Rickettsia buchneri sp. nov., a rickettsial endosymbiont of the blacklegged tick *Ixodes scapularis*

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We obtained a rickettsial isolate from the ovaries of the blacklegged tick, *Ixodes scapularis*. The isolate (ISO7^T) was grown in the *Ixodes ricinus* embryonic cell line IRE11. We characterized the isolate by transmission electron microscopy and gene sequencing. Phylogenetic analysis of 11 housekeeping genes demonstrated that the isolate fulfils the criteria to be classified as a representative of a novel rickettsial species closely related to '*Rickettsia monacensis*'. These rickettsiae form a clade separate from other species of rickettsiae. Gene sequences indicated that several genes important in rickettsial motility, invasiveness and temperature adaptation were mutated (e.g. *sca2*, *rickA*, *hsp22*, *pldA* and *htrA*). We propose the name *Rickettsia buchneri* sp. nov. for this bacterium that infects the ovaries of the tick *I. scapularis* to acknowledge the pioneering contributions of Professor Paul Buchner (1886–1978) to research on bacterial symbionts. The type strain of *R. buchneri* sp. nov. is strain ISO-7^T (=DSM 29016^T=ATCC VR-1814^T).

Ticks harbour non-pathogenic endosymbiotic bacteria that are transovarially transmitted (Buchner, 1926; Cowdry, 1925; Noda *et al.*, 1997). In his seminal publications, Paul Buchner (Buchner, 1926, 1965) described bacterial endosymbionts within oocytes of *Ixodes hexagonus*, a tick that parasitizes the European hedgehog. In North America, *Ixodes scapularis*, the blacklegged tick, is an important vector of bacterial pathogens (Munderloh & Kurtti, 2011), but it has not been associated with transmission of pathogenic rickettsiae. Nevertheless, presumably non-pathogenic endosymbiotic rickettsiae are frequently detected in this tick by microscopy (Magnarelli *et al.*, 1991) and PCR assays (Benson *et al.*, 2004; Moreno *et al.*, 2006; Noda *et al.*, 1997; Weller *et al.*, 1998). Several different names have been given to these rickettsiae, i.e. '*Rickettsia cooleyi*' (Billings *et al.*, 1998), '*Rickettsia midichlorii*' (Troughton & Levin, 2007) and the rickettsial endosymbiont of *I. scapularis* (REIS) (Felsheim *et al.*, 2009). Analysis of data generated by PCR-mediated amplification of selected gene sequences indicate that the *I. scapularis* rickettsia is a single species that displays limited sequence divergence indicating it is ancestral to the spotted fever group rickettsiae (Gillespie *et al.*, 2012). A draft genome of REIS obtained from the project to sequence the genome of *I. scapularis* (Gillespie *et al.*, 2012) revealed that REIS was closely related to rickettsiae detected in other *Ixodes* ticks: *Ixodes pacificus* in California (Phan *et al.*, 2011), *Ixodes ricinus* in Europe (Corrain *et al.*, 2012; Madeddu *et al.*, 2012; Schicht *et al.*, 2012; Simser *et al.*, 2002), *Ixodes nipponensis* in Korea (Lee *et al.*, 2013; Shin *et al.*, 2013) and *Ixodes boliviensis* in Central America (Troyo *et al.*, 2013).

We have observed rickettsiae, by light and electron microscopy, which are restricted to the ovaries of field collected and laboratory reared *I. scapularis* females (Munderloh *et al.*, 2005). Here we describe the isolation and cultivation of a rickettsial species from ovaries of *I. scapularis* in a tick cell line. We examined the isolate, ISO7^T (*I. scapularis* ovary from tick 7), by transmission electron microscopy and confirmed that the culture isolate displayed the ultrastructural features typical of rickettsiae. Pulsed field gel electrophoresis (PFGE) (Baldridge *et al.*, 2010) and PCR data of the plasmids matched those described for REIS (Gillespie *et al.*, 2012). In addition, a draft genome sequence confirmed the identity of isolate

Abbreviations: PFGE, pulsed field gel electrophoresis; REIS, rickettsial endosymbiont of *Ixodes scapularis*.

The GenBank/EMBL/DDBJ accession number for the whole genome shotgun sequence of strain ISO7^T is JFKF01000000. Those for the 16S rRNA, 17 kDa, gltA, ompA, ompB, sca2, rickA, hsp22, pldA, htrA, htpG, infB, rpoA, rpoB, thrS, polA, groEL, gyrB, recA, pnp and dnaE gene sequences of strain ISO7T are REISMN_05090, KDO03625, KDO03019, KDO02351, KDO02445, KDO02274 (2 copies), REISMN_06330 (pseudogene), KDO02374, JFK01000034 (missing from contig), REISMN_07520 (pseudogene), KDO03169, KDO03029, KDO02424, KDO03553, KDO03152, KDO03503, KDO02952, KDO03587, KDO03340, KDO02955, KDO02387, KDO02840, respectively.

One supplementary table is available with the online Supplementary Material.

ISO7^T as REIS. In honour of the first description and analysis of presumed rickettsial endosymbionts by Paul Buchner (Buchner, 1926, 1965), we propose the name *Rickettsia buchneri* sp. nov. to accommodate isolate ISO-7. A comparative phylogenetic analysis of 11 protein sequences from a draft genome sequence of ISO7^T generated by us and a draft genome sequence of '*R. monacensis*' (NZ_CBUA000000000) isolated from *I. ricinus* (Simser *et al.*, 2002) confirmed that these two rickettsiae form a clade separate from other rickettsiae, as noted by others (Gillespie *et al.*, 2012; Lee *et al.*, 2013). Preliminary data from our laboratory indicate that this clade will include rickettsial endosymbionts from other *Ixodes* species as well.

ISO7^T (*I. scapularis* ovary from tick 7) was isolated from a female tick removed from a dog in October 2007 (GPS position; 45° 16′ 07 N 93° 04′ 51 W, Columbus, MN, USA). The tick was surface disinfected 1 week after collection (Kurtti *et al.*, 1996) and the developing ovaries were extirpated, minced and inoculated into the wells of a 24-well plate previously seeded with tick cell lines IRE11 or ISE6 derived from embryos of *I. ricinus* (Simser *et al.*, 2002) and *I. scapularis* (Munderloh *et al.*, 1999), respectively. Infected and uninfected tick cells were cultured in modified Leibovitz's L15 medium supplemented with fetal bovine serum and tryptose phosphate broth as described elsewhere (Munderloh & Kurtti, 1989; Oliver *et al.*, 2014). The plate was incubated in a humidified candle jar at 26–28 °C. After 3–4 weeks, ovarian fragments and infected cells were transferred to IRE11 or ISE6 cell cultures in 12.5 cm^2 flasks (vented caps) and maintained in a humidified candle jar for six subcultures. Initially, ISO7^T was sensitive to atmospheric conditions, growing best in a hypoxic and CO_2-enriched atmosphere. Once adapted, isolate ISO7^T was maintained using ambient air. Monthly subcultures were made by transferring 1 ml of heavily infected cells (>95 % infected) onto a fresh layer of uninfected tick cells in 5 ml of medium in 25 cm^2 tissue culture flasks. The isolate did not form plaques but heavily infected cells detached from the substrate. Optimal growth of ISO7^T was obtained in line IRE11 incubated at 25–28 °C, temperatures lower than those routinely used with pathogenic rickettsiae. When ISO7^T was incubated at 32 °C or higher, rickettsial growth and cross infection of uninfected cells ceased (Fig. 1). ISO7^T was able to grow in ISE6 cells, but cell-to-cell spread was considerably slower than in IRE11 cultures, and rickettsial numbers declined when cultures were incubated at more than 25 °C.

The ultrastructure of ISO7^T in IRE11 cells (Fig. 2) was similar to that of rickettsiae found in *I. scapularis* ovarian cells (Munderloh *et al.*, 2005). Infected cells contained rickettsiae in tightly packed colonies within the cytoplasm only (Fig. 2a, black arrowheads) or dispersed within large vacuoles (Fig. 2a, black arrows), similar to observations reported for *Rickettsia peacockii* (Balraj *et al.*, 2008; Simser *et al.*, 2001). ISO7^T was generally in direct contact with the cytoplasm, but some were being degraded within membrane bound vesicles (presumed lysosomes). In both the

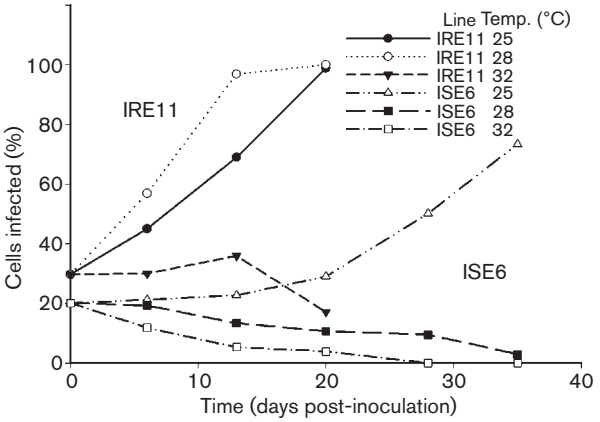

Fig. 1. Growth of ISO7^T in cell lines IRE11 and ISE6. Infected IRE11 cells were diluted 1 : 5 with uninfected cells and inoculated into replicated cultures. Infection was monitored by preparing cytocentrifuge slides from each culture at selected times for staining with Giemsa stain. Spread of infection was measured by determining the proportion of infected cells at each time point. The values at each time point are the mean of two replicated cultures.

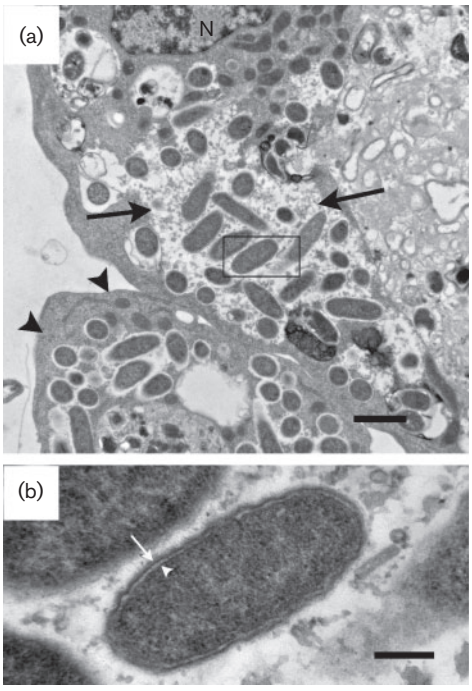

Fig. 2. Transmission electron microscopy of IRE11 cells infected with ISO7^T. (a) *I. ricinus* (IRE11) cell with ISO7^T in cytoplasm (arrowheads) and in a vacuole (arrows). N, host cell nucleus. Bar, 1 μm. (b) Rickettsiae free in the cytoplasm showing the outer microcapsular layer, cell wall (arrow), electron-lucent periplasmic space and the periplasmic membrane (white arrowhead). Bar, 0.2 μm. The rectangle in (a) delineates the area shown in (b).

ovary and IRE11 cells, rod-shaped rickettsiae were 1.0 to 1.5 μm by 0.4 to 0.5 μm (Fig. 2b). The mottled cytoplasm was surrounded by a narrow periplasmic space of 5 to 10 nm enclosed by a trilaminar cell wall (15–20 nm) (arrow). The inner leaflet was slightly thicker or similar in thickness to the outer leaflet. The microcapsular layer was not prominent and the halo (slime layer) of intracytoplasmic rickettsiae was thin (30 nm or less). ISO7^T was never found within host cell nuclei or pseudopodia.

The DNA sequences of PCR-products targeting *ompA*, *ompB*, 17 kDa and *gltA* genes, obtained from ovaries of *I. scapularis* and whole female *I. scapularis* previously collected in Minnesota, matched sequences of the same genes amplified from the isolate ISO7^T and REIS. The DNA sequences of *ompA*, 17 kDa and *gltA* were identical to those reported for REIS (Gillespie *et al.*, 2012) but the *ompB* sequences indicated the gene was not mutated in ISO7^T as previously reported for REIS (Gillespie *et al.*, 2012).

REIS carries four plasmids, pREIS1, pREIS2, pREIS3 and pREIS4 that are 55, 67, 50 and 34 kbp, respectively (Gillespie *et al.*, 2012). PCR using plasmid-specific *parA* gene primers (Baldridge *et al.*, 2010) demonstrated that each of the four plasmids was present in ISO7^T. End point dilution of host-cell-free *R. buchneri* sp. nov. was used to purify isolate ISO7^T in an effort to generate clonal populations instead of the standard rickettsial plaque assay due to the sensitivity of *R. buchneri* sp. nov. to suboptimal growth conditions. ISO7^T plasmids were resolved using PFGE and subjected to Southern blot as previously described (Burkhardt *et al.*, 2011); this enabled comparison of the plasmid profiles from the parental isolate ISO7^T and clones derived from it. ISO7^T (P) and clone (B8) had similar PFGE profiles with three prominent plasmid bands ranging from 50 to 70 kbp (Fig. 3). Southern blots of gels probed with digoxigenin labelled *parA* probes specific for pREIS1, pREIS2, pREIS3 and pREIS4 confirmed the presence of all four plasmids in uncloned ISO7^T and the absence of pREIS4 in clone B8 (Fig. 3b). These results suggested that pREIS4 was present only in a subpopulation of ISO7^T.

We sequenced the genome of ISO7^T clone B8 using DNA extracted from rickettsiae purified from IRE11 cells and compared it to the draft genome sequence of REIS (Gillespie *et al.*, 2012). Genomic DNA was pyrosequenced (454 GS FLX titanium platform; Roche) and sequences assembled using Newbler software (Roche). We predicted potential coding sequences using the annotation pipeline Prokka (http://vicbioinformatics.com/) (Seemann, 2014) together with the Artemis Genome Browser (http://www.sanger.ac.uk/resources/software/artemis/) (Rutherford *et al.*, 2000) and BLAST to detect split and non-predicted genes. The whole genome shotgun sequence for ISO7^T is available in GenBank (JFKF01000000). In summary, the genome assembly of ISO7^T B8 comprises 1.66 Mb (1642 ORFs in 207 contigs). Taken together, our PFGE and 454 sequencing data demonstrate that the genome includes the chromosome and four plasmids (pREIS1, pREIS2, pREIS3 and pREIS4). The genome has a DNA G+C content of 32.5 mol%. For comparison, we also sequenced the genome of '*R. monacensis*' strain IrR/Munich (GenBank, CBUA000000000.1; DSM 29017 available from Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures).

To analyse the phylogenetic position of *R. buchneri* sp. nov. (ISO7^T) and identify factors germane to its phenotypic characteristics, we compared its draft genome sequence with the genomes of other rickettsial species. The draft genome placed ISO7^T into a lineage close to '*R. monacensis*' and both resided in a clade separate from other rickettsiae. The draft genome sequence also confirmed the absence of pREIS4 in clone B8. Several genes important in rickettsial motility,

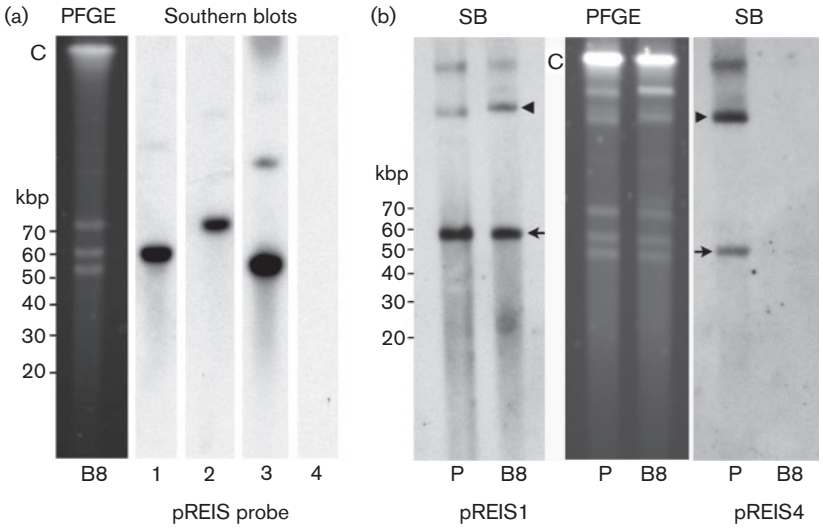

Fig. 3. PFGE and Southern blot (SB) analysis of parental isolate ISO7^T and clone B8. Cells were released by passing infected IRE11 cells through a 25 G needle. The suspension was filtered through a sterile 2 μm syringe filter and prepared for PFGE and Southern blot analysis (Burkhardt *et al.*, 2011). (a) B8, PFGE and Southern blots probed with digoxigenin-labelled *parA* probes specific for plasmid pREIS1, -2, -3 or -4. Note absence of pREIS4. (b) Parental ISO7^T (P) and clone B8 PFGE gel and Southern blots. Blots were probed with digoxigenin-labelled *parA* probes specific for plasmid pREIS1 or pREIS4. Note presence of pREIS4 in the parent (P). Arrows mark putative monomers of each plasmid and arrowheads indicate their conformational isomers. C, chromosomal DNA. Linear DNA marker positions are to the left of panels (a) and (b).

invasiveness and temperature adaptation were mutated in ISO7^T [e.g. *sca2* (Kleba *et al.*, 2010), *rickA* (Simser *et al.*, 2005), *hsp22* (Bechah *et al.*, 2010), *pldA* (Rahman *et al.*, 2013) and *htrA* (Zhang *et al.*, 2006)]. Additionally, we conducted a neighbour-joining analysis (Saitou & Nei, 1987) of *R. buchneri* sp. nov. (ISO7^T) using the deduced amino acid sequences (concatenated) from 11 housekeeping genes, i.e. *htpG*, *infB*, *rpoA*, *rpoB*, *polA*, *thrS*, *groEL*, *gyrB*, *recA*, *dnaE* and *pnp* (Fig. 4 and Table S1, available with the online Supplementary Material). The phylogenetic tree corroborated results obtained using whole genome sequences.

A multigenic approach is used to define new rickettsial species (Duh *et al.*, 2010; Fournier *et al.*, 2003) and improve phylogenetic resolution. The genes commonly analysed are 16S rRNA (Roux & Raoult, 1995), *gltA* (Roux *et al.*, 1997), 'geneD' (Sekeyova *et al.*, 2001), *ompA* and *ompB* (Roux & Raoult, 2000). According to the classification scheme proposed by Fournier *et al.* (2003), ISO7^T is a representative of a novel species closely related to '*Rickettsia monacensis*' that we propose to name *Rickettsia buchneri* sp. nov. Our proposal is reinforced by the approach of using concatenated gene sequences. Vitorino *et al.* (2007) have demonstrated that concatenation of nucleotide sequences of eight loci (*atpA*, *recA*, *virB4*, *dnaA*, *dnaK*, *rrl–rrf* internal transcribed spacer, *ompA* and *gltA*) gave improved discrimination between rickettsial species. Our phylogenetic placement obtained when concatenating 11 housekeeping genes agrees with that of Gillespie *et al.* (2012) who concatenated the amino acid sequence of 191 proteins and suggested that REIS was ancestral to spotted fever group rickettsiae (Gillespie *et al.*, 2012).

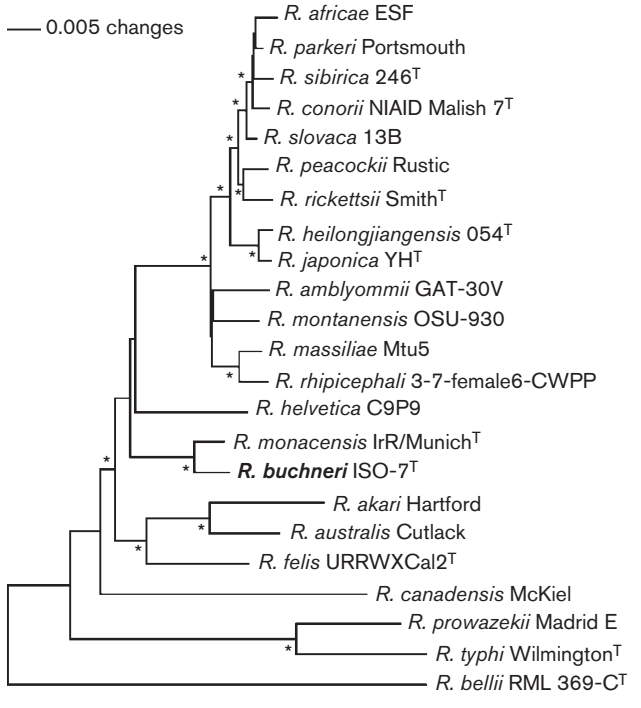

Fig. 4. Phylogram showing that ISO7^T and '*R. monacensis*' are closely related and reside in a clade separate from other rickettsiae. Neighbour-joining tree is based on amino acid sequences of 11 concatenated proteins (Table S1). Protein sequences were concatenated in the direction of amine to carboxyl group in the order of proteins HtpG, InfB, RpoA, RpoB, PolA, ThrS, GroEL, GyrB, RecA, DnaE and Pnp. Sequences were aligned using MUSCLE (Edgar, 2004) set at default parameters. The alignments were transferred into the PAUP* (Swofford, 2002) program as nexus files, and maximum-likelihood and neighbour-joining trees were reconstructed. Trees were rooted by making the outgroup (*Rickettsia bellii*) paraphyletic with respect to the ingroup. The robustness of clade designations was tested with a full heuristic search and 1000 bootstrap replicates, and nodes with asterisks are supported at values of $\geqslant$99 %.

Description of *Rickettsia buchneri* sp. nov.

Rickettsia buchneri (buch′ne.ri. N.L. gen. masc. n. *buchneri* of Buchner, named in honour of Dr Paul Buchner, a German biologist who made pioneering contributions to the identification of non-pathogenic tick endosymbionts that are transovarially transmitted).

An obligate intracellular bacterium found in ovaries of the blacklegged tick *I. scapularis*. The rickettsiae can be grown in tick cell lines IRE11 and ISE6 at 25–28 °C. The *I. ricinus* and *I. scapularis* host cells are grown in a medium formulated for tick cell culture. The ultrastructural appearance of the culture isolate is similar to rickettsiae found in the ovaries of *I. scapularis* and is typical for the genus *Rickettsia*. The cell wall and cytoplasmic membrane are separated by a thin periplasmic space. Cells infected with coccobacillary rickettsiae can be seen with Giemsa stain. Grows extensively in the cytoplasm of host cells and causes hypertrophy. To date, *R. buchneri* has only been isolated from *I. scapularis* but closely related bacteria are found in other *Ixodes* ticks. Widely distributed among North American *I. scapularis* ticks. The draft 1.66 Mb genome sequence indicates that *R. buchneri* is closely related to '*R. monacensis*' found in *I. ricinus* ticks in Europe and forms a clade separate from rickettsiae found in other tick species.

The type strain, ISO7^T (=DSM 29016^T=ATCC VR-1814^T), was isolated from a partially engorged *I. scapularis* female collected in Minnesota, USA, October 2007.

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