Legionella dresdenensis sp. nov., isolated from river water

Paul Christian Lück,1 Enno Jacobs,1 Isolde Röske,2 Ute Schröter-Bobsin,1 Roger Dumke1 and Sabine Gronow3

1Institut für Medizinische Mikrobiologie und Hygiene, TU Dresden, 01307 Dresden, Germany
2Institut für Mikrobiologie, TU Dresden, 01062 Dresden, Germany
3DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany

Legionella-like isolates, strains W03-356T, W03-357 and W03-359, from three independent water samples from the river Elbe, Germany, were analysed by using a polyphasic approach. Morphological and biochemical characterization revealed that they were Gram-negative, aerobic, non-spore-forming bacilli with a cut glass colony appearance that grew only on L-cysteine-supplemented buffered charcoal yeast extract agar. Phylogenetic analysis based on sequence comparisons of the 16S rRNA, macrophage infectivity potentiator (mip), gyrase subunit A (gyrA), ribosomal polymerase B (rpoB) and RNase P (rnpB) genes confirmed that the three isolates were distinct from recognized species of the genus Legionella. Phenotypic characterization of strain W03-356T based on fatty acid profiles confirmed that it was closely related to Legionella rubrilucens ATCC 35304T and Legionella pneumophila ATCC 33152T, but distinct from other recognized species of the genus Legionella. Serotyping of the isolates showed that they were distinct from all recognized species of the genus Legionella. Strains W03-356T, W03-357 and W03-359 are thus considered to represent a novel species of the genus Legionella, for which the name Legionella dresdenensis sp. nov. is proposed. The type strain is W03-356T (=DSM 19488T=NCTC 13409T).

In 1976, an outbreak of severe pneumonia among participants of an American Legion convention led to the discovery of a new bacterial genus, Legionella (Brenner et al., 1979). At the time of writing, the genus comprised 50 recognized species (Euzéby, 2009). Members of the genus Legionella are Gram-negative bacteria and normally occupy natural aquatic environments where they survive as intracellular parasites of protozoa (Steinert et al., 2002). Human infections occur as sporadic or epidemic disease that may be acquired from different environmental sources such as warm water supplies, cooling towers or evaporative condensers. Most infections are caused by Legionella pneumophila (Diederen, 2008).

Legionellae have been isolated from a wide diversity of samples: human clinical specimens, animals (Fabbi et al., 1998), natural and treated water (Diederen, 2008; Steinert et al., 2003) and protozoa (Dey et al., 2009). However, some species, such as Legionella-like amoebal pathogens, are unculturable on the buffered charcoal yeast extract (BCYE) agar normally used to grow legionellae as well as on all other media tested (La Scola et al., 2004).

Routine identification of legionellae is based on phenotypic properties, mainly by serotyping, but molecular biological techniques have resulted in the development of new methods that allow more reliable characterization of species of the genus Legionella (Lück et al., 2006). Genotyping methods such as sequencing of genes coding for 16S or 5S rRNA (Adeleke et al., 2001; Birtles et al., 1996), macrophage infectivity potentiator (mip) (Fry et al., 2007; Ratcliff et al., 1998), gyrase subunit A (gyrA) (Feddersen et al., 2000), ribosomal polymerase B (rpoB) (Ko et al., 2002) and RNase P (rnpB) (Rubin et al., 2005) have been shown to be useful in classifying isolated Legionella strains to the species level.

In a study aimed at detecting human pathogens in natural water samples, a Legionella genus-specific PCR, based on amplification of 16S rRNA genes (Miyamoto et al., 1997), revealed sequences that showed less than 93 % similarity to...
all recognized species of the genus *Legionella*. We therefore suspected that a novel species might be present in these water samples. Cultivation led to the isolation of three *Legionella*-like isolates from three different water specimens collected on the same day in May 2003 from the river Elbe near the city of Dresden, Germany. After preliminary phenotypic characterization, the three isolates (W03-356<sup>T</sup>, W03-357 and W03-359) were completely typed by serological and genotypic methods. The results of these experiments showed that these isolates represented a novel species of the genus *Legionella*. Cellular fatty acids and DNA G+C content were analysed for strain W03-356<sup>T</sup>.

### Isolation procedures

Water samples (1–2 litres) were collected from the river Elbe in Dresden, Germany. These samples were concentrated by continuous centrifugation (2500 g, 30 min, 4 °C) followed by filtration to a volume of 10 ml and were either left untreated or treated by acidification and heating (3 min, 60 °C) to suppress heterotrophic bacteria according to standard international procedures (International Organization for Standardization, 1998). Finally, the samples were cultured on selective BCYE agar medium (Oxoid) (Wadowsky & Yee, 1981). Plates were incubated at 37 °C in 5% CO<sub>2</sub> and at a relative humidity of 95%. Suspected *Legionella* isolates were subcultured on BCYE agar and blood agar. As expected for members of the genus *Legionella*, all isolates grew on the former but not on the latter. Several strains of *L. pneumophila* were also recovered.

### Phenotypic characterization

Each putative *Legionella* strain tested grew well on selective and non-selective BCYE agar. All required cysteine for growth on BCYE medium. Blue–white autofluorescence under UV light (366 nm) was not observed for any of the three isolates. However, the isolates showed a weak reddish fluorescence that was less remarkable than that of the type strains of *Legionella* erythra and *Legionella rubrilucens* (data not shown). Biochemical tests were positive for the presence of catalase and gelatinase, and negative reactions were obtained for oxidase, urease, carbohydrate fermentation, nitrate reduction and hippurate hydrolysis (Brenner et al., 1988).

### Serological identification

The three new isolates reacted strongly when tested in indirect immunofluorescence assays with a rabbit antisera prepared against strain W03-356<sup>T</sup>. Furthermore, this antisera did not react with any of the 63 type strains tested here, including all serogroups of recognized species of the genus *Legionella*. A full list of the 63 type strains used is presented in Supplementary Table S1 (available in IJSEM Online). In contrast, the three new isolates tested negative by using antisera prepared against 46 strains of the genus *Legionella* (for the full list of strains used, see Supplementary Table S2).

The commercially available Duopath identification kit based on monoclonal antibodies against *Legionella* (Merck) clearly identified the three new isolates as members of the genus *Legionella* (Helbig et al., 2006; data not shown).

### Cellular fatty acid composition

For analysis of fatty acids, cells of strain W03-356<sup>T</sup> were grown on BYCE agar for 48 h at 37 °C. Fatty acid methyl esters were obtained by saponification, methylation and extraction as described by Kämpfer & Kroppenstedt (1996) and were separated by GC (model 5898A; Hewlett Packard). Peaks were automatically integrated and fatty acid components and their proportions were determined by using the Microbial Identification standard software package MIDI (Sasser, 1990). The predominant fatty acids of strain W03-356<sup>T</sup> were iso-C<sub>16 : 0</sub> (25.3%), C<sub>16 : 1</sub>ω<sub>7c</sub> (24.4%) and anteiso-C<sub>15 : 0</sub> (15.7%). The presence of anteiso-C<sub>17 : 0</sub> (8.5%) as a significant component was also characteristic. No cyclic fatty acids were detected. A dendrogram of Euclidian distances positioned strain W03-356<sup>T</sup> in closest neighbourhood to *L. pneumophila* subsp. *pneumophila* and *L. rubrilucens* (data not shown).

### Genotypic characterization

#### Fluorescence in situ hybridization (FISH)

A commercially available FISH assay (Vermicon) was performed according to the manufacturer’s recommendations and showed strong hybridization of the new isolates with the *Legionella* genus-specific probe but not with the *L. pneumophila*-specific probe (data not shown).

#### DNA amplification and sequencing.

Two isolates (W03-356<sup>T</sup> and W03-359) were subjected to 16S rRNA gene sequence analysis. DNA amplification of the 16S rRNA gene was performed by using oligonucleotide primers 28 forward and 1508 reverse (*Escherichia coli* numbering) (Brosius et al., 1978). In addition, the *Legionella*-specific primers described by Miyamoto et al. (1997) were used for sequence analysis. Sequencing of the *mip* gene was performed for all three isolates essentially as described by Ratcliff et al. (1998). In a similar fashion, selected regions of the *gyrA* (Feddersen et al., 2000), *rpoB* (Ko et al., 2002) and *rnpB* (Rubin et al., 2005) genes were amplified and sequenced for strains W03-356<sup>T</sup> and W03-359.

### Phylogenetic analyses.

16S rRNA gene sequences for isolates W03-356<sup>T</sup> and W03-359 were compared with previously published sequences available in the EMBL and GenBank databases (http://www.ncbi.nlm.nih.gov) by using the gapped BLAST program (Altschul et al., 1997). A comparison of 1420 bp aligned 16S rRNA gene sequences showed that strains W03-356<sup>T</sup> and W03-359 shared highest similarity with the type strain of *Legionella birminghamensis* ATCC 700508<sup>T</sup>
Table 1. Phylogenetic relationships between *Legionella dresdenensis* sp. nov. and other closely related members of the genus *Legionella*

<table>
<thead>
<tr>
<th>Sequence comparison gene/database (length of fragment analysed)</th>
<th>Closest related <em>Legionella</em> species (gene sequence similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA/NCBI BLAST (1466 bp)</td>
<td><em>L. birminghamensis</em> (97 %), <em>L. pneumophila</em> (96 %), <em>L. taurinensis</em> (96 %), <em>L. rubrilucens</em> (96 %), <em>L. erythra</em> (96 %)</td>
</tr>
<tr>
<td>mip/NCBI BLAST (574 bp)</td>
<td><em>L. rubrilucens</em> (79 %), <em>L. taurinensis</em> (79 %), <em>L. erythra</em> (77 %), <em>L. fcelei</em> (78 %), <em>L. jamestownensis</em> (78 %)</td>
</tr>
<tr>
<td>mip/(EWGLI*) (574 bp)</td>
<td><em>L. taurinensis</em> (76.8 %), <em>L. rubrilucens</em> (76.2 %), <em>L. erythra</em> (75.7 %), <em>L. gresilens</em> (74.2 %), <em>L. jamestownensis</em> (74.2 %)</td>
</tr>
<tr>
<td>rpoB/NCBI BLAST (342 bp)</td>
<td><em>L. gormanii</em> (83 %), <em>L. steigerwaltii</em> (83 %), <em>L. saintelhensi</em> (83 %), <em>L. cherrii</em> (82 %), <em>L. bozemanae</em> (81 %)</td>
</tr>
<tr>
<td>gyrA/NCBI BLAST (300 bp)</td>
<td><em>L. pneumophila</em> (84 %), <em>L. birminghamensis</em> (84 %), <em>L. spiritensis</em> (84 %), <em>L. shakespearei</em> (83 %)</td>
</tr>
<tr>
<td>rnpB/NCBI BLAST (527 bp)</td>
<td><em>L. jordanis</em> (89 %), <em>L. lansingensis</em> (84 %), <em>L. micladei</em> (86 %), <em>L. hackelae</em> (85 %), <em>L. pneumophila</em> (84 %)</td>
</tr>
</tbody>
</table>

*A European Working Group on Legionella Infections (Fry et al., 2007).*

(97 %), followed by those of *Legionella taurinensis* ATCC43702T, *L. pneumophila* ATCC 33152T, *L. rubrilucens* ATCC 35304T and *Legionella erythra* ATCC 35303T (Table 1).

A dendrogram showing the phylogenetic relationship between strains W03-356T, W03-357 and W03-359 and recognized species of the genus *Legionella* was derived from an alignment of *mip* gene sequences. It was apparent that the new isolates were related to the red fluorescing species *L. erythra* and *L. rubrilucens*, as well as to *L. taurinensis* (Fig. 1).

Sequences of the *gyrA* (301 bp), *rpoB* (342 bp) and *rnpB* (527 bp) genes from strains W03-356T, W03-357 and W03-359 were also significantly different from those of all recognized species of the genus *Legionella* (Table 1). However, the phylogenetic relationship was not completely congruent with that determined using the 16S rRNA and *mip* gene sequences. The *mip* and 16S rRNA gene sequences showed that the new isolates were most closely related to the red fluorescing species *L. erythra* and *L. rubrilucens* but also to the non-fluorescing species *L. taurinensis*. In contrast, the *rpoB*, *gyrA* and *rnpB* gene sequences did not show this relatedness. It therefore appears that the red fluorescence phenotype has no strong correlation at the gene sequence level for all genes analysed.

**Uptake and multiplication in Acanthamoeba castellanii**

Uptake and multiplication rate of strain W03-356T were determined with the gentamicin protection assay in *Acanthamoeba castellanii* (Lück et al., 1998). The multiplication rate was estimated after 6, 16, 40 and 70 h. Strain W03-356T multiplied by 1–1.5 logs within 70 h. By staining infected amoeba with the polyclonal antiserum specific for strain W03-356T, clusters of intracellular bacteria could be detected within the amoebal cells (Fig. 2).

Based on the above results, strains W03-356T, W03-357 and W03-359 are considered to represent a novel species of the genus *Legionella*, for which the name *Legionella dresdenensis* sp. nov. is proposed.

**Discussion**

The presence of *Legionella* isolates in potable and non-potable water available to the public is associated with a health risk for the population and frequently results in surveys of these water systems. During routine water sampling, several atypical *Legionella* isolates were recovered from the river Elbe, Germany. On the basis of cultural criteria, these strains appeared to belong to the genus *Legionella*, but remained untypeable according to routine techniques such as serotyping. For identification of species of the genus *Legionella*, gene sequence analysis is much faster and more reliable than the phenotypic methods currently available. The results obtained here show clearly that the identification of *L. dresdenensis* sp. nov. by *mip* gene sequencing was excellent (Fry et al., 2007). Based on the 16S rRNA gene and other targets, e.g. the *gyrA*, *rpoB* or *rnpB* genes, *L. dresdenensis* sp. nov. is clearly different from all other recognized species of the genus *Legionella* (Kuroki et al., 2007; Park et al., 2003, 2004).

The phenotypic characteristics of the new isolates were in accordance with the genotypic results. As none of the recognized species of the genus *Legionella* tested reacted with an antiserum specific for *L. dresdenensis* sp. nov. and none of the antisera directed against other *Legionella* species or serogroups revealed any serological cross-reaction with the new isolates, *L. dresdenensis* sp. nov. can be clearly identified by serological methods. Analysis of fatty acids also confirmed the affiliation of the novel species to the genus *Legionella*; the profile consisting predominantly of branched-chain components (Diogo et al., 1999). Biochemical and growth characteristics of isolates W03-356T, W03-357 and W03-359 were typical of those of members of the family *Legionellaceae* (Brenner et al., 1988).

In addition, the new isolates could be identified as
Fig. 1. Neighbour-joining tree showing the relationship between strains W03-356, W03-357 and W03-359 and other members of the family Legionellaceae based on partial sequencing of the mip gene (572 bp) created by using the BioNumerics software. Numbers at nodes are cophenetic correlations that express the consistency of a cluster, thus estimating the faithfulness of each cluster of the dendrogram. GenBank accession numbers are given in parentheses. Bar, % similarity.
members of the genus Legionella based on commercially available identification assays, i.e., FISH and a chromato-
graphic immunoassay based on monoclonal antibodies
(Helbig et al., 2006). As a typical characteristic of members
of the genus Legionella, strain W03-356T was able to
multiply in A. castellanii.

As legionelliae are of public health importance, the
description of a novel species is significant in the
identification of Legionella infections. We have no evidence
that L. dresdenensis sp. nov. causes infections in humans
(von Baum et al., 2008). However, diagnostic assays must
be able to detect and differentiate all currently recognized
species of the genus Legionella.

Description of Legionella dresdenensis sp. nov.

Legionella dresdenensis (dres.den’ensis. N.L. fem. adj.
dresdenensis pertaining to Dresden, Germany, where the
type strain was isolated).

Gram-negative rods. Grows on BCYE agar, but not on
sheep blood agar or on BCYE agar lacking L-cysteine.
Positive in tests for catalase and gelatinase. Negative in tests
for oxidase, urease, carbohydrate fermentation, nitrate
reduction and hippurate hydrolysis. Serological typing
indicates no serological cross-reaction with all recognized
species of the genus Legionella. 16S rRNA, mip, rpoB, gyrA
and rnpB gene sequences differ significantly from all
recognized species of the genus Legionella. The DNA G+C
content of the type strain is 42.5 mol%.

The type strain, W03-356T (=DSM 19488T=NCTC 13409T),
was isolated from water samples from the river Elbe.

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