Legionella dresdenensis sp. nov., isolated from river water

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Legionella-like isolates, strains W03-356^T, 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 W03-356^T based on fatty acid profiles confirmed that it was closely related to *Legionella rubrilucens* ATCC 35304^T and *Legionella pneumophila* ATCC 33152^T, but distinct from other species of the genus *Legionella*. Serotyping of the isolates showed that they were distinct from all recognized species of the genus *Legionella*. Strains W03-356^T, 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 W03-356^T (=DSMZ 19488^T=NCTC 13409^T).

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* (Diederer, 2008).

Legionellae have been isolated from a wide diversity of samples: human clinical specimens, animals (Fabbi *et al.*,

1998), natural and treated water (Diederer, 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

Abbreviations: BCYE, buffered charcoal yeast extract; FISH, fluorescence *in situ* hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, partial *mip*, partial *gyrA*, partial *rpoB* and partial *rnpB* gene sequences of strain W03-356^T are AM747393, AM600957, AM600958, AM600959 and AM600960, respectively.

Supplementary tables detailing the strains used for indirect immunofluorescence assays are available with the online version of this paper.

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^T, 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^T.

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% CO₂ 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 anti-serum prepared against strain W03-356^T. Furthermore, this antiserum 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^T 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^T were iso-C_{16:0} (25.3%), C_{16:1ω7c} (24.4%) and anteiso-C_{15:0} (15.7%). The presence of anteiso-C_{17:0} (8.5%) as a significant component was also characteristic. No cyclic fatty acids were detected. A dendrogram of Euclidian distances positioned strain W03-356^T 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^T 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^T and W03-359.

Phylogenetic analyses. 16S rRNA gene sequences for isolates W03-356^T 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^T and W03-359 shared highest similarity with the type strain of *Legionella birminghamensis* ATCC 700508^T

Table 1. Phylogenetic relationships between *Legionella dresdenensis* sp. nov. and other closely related members of the genus *Legionella*

Sequence comparison gene/database (length of fragment analysed)	Closest related <i>Legionella</i> species (gene sequence similarity)
16S rRNA/NCBI BLAST (1466 bp)	<i>L. birminghamensis</i> (97 %), <i>L. pneumophila</i> (96 %), <i>L. taurinensis</i> (96 %), <i>L. rubrilucens</i> (96 %), <i>L. erythra</i> (96 %)
<i>mip</i> /NCBI BLAST (574 bp)	<i>L. rubrilucens</i> (79 %), <i>L. taurinensis</i> (79 %), <i>L. erythra</i> (77 %), <i>L. feeleei</i> (78 %), <i>L. jamestowniensis</i> (78 %)
<i>mip</i> /(EWGLI*) (574 bp)	<i>L. taurinensis</i> (76.8 %), <i>L. rubrilucens</i> (76.2 %), <i>L. erythra</i> (75.7 %), <i>L. gresilensis</i> (74.2 %), <i>L. jamestowniensis</i> (74.2 %)
<i>rpoB</i> /NCBI BLAST (342 bp)	<i>L. gormanii</i> (83 %), <i>L. steigerwaltii</i> (83 %), <i>L. sainthelensi</i> (83 %), <i>L. cherrii</i> (82 %), <i>L. bozemanii</i> (81 %)
<i>gyrA</i> /NCBI BLAST (300 bp)	<i>L. pneumophila</i> (84 %), <i>L. birminghamensis</i> (84 %), <i>L. spiritensis</i> (84 %), <i>L. shakespearei</i> (83 %)
<i>rnpB</i> /NCBI BLAST (527 bp)	<i>L. jordanis</i> (89 %), <i>L. lansingensis</i> (84 %), <i>L. micdadei</i> (86 %), <i>L. hackeliae</i> (85 %), <i>L. pneumophila</i> (84 %)

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

(97 %), followed by those of *Legionella taurinensis* ATCC43702^T, *L. pneumophila* ATCC 33152^T, *L. rubrilucens* ATCC 35304^T and *Legionella erythra* ATCC 35303^T (Table 1).

A dendrogram showing the phylogenetic relationship between strains W03-356^T, 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 W03-356^T, 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 W03-356^T 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 W03-356^T multiplied by 1–1.5 logs within 70 h. By staining infected amoebae with the polyclonal antiserum specific for strain W03-356^T, clusters of intracellular bacteria could be detected within the amoebal cells (Fig. 2).

Based on the above results, strains W03-356^T, 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 W03-356^T, 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^T, 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.

Fig. 2. Immunofluorescence micrograph of strain W03-356^T within *Acanthamoeba castellanii* 70 h after infection. The replicative phagosome is approximately 6 µm in size.

members of the genus *Legionella* based on commercially available identification assays, i.e. FISH and a chromatographic immunoassay based on monoclonal antibodies (Helbig *et al.*, 2006). As a typical characteristic of members of the genus *Legionella*, strain W03-356^T was able to multiply in *A. castellanii*.

As legionellae 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, W03-356^T (=DSM 19488^T=NCTC 13409^T), was isolated from water samples from the river Elbe.

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