**Legionella norrlandica** sp. nov., isolated from the biopurification systems of wood processing plants

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Fourteen isolates of an unknown species identified as belonging to the genus *Legionella* by selective growth on BCYE agar were isolated from the biopurification systems of three different wood processing plants. The *mip* gene sequence of all 14 isolates was identical and a close match alignment revealed 86 % sequence similarity with *Legionella pneumophila* serogroup 8. The whole genome of isolate LEGNT was sequenced, and a phylogenetic tree based on the alignment of 16S rRNA, *mip*, *rpoB*, *rplB* and the 23S–5S intergenic region clustered LEGNT with *L. pneumophila* ATCC 33152T. Analysis of virulence factors showed that strain LEGNT carries the majority of known *L. pneumophila* virulence factors. An amoeba infection assay performed to assess the pathogenicity of strain LEGNT towards *Acanthamoeba castellanii* showed that it can establish a replication vacuole in *A. castellanii* but does not significantly affect replication of amoebae. Taken together, the results confirm that strain LEGNT represents a novel species of the genus *Legionella*, for which the name *Legionella norrlandica* sp. nov. is proposed. The type strain is LEGNT (=ATCC BAA-2678T=CCUG 65936T).

*Legionella* is a genus of Gram-negative bacilli that belong to the family *Legionellaceae*. A total of 58 *Legionella* species have been characterized to date (Parte, 2014). At least 24 of these species have been isolated from, or linked to, human infections, where they were believed to be the causative agent of disease (Newton et al., 2010). *Legionella pneumophila* is the main causative agent of legionellosis or Legionnaires’ disease, a severe pneumonia. *Legionella* are ubiquitous freshwater environmental bacteria; as facultative intracellular bacteria, *Legionella* exploit amoebae as natural hosts, and free-living amoebae function as a reservoir for these intracellular bacteria (Diederen, 2008; Gomez-Valero & Buchrieser, 2013; Richards et al., 2013).

In Sweden, legionellosis is reportable under the communicable disease act and source tracing is conducted for all diagnosed cases. The Public Health Agency of Sweden performs water quality analysis of freshwater systems, which includes detection and identification of species of the genus *Legionella*. When using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS (Microflex LT; Bruker Daltonics) with an in-house *Legionella* database for species identification (Gaia et al., 2011), we detected 14 isolates of an unknown *Legionella* species from biopurification systems of three different wood processing plants located in northern Sweden. The isolates were classified as belonging to the genus *Legionella* according to ISO 11731 of the International Organization for Standardization but the species could not be identified by MALDI-TOF MS. A close match alignment of the *mip* gene sequence (Ratcliff et al., 1998), analysed at http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/mip/mip_id.cgi, revealed 86 % sequence similarity with *L. pneumophila* serogroup 8. All 14 isolates had 100 % identical *mip* gene sequences (data not shown) and were deemed to represent the same species. The low *mip* gene sequence similarity to *L. pneumophila* and the poor match against our in-house *Legionella* spectra database (available for download from http://spectra.folkhalsomyndigheten.se) suggested that this was a novel species of the genus *Legionella*.

All strains required cysteine for growth on buffered charcoal yeast extract (BCYE) agar; no growth was detected on 5 % sheep blood agar plates. Bacterial growth was tested at a temperature range from 25 to 42 °C on BCYE agar. Optimal growth was observed with and without 5 % CO₂ at 30 and 37 °C, respectively, while no growth was observed at 25 °C. Cells were Gram-stain negative rods without pigmentation and no autofluorescence under UV light; they were also negative for oxidase (Bactident oxidase; Merck).
As *L. pneumophila*, the main causative agent of legionellosis, was identified as the closest match, we performed whole genome sequencing on the randomly selected isolate LEGNT to identify possible virulence factors, resistance mechanisms and conserved regions to generate a phylogenetic tree. One microgram of DNA was used for whole genome sequencing,

Table 1. Genomic analysis of strain LEGNT\(^T\) and the type strains of publicly available species of the genus *Legionella*

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA G+C content (mol%)</th>
<th>Genome size (Mb)</th>
<th>Percentage of annotation matches against the whole <em>L. pneumophila</em> ATCC 33152(^T) genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEGNT(^T)</td>
<td>37.6</td>
<td>3.07</td>
<td>51</td>
</tr>
<tr>
<td><em>L. anisa</em> ATCC 35292(^T)</td>
<td>38.2</td>
<td>4.32</td>
<td>12</td>
</tr>
<tr>
<td><em>L. shakespearei</em> DSM 23087(^T)</td>
<td>41.7</td>
<td>3.51</td>
<td>13</td>
</tr>
<tr>
<td><em>L. dumoffii</em> ATCC 33279(^T)</td>
<td>38.1</td>
<td>3.91</td>
<td>11</td>
</tr>
<tr>
<td><em>L. drancourtii</em> LLAP 12(^T)</td>
<td>39.2</td>
<td>3.99</td>
<td>9</td>
</tr>
<tr>
<td><em>L. moravica</em> DSM 19234(^T)</td>
<td>40.0</td>
<td>3.76</td>
<td>7</td>
</tr>
<tr>
<td><em>L. tunisensis</em> LegMT(^T)</td>
<td>39.9</td>
<td>3.62</td>
<td>6</td>
</tr>
<tr>
<td><em>L. oakridgensis</em> ATCC 33761(^T)</td>
<td>40.9</td>
<td>2.98</td>
<td>5</td>
</tr>
<tr>
<td><em>L. longbeachae</em> NSW150</td>
<td>37.1</td>
<td>4.15</td>
<td>1</td>
</tr>
<tr>
<td><em>L. pneumophila</em> ATCC 33152(^T)</td>
<td>38.3</td>
<td>3.40</td>
<td>-</td>
</tr>
</tbody>
</table>
which was performed on an Ion Torrent PGM sequencer, using Library Builder and Ion Chef according to the manufacturer’s protocol (Thermo Fisher Scientific).

A concatenation of the sequences for the mip (Ratcliff et al., 1998), 16S rRNA (Birtles et al., 1996), rpoB (Ko et al., 2002), rnpB (Rubin et al., 2005) and 23S–5S intergenic spacer region (Grattard et al., 2006) genes of strain LEGN\textsuperscript{T} and the type strains of publicly available species of the genus Legionella were aligned and a neighbour-joining tree, with bootstrap values based on 1000 calculations, was generated using CLC genomics workbench version 6.5.1 (CLCbio). Strain LEGN\textsuperscript{T} clustered with L. pneumophila ATCC 33152\textsuperscript{T} and this cluster was supported by a bootstrap value of 100 % (Fig. 1).

The genomes of strain LEGN\textsuperscript{T}, and the type strains of Legionella anisa, Legionella longbeachae, Legionella oakridgensis, Legionella tucsonensis, Legionella dumoffii, Legionella drancourtii and Legionella tunisiensis were compared with the annotated genome of L. pneumophila Philadelphia-1\textsuperscript{T} (=ATCC 33152\textsuperscript{T}). Intersection with the reference annotation was determined by a word size of ≥ 20 bases, with an E-value/false discovery rate ≥ 0.0001 and minimum match size 100 bases. –, indicates that no virulence factors analyzed would fall into that category.

<table>
<thead>
<tr>
<th>Virulence factor function</th>
<th>LEGN\textsuperscript{T}</th>
<th>Match</th>
<th>LO</th>
<th>NI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dot/icm substrate</td>
<td>15/68</td>
<td>7/68</td>
<td>46/68</td>
<td></td>
</tr>
<tr>
<td>Enhance protozoan infection</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Enhanced entry</td>
<td>3/3</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Flagellum</td>
<td>1/29</td>
<td>1/29</td>
<td>27/29</td>
<td></td>
</tr>
<tr>
<td>Host gene regulation</td>
<td>4/5</td>
<td>–</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>hel locus inner membrane heavy metal transporter</td>
<td>3/3</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Hsp60, enhanced epithelial cell invasion</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Infection enhancement</td>
<td>4/5</td>
<td>–</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Iron uptake</td>
<td>5/5</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Legionella virulence region</td>
<td>5/5</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Legionella virulence homologue region</td>
<td>11/11</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Lipid A modification</td>
<td>–</td>
<td>1/1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Macrophage infection</td>
<td>–</td>
<td>–</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>Macrophage infectivity potentiator</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>MOMP (major outer-membrane protein)</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Nutrient acquisition</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Pore-forming toxin</td>
<td>4/5</td>
<td>–</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Promotion of macrophage infection and virulence</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Required for replication in protozoa</td>
<td>–</td>
<td>1/1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Type II secretion-dependent substrates</td>
<td>13/21</td>
<td>6/21</td>
<td>2/21</td>
<td></td>
</tr>
<tr>
<td>Type II secretion system</td>
<td>10/10</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Type IV pilus</td>
<td>3/3</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Type IV secretion system dot/icm</td>
<td>17/17</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

\textit{L. pneumophila} Philadelphia-1\textsuperscript{T} annotations were matched to the LEGN\textsuperscript{T} genome compared with approximately 10 % for the other species analysed (Table 1), confirming that strain LEGN\textsuperscript{T} is closely related to \textit{L. pneumophila}.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Etest (µg ml\textsuperscript{–1})</th>
<th>Disc zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>0.38</td>
<td>–</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>–</td>
<td>44</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>8\textsuperscript{±}; 12\textsuperscript{†}</td>
<td>–</td>
</tr>
<tr>
<td>Trimethoprim–sulfamethoxazole (25 µg)</td>
<td>–</td>
<td>22</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&lt;0.016</td>
<td>–</td>
</tr>
<tr>
<td>Imipenem/inhibitor</td>
<td>No growth</td>
<td>–</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.032</td>
<td>–</td>
</tr>
</tbody>
</table>

\textit{–}, not tested, as the susceptibility test was either performed by disc diffusion or Etest.

\textsuperscript{†}Results after 4 and 5 days.

\textsuperscript{†}Result after 3 days.
The virulence of *L. pneumophila* is not mediated by specific toxins but rather by a variety of virulence factors that affect all steps of molecular pathogenesis, from infection, intracellular survival and replication to host cell lysis (Cianciotto, 2001; Newton *et al.*, 2010). The close phylogenetic relationship between strain LEGN<sup>T</sup> and *L. pneumophila*, the main causative agent of legionellosis, prompted us to analyse the occurrence and the genetic relationship of known *L. pneumophila* virulence factors. Mean sequence coverage for the virulence factors was 97.3% and mean sequence ID was 83.8%. The complete list of the virulence genes analysed is given in Table S2. Strain LEGN<sup>T</sup> harbours the majority of known virulence mechanisms present in *L. pneumophila*, including all the genes encoding the type IV (Doc/1cm) and type II secretion systems as well as the type IV pilus (Table 2). Strain LEGN<sup>T</sup> lacks all but two of the genes coding for the presence of flagellum, and only 22 of 68 analysed dot/icm-dependent substrates were found having a match or low genetic overlap. By contrast, the majority (19 of 21) of the type II secretion system-dependent substrates analysed were identified in strain LEGN<sup>T</sup>.

A genome-wide analysis for known antibiotic resistance genes was conducted and strain LEGN<sup>T</sup> was found to carry genetic sequences matching *macA*, *macB* and *mtrE*, *sul1* and *sul2*, and *CmlA*, genes known to confer resistance against macrolides, sulfonamide and chloramphenicol, respectively (Bissonnette *et al.*, 1991; Rouquette-Loughlin *et al.*, 2005; Sköld, 2000). An antibiotic susceptibility assay was performed to assess the phenotypic antibiotic resistance pattern. A sterile swab dipped in a 1 McFarland unit inoculate of strain LEGN<sup>T</sup> suspended

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**Fig. 2.** Effect of strain LEGN<sup>T</sup> infection on *A. castellanii* cells. (a) Number of intact *A. castellanii* cells 24 and 48 h after infection with strain LEGN<sup>T</sup> or *L. pneumophila*; uninfected *A. castellanii* cells were used as negative control. ***P* ≤ 0.0001 (unpaired *t*-test). Error bars show SD. (b) Light micrographs of uninfected and infected *A. castellanii* cells 24 and 48 h after infection with strain LEGN<sup>T</sup> or *L. pneumophila*. Black arrows indicate *Legionella*-containing vacuoles, and the grey arrow indicates an aggregation of bacteria. All images were taken at 100× magnification. The experiment was conducted twice in triplicate, and the figure shows data from one representative experiment.
in BYE broth was spread on BCYE plates and antibiotic discs or Etest strips were applied. Plates were incubated at 37 °C with 5 % CO2 and checked after 3, 4 and 5 days. MIC values and inhibition zones are reported in Table 3. Although very little is known about antibiotic susceptibility in Legionella, the genetic markers detected appear not to confer phenotypic antibiotic resistance (Bruin et al., 2012).

To assess if strain LEGNT is as pathogenic to amoebae as L. pneumophila, we performed an amoeba infection assay (Tezcan-Meridol et al., 2004). Briefly, axenically grown Acanthamoeba castellanii cells (Winiecka-Krusnell et al., 2002) in PYG medium were dispensed into multi-well sterile plates, approximately 25,000 cells per well in 2 ml PYG medium. The plates were centrifuged for 2 min at 500 g and the cells were allowed to adhere for 2 h at 30 °C. Strain LEGNT and L. pneumophila ATCC 33152T were grown in liquid BYE medium to exponential growth phase (Byrne & Swanson, 1998). The bacterial cell cultures were then washed in PYG medium and strain LEGNT or L. pneumophila was added to A. castellanii at a 50:1 ratio. A. castellanii without bacteria were used as a negative control. After addition of bacteria, the plates were centrifuged for 5 min at 500 g to allow contact between A. castellanii and the bacterial cells; this was considered time point zero of infection. Intact amoeba cells were counted 24 and 48 h after infection using a Bürker chamber. Cells were also fixed in methanol for 5 min and stained with Giemsa stain for 10 min at 24 and 48 h after infection. The experiment was repeated twice in triplicate with comparable results; the data shown are from one representative experiment (Fig. 2). Round, non-attached, amoebas were observed in the wells containing L. pneumophila and strain LEGNT both at 24 and at 48 h, but not in the negative controls. L. pneumophila had a significant effect on A. castellanii cell growth at both time points (unpaired t-test, P ≤ 0.0001), while no significant difference was observed for strain LEGNT compared with the negative control at 24 and 48 h (Fig. 2a). Giemsa staining of the cells revealed that strain LEGNT is able to establish a replication vacuole in A. castellanii, although most of the cells at both time points were not infected (Fig. 2b). The few intact A. castellanii cells found after 24 h of incubation with L. pneumophila were all infected and displayed multiple Legionella-containing vacuoles. After 48 h of infection with L. pneumophila most A. castellanii cells were lysed and large aggregations of bacteria were observed.

The results indicate that strain LEGNT is less virulent towards A. castellanii than L. pneumophila, although strain LEGNT carries many known virulence factors with a high sequence similarity to those of L. pneumophila. It is thus possible that strain LEGNT might not be as pathogenic to humans as L. pneumophila, which is also supported by the fact that no clinical data have linked it to human infections.

Based on the data presented, the 14 isolates from wood processing plants are considered to represent a novel species of the genus Legionella, for which the name Legionella norrlandica sp. nov. is proposed.

**Description of Legionella norrlandica sp. nov.**

Legionella norrlandica (norr.lan.di’ca. N.L. fem. adj. norrlandica pertaining to Norrland, the northern region of Sweden where the type strain was isolated).

Gram-negative rods that require L-cysteine for growth on BCYE agar with or without 5 % CO2 oxidase-negative and negative for auto-fluorescence. Grows at temperatures between 28 and 42 °C, with optimal growth at 30 and 37 °C with and without 5 % CO2, respectively. Able to establish a replication vacuole in A. castellanii, but has no significant negative effect on growth or replication of the amoeba.

The type strain is LEGNT (=ATCC BAA-2678T=CCUG 65936T), which was isolated from the biopurification system of a wood processing plant in Piteå, Sweden. The DNA G+C content of the type strain is 37.6 mol%. Thirteen other isolates from wood processing plants are members of the species.

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**References**


phylogeny to direct identification of isolates at the species level from clinical specimens. *Microbes Infect* 8, 73–83.


