Identification and sequencing of the *groE* operon and flanking genes of *Lawsonia intracellularis*: use in phylogeny

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Proliferative enteropathy (PE) is a complex of diseases of commercial importance to the pig industry. The obligate intracellular bacterium *Lawsonia intracellularis* is consistently associated with PE and pure cultures of this bacterium have been used to reproduce PE in pigs. In this study *L. intracellularis* bacteria were purified directly from PE-affected tissue. DNA extracted from purified bacteria was used to construct a partial genomic library which was screened using sera from *L. intracellularis*-immunized rabbits. Two seroreactive recombinant clones were identified, one of which expressed proteins of 10 and 60 kDa. The sequence of the insert from this clone, pISI-2, revealed ORFs with sequence similarity to the *groES/EL* operon of *Escherichia coli*, the 50S ribosomal proteins L21 and L27 of *E. coli*, a GTP-binding protein of *Bacillus subtilis* and a possible protoporphyrinogen oxidase, HemK, of *E. coli*. Primers designed from unique sequences from the pISI-2 insert amplified DNA from infected, but not non-infected, porcine ilea; the amplicon sequence obtained from tissue-cultured *L. intracellularis* was identical to the corresponding sequence in pISI-2, confirming the origin of the clone. The sequence of *L. intracellularis* GroEL and other GroEL sequences in the databases were used to construct a partial phylogenetic tree. Analysis of the GroEL sequence relationship suggested that *L. intracellularis* is not significantly related to other organisms whose GroEL sequences are held in the databases and supports previous data from 16S sequence analyses suggesting that *L. intracellularis* is a member of a novel group of enteric pathogens.

Keywords: proliferative enteropathy, *Lawsonia intracellularis*, *groE* operon, heat-shock protein

INTRODUCTION

Proliferative enteropathy (PE) is a commercially important disease in the pig industry, with the potential to affect large numbers of pigs, particularly those reared intensively. The disease is characterized by proliferation of intestinal mucosa enterocytes leading to gross thickening of the ileal wall (Rowland & Lawson, 1974). This

pathology is accompanied by a marked reduction in weight gain by affected pigs. Severely affected pigs scour and sudden death is not uncommon.

A consistent feature of PE is the presence of curved Gram-negative bacilli within the cytoplasm of immature crypt epithelial cells of the ileum (Rowland & Lawson, 1974). These intracellular bacteria have not yet been cultured in cell-free media but can be maintained in a rat enterocyte cell line (McOrist *et al.*, 1990; Lawson *et al.*, 1993). Bacterial isolates grown *in vitro* have been used to reproduce PE in pigs (McOrist *et al.*, 1993) and hamsters (Jasni *et al.*, 1994). Other bacteria may have a role in the establishment of PE, as attempts at reproducing the disease in gnotobiotic pigs using a pure bacterial inoculum have failed (McOrist *et al.*, 1993, 1994).

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Abbreviation: PE, proliferative enteropathy.

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However, the oxygen tension and crypt architecture in the intestines of gnotobiotic pigs may be responsible for this effect (McOrist *et al.*, 1993, 1994).

The taxonomy of the bacterium and the relationship between the organism and the pathology of PE have not been fully resolved. Previous studies of this intracellular bacterium isolated from lesions in pigs indicate that it belongs to a taxonomic group distinct from all known bacteria (McOrist *et al.*, 1990; Gebhart *et al.*, 1993). Based on classification similarities, this organism was formerly known as *Campylobacter*-like organism, but was given the vernacular name 'ileal symbiont intracellularis' until further clarification of its taxonomic position (Gebhart *et al.*, 1993). Analysis of the 16S rRNA sequence from 'ileal symbiont intracellularis' has revealed the highest sequence similarity, 91% (Gebhart *et al.*, 1993; McOrist *et al.*, 1995), to that of *Desulfovibrio desulfuricans* and greater than 86% sequence similarity to 16S rRNA sequences of all members of the family *Desulfovibrionaceae* (Gebhart *et al.*, 1993). Similar results have been found by Fox *et al.* (1994). However, physiological classification characteristics of *D. desulfuricans* indicate that this species is considerably different to 'ileal symbiont intracellularis' (Gebhart *et al.*, 1993; Fox *et al.*, 1994; McOrist *et al.*, 1995). McOrist *et al.* (1995) have suggested that 'ileal symbiont intracellularis' belongs to a new genus and have renamed this bacterium *Lawsonia intracellularis*. The identification of genes from *L. intracellularis* may provide a further insight into classification of this bacterium and its relationship with PE.

Although there are reports from research groups successfully co-culturing *L. intracellularis*, in our experience co-culture of this bacterium in rat enterocytes does not produce large numbers and alternative methods are being sought to generate material for taxonomic, diagnostic and pathogenesis studies. The diagnosis of disease caused by newly identified pathogens which are difficult to culture, such as *L. intracellularis*, requires the generation of reagents which may include recombinant proteins for serological investigations and/or unique DNA sequences for use in PCR or DNA probe-based assays. Characterization of the gene products from *L. intracellularis* may also assist studies to identify the mechanisms that influence the progression of infection. In this study, we have constructed a partial library of *L. intracellularis* and have begun analysing *L. intracellularis* gene sequences. We report on several previously unidentified gene sequences from this novel pathogen.

METHODS

Pig ilea samples. Sections of grossly thickened pig ilea were collected post-mortem and serious generalized lesions associated with PE confirmed by histopathology. The presence of *L. intracellularis* in the ilea was confirmed using modified acid-fast Ziehl-Neelsen staining and immunofluorescence staining using specific mAbs from Dr Steve McOrist (University of Edinburgh, UK) as described by McOrist *et al.*

(1987). Ileal samples from five pigs not infected with the disease were obtained from a quarantined piggery which has had only isolated cases of PE (Victorian Institute of Animal Science, Werribee, Australia).

Isolation of *L. intracellularis* from infected porcine ilea. Infected ilea were collected from pigs and stored at -80°C . Ileal samples were thawed and *L. intracellularis* bacteria were isolated from mucosal scrapings taken from the infected intestinal wall. Infected mucosa were homogenized repeatedly with PBS in an omnimixer (Sorvall) to release the intracellular bacteria and contaminating cellular debris was removed by low-speed centrifugation steps as described by Holyoake *et al.* (1994). The supernatant obtained was filtered through 3, 1.2 and 0.8 μm filters (Millipore) and the filtrate centrifuged at 8000 g for 30 min, resulting in a small pellet of *L. intracellularis* bacteria. The bacteria were further purified using a Percoll (Pharmacia) gradient as described by Holyoake *et al.* (1994). The purity of the isolated bacteria (>95%) was assessed by reacting a portion with mAbs to *L. intracellularis* (McOrist *et al.*, 1987) and by phase-contrast microscopy to reveal any contaminating bacteria or gut debris present.

Cell line. *L. intracellularis* bacteria purified from the ilea of infected pigs were co-cultured with the rat ileum enterocyte cell line IEC 18 and kindly supplied by Alison Collins (Department of Agriculture, Bendigo, Australia). The isolates used and the conditions for growth and maintenance of this co-culture are described by Collins *et al.* (1996).

Isolation of genomic DNA. *L. intracellularis* genomic DNA was isolated from Percoll-gradient-purified bacteria using the method described by Anderson *et al.* (1984). This DNA preparation was used to construct a genomic library (as described below). For use in PCR, DNA was isolated from PBS-washed mucosa taken from normal and proliferated pig ilea. Co-cultured *L. intracellularis* within the IEC rat enterocyte cell line was boiled for 10 min with 0.3% SDS followed by phenol/chloroform extraction and ethanol precipitation of DNA before use in PCR.

Antisera. Antisera to *L. intracellularis* were raised in rabbits and pigs. Rabbits were injected intramuscularly with a preparation of Percoll-gradient-purified *L. intracellularis* bacteria mixed at a ratio of 1:1 with a double-emulsion made by processing with oil adjuvant (Freund's incomplete adjuvant) and then with an equal volume of Tween 80 enhancer. Two 3 ml injections, containing approximately 9 mg protein, were given 4 weeks apart. Blood samples were collected from the ear vein prior to immunization and 2 weeks following the second injection. A 6-week-old pig was hyperimmunized by intramuscular injection of Percoll-gradient-purified *L. intracellularis* bacteria prepared with Freund's incomplete adjuvant. Three injections of the prepared antigen were administered 4 weeks apart and blood was collected from the anterior vena cava 2 weeks following the final injection.

Library construction and immunological screening. *L. intracellularis* DNA was digested with *EcoRI* (Promega) and then ligated to *EcoRI*-digested and calf intestinal phosphatase (Promega)-treated λ ZAP II (Stratagene), and packaged using Gigapack packaging extracts (Stratagene) according to the manufacturer's recommendations. Phage-infected *E. coli* XL-1 Blue (Stratagene) was grown overnight on Luria-Bertani (LB) agar at a density of 2000 plaque-forming units (p.f.u.) per 150 mm plate and phage were transferred to nitrocellulose filters (Hybond C; Amersham). Transferred phage were denatured and prepared for immunohybridization as described by Moses *et al.* (1989). Filters were screened using the

rabbit antiserum (1:100 dilution in 1% gelatin/PBS). Horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Silenus; 1:3000 dilution in 1% gelatin/PBS) was used to detect seroreactive plaques followed by colour development in a solution of 0.05% 4-chloro-1-naphthol (Sigma)/0.015% H_2O_2 in a 5:1 solution of Tris-buffered-saline/methanol. Positive plaques identified in a primary screen were picked, replated at a lower density and rescreened until individual positive plaques were identified. Phagemid clones were excised according to the manufacturer's protocols (Stratagene).

SDS-PAGE. *E. coli* transformed with pBluescript SK(–) (Stratagene) and clones pISI-1 and pISI-2 were grown to stationary phase in 10 ml LB/ampicillin broth. The cell pellets from 0.2 ml aliquots of culture were resuspended in 50 µl sample buffer (62.5 mM HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, pH 6.8) and heated to 95 °C for 5 min before separating solubilized proteins electrophoretically on a 12% SDS-PAGE vertical slab gel (Laemmli, 1970). Proteins were visualized by staining with Coomassie blue R250.

Western immunoblotting. Proteins were electrophoretically transferred to Immobilon-P PVDF membrane (Millipore) in a buffer containing CAPS (pH 11) and 10% methanol as described by Towbin *et al.* (1979). The membranes were blocked with 10% skim milk powder in PBS containing 0.05% Tween-20 (PBST) and then incubated in either rabbit or pig antiserum for 1 h followed by washing twice in 0.1% skim milk powder/PBST. Rabbit antisera were used at a dilution of 1:2000 in 1% skim milk powder, PBST. Diluted pig sera (1:1000) were pre-absorbed with an *E. coli* (DH5α) lysate for 1 h at room temperature prior to adding to the membranes at a final dilution of 1:10000. HRP-conjugated goat anti-rabbit immunoglobulins (Silenus), diluted 1:3000, were used to detect rabbit antibodies. Similarly, HRP-conjugated swine immunoglobulins (DAKO) were applied at a dilution of 1:1000 to detect porcine antibodies. Seroreactive products were detected by Enhanced Chemiluminescence (ECL; Amersham) according to the manufacturer's protocols.

Exonuclease deletions. The plasmid clones pISI-2 and pISI-42, containing identical 5 kb inserts in opposite orientations, were subjected to deletion analysis using the Erase-a-Base Kit and the protocol supplied (Promega). The unique *KpnI* and *SalI* sites in the polylinker of pBluescript were used to provide the overhangs required for exonuclease III digestion. After treatment with these enzymes the linearized DNA was digested with exonuclease at 37 °C and stopped at intervals of 45 s, religated and transformed into *E. coli* DH5α.

Plasmid DNA isolation and analysis. Plasmid DNA was purified from 5 ml *E. coli* DH5α cultures by the modified alkaline lysis, PEG precipitation protocol supplied with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Restriction endonucleases, other enzymes and reaction buffers were from Promega. Methods used for DNA treatments with restriction endonucleases or modification enzymes were from Sambrook *et al.* (1989).

DNA sequencing. Plasmid DNA was isolated and sequenced using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Clones were sequenced using the M13 universal sequencing primer (Pharmacia). Sequence analysis was performed using the Australian National Genomic Information Service (ANGIS) and DNA and protein database searches were performed using the BLAST (Altschul *et*

al., 1990) network services. GCG programs (Wisconsin Package Version 8) were used to produce contiguous sequence and CLUSTAL W (Thompson *et al.*, 1994) was used to align the cloned sequences with similar bacterial sequences currently available in the databases.

PCR. Primers designed to amplify a 720 bp fragment of pISI-2, including sequence of a putative 50S ribosomal protein L21–L27 gene cluster, were ISI50S2F (5' GAAGAAGTC-ATTACACTCC) and ISI50S2R (5' CATTCTAAAGATA-AAGTTCC). Cycling was performed using the GeneAmp PCR System 9600 (Perkin Elmer) with the following conditions: 95 °C for 10 s, 80 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 50 °C for 15 s and 72 °C for 30 s. Each 50 µl reaction contained 200 µM dNTPs, 1× *Tth* reaction buffer, 1 µM each primer and DNA. Two units of *Tth* thermostable polymerase (Toyobo) was added to each of the reaction mixtures held at 80 °C prior to cycling. The PCR product obtained from co-cultured *L. intracellularis* was sequenced directly to confirm the origin of the library clone (pISI-2). Following amplification, salts and nucleotides were removed from the PCR reaction using Wizard DNA clean-up resin (Promega) and the 720 bp amplicon was sequenced using the same primers.

Phylogenetic tree analysis. GroEL protein alignments and evolutionary distances were kindly provided by Dr Alejandro Viale (Universidad Nacional de Rosario, Argentina; Ferreyra *et al.*, 1993; Viale *et al.*, 1994). The Alignment Editor 2 (AE2) program was used to insert the GroEL sequence from *L. intracellularis* and other GroEL sequences submitted recently to GenBank. Following visual inspection, gaps were inserted into sequences to improve the alignment. An aligned column of amino acid residues which included a gap in any one sequence was excluded from the alignment prior to phylogenetic tree construction. This included the last 21 amino acid residues of each sequence in the alignment (numbering according to the *E. coli* GroEL sequence). Comparisons were made from an alignment of 506 predicted amino acids for each entry. The GenBank database accession numbers and sources of GroEL sequences used for this phylogenetic study are given in Table 1.

Phylogenetic trees based on GroEL amino acid sequences were constructed using the PHYLIP programs (J. Felsenstein, University of Washington, USA). The GroEL alignment was bootstrapped 100 times using SEQBOOT. The 100 data sets generated were put into the distance matrix program PROTDIST. Distance matrix data were arranged into a tree file using the neighbour-joining method (NEIGHBOR). A consensus tree of the data set was produced by the program CONSENSE. The phylogenetic tree was finally arranged and edited using TreeTool 2.0 (Mike Maciukenas, University of Illinois, USA). The partial prokaryotic 16S rRNA tree was extracted from the Ribosomal Database Project (RDP, University of Illinois, USA).

RESULTS

Library screening

DNA isolated from Percoll-gradient-purified *L. intracellularis* bacteria was fully digested with *EcoRI* and ligated to *EcoRI*-digested λ ZAP II to produce a partial genomic library. Approximately 2000 plaques were transferred to nitrocellulose and reacted with rabbit antiserum specific for *L. intracellularis*. Plasmids were excised from 18 plaques which were positive in this

Table 1. Sources of GroEL sequences used for sequence alignments and phylogenetic tree

Organism	GenBank accession no.	Reference
<i>Lawsonia intracellularis</i>	U45241	This publication
<i>α-Proteobacteria</i>		
<i>Sinorhizobium meliloti</i>	M94192	Rasunganwa & Gupta (1993)
<i>Agrobacterium tumefaciens</i>	X68263	Segal & Ron (1993)
<i>Rhizobium leguminosarum</i>	L20775	Wallington & Lund (1994)
<i>Brucella abortus</i>	L09273	Gor & Mayfield (1992)
<i>Bartonella bacilliformis</i>	M98257	Unpublished data
<i>Bradyrhizobium japonicum</i>	Z22604	See Viale <i>et al.</i> (1994)
<i>Zymomonas mobilis</i>	L11654	Barbosa <i>et al.</i> (1994)
<i>Ehrlichia chaffeensis</i>	L10917	See Viale <i>et al.</i> (1994)
<i>Cowdria ruminantium</i>	U13638	Lally <i>et al.</i> (1995)
<i>β-Proteobacteria</i>		
<i>Neisseria flavescens</i>	Z22955	Pannekoek <i>et al.</i> (1995)
<i>Neisseria meningitidis</i>	Z22956	Pannekoek <i>et al.</i> (1995)
<i>Neisseria gonorrhoeae</i>	Z23008	Pannekoek <i>et al.</i> (1995)
<i>γ-Proteobacteria</i>		
<i>Chromatium vinosum</i>	M99443	Ferreyra <i>et al.</i> (1993)
<i>Legionella micdadei</i>	X57520	See Ferreyra <i>et al.</i> (1993)
<i>Legionella pneumophila</i>	M31918	See Viale <i>et al.</i> (1994)
<i>Coxiella burnetii</i>	M20482	See Ferreyra <i>et al.</i> (1993)
<i>Salmonella typhi</i>	U01039	Lindler & Hayes (1994)
<i>Escherichia coli</i>	X07850	See Viale <i>et al.</i> (1994)
<i>Yersinia enterocolitica</i>	X68526	Unpublished data
Symbiont of <i>Acyrtosiphon pisum</i>	X61150	See Viale <i>et al.</i> (1994)
<i>Haemophilus ducreyi</i>	M91030	See Viale <i>et al.</i> (1994)
<i>Pseudomonas aeruginosa</i>	M63957	See Viale <i>et al.</i> (1994)
<i>δ/ε-Proteobacteria</i>		
<i>Helicobacter pylori</i>	X73840	See Viale <i>et al.</i> (1994)
<i>Bacteroides</i>		
<i>Porphyromonas gingivalis</i>	D17398	Maeda <i>et al.</i> (1994)
<i>Chlamydiae</i>		
<i>Chlamydia trachomatis</i>	M31739	See Ferreyra <i>et al.</i> (1993)
<i>Chlamydia psittaci</i>	X51404	See Ferreyra <i>et al.</i> (1993)
<i>Chlamydia pneumoniae</i>	M69217	See Viale <i>et al.</i> (1994)
Firmicutes (Gram-positive bacteria)		
(Low G + C content)		
<i>Clostridium perfringens</i>	X62914	See Ferreyra <i>et al.</i> (1993)
<i>Clostridium acetobutylicum</i>	M74572	See Ferreyra <i>et al.</i> (1993)
<i>Bacillus stearothermophilus</i>	L10132	See Viale <i>et al.</i> (1994)
<i>Bacillus subtilis</i>	M81132	See Ferreyra <i>et al.</i> (1993)
<i>Staphylococcus aureus</i>	D14711	Ohta <i>et al.</i> (1993)
<i>Lactococcus lactis</i>	X71132	Kim & Batt (1993)
(High G + C content)		
<i>Streptomyces coelicolor</i>	X75206	Duchene <i>et al.</i> (1994)
<i>Streptomyces albus</i>	M76658	See Viale <i>et al.</i> (1994)
<i>Mycobacterium tuberculosis</i>	M17705	Thole <i>et al.</i> (1987)
<i>Mycobacterium leprae</i>	M14341	See Viale <i>et al.</i> (1994)
<i>Mycobacterium paratuberculosis</i>	X74518	Colston <i>et al.</i> (1994)
<i>Cyanobacteria</i>		
<i>Cyanidium caldarium</i>	X62578	See Viale <i>et al.</i> (1994)
<i>Synechococcus</i> sp.	M58751	See Ferreyra <i>et al.</i> (1993)
<i>Synechocystis</i> sp.	M57517	See Ferreyra <i>et al.</i> (1993)
<i>Spirochaetes</i>		
<i>Treponema pallidum</i>	X54111	See Viale <i>et al.</i> (1994)
<i>Borrelia burgdorferi</i>	X54059	See Viale <i>et al.</i> (1994)
<i>Leptospira interrogans</i>	L14682	See Viale <i>et al.</i> (1994)

Fig. 1. SDS-PAGE analysis of pISI-1 and pISI-2. (a) Coomassie blue R250-stained gel showing the profile of proteins expressed by DH5 α transformed with the vector pBluescript (lane 1), pISI-1 (2) and pISI-2 (3). (b) Western immunoblot screened by antisera raised in rabbits. The antigens of approximately 10 and 60 kDa expressed by pISI-2 in DH5 α are indicated. Low molecular mass standards (lanes M) are rabbit muscle phosphorylase b (94000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), bovine erythrocyte carbonic anhydrase (30000 Da) and bovine α -lactalbumin (14000 Da).

Fig. 2. Restriction map of pISI-2. (a) Exonuclease deletions derived from pISI-2 are indicated as bold lines below the restriction map. (b) SDS-PAGE profile of the nested deletions stained with Coomassie blue R250. Expression of the 60 kDa protein from *L. intracellularis* in DH5 α is not evident when 1.4 kb was deleted, corresponding to deletion derivative Δ ISI-29. Proteins expressed by DH5 α transformed with pBluescript are shown.

assay and produced one of two distinct *Eco*RI restriction patterns. These *E. coli* XL-1 Blue plasmids contained inserts of either 9 or 5 kb. An *E. coli* clone from each class was chosen for further analysis and named pISI-1 (9 kb insert) and pISI-2 (5 kb insert). Based on restriction enzyme digests, a third clone pISI-42, which contained an identical insert to pISI-2 in the opposite orientation, was also chosen for further analysis.

The *E. coli* clones were analysed by SDS-PAGE and Western immunoblotting. Repeated analysis of *E. coli*(pISI-1) did not reveal any novel antigens in the profile produced by either SDS-PAGE or Western immunoblotting (Fig. 1). *E. coli*(pISI-2) expressed antigens of approximately 10 and 60 kDa. These were detected by staining with Coomassie blue R250 (Fig. 1a) and with either the rabbit antisera (Fig. 1b) or sera raised

Fig. 3. Gene arrangement of the 5 kb clone from *L. intracellularis*. The position and relative size of the ORFs are given by the large arrows under the restriction map. The names given to each ORF have been derived from homology searches with sequences submitted to GenBank. ORF1, ORF2 and ORF3 are contiguous as are ORF4, ORF5 and ORF6. Small facing arrows indicate the positions of the primers used to amplify a PCR product which includes the gene cluster with sequence similarity to the 50S ribosomal proteins L21 and L27.

in the hyperimmunized pig (data not shown). The 10 kDa protein was not detected in an *E. coli* control containing pBluescript without an insert. However, there was some cross-reactivity of the antisera with an *E.*

coli protein of approximately 60 kDa (Fig. 1b). *E. coli*(pISI-42), in which the insert orientation was inverted relative to the *lacZ* promoter of pBluescript, also expressed the 60 kDa protein (data not shown).

Identification of the 60 kDa protein encoded by pISI-2

To identify the gene encoding the 60 kDa protein and other gene sequences, a restriction map of the 5 kb insert of pISI-2 was generated (Fig. 2) and the clone was subjected to deletion analysis (Fig. 2a). The deletion derivatives were analysed by SDS-PAGE for expression of the 60 kDa protein (Fig. 2b). Expression of the 60 kDa protein was not detected when 1.4 kb were deleted, corresponding to deletion derivative Δ ISI-29 (Fig. 2). The nucleotide sequences of deletion derivatives Δ ISI-26, Δ ISI-29 and Δ ISI-32 were compared with sequences held by the NCBI database using the BLAST network service (Altschul *et al.*, 1990) and revealed sequence similarity to a gene encoding the molecular chaperonin GroEL of *E. coli* and of many other species. Further sequencing revealed that a homologue of the *groES/EL* operon of *E. coli* was present within the 5 kb insert of pISI-2 (Fig. 3).

Fig. 4. Alignment of the predicted amino acid sequences of the GroEL-like protein from *L. intracellularis* (LI) and *E. coli* (EC), *H. pylori* (HP), the endosymbiont of *A. proteus* known as X-bacteria (XB) and *S. meliloti* (SM). Sequences were extracted from the GenBank database using the appropriate accession numbers listed in Table 1 and were aligned using CLUSTAL W (Thompson *et al.*, 1994). Boxes indicate identical amino acids in all sequences.

Fig. 5. Alignments of predicted amino acid sequences. (a) GroES from *L. intracellularis* (LI) and *B. japonicum* (BJ). (b, c) 50S rRNA proteins L21 (b) and L27 (c) from *L. intracellularis* (LI), *E. coli* (EC) and *B. subtilis* (BS). (d) Partial sequence from the putative GTP-binding protein of *L. intracellularis* (LI) and sequence from the GTP-binding protein of *B. subtilis* (BS) and the ORF of *E. coli* (EC). (e) Possible protoporphyrinogen oxidase from *L. intracellularis* (LI) and *E. coli* (EC). Boxes indicate identical amino acids in all sequences.

The molecular chaperonins GroES and GroEL of *E. coli* have molecular masses of 10.4 and 57.3 kDa, respectively (Hemmingsen *et al.*, 1988). The 60 kDa protein of *L. intracellularis* expressed by the *E. coli*(pISI-2) clone (Fig. 1) is consistent with the predicted size of GroEL. Sequence analysis revealed that the deletion derivative Δ ISI-29, which does not express the 60 kDa protein (Fig. 2), has the predicted termination codon removed from the *groEL*-like sequence. The high level of expression of the 60 kDa protein is halted, presumably due to the continuation of transcription through to some point within pBluescript whereby the accumulation of the 60 kDa protein is not seen on the SDS-PAGE profile. Hence, it is likely that the 60 kDa polypeptide expressed

by *E. coli*(pISI-2) (Fig. 1) is an *L. intracellularis* homologue of *E. coli* GroEL.

Sequence analysis of the 5 kb clone

The exonuclease derivatives of pISI-2 and pISI-42 were sequenced. Six ORFs (Fig. 3) were identified on pISI-2 and compared with sequences held by the NCBI database as described above.

ORF1 (Fig. 3) is 309 nt long with a deduced amino acid sequence homologous to a 50S ribosomal protein, L21, of *E. coli*. It is predicted to encode a peptide of 102 aa with a molecular mass of 11 516 Da. ORF2 has an ATG start codon 9 bp downstream from the putative TAA

Fig. 6. PCR amplicons generated using primers ISI50S2F and ISI50S2R. The amplicons in lane 1 are from tissue-cultured *L. intracellularis* in the IEC rat enterocyte cell line. PCR products were not amplified from the IEC cell line cultured without *L. intracellularis* bacteria (lane 2). Lanes 3–6 show amplicons from DNA extracted from the ilea of four pigs with characteristic lesions of PE. Lanes 7–11 show that a product was not amplified from DNA extracted from the ilea of five pigs which had no symptoms of an infection of *L. intracellularis*. The PCR product shown in lane 12 was amplified from pISI-2 as a positive control.

stop codon of ORF1. ORF2 is 267 nt long and is predicted to encode a peptide of 88 aa with a molecular mass of 9816 Da. The predicted amino acid sequence shares sequence similarity to L27, another 50S ribosomal protein of *E. coli*. The sequences of the ribosomal proteins L21 (GenBank accession no. P02422) and L27 (P02427) of *E. coli* encode polypeptides of 11·6 and 9 kDa, respectively. According to sequence comparisons obtained through the BLAST network server, the highest sequence similarity was found between the *Bacillus subtilis* L21 homologue (X59528) and ORF1, with 57 and 43 % conservation of the nucleotide and predicted amino acid sequences, respectively. Sequence similarity was highest between *E. coli* L27 and ORF2 with 63 % matching nucleotides and 58 % conservation of the predicted amino acid sequence.

Contiguous with ORF1 and ORF2, ORF3 is a partial sequence of 345 bp with an ATG start codon 162 bp downstream from a TAA putative stop codon of ORF2. The deduced amino acid sequence of ORF3, which appears to be incomplete, extending beyond the *Eco*RI cloning site, shares sequence similarity to the 5' region of the gene encoding an essential GTP-binding protein, Ogb, of *B. subtilis* (Trach & Hoch, 1989; Welsh *et al.*, 1994), and to an ORF of *E. coli* (U18997) with matches of 45 and 50 %, respectively. The incomplete ORF encodes 115 residues which share sequence similarity with the 428-aa Ogb protein of *B. subtilis* which has a calculated molecular mass of 47 688 Da.

ORF4, ORF5 and ORF6 are contiguous and in the opposite orientation to ORF1, ORF2 and ORF3 (Fig. 3). ORF4 (306 nt) is predicted to encode a peptide of 101 aa with a molecular mass of 10942 Da. ORF4 is homologous to *E. coli* GroES and to other GroES sequences held in the database. The nucleotide sequence sharing the highest level of similarity to ORF4 (66 %) is the GroES homologue of the facultative intracellular bacterium *Francisella tularensis* (X98853). The predicted amino acid sequence of ORF4 shares the highest level of sequence similarity (60 %) with *Bradyrhizobium japonicum* (P35864) (see Fig. 5).

ORF5 is 1647 nt long, starting with an ATG start codon 147 bp downstream from the TGA stop codon of ORF4. ORF5 is predicted to encode a polypeptide of 548 aa

with a molecular mass of 58602 Da. The predicted amino acid sequence is homologous to that of *E. coli* GroEL. The nucleotide sequence of ORF5 is most similar to that of X-bacteria (68 %), an endosymbiont of *Amoeba proteus* (M86549) and the predicted amino acid sequence is most similar (69 %) to that of *Sinorhizobium meliloti* (M94192).

ORF6 is 882 nt long, starting 126 bp downstream of the TAG stop codon of ORF5. The predicted polypeptide encoded by ORF6 is 293 aa long with a molecular mass of 33 166 Da. Homology searches with sequences held in GenBank revealed similarity with a possible proto-porphyrinogen oxidase, HemK, of *E. coli* (U18555) and with a number of other bacterial species.

An alignment of predicted amino acid sequences encoded by ORF5 and the GroEL sequence of *E. coli* and other bacterial sequences sharing highest sequence similarity, X-bacteria, *S. meliloti* and *Helicobacter pylori*, is shown in Fig. 4. Alignments of predicted amino acid sequences encoded by ORF1, ORF2, ORF3, ORF4 and ORF6 with sequences held within GenBank sharing the highest similarity to each is given in Fig. 5. The sequence of the insert of pISI-2 revealed a G + C content of 34·2 mol %.

PCR and sequence confirmation of the source of the pISI-2 DNA

PCR primers were designed to match regions flanking the L21 and L27 gene sequences (small facing arrows, Fig. 3) and used to amplify sequences present in co-culture-derived *L. intracellularis* (Collins *et al.*, 1996) and from *L. intracellularis* present within infected porcine ilea. The expected 720 bp amplicon was obtained from pre-boiled IEC18 rat enterocytes containing *L. intracellularis* (Fig. 6, lane 1) but not from IEC18 cells alone (Fig. 6, lane 2). Ileum samples from nine pigs, four affected by PE and five histologically normal, were also analysed by PCR. Only DNA extracted from the ilea of pigs affected by PE produced the 720 bp amplicon following PCR (Fig. 6, lanes 3–6). An identical sequence obtained from the amplicon produced from the co-cultured *L. intracellularis* to that

Fig. 7. Phylogenetic tree based on GroEL relationships. The arrangement is based on the distance matrix method using an alignment of predicted amino acid residues. The numbers given at the ends of branches are obtained by the bootstrap procedure from 100 replicated trees, indicating the number of times the tree branched in this order. The phylogenetic tree based on GroEL relationships is shown here aligned to a partial 16S rRNA tree to demonstrate that the bacteria are similarly assigned in both trees. Some of the branches of the 16S rRNA tree have been crossed, keeping the horizontal distances the same, to fit in with the order given by the phylogenetic tree based on GroEL sequences.

of the corresponding sequence within pSI-2 verified that the 5 kb clone was isolated from *L. intracellularis*.

Taxonomic classification of *L. intracellularis* using the predicted amino acid sequence of the GroEL homologue

The unrooted phylogenetic tree based on predicted GroEL amino acid sequences in Fig. 7 (sources of sequences given in Table 1) was produced using the PHYLIP programs PROTDIST, FITCH and CONSENSE. Similar results were obtained when parsimony analysis was used (data not shown). The phylogenetic tree was compared with a tree produced using 16S rRNA sequences. Both trees show that *L. intracellularis* is taxonomically isolated from other bacteria whose sequences are represented. The nearest neighbour to *L. intracellularis*, according to *groEL* sequences held in databases, is *H. pylori*, an intestinal pathogen associated with gastric ulcers. Other close neighbours include the obligate intracellular pathogens of the genus *Chlamydia*.

CLUSTAL W analysis of deduced amino acid sequences of GroEL from *L. intracellularis* and *H. pylori* (Fig. 4) shows dispersed regions of sequence similarity, including strong sequence conservation at the carboxy terminus of the proteins.

DISCUSSION

The diagnosis of PE is usually made retrospectively after animals exhibiting symptoms are slaughtered. The pathogen associated with the disease, *L. intracellularis*, is difficult to culture and no diagnostic reagents, apart from PCR primers (Jones *et al.*, 1993) and a specific mAb (McOrist *et al.*, 1987) have been developed. Our aim was to use recombinant techniques to produce proteins for use in sero-diagnosis of PE and to identify proteins which may be used for further characterization of this recently identified intracellular bacterium.

At present, co-culture of *L. intracellularis* is insufficient to allow construction of a representative genomic library. We used bacteria purified from infected ilea of

pigs to obtain sufficient DNA to construct a partial genomic library. Two seroreactive clones, pISI-1 and pISI-2, were identified when the library was screened with rabbit antisera raised against *L. intracellularis*. Proteins of approximately 10 and 60 kDa were expressed by *E. coli*(pISI-2) as shown by SDS-PAGE and reacted with rabbit (Fig. 1) and pig anti-sera by Western analysis, prompting further analysis of this clone. The 9 kb insert of *E. coli*(pISI-1) has not yet been analysed further as seroreactive proteins were not obvious from Western immunoblots of *E. coli*(pISI-1).

Sequence analysis of pISI-2 revealed that it contained an operon encoding homologues of the *E. coli* chaperonins, GroES and GroEL. Molecular chaperones make up a family of proteins which are essential for the folding and correct assembly of mature polypeptides in all types of organisms (Ellis, 1987). Within this family, the chaperonins GroES and GroEL are highly related by sequence and found in all eubacteria, mitochondria and plastids examined (Zeilstra-Ryalls *et al.*, 1991; Ellis, 1992). Homologues of GroES and GroEL, also known as Hsp10 and Hsp60, have been identified from many different organisms, partly attributed to the recent interest in the clinical and biological significance of these proteins.

Given that the library was constructed from material extracted from the ileum of the pig, we established by PCR and sequence analysis that pISI-2 was derived from *L. intracellularis*. Unique PCR primers designed from sequences external to the 50S ribosomal protein gene cluster on pISI-2 generated PCR amplicons from co-cultured *L. intracellularis* (Collins *et al.*, 1996) and from DNA purified from the ilea of pigs identified with characteristic lesions of PE. The 720 bp PCR amplicon from co-cultured *L. intracellularis* was partially sequenced and was identical to the pISI-2 sequence from which the primers were designed, confirming the origin of the *E. coli*(pISI-2) clone. PCR amplicons were not generated from DNA extracted from the ilea of pigs showing no symptoms of PE, suggesting the primers may be used to identify *L. intracellularis*-infected pig ilea.

Four ORFs were identified on pISI-2 in addition to the *groES/EL* operon. The genes for the 50S ribosomal proteins L21 and L27 of *E. coli* form a gene cluster separated from other ribosomal protein genes (Kitakawa *et al.*, 1979). The same gene arrangement has been described for *B. subtilis* (Dabbs, 1983) and now a similar arrangement is shown in Fig. 3 for *L. intracellularis*. The sequence downstream of this gene cluster in *L. intracellularis* strongly resembles the sequence of the amino terminus of a GTP-binding protein, Obg, of *B. subtilis* (Fig. 4). The gene encoding Obg is present in an operon structure with the sporulation gene *spo0B* and is essential for growth (Kok *et al.*, 1994). The gene for L27 is upstream of the *spo0B-obg* operon of *B. subtilis* (Ferrari *et al.*, 1985; Trach & Hoch, 1989). However, sequence sharing homology with the *spo0B* gene was not present between the ORFs sharing

sequence similarity with L27 and Obg in the *L. intracellularis* clone, pISI-2.

An ORF with sequence similarity to a possible protoporphyrinogen oxidase was found downstream of the *groES/EL* operon within the *L. intracellularis* clone pISI-2. The gene encoding a possible protoporphyrinogen oxidase of *E. coli*, *hemK*, has been described (Nakayashiki *et al.*, 1995). In the final stages of haem synthesis, protoporphyrinogen is oxidized to protoporphyrin by protoporphyrinogen oxidase. The pathway for haem synthesis involves a number of genes which have been mapped and characterized, and is highly conserved among many different species. In *E. coli*, *hemK* forms part of the *hemA-prfA-hemK* operon involved in the biosynthesis of haem (Nakayashiki *et al.*, 1995). However, little is known of the involvement of *hemK* in the synthesis of haem.

The size and highly conserved function of GroEL permit its use as a valuable evolutionary chronometer (Viale *et al.*, 1994). Comparisons of 16S rRNA sequences have provided a simple way to classify and relate organisms (Woese, 1987). Based on 16S rRNA phylogenetic studies, *L. intracellularis* is most closely related to *Desulfovibrio desulfuricans* (McOrist *et al.*, 1995), sharing 91% sequence similarity. However, members of this family of sulfate-reducing bacteria belong to the δ subclass of the *Proteobacteria*, a subclass in which there are no known intracellular pathogens of vertebrate hosts (Woese, 1987; Devereux *et al.*, 1989). The use of more macromolecules demonstrating features of molecular chronometers, such as GroEL, may help resolve evolutionary relationships between prokaryotes (Woese, 1987; Viale *et al.*, 1994) and place organisms which are difficult to classify, such as *L. intracellularis*, with more confidence.

Our taxonomic analysis using predicted GroEL amino acid sequences available in the databases revealed very similar genetic relationships to those inferred by the 16S rRNA sequence database project. That is, the bacterial species clustered into similar divisions and subdivisions as those defined by the phylogenetic arrangement of 16S rRNA sequences and shown in Fig. 7. However, the lack of GroEL sequences from the bacteroides and the δ/ϵ -*Proteobacteria* species in the database means that bootstrap analysis cannot provide enough confidence to assign these affiliations (Viale *et al.*, 1994) which may account for some of the differences between the trees. The phylogenetic tree based on GroEL sequences shows *L. intracellularis* to be genetically isolated from the other bacteria represented. The closest neighbour to *L. intracellularis* within the incomplete tree is *H. pylori* of the δ/ϵ subclass of the *Proteobacteria*, which is consistent with the classification by 16S rRNA relationships. Unfortunately, a comparison between *Desulfovibrio* spp. and *L. intracellularis* could not be made as there are no GroEL sequences from *Desulfovibrio* spp. present in the databases. Similarly, GroEL sequences from *Campylobacter* spp. are not available. Interestingly, the G + C content of the 5 kb insert of *E. coli*(pISI-2) is 34.2 mol%, which is considerably lower than that of members of the *Desulfovibrionaceae*. *D. desulfuricans*

has a G + C content of 59 mol% (Stackebrandt *et al.*, 1995) and few *Desulfovibrio* spp. have a G + C content of less than 50 mol%, with none reported having less than 48 mol% (Stackebrandt *et al.*, 1995).

With the *groE*-operon sequence of *L. intracellularis* now available, it may be possible to identify epitopes specific to *L. intracellularis* that can be produced by recombinant techniques for diagnostic use. Lally *et al.* (1995) have demonstrated the use of species-specific GroEL antibodies produced during infection for sero-diagnosis of a disease caused by the intracellular bacterium *Cowdria ruminantium*, the causative agent of heartwater in ruminants. There have also been a number of reports describing the use of heat-shock proteins as vaccine antigens against intracellular pathogens such as *H. pylori* (Ferrero *et al.*, 1995) and *Mycobacterium tuberculosis* (Lowrie *et al.*, 1994). Despite their conservation amongst the different kingdoms, heat-shock proteins can activate the immune response following natural infective delivery or when given as vaccines. Hsp65, the GroEL homologue of *Mycobacteria*, is a prominent antigen of *M. leprae* and *M. tuberculosis* infections. Hsp65 has been used in vaccine trials against *Mycobacterium* infections and has conferred protective immunity in animal models (Lowrie *et al.*, 1994; Silva & Lowrie, 1994; Silva *et al.*, 1994). Studies are in progress to determine whether the GroEL homologue from *L. intracellularis* can act as a protective vaccine antigen against porcine PE.

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