻¹, RNAase to 100 µg ml⁻¹ and SDS to 0.055%, and digestion continued overnight at 37 °C. RNAase was then added to 160 µg ml⁻¹ and digestion continued at 37 °C for 90 min. Pronase (35 µg ml⁻¹) was added and digestion continued for 90 min at 37 °C. After addition of SDS to 1%, the DNA was extracted twice with phenol and twice more with chloroform. The purified DNA was precipitated with ethanol. The DNA was digested with limiting amounts of restriction endonuclease *Eco*RI to produce fragments with an apparent average size on agarose gels of 2 kb. Plasmid pUC9 (Vieira & Messing, 1982) was digested to completion with *Eco*RI and digested with calf intestine alkaline phosphatase. The two were ligated together in a 10 µl mixture with cut and dephosphorylated pUC9 (56 µg ml⁻¹) and digested *Brucella* DNA (either 60 or 120 µg ml⁻¹). After incubation at 15 °C overnight, the mixture was

transformed (Hanahan, 1983) into *E. coli* strain RE484. Transformants were plated on LM plates containing 50 µg ampicillin ml⁻¹, 33 µg 5-bromo-4-chloro-3-indolyl β-D-galactoside ml⁻¹ and 33 µM-isopropyl β-D-thiogalactoside. A total of 1800 colonies was plated, of which two-thirds had inserts. Plates were scraped and plasmids isolated by the alkaline-SDS method (Ish-Horowitz & Burke, 1981).

Enzyme assays. γ-Glutamylphosphate reductase was assayed by the procedure of Hayzer & Leisinger (1980), after separation from L-pyrroline-5-carboxylate reductase on DEAE-cellulose. Cells were grown overnight on LB, harvested, washed in 50 mM-Tris/HCl, pH 7.2 containing 1 mM-DDT, then resuspended in 1/100 volume of the same buffer. This concentrated cell suspension was sonicated for 60 s in 15 s bursts with 30 s cooling periods. Cell debris and unbroken cells were removed by centrifugation at 27000 g for 15 min. DL-1-Pyrroline-5-carboxylate was synthesized by the method of Williams & Frank (1975). β-Isopropylmalate dehydrogenase was assayed by the method of Searles & Calvo (1988), except that sonic extracts of strains were

used. Cells were grown on minimal medium containing leucine to a Klett reading of 100 (red filter), then washed and resuspended in fresh minimal medium lacking leucine. They were starved with shaking at 37 °C for 2 h, then harvested, washed and resuspended in 0.075 vols 50 mM-potassium phosphate buffer, pH 7.4. The cells were sonicated and centrifuged as above. β -Isopropylmalate was a generous gift from J. M. Calvo. Protein was determined by the dye binding assay of Bradford (1976), using bovine serum albumin as standard.

Subcloning and deletion construction. Plasmids were digested with restriction enzymes to generate the desired fragments and these were separated on low melting point agarose (Sea-Plaque, FMC) gels. The fragments were identified by fluorescence and cut out. If used, a vector was similarly cut and electrophoresed and excised. Ligation was done in melted agarose (Murray, 1986). The constructions were confirmed by restriction mapping. Phenotypes were determined by growth on minimal media after transformation into the appropriate strains.

Deletions were constructed in pBluescript subclones by digestion from either end of the insert using exonuclease III and mung bean nuclease (Heinrich, 1988). The 3' overhang was generated using *Apal* or *SacI* depending on which end was to be deleted. The 5' overhang was generated with the cloning enzyme, if possible, or any other in the multi-cloning site in the appropriate location. After ligation, the plasmids were transformed into RE521. The extent of the deletion was determined by digestion with *PvuII*, then more accurately with *BglI*. Phenotypes were determined by growth on appropriately supplemented minimal media.

Genomic digests and hybridization to cloned genes. *B. abortus* strain 2308 cells were encapsulated in agarose (InCert, FMC) beads (Jackson & Cook, 1985) and digested to free the chromosomal DNA (Smith & Cantor, 1987). The cells from one plate were encapsulated in approximately 1 ml of beads. The encapsulated DNA was digested in a sample of beads with restriction endonucleases *XhoI*, *XbaI* or both after soaking the beads in the recommended buffer three times for 15 min (200 μ l beads were digested with 50 units enzyme overnight at 37 °C). DNA bands were separated by field inversion gel electrophoresis (Carle *et al.*, 1986) at 12 °C in 7.5 $\times$ 15 cm 1% agarose gels at 4.6 V cm⁻¹ using reversal times increasing linearly from 1.5 s forward/0.5 s reverse to 21 s forward/7 s reverse over 18 h. Bands were visualized after staining in 0.5 μ g ethidium bromide ml⁻¹. A bacteriophage λ ladder for molecular mass calibration was made by encapsulating a high-titre lysate ($\geq 10^{12}$ p.f.u. ml⁻¹) of λ cl1857Sam7 in agarose beads and digesting with 50 μ g proteinase K ml⁻¹ in 0.5 M-EDTA, pH 8.0, 1% *N*-lauroylsarcosine. In other experiments, *Brucella* DNA was digested with restriction enzymes and run on 0.8% agarose under normal constant voltage conditions, then transferred and hybridized as described.

The DNA was transferred to nylon (Nytran; Schleicher and Schuell) in base (Chomczynski & Qasba, 1984) and hybridized to plasmid clones labelled with biotin using a photoactivated reagent (Sigma) (Forster *et al.*, 1985) in 50% formamide, 6 $\times$ SSPE (1 $\times$ SSPE is 0.18 M-NaCl, 10 mM-sodium phosphate, pH 7.7, 1 mM-EDTA) at 42 °C overnight. The blot was washed with a final high stringency wash in 0.16 $\times$ SSPE at 65 °C twice for 15 min. The location of hybridizing bands was detected with the BlueGene detection kit (BRL).

Identification of gene products in maxicells. Plasmids were transformed into strain CSR603, and proteins expressed after UV irradiation labelled with a ³⁵S protein labelling mix (Expre³⁵S³⁵S, New England Nuclear) (Sancar *et al.*, 1981). Proteins were separated by one-dimensional SDS-PAGE on 12% gels (Laemmli, 1970) or by two-dimensional gel electrophoresis (Ames & Nikaido, 1976). One-dimensional gels were loaded with equal amounts of radioactivity in each lane. Gels were soaked in Autofluor (National Diagnostics) and exposed to Kodak XAR-5 film at -70 °C.

Results

Isolation of clones complementing amino acid biosynthesis deficiencies

A library of *B. abortus* strain 19 genomic DNA was transformed into *E. coli* strain RE521, which has several deficiencies in amino acid and nucleotide biosynthesis. Clones able to grow in the absence of proline or leucine arose with a frequency of 5/8500, while 0/8500 could grow without adenine or lysine. On rough restriction mapping the proline-complementing clones appeared to be the same, as did the leucine-complementing ones. One *proA*-complementing clone was chosen and numbered pRE63, and one *leuB*-complementing clone was numbered pRE64. These clones could complement the deficiencies in strain JC10289, but, curiously, not the identical alleles in strain JC7623. Assay demonstrated that pRE63 restored activity for γ -glutamylphosphate reductase (EC 1.2.1.41) in strain RE521 (Table 2) and that pRE64 restored activity for β -isopropylmalate dehydrogenase (EC 1.1.1.85, Table 3).

These clones were tested to see if they carried any other genes in the pathway. pRE63 failed to complement the *proB* strain RE603 or *proC* strain RE604. pRE64 failed to complement *leuA* strain RE623, *leuC* strain RE799 or *leuD* strain RE625.

Table 2. γ -Glutamylphosphate reductase activity in *E. coli* RE521 carrying *Brucella* clones

Values are means $\pm$ SE.

Plasmid	Activity [μ mol min ⁻¹ (mg protein) ⁻¹]
pBluescript	-0.08 $\pm$ 0.02 (<i>n</i> = 3)
pRE63	0.97 $\pm$ 0.18 (<i>n</i> = 4)
pRE93	0.73 $\pm$ 0.05 (<i>n</i> = 4)
W3110*	0.79 $\pm$ 0.11 (<i>n</i> = 4)

* Wild-type strain W3110 carrying no plasmid.

Table 3. β -Isopropylmalate dehydrogenase activity in *E. coli* RE521 carrying *Brucella* clones

Values are means $\pm$ SE.

Plasmid	Activity [nmol min ⁻¹ (mg protein) ⁻¹]
pBluescript	1.9 $\pm$ 0.2 (<i>n</i> = 8)
pRE64	58.0 $\pm$ 2.3 (<i>n</i> = 8)
pRE89	22.3 $\pm$ 0.3 (<i>n</i> = 8)
W3110*	58.6 $\pm$ 1.6 (<i>n</i> = 8)

* Wild-type strain W3110 carrying no plasmid.

Fig. 1. Map of pRE63, subclones and deletions. The top line is a restriction map of the *Brucella* DNA insert of pRE63, showing sites for cleavage by *AffIII* (A), *ClaI* (C), *EcoRI* (E), *BglII* (G), *HindIII* (H), *HincII* (I) and *PvuII* (V). Next is shown the extent of the subclones, then the extents of the ExoIII-generated deletions of pRE93. The ProA phenotype conferred on strain RE521 is indicated for each subclone or deletion. The hatched box shows the inferred extent of the *Brucella proA* gene.

Mapping and characterization of genes

The clones were mapped with restriction enzymes as shown in Fig. 1 for pRE63 and Fig. 2 for pRE64. Various fragments were isolated and recloned to localize the genes on these plasmids. From Fig. 1, one can see that the *proA*-complementing region of pRE63 was on a 3300 bp *EcoRI*-*HindIII* fragment (subcloned as pRE93). This fragment could complement the *proA* mutation in both orientations with respect to the vector. It also restored γ -glutamylphosphate reductase activity to strain RE521. The complementing region was further localized by making deletions from each end. pRE127, with 1390 bp deleted from the *EcoRI* site, was still Pro⁺, but pRE135, with 1590 bp deleted, was not. All deletions from the *HindIII* end were Pro⁻, except the 700 bp deletion of pRE159. The *leuB*-complementing activity of pRE64 was found on the 2020 bp *HindIII*-*NheI* fragment (Fig. 2). This fragment likewise could complement the *leuB* mutation in either orientation and restore enzyme activity to strain RE521. Deletions of 590 bp, but not 1140 bp from the *HindIII* site retained it. All deletions from the *NheI* end were Leu⁻.

B. abortus DNA was encapsulated in agarose, digested with *XhoI* and *XbaI* and separated by field-inversion gel electrophoresis. Southern blots localized DNA found in pRE63 and 64 to single bands in each single digest and the double digest. In other experiments, these plasmids hybridized with single *EcoRI* fragments of the expected size.

Fig. 2. Map of pRE64, subclones and deletions. The top line is a restriction map of the *Brucella* DNA insert in pRE64, showing sites for cleavage by enzymes as given in Fig. 1 except *BamHI* (B), *NheI* (N), *PvuI* (V) and *RsaI* (R). The extent of subclones and ExoIII-generated deletions of pRE89 are indicated below. The LeuB phenotype conferred on strain RE521 is indicated. The hatched box shows the inferred extent of the *Brucella leuB* gene.

Fig. 3. One-dimensional SDS-PAGE of proteins labelled in maxicells containing pRE93 and various deletions. The positions of the prestained protein standards and their apparent molecular masses are indicated. Plasmids were pRE93 (lanes A and I), pRE158 (B and G), pRE159 (C), pRE160 (D), pRE127 (E), pBluescript (F) and pRE135 (H).

Fig. 4. Two-dimensional gel electrophoresis of proteins labelled in maxicells containing pRE93 (a), pRE89 (b) and pBluescript (c). The positions and apparent molecular masses of the prestained protein standards are shown. The position and pI of the first, sixth, eleventh, sixteenth and eighteenth [for (a) and (c) only] carbamylated carbonic anhydrase spots are indicated at the bottom of each gel.

Fig. 5. One-dimensional SDS-PAGE of proteins labelled in maxicells containing pRE89 and various deletions. The positions of the prestained protein standards and their apparent molecular masses are indicated. Plasmids used were pBluescript (lane A), pRE89 (B), pRE126 (C), pRE131 (D) and pRE138 (E).

Identification of gene products

In maxicells, *proA* subclone pRE93 directed the synthesis of β -lactamase and another protein at 48 000 Da (Fig. 3). On two-dimensional gels, this protein had an isoelectric point similar to that of β -lactamase (Fig. 4). In deletion strains, the 48 000 Da protein was present in those strains that were Pro^+ and not in those that were Pro^- . The *leuB* subclone pRE89 gave a protein of 46 000 Da in addition to β -lactamase on one-dimensional gels (Fig. 5). This protein was much fainter than β -lactamase. On two-dimensional gels, the 46 000 Da protein was neutral, like β -lactamase. In deletions, the presence of the 46 000 Da protein was correlated with the ability of the clone to complement *leuB*.

Discussion

A few genes have been cloned previously from *Brucella abortus*. These include genes for proteins expected to be

antigens, such as the porin in the outer membrane (Ficht *et al.*, 1988) and proteins released by high salt wash (Mayfield *et al.*, 1988). One enzyme, superoxide dismutase, has also been cloned (Bricker *et al.*, 1990), but its identity was discovered by sequencing. The present report is the first to describe the cloning of *Brucella* genes by functional complementation.

The *proA* and *leuB* genes have been isolated from a number of bacteria, largely because the commonly used cloning strain HB101 carries these lesions. In most cases, only the cloning was noted, but a few of the genes have been studied in some detail. DNA sequences have been obtained for *proA* from *Serratia marcescens* (Omori *et al.*, 1991) and *E. coli* (Deutch *et al.*, 1984), and for *leuB* from several organisms (Sekiguchi *et al.*, 1986, 1987; Imai *et al.* 1987; Kirino & Oshima, 1991). We have isolated *B. abortus* DNA clones that restore enzyme activity for γ -glutamylphosphate reductase and β -isopropylmalate dehydrogenase in *E. coli* strains deficient in these enzymes. These enzymes are expressed from *Brucella* promoters at a rate sufficient to give activity similar to that of the *E. coli* enzyme in wild-type cells. These clones direct the synthesis of proteins of 48 000 Da for *proA* and 46 000 Da for *leuB*. The gene is about 1300 bp for *proA*, which is what would be expected for a protein of the size seen. The protein is similar in size to the subunit molecular mass of 42 000 Da seen for the *E. coli* enzyme (Hayzer & Leisinger, 1982) or 44 000 Da for the *Serratia* enzyme (Omori *et al.*, 1991). For *leuB*, the gene is 1400 bp, which could code for a protein of 51 000 Da, more than the 46 000 Da observed. This is somewhat larger than the subunit molecular mass of 35 000 Da reported for *E. coli* (Parsons & Burns, 1969) or 40 000 Da for *B. coagulans* (Sekiguchi *et al.*, 1986).

In *E. coli* and *Salmonella typhimurium*, the leucine genes are arranged in a single operon (Umbarger, 1987). We found no evidence of any gene but *leuB* on our clone, though it extends far enough in both directions that one would expect to detect other genes if they were present. This suggests the arrangement of genes in *Brucella* is different from that in *E. coli*. Likewise, *proA* and *B* are adjacent in *E. coli* and *S. typhimurium* (Leisinger, 1987) and in *S. marcescens* (Omori *et al.*, 1991), but again, there is no evidence for *proB* on our *proA* clone.

The presence of these enzymes suggests that *B. abortus*, like most bacteria, has the isopropylmalate pathway for leucine biosynthesis (Stieglitz & Calvo, 1974). Likewise, it would appear to possess the γ -glutamylphosphate pathway for proline biosynthesis (Csonka & Baich, 1983). Since *B. abortus* strain 19 has these two enzymes, it seems very likely that it can synthesize proline and leucine. It will be interesting to determine what controls operate in these pathways in this intracellular pathogen.

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