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 μg 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 *phaA8* deletion from strain JF4281 by cotransduction with the transposon from strain χ2844. 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 χ462 in which the *leuB2* lesion was replaced by wild-type by transduction, leaving the strain *aro-14*. The *leuA, C* and *D* lesions from strains CV312, 522 and 526, respectively, were transduced in by selecting for *Ara*⁺. Finally, the *recA* deletion was put in by mating with strain RES06 (RE799) or RES07 (RE623 and 625), selecting for tetracycline resistance.

*General procedures.* Cells were transformed by the PEG-DMO 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
Table 1. *E. coli* strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference*</th>
</tr>
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<tbody>
<tr>
<td>JM107</td>
<td>F′traD36 proAB′lacPZAM15(lac−pro) thi gyrA96 endA1 hisDR17 relA1 supE44</td>
<td>C. O. Gardner, Jr* (Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td>JF4281</td>
<td>HfrC ΔphaA8 faDl701 relA1 pit-10 spoT1 tonA22 ompF627 Δ</td>
<td>J. D. Friesen† (Hayashi et al., 1964)</td>
</tr>
<tr>
<td>χ2844</td>
<td>tsex-462::Tn10, λ</td>
<td>GCSC 6683</td>
</tr>
<tr>
<td>WL66</td>
<td>hsdR recB21C22 supE supF metB trpR</td>
<td>Amersham</td>
</tr>
<tr>
<td>NK526</td>
<td>hisG215::Tn10 IN(rrnD−rrnE11)</td>
<td>CGSC 6416</td>
</tr>
<tr>
<td>RE484</td>
<td>F′ traD36 proAB′lacPZAM15(lac−pro) thi gyrA96 endA1 hisDR17 relA1 supE44 ΔphaA8 recBC sbeB15</td>
<td>See text</td>
</tr>
<tr>
<td>JC7623</td>
<td>thr-1 ara-14 leuB6 Δgpt−proA62 lacY1 tss-33 supE44 galK2 rac sbeB15 hisG4, rfbD1 recB21 recC22 pkoL31 kgdk51 xyl-5 mtl-1 argE3 thi-1 thr-1 metE70 lysA23 trpE38 purE42 proA leuB6 mtl-1 xyl-5 ara-14 lacZ66 ari-6 rpsL109 tonA23</td>
<td>M. C. Jones-Mortimer† (Berg &amp; Curtiss, 1967)</td>
</tr>
<tr>
<td>χ462</td>
<td>as χ462, but Δ(recA−srlR)306 srlR301::Tn10−84</td>
<td>CGSC 5188 (Kushner et al., 1971)</td>
</tr>
<tr>
<td>χ474</td>
<td>as χ462, but pro91</td>
<td>CGSC 5395 (Broda et al., 1972)</td>
</tr>
<tr>
<td>RE603</td>
<td>as χ474, but Δ(recA−srlR)306 srlR301::Tn10−84</td>
<td>R. D. Porter* (Csonka &amp; Clark, 1979)</td>
</tr>
<tr>
<td>χ478</td>
<td>as χ462, but proC32</td>
<td>CGSC 6452 (Berg &amp; Curtiss, 1967)</td>
</tr>
<tr>
<td>RE604</td>
<td>as χ478, but Δ(recA−srlR)306 srlR301::Tn10−84</td>
<td>χ478 mated with RE506, selecting Te*</td>
</tr>
<tr>
<td>RE623</td>
<td>as RE521, but ara* leuA371</td>
<td>CGSC 5539 (Somers et al., 1973)</td>
</tr>
<tr>
<td>RE622</td>
<td>as RE522, but ara* leuA371</td>
<td>See text</td>
</tr>
<tr>
<td>RE609</td>
<td>as RE521, but ara* leuA371</td>
<td>CGSC 5543 (Somers et al., 1973)</td>
</tr>
<tr>
<td>RE625</td>
<td>as RE521, but ara* leuA371</td>
<td>See text</td>
</tr>
<tr>
<td>CSR603</td>
<td>thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uraA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tss-33 supE44</td>
<td>J. D. Friesen* (Sancar &amp; Rupert, 1978)</td>
</tr>
</tbody>
</table>

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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 0.05%, 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 limited amounts of restriction endonuclease EcoRI to produce fragments with an apparent average size on agarose gels of 2 kb. Plasmid pUC9 (Vieira & Messing, 1982) was digested to completion with EcoRI 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-Horowicz & Burke, 1981).

**Enzyme assays.** γ-Glutamylphosphate reductase was assayed by the procedure of Hayzer & Leisinger (1980). After separation from L-proline-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-DTT, 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-Proline-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 5' overhang was generated using Apal or SacI depending on which end was to be deleted. The 3' 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 PstI, then more accurately with BglII. 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 µl 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 × 15 cm 1% agarose gels at 46 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 µg ethidium bromide ml⁻¹. A bacteriophage λ ladder for molecular mass calibration was made by encapsulating a high-titre lysate (≥ 10¹⁰ p.f.u. ml⁻¹) of λcl857Sam7 in agarose beads and digesting with 50 µg 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; Scheicher 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 × SSPE (1 × 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 x 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 CSRE603, and proteins expressed after UV irradiation labelled with a 35S protein labelling mix (Expre³⁵⁵, 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Activity [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript</td>
<td>0.08 ± 0.02 (n = 3)</td>
</tr>
<tr>
<td>pRE63</td>
<td>0.97 ± 0.18 (n = 4)</td>
</tr>
<tr>
<td>pRE93</td>
<td>0.73 ± 0.05 (n = 4)</td>
</tr>
<tr>
<td>W3110*</td>
<td>0.79 ± 0.11 (n = 4)</td>
</tr>
</tbody>
</table>

* Wild-type strain W3110 carrying no plasmid.

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Activity [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript</td>
<td>1.9 ± 0.2 (n = 8)</td>
</tr>
<tr>
<td>pRE64</td>
<td>5.80 ± 2.3 (n = 8)</td>
</tr>
<tr>
<td>pRE93</td>
<td>22.3 ± 0.3 (n = 8)</td>
</tr>
<tr>
<td>W3110*</td>
<td>58.6 ± 1.6 (n = 8)</td>
</tr>
</tbody>
</table>

* Wild-type strain W3110 carrying no plasmid.
**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.

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**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 *AflIII* (A), *CiaI* (C), *EcoRI* (E), *BglI* (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.

**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 *Rsal* (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.
Identification of gene products

In maxicells, proA subclone pRE93 directed the synthesis of β-lactamase and another protein at 48000 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 48000 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 46000 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 46000 Da protein was neutral, like β-lactamase. In deletions, the presence of the 46000 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 and A 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 (Stieglietz & 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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References


