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Legionella shakespearei sp. nov., Isolated From Cooling Tower Water

USHA K. VERMA,[1]† DON J. BRENNER,[2] W. LANIER THACKER,[2] ROBERT F. BENSON,[2] GRAHAM VESEY,[3]‡ JOHN B. KURTZ,[4] P. J. L. DENNIS,[3]‡ ARNOLD G. STEIGERWALT,[2] JIM S. ROBINSON,[1]* AND C. WAYNE MOSS[2]

Houseman Ltd., The Priory, Burnham, Slough SL1 7LS, United Kingdom[1]; Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]; Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, United Kingdom[3]; and Department of Virology, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom[4]

A *Legionella*-like organism (strain 214^T [T = type strain]) was isolated from a cooling tower in Stratford-upon-Avon, England. This strain required L-cysteine and contained cellular branched-chain fatty acids that are typical of the genus *Legionella*. Strain 214^T produced pink colonies on buffered charcoal-yeast extract agar. Ubiquinone Q-12 was the major quinone. Strain 214^T was serologically distinct from other legionellae as determined by a slide agglutination test. The results of DNA hybridization studies showed that strain 214^T (= ATCC 49655^T) is a member of a new *Legionella* species, *Legionella shakespearei*.

Members of the genus *Legionella* are ubiquitous inhabitants of freshwater environments (12, 18). These organisms flourish in natural habitats wherever water is present and also colonize engineered water systems, in particular hot- and cold-water services in large buildings and recirculating cooling water systems associated with central air-conditioning plants (11, 13, 23). The mode of transmission to humans is by inhalation of aerosols (2, 17) created by running water in taps or showers (9) and by sprays drifting from cooling towers and evaporative condensers (1, 10).

Legionellae are fastidious, require iron and cysteine, and are often found in symbiotic relationships with other microorganisms, such as protozoa, algae, cyanobacteria, and other bacteria (22, 24, 25). Legionellae can grow on microbial organic waste products, which enables them to survive and proliferate in biofilms (8).

To date, 51 serogroups representing 32 *Legionella* species have been described (3, 5, 20). Most of these organisms have been isolated from human clinical specimens from patients with pneumonia or a mild febrile disease, but some species and serogroups have not yet been implicated in causing disease. Characterization of *Legionella* species since the description of *Legionella pneumophila* has involved the use of both phenotypic methods (substrate utilization, serologic reactivity, cellular fatty acid composition, isoprenoid quinone content) and genotypic methods (restriction fragment length polymorphism analysis, DNA-DNA homology). The biochemical approach to phenotypic distinctions has at best allowed only grouping of species, although recent work has shown that phenotypic differentiation of *Legionella* species on the basis of substrate utilization patterns is possible (16). Because serologic reactivity, the most widely used method for species identification, may be unreliable as a means of identifying strains, genotypic methods of analysis should be used if the identity of a *Legionella* species needs to be established (19).

In this paper we describe an isolate (strain 214^T [T = type strain]) of a new species that was obtained from water taken from an evaporative cooling tower. This organism is unique in that it is the first recognized pigmented *Legionella* species. Strain 214^T forms light pink colonies when it is cultured on buffered charcoal-yeast extract (BCYE) agar. The name *Legionella shakespearei* is proposed for this new species.

MATERIALS AND METHODS

Isolation procedure. A 1-liter sample of water was taken from a cooling tower (Houseman Ltd. laboratory reference no. 86030153) at a site in Stratford-upon-Avon for routine testing. The sample was processed by using protocols developed by Houseman Ltd. in conjunction with the Public Health Laboratory Service. Bacteria from the 1-liter sample were concentrated on membrane filters (diameter, 142 mm; pore size, 0.45 μm). Each filter was then aseptically comminuted with scissors and suspended in a tube containing 20 ml of sterile distilled water. The tube was tightly capped and shaken vigorously by hand for 1 min to suspend the microorganisms.

A 1-ml sample of the concentrated suspension was heat treated at 50°C for 30 min. Another 10 ml of the suspension was concentrated further by centrifugation at 4,000 rpm for 30 min, and 9 ml of the supernatant was removed and discarded; the remaining 1 ml was acidified with 9 ml of KCl-HCl buffer to pH 2.2 (4) and allowed to stand for 5 min. Portions (0.1 ml) of the original and heat- and acid-treated suspensions were spread over the surfaces of three separate BCYE agar plates supplemented with glycine (3 g/liter), polymyxin B sulfate (79,200 IU/liter), vancomycin (5 mg/liter), and cycloheximide (80 mg/liter).

The plates were incubated at 35°C in the presence of high relative humidity for 7 days. At 2-day intervals, the plates were examined with a stereoscopic dissecting microscope for colonies that had the ground-glass appearance that is typical of legionellae. A selection of such colonies was subcultured onto BCYE agar and nutrient agar plates. Isolates which grew on BCYE agar but not on nutrient agar

* Corresponding author.
† Present address: BioConsultancy Services, Chalvey, Slough SL1 2TW, United Kingdom.
‡ Present address: Thames Water Utilities, Spencer House Laboratory, Reading, Berkshire RG2 0JN, United Kingdom.

after 48 h of incubation at 35°C were presumptively identified as *Legionella* species.

The serologic reactivity of the *Legionella*-like organisms was tested by using the indirect immunofluorescence technique. Rabbit antisera against *Legionella* species were obtained from the Division of Microbiological Reagents and Quality Control, Central Public Health Laboratory, Colindale, London, United Kingdom, and the sheep conjugate was obtained from the Wellcome Foundation, London, United Kingdom.

Monovalent antisera against *L. pneumophila* serogroups 1 through 13, *L. feeleii* serogroups 1 and 2, *L. hackeliae* serogroups 1 and 2, *L. israelensis*, *L. jordanis*, *L. jamestowniensis*, *L. longbeachae* serogroups 1 and 2, *L. micdadei*, *L. oakridgensis*, *L. sainthelensi*, *L. santicrucis*, *L. gormanii*, *L. rubrilucens*, and *L. steigerwaltii* were tested with the presumptive *Legionella* isolates by using whole cells as the antigen.

A representative isolate (strain 214^T), which did not react with the antisera listed above, was sent to the Public Health Laboratory Service facilities at Oxford and Porton Down, United Kingdom, and subsequently to the Centers for Disease Control, Atlanta, Ga., for further study.

Growth and biochemical tests. Strain 214^T was grown on BCYE agar for all of the tests except the tests for autofluorescence and L-cysteine requirement. The buffer was omitted from the medium when autofluorescence was determined, and L-cysteine was omitted when the L-cysteine requirement was determined (26). The biochemical test methods which we used have been described previously (7).

Slide agglutination test. Strain 214^T was tested with antisera against all previously characterized *Legionella* species (n = 32) and serogroups (n = 51) (3, 5, 20). Antiserum to strain 214^T was prepared and tested as previously described (21).

Cellular fatty acids and isoprenoid quinones. Strain 214^T cells were harvested from BCYE agar plates after 48, 72, or 96 h of incubation at 35°C. The growth from one plate was harvested with approximately 1 ml of distilled water and placed in a screw-cap tube (13 by 100 mm). The cellular lipids were saponified with 1 ml of 15% NaOH in 50% aqueous methanol, and the liberated fatty acids were converted to fatty acid methyl esters as described previously (14). Cells were also hydrolyzed by heating them at 80°C for 16 h with 2.5 ml of a mixture containing methanol, toluene, and H_2SO_4 (30:15:1), and the resulting fatty acid methyl esters were extracted with 2 ml of hexane, followed by 2 ml of a 1:1 mixture of ether and hexane. The combined organic layers were concentrated to 0.6 ml with N_2 and washed with buffer (14). Fatty acid methyl ester samples were analyzed by capillary gas-liquid chromatography, and the identities of the acids were confirmed as described previously (14). The isoprenoid quinone content of strain 214^T was determined as described previously (14).

DNA methods. The guanine-plus-cytosine content of strain 214^T DNA was determined spectrophotometrically by using the method of Marmur and Doty (15). The methods which we used for isolation and purification of DNA have been described previously (6). DNAs from strain 214^T and from the species indicated in Table 1, footnote *a*, were labeled enzymatically in vitro as described previously (7). Levels of DNA relatedness were determined by using the free-solution hydroxyapatite method performed at 60°C (6). We tested all of the previously described *Legionella* species except *L. anisa*, *L. rubrilucens*, *L. cherrii*, *L. steigerwaltii*, and *L. santicrucis* (7); each of these five species is at least 50% related to a species that was tested and that exhibited less than 10% relatedness to strain 214^T. Therefore, it was not necessary to test them directly against strain 214^T. *Legionella* species DNAs were obtained from the type strains in all cases.

TABLE 1. Levels of DNA relatedness between strain 214^T and other legionellae

Strain[a]	% Relatedness at 60°C to DNA from strain 214^T
L. shakespearei 214^T	100
L. jordanis BL-540	25
L. pneumophila Philadelphia 1	8
L. moravica 316-36[a]	8
L. fairfieldensis 1725-AUS-E	8
L. dumoffii NY-23	7
L. gormanii LS-13	7
L. bozemanii WIGA	6
L. longbeachae Long Beach 4	6
L. wadsworthii 81-716A	6
L. parisiensis PF-209C-C2	6
L. micdadei TATLOCK	5
L. feeleii WO-44C-C3	5
L. sainthelensi Mount Saint Helens 4	5
L. erythra SE-32A-C8	5
L. tucsonensis 1087-AZ-H	5
L. gratiana Lyon 8420412	5
L. hackeliae Lansing 2	4
L. cincinnatiensis 72-OH-H	4
L. oakridgensis Oak Ridge 10	3
L. maceachernii PX-1-G2-E2	3
L. jamestowniensis JA-26-G1-E2	3
L. spiritensis Mount Saint Helens 9	3
L. israelensis Bercovier 4	3
L. quinlivanii 1442-AUS-E	3
L. adelaidensis 1762-AUS-E	3
L. birminghamensis 1407-AL-H	1
L. brunensis 441-1	1

[a] In most experiments the labeled DNA which we used was strain 214^T DNA. The exceptions were the experiments in which we hybridized unlabeled strain 214^T DNA with labeled DNAs from *L. moravica* 316-36, *L. fairfieldensis* 1725-AUS-E, *L. tucsonensis* 1087-AZ-H, *L. gratiana* Lyon 8420412, *L. cincinnatiensis* 72-OH-H, *L. quinlivanii* 1442-AUS-E, *L. adelaidensis* 1762-AUS-E, *L. birminghamensis* 1407-AL-H, and *L. brunensis* 441-1.

RESULTS

A total of 17 *Legionella*-like colonies were isolated on the BCYE agar plate that was inoculated with the acid-treated concentrate obtained from the water sample (Houseman Ltd. laboratory reference no. 86030153); the only observable difference among these colonies was that some of the colonies which had the ground-glass appearance that is typical of legionellae were light pink across the whole colony. Young colonies that were visible only under the dissecting microscope were also pink, suggesting that production of the pigment was not an age-related process. The pink colonies did not react with any of the antisera used in indirect immunofluorescence assays. Nonpigmented colonies were not identified.

Growth characteristics and biochemical tests. Strain 214^T was chosen for further study as a typical representative of the pink colonies. This organism required L-cysteine and iron for growth; autofluorescence was not observed when it was exposed to long-wavelength (365-nm) UV light. Microscopically, the cells were gram-negative bacilli with a single polar flagellum. Strain 214^T was positive in tests for catalase,

gelatinase, and β-lactamase and weakly positive in tests for oxidase. It was negative in tests for glucose fermentation, browning of tyrosine-supplemented agar, hippurate hydrolysis, nitrate reduction, and urease production.

Slide agglutination test. No agglutination was observed when strain 214^T was tested with antisera to the 32 previously recognized species and 51 serogroups of the genus *Legionella*. Antiserum prepared against strain 214^T, at the optimal working dilution of 1:8, gave a 4+ agglutination reaction with its homologous antigen (whole cells) and was negative with all other *Legionella* antigens.

Cellular fatty acids. Like other legionellae, strain 214^T contained large amounts (>50%) of branched-chain acids and only trace to small amounts of hydroxy acids. The major acids were 14-methylpentadecanoic acid ($C_{i\text{-}16:0}$) (29%), hexadecenoate ($C_{n\text{-}16:1}$) (16%), hexadecanoate ($C_{n\text{-}16:0}$) (11%), 12-methyltetradecanoate ($C_{a\text{-}15:0}$) (10%), and 12-methyltridecanoate ($C_{i\text{-}14:0}$) (9%). Other acids detected were pentadecenoate ($C_{n\text{-}15:1}$) (5%), pentadecanoate ($C_{n\text{-}15:0}$) (4%), heptadecanoate ($C_{n\text{-}17:0}$) (4%), 14-methylhexadecanoate ($C_{a\text{-}17:0}$) (3%), 13-methyltetradecanoate ($C_{i\text{-}15:0}$) (2%), 15-methylhexadecanoate ($C_{i\text{-}17:0}$) (1%), octadecanoate ($C_{n\text{-}18:0}$) (1%), and 3-hydroxy-12-methyltridecanoate ($C_{i\text{-}3\text{-}OH\text{-}14:0}$) (1%). Essentially identical profiles were obtained with five separate strain 214^T cell preparations, as well as with cells processed after 48, 72, and 96 h of incubation. Acid-hydrolyzed cells yielded increased amounts of $C_{i\text{-}3\text{-}OH\text{-}14:0}$ and small amounts (1 to 2%) of three hydroxy acids ($C_{3\text{-}OH\text{-}16:0}$, $C_{3\text{-}OH\text{-}17:0}$, and $C_{3\text{-}OH\text{-}18:0}$) that were not detected in saponified cells.

Isoprenoid quinones. Strain 214^T contained ubiquinones with 10, 11, 12, and 13 isoprene units (Q-10, Q-11, Q-12, Q-13, respectively) in the polyprenoid side chain. Q-12 was the major ubiquinone and was present at a concentration which was approximately three times the concentrations of Q-10, Q-11, and Q-13.

DNA studies. The guanine-plus-cytosine content of DNA from strain 214^T was 45.5 mol%. DNA from strain 214^T was 25% related to DNA from *L. jordanis* and less than 10% related to DNAs from other *Legionella* species.

DISCUSSION

Strain 214^T was identified as a member of the genus *Legionella* on the basis of its cultural and biochemical characteristics, its cellular fatty acid composition, and its isoprenoid quinone content. Strain 214^T did not react with antiserum to any of the 51 previously described serogroups of *Legionella* species. This meant that strain 214^T was a member of either a new species or a new serogroup of an existing species. We confirmed that this organism was a member of a new species on the basis of its levels of DNA relatedness to previously described species (25% or less).

The species represented by strain 214^T can be identified serologically, by a combination of its cellular fatty acids and isoprenoid quinone content, and by DNA hybridization. The production of the pink pigment by this new species, which persisted when the organism was subcultured, should aid in its identification.

The presence of $C_{i\text{-}16:0}$ as the major cell wall acid distinguishes strain 214^T from all other legionellae except *L. pneumophila*, *L. oakridgensis*, and *L. spiritensis* (14). Strain 214^T contains smaller amounts of $C_{n\text{-}18:0}$ and larger amounts of $C_{i\text{-}14:0}$ and $C_{a\text{-}15:0}$ than *L. oakridgensis*. It differs from *L. pneumophila* and *L. spiritensis* by the presence of $C_{i\text{-}15:0}$ and the absence of a branched-chain monounsaturated 16-carbon acid ($C_{i\text{-}16:1B}$) and a 17-carbon cyclopropane acid ($C_{n\text{-}17:0cyc}$).

Other than strain 214^T, only *L. pneumophila*, *L. erythra*, and *L. rubrilucens* contain Q-12 as the major quinone. These species are differentiated from strain 214^T by the absence (or presence of only trace amounts) of Q-10 (14).

We propose the name *Legionella shakespearei* for the *Legionella* species represented by strain 214^T. The species description is given below.

Description of *Legionella shakespearei* sp. nov. *Legionella shakespearei* (shakes.pear. e′ i. N. L. gen. n. *shakespearei*, named after the playwright William Shakespeare because the organism was isolated in Stratford-upon-Avon) is a weakly oxidase-positive, catalase-positive, β-lactamase-positive, gelatinase-positive, gram-negative bacillus that has a single polar flagellum and does not autofluoresce. Colonies grown on BCYE agar are light pink across the entire colony and have a ground-glass appearance. The organism requires L-cysteine and does not ferment glucose, brown tyrosine-supplemented agar, hydrolyze hippurate, reduce nitrate, or produce urease. $C_{i\text{-}16:0}$ is the predominant cell wall fatty acid; other major fatty acids are $C_{n\text{-}16:1}$, $C_{n\text{-}16:0}$, $C_{a\text{-}15:0}$, and $C_{i\text{-}14:0}$. The predominant quinone has 12 isoprene units in the polyprenoid side chain; other major quinones have 10, 11, or 13 side units. The type strain is strain 214 (= ATCC 49655), which has a guanine-plus-cytosine content of 45.5 mol%.

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