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Legionella

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Edited by

Carmen Buchrieser

Biology of Intracellular Bacteria, Institut Pasteur, Paris, France

Hubert Hilbi

Max von Pettenkofer Institute, Ludwig-Maximilians-University, Munich, Germany

Editors

Carmen Buchrieser
Biology of Intracellular Bacteria
Institut Pasteur
Paris, France

Hubert Hilbi
Max von Pettenkofer Institute
Ludwig-Maximilians-University
Munich, Germany

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Cover photograph of cells of *Dictyostelium* infected with *Legionella pneumophila* (in red) by Mariella Lomma.

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***Legionella*: A Versatile Model to Study Host–Pathogen Interactions**

Hubert Hilbi and Carmen Buchrieser

1. Summary

Legionnaires' disease is a severe form of pneumonia that can occur in epidemics of several hundred cases. The atypical pneumonia is characterized by a high mortality rate and affects primarily elderly and immunocompromised individuals. The causative agent of Legionnaires' disease, the amoebae-resistant environmental bacterium *Legionella pneumophila*, was identified 35 years ago in the aftermath of an epidemic of pneumonia that spread during a convent of the American Legion. Intense research on *L. pneumophila* over the last two decades provided insights into the evolution, ecology, epidemiology, and pathogenesis of this opportunistic pathogenic, facultative intracellular bacterium. This book assembles state-of-the-art protocols and methods to study *L. pneumophila* and other clinically relevant *Legionella* species. Specifically, the volume includes chapters on identification and typing of *Legionella* spp., bacterial physiology and genetics, cellular pathogen–host interactions, biochemical functions of effector proteins, host response and immunology, as well as genomics and transcriptomics. An in-depth understanding of the ecology and virulence of *Legionella* spp. will contribute to an efficient and sustainable elimination of the bacteria from anthropogenic water systems and might lead to the development of novel therapeutics.

2. Introduction

Legionella pneumophila was identified 35 years ago in the aftermath of an epidemic of pneumonia that spread during a convent of the American Legion in Philadelphia, USA (1, 2). Accordingly, the severe and atypical pneumonia with a high mortality rate of 15% was termed “Legionnaires' disease.” Legionnaires' pneumonia primarily affects elderly and immunocompromised individuals (3), two demographic groups that are steadily increasing in size, particularly in developed countries.

Shortly after its identification, *L. pneumophila* was characterized as a ubiquitous waterborne bacterium that parasitizes free-living environmental protozoa (4). This seminal discovery paved the way for the concept that the ecology and pathogenesis of *L. pneumophila* are intimately linked. *L. pneumophila* is able to grow intracellularly in a range of environmental protozoa, including *Acanthamoeba*, *Hartmannella*, or *Naegleria* spp. (5), as well as—at least under laboratory conditions—in the social soil amoeba *Dictyostelium discoideum* (6, 7). It is believed that the evolutionary adaptation of *Legionella* spp. to bactericidal amoebae did select for virulence traits, which allow the bacteria to also infect and replicate in mammalian cells, in particular alveolar lung macrophages (8–10). Macrophage resistance is a prerequisite for the development of Legionnaires' disease.

Further highlighting the importance of bacterial ecology for pathogenesis of *Legionella* spp., an infection occurs solely through the inhalation of bacteria-laden aerosols, produced by air conditioning systems, cooling towers, showers, or other technical devices; person-to-person transmission has never been observed (3, 11). More than 50 *Legionella* spp. have been identified to date, and approximately half of these species have been linked to clinical manifestations of Legionnaires' disease. However, with distinct geographical incidence patterns, *L. pneumophila* (serogroup 1) or *L. longbeachae* (serogroup 1) are responsible for the vast majority of clinical cases in Europe and the USA, or in Australia and New Zealand, respectively (3).

L. pneumophila is by far the best-studied *Legionella* species. Intense research on *L. pneumophila* over the last two decades focused on the evolution, ecology, epidemiology, and pathogenesis of this opportunistic pathogen. This book covers protocols and methods to address the identification and typing of *Legionella* spp., bacterial physiology and genetics, cellular pathogen–host interactions, biochemical functions of effector proteins, host response and immunology, as well as genomics and transcriptomics.

3. Identification and Typing of *Legionella* in the Environment and During Disease

Legionella spp. are ubiquitous, waterborne environmental bacteria that only accidentally infect and sicken people. *Legionella*-contaminated aerosols can spread over wide distances from the contaminated source and infect people living in a diameter of several kilometers (12). Thus, the isolation and identification of *Legionella* from the environment is crucial for outbreak investigations and epidemiological studies. *Legionella* spp. can be isolated

not only from technical and natural water sources but also from plant material, compost, and potting soil (13–15).

Various culture-independent assays for the detection and typing of *L. pneumophila* and other *Legionella* spp. in environmental and clinical samples have been developed, including urinary antigen detection, monoclonal antibody subtyping, quantitative PCR amplification, fluorescent in situ hybridization (FISH), and genotyping (13, 16–19). Moreover, mass spectrometry is increasingly used for the identification of *Legionella* spp. (20). Finally, in culture-negative samples amoebic coculture has been reported to recover *Legionella* spp. (21).

4. Physiology and Genetics of *Legionella*

Legionella spp. are fastidious organisms. The bacteria can be grown in complex medium, buffered charcoal yeast extract (BCYE) medium being the most commonly used (22), as well as in chemically defined medium (CDM) (23). CDM has been used to study the production of the *L. pneumophila* siderophore legiobactin (24) and the secreted brown pyomelanin pigment (25). Recently, the metabolic pathways and fluxes employed by extra- or intracellular *L. pneumophila* were analyzed by isotopologue profiling experiments using ^{13}C -labeled compounds (26). The incorporation of label into amino acids or other metabolites allows studying catabolic pathways in detail.

Like many other bacteria, *L. pneumophila* is naturally competent, i.e. “naked” DNA is taken up. Natural competence of *L. pneumophila* is dependent on type IV pili (27) and represents an important mechanism of horizontal gene transfer and evolution of *Legionella* spp. (28). UV radiation and genotoxic fluoroquinolone antibiotics induce competence of *L. pneumophila* (29). Natural competence can be used in the laboratory to transform *Legionella* spp. and construct mutants. Unmarked gene deletions in the genome of *L. pneumophila* are generated by constructing a mutant allele in *E. coli* using λ -Red recombination, integrating the allele into the chromosome by natural transformation, and finally removing the selectable marker with the Flp site-specific recombinase (30).

In addition to a planktonic life style, *L. pneumophila* also forms and grows in biofilms. The formation and colonization of biofilms can be analyzed with multi-species or mono-species bacterial aggregates formed in complex AYE medium (31, 32) or in chemically defined medium (33). To secrete cell envelope components into the environment, *L. pneumophila* produces and releases outer membrane vesicles (OMVs). These vesicles are enriched from the

supernatant of *L. pneumophila* liquid cultures by differential centrifugation steps. Isolated OMVs can be analyzed for their molecular components by proteomics (34) and regarding their interactions with bacterial or eukaryotic cells (35, 36).

5. Cellular Microbiology of *Legionella*

Within environmental amoebae and mammalian phagocytes *L. pneumophila* forms a distinct membrane-bound replicative niche, the *Legionella*-containing vacuole (LCV). Formation of LCVs is a complex and robust process that requires the Icm/Dot type IV secretion system (T4SS) and involves more than 250 “effector” proteins (37, 38). The effector proteins are translocated into host cells, where they subvert signal transduction and vesicle trafficking pathways.

A plethora of techniques have been adopted to study on a cellular level the interactions of *Legionella* spp. with protozoan and metazoan phagocytes. Flow cytometry can be used to analyze uptake, intracellular replication, and cytotoxicity of *L. pneumophila* wild-type and defined mutant strains (39, 40). For the investigation of host cell factors involved in LCV formation, gene silencing by RNA interference in *Drosophila melanogaster* phagocytic cells has been instrumental (41–43). *L. pneumophila* strains producing green or red fluorescent proteins are not impaired for virulence, and therefore, are useful tools to analyze infected host cells by flow cytometry or fluorescence microscopy (44, 45). Antibodies against host or bacterial factors, or ectopically produced proteins fused to fluorescent proteins allow the spatiotemporal analysis of LCV formation and intracellular replication of *Legionella* spp. (39, 46, 47). Using live cell microscopy and GFP fusions of host proteins, the dynamics of LCV formation can be studied in real time (48). Finally, (immuno-gold) electron microscopy provides a superior resolution to analyze the morphology and ultrastructure of *Legionella* and *Legionella*-infected eukaryotic cells (49, 50).

Recently, intact LCVs were purified using two different approaches. One method exploits the fact that some Icm/Dot-translocated effectors specifically decorate the LCV membrane (43, 51). Here, the LCVs are enriched in a first step by immuno-magnetic separation using an antibody against the effector localizing to the LCV, followed by further purification by density gradient centrifugation. In another protocol LCVs were enriched by density gradient centrifugation after selective removal of mitochondrial and lysosomal contamination (52). The purified LCVs can be characterized by proteomics and other biochemical approaches.

L. pneumophila strains lacking a functional Icm/Dot T4SS do not form LCVs, are degraded in acidic phagolysosomes, and are avirulent (53, 54). In 1998, the Icm/Dot T4SS was identified as a key *L. pneumophila* virulence factor. However, after that it still took 4 years for the first Icm/Dot substrate to be discovered and characterized as an effector protein (46). To date, 275 different Icm/Dot substrates have been identified by various approaches and experimentally verified as Icm/Dot-translocated proteins (55, 56). The subcellular localization of Icm/Dot-translocated proteins in host cells provides insight into their function and is defined by co-staining with host markers. To analyze their localization within eukaryotic cells, Icm/Dot substrates can be ectopically expressed as fluorescent fusion proteins in host cells or overexpressed in *L. pneumophila* as epitope-tagged proteins that are translocated upon infection (57, 58).

6. Biochemical Assays to Study *Legionella* Effectors and Enzymes

Only few of the more than 250 different Icm/Dot substrates have been characterized biochemically, and the function of most of these proteins is unknown. Similar to other intracellular bacteria, the *L. pneumophila* effector proteins that have been studied mimic and/or exploit eukaryotic proteins to manipulate or subvert host cell signaling or trafficking pathways (38). A number of *L. pneumophila* effector proteins including LubX and AnkB show ubiquitin ligase activity (59–62) or glycosyltransferase activity (63). Other *L. pneumophila* effectors target the host small GTPases Arf1 or Rab1 (38). These effectors act as guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), or they covalently modify the small GTPases with adenosine monophosphate (AMPylation) or phosphocholine (phosphocholination). De-AMPylation or de-phosphocholination is catalyzed by distinct Icm/Dot substrates.

Type II-secreted or type IV-translocated *L. pneumophila* effectors also target the host cell's lipid metabolism. Several class A phospholipases (PLA) are implicated in intracellular replication and pathogenesis of *L. pneumophila* (64). A PLA termed PlaB is a cell-associated hemolytic phospholipase with a catalytic serine-aspartate-histidine triad (65). PlaB hydrolyzes the eukaryotic membrane lipid phosphatidylcholine, indicating a role for the enzyme in bacterial virulence. Moreover, *L. pneumophila* produces a number of Icm/Dot substrates that selectively bind host phosphoinositide (PI) lipids on the LCV membrane (66, 67). The effectors SidC (39, 57) and SidM (42) bind phosphatidyl-4-phosphate (PtdIns(4)P) and promote the interaction of LCVs with the ER and the secretory vesicle trafficking pathway, respectively. *L. pneu-*

mophila PI-binding effectors can be characterized using PI-coupled beads, protein–lipid overlay assays and ectopic production in eukaryotic cells.

Finally, *Legionella* spp. produce a peculiar, noninflammatory lipopolysaccharide (LPS), which is the molecular basis of serogroup classification (68). The lipid A moiety of *L. pneumophila* LPS is very hydrophobic due to its acylation by long chain fatty acids, and the O-antigen consists of homopolymeric legionaminic acid. *L. pneumophila* LPS is an immuno-dominant antigen, and LPS phase variation has been implicated in the bacterial life cycle and virulence (69, 70).

7. Immunity and Host Response Against *Legionella*

Legionella spp. are versatile microorganisms that colonize a variety of environmental niches, including predators such as protozoa and nematodes that feed on bacteria. These nonmammalian hosts are infected by *L. pneumophila*, and therefore, represent suitable models to investigate bacterial virulence traits and pathogenesis (7, 71). Ciliates and amoeba of the genera *Tetrahymena*, *Hartmanella*, and *Acanthamoeba* play an important role in the pathogenicity, ecology, and evolution of *Legionella* spp. (72–75). These protozoa contribute to the protection, amplification, and spread of *Legionella* (11), and they might represent an “evolutionary crib” for horizontal gene transfer and the acquisition of virulence traits of *Legionella* and other protozoa-resistant human pathogens (8, 9).

Even though presumably not a natural host of *Legionella* spp., the social soil amoeba *Dictyostelium discoideum* has emerged over the past decade as a powerful model to analyze LCV formation and *L. pneumophila* virulence (6, 76). *D. discoideum* amoeba are easily cultivated axenically, and a high-quality genome sequence, as well as excellent molecular genetic, biochemical, and cell biological tools are available (39, 77, 78). Noteworthy, many defined deletion mutants of this haploid amoeba have been produced, and the production of fluorescence-tagged proteins allows investigating in real time the dynamics of *L. pneumophila* uptake, LCV formation, and intracellular replication (48).

Recently, the environmental nematode *Caenorhabditis elegans* was established as an infection model for *L. pneumophila*. The bacteria colonize and grow in a noninvasive and Icm/Dot-independent manner in the intestinal tract of *C. elegans*, leading to the death of the host (79, 80). The susceptibility of nematodes to *L. pneumophila* was found to be regulated by innate immune signaling pathways involving p38 mitogen-activated protein kinase and insulin/insulin growth factor-1 receptor. Interestingly, *L. pneumophila* is

released from the nematodes in a cyst-like form resembling the “mature intracellular form” (MIF) observed in nonphagocytic mammalian cells. Therefore, the worms might serve as an environmental reservoir for the dissemination of the pathogen.

L. pneumophila preferentially infects environmental and immune phagocytic cells, yet upon (inefficient) infection of nonphagocytic mammalian cells, the invasion process and morphological aspects of the bacterial developmental cycle can be studied (81). At late infection stages, *L. pneumophila* differentiates into a resilient, cyst-like and highly infectious form termed MIF, which contains poly- β -hydroxybutyrate (PHB) storage granules (82).

Macrophages play a pivotal role in initiating the host response to *Legionella* infection, triggering inflammation and promoting pathogenesis. These leukocytes produce caspase-1, which by processing the cytokines pro-IL-1 β and pro-IL-18 to their mature form is a critical factor in the innate immune response to *L. pneumophila*. Caspase-1 activation by *L. pneumophila* occurs in an “inflammasome” complex that contains as an adapter protein either Asc or the Nod-like pattern recognition receptor (NLR) protein Ipaf (Nlrc4) (83, 84). Activation of the inflammasome restricts *L. pneumophila* replication (85, 86). The assembly of the Ipaf inflammasome is triggered by direct binding of flagellin to Naip5 (also known as Birc1e) (87), an NLR protein that determines *L. pneumophila* replication in mouse macrophages (88–90).

Mouse models of Legionnaires’ disease faithfully mimic the human pathology and acute inflammation triggered by *L. pneumophila* infection. The A/J mouse strain is permissive for *L. pneumophila* infection, since it harbors a Naip5/Birc1e allele that does not recognize flagellin. C57BL/6 mice and the many genetically modified strains generated in this genetic background can be used to study infection with non-flagellated *L. pneumophila* strains. Upon pulmonary infection, innate immune cells such as neutrophils quickly invade the lung and clear the infection within days depending on IFN γ produced by natural killer cells (91).

The guinea pig intratracheal model was the first animal model of Legionnaires’ disease to be established (92). This pneumonia model provided evidence that *L. pneumophila* does not directly spread among mammalian hosts (93), and it has been used to assess the relative virulence of *L. pneumophila* mutant strains (94). Finally, while human susceptibility to Legionnaires’ disease is largely determined by predisposing illness, life style, and age, genetic factors may also play a role. Toll-like receptor (TLR) 4 and 5 are associated with susceptibility to Legionnaires’ disease (95, 96). However, a comprehensive understanding of genetic susceptibility factors remains an elusive and important goal.

8. Genomics, Transcriptomics, and Bioinformatics of *Legionella*

Genomics, comparative genomics, functional genomics, and related technologies helped substantially to unravel the molecular basis of evolution, pathogenesis, and phenotypic differences among different *Legionella* species, *L. pneumophila* strains, and clones. The elucidation and publication of the first complete genome sequences of *L. pneumophila* and *L. longbeachae* isolates paved the way for major breakthroughs in understanding the biology of these organisms (97–100). The genome sequences uncovered potential virulence genes and identified eukaryotic-like proteins as a particular feature of *Legionella*, witnessing the long coevolution of the bacteria with protozoa (98). Work of many different groups around the world has now shown that these eukaryotic-like proteins are major players in the process of host function subversion by *L. pneumophila* to its advantage (10, 38, 101).

The availability of the genome sequences allowed constructing whole genome microarrays to study *L. pneumophila* gene expression on the genome level. Such microarrays were used to analyze the gene expression program of *L. pneumophila* at different time points during intracellular growth in *A. castellanii*, showing distinct gene expression patterns during the replicative and transmissive phase (102). Moreover, the gene expression of *L. pneumophila* in human macrophages was analyzed with microarrays and an RNA amplification procedure called selective capture of transcribed sequences, which detects small amounts of mRNA from low numbers of intracellular bacteria (103). This study showed that in macrophages genes involved in catabolism of glycerol or amino acid biosynthetic pathways leading to L-arginine, L-histidine, or L-proline, as well as many amino acid and iron uptake systems were induced.

Recently, the advent of next generation sequencing (NGS) has significantly changed biological research and has opened exciting new possibilities allowing, for example, to (1) study entire bacterial communities, (2) analyze the dynamics and evolution of bacterial populations, (3) characterize the entire bacterial transcriptome under different conditions, or (4) identify binding partners of DNA- or RNA-binding proteins by sequencing (104, 105). In genomics and comparative genomics, NGS has been applied to the study of different *L. pneumophila* or *L. longbeachae* strains to identify genetic differences and horizontal gene transfer (28, 97, 106, 107). However, an analysis by NGS of *L. pneumophila* gene expression in *A. castellanii* has been reported only once to date (108). Thus, many different conditions remain to be analyzed to get a complete picture of the transcriptional changes allowing *L. pneumophila* to adapt to diverse intra- and extracellular environments.

Furthermore, the targets of major *L. pneumophila* regulatory proteins, such as the DNA-binding regulators LetA or RpoS, the RNA-binding regulators Hfq or CsrA, or the response regulator LqsR (40) also await an analysis by NGS on a genome-wide level.

Much of the power of the different genomics methods is based on the development of high-performance bioinformatics tools. The recent progress made in bioinformatics also led to the development of improved predictive tools. One burning question in the field of *Legionella* research is the identification of secreted proteins that promote intracellular bacterial replication and disease in humans. The major secretion system delivering proteins to the host cell is the Icm/Dot T4SS (109, 110). Machine learning algorithms trained on features defined for known Icm/Dot substrates identified 40 new *L. pneumophila* effectors. These putative Icm/Dot substrates were experimentally confirmed, proving the power of the bioinformatics approach (111). NGS applied to DNA and RNA sequencing and to ChIPseq and RIPseq, together with novel developments in bioinformatics, are revolutionizing our understanding of the complexity, plasticity, and regulation of *Legionella* genomes.

9. Conclusions and Perspectives

During thousands of years of coevolution with aquatic protozoa, the environmental bacteria *L. pneumophila* and *L. longbeachae* have developed a vast array of secreted proteins, allowing them to exploit a wide variety of host functions to replicate intracellularly. Genetic diversity and plasticity has led to the adaptation to diverse environments and conditions, and distinct regulatory mechanisms, many of which likely uncharacterized, allow the spatiotemporal regulation of effector proteins and virulence factors. Horizontal gene transfer among bacteria and from eukaryotes to *L. pneumophila*, as well as recombination between strains, allowed the evolution of predominant disease clones that might prevail only within a relatively short period of time. Thus, exact typing and constant surveillance is necessary to prevent and contain human disease. The many different methods described in this book are all specifically adapted and developed for the study of distinct features of *L. pneumophila*, and have already led to exciting discoveries. Most of these techniques can also be applied to the study of other bacterial pathogens, in particular intracellular pathogens like *Shigella*, *Salmonella*, *Listeria*, or *Mycobacterium*. Thus, this book will be useful not only for research groups studying *Legionella* but also for a broader scientific community studying the epidemiology, typing, physiology, pathogenesis, immunity, genetics, and evolution of other bacterial pathogens.

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Contributors

ZACHARY D. ABBOTT • *Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA*

SHARON G. BERK • *Center for Management, Utilization and Protection of Water Resources, Tennessee Technological University, Cookeville, TN, USA*

WILLIAM R. BERRINGTON • *Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA*

SALVATORE BOZZARO • *Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy*

ANN KAREN C. BRASSINGA • *Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada*

ANDREW BRYAN • *Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA*

CARMEN BUCHRIESER • *Biology of Intracellular Bacteria, Institut Pasteur, Paris, France CNRS UMR 3525, Paris, France*

CHRISTOPHER L. CASE • *Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven CT, USA*

XAVIER CHARPENTIER • *Unité de Microbiologie, Adaptation et Pathogénie, Villerubanne, France*

CHRISTA H. CHATFIELD • *Department of Microbiology and Immunology, Northwestern University Medical School, Chicago, IL, USA*

NICHOLAS P. CIANCIOOTTO • *Department of Microbiology and Immunology, Northwestern University Medical School, Chicago, IL, USA*

SHANDRA R. DAY • *Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, VA, USA*

DENNISE A. DE JESÚS • *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*

GHISLAINE DESCOURS • *National Reference Center of Legionella, Centre de Biologie Est, Hospices Civils de Lyon, Lyon, France; Université de Lyon, Lyon, France*

STEPHANIE DOLINSKY • *Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany*

PAUL H. EDELSTEIN • *Clinical Microbiology Laboratory, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*

LUDWIG EICHINGER • *Medical Faculty, Center for Biochemistry, University of Cologne, Cologne, Germany*

WOLFGANG EISENREICH • *Lehrstuhl für Biochemie, Technische Universität München, Garching, Germany*

JEROME ETIENNE • *National Reference Center of Legionella, Centre de Biologie Est, Hospices Civils de Lyon, Lyon, France; Université de Lyon, Lyon, France*

- SEBASTIEN P. FAUCHER • *Faculty of Agricultural and Environmental Sciences, Department of Natural Resource Sciences, McGill University, Quebec, Canada*
- GARY FAULKNER • *Department of Microbiology and Immunology, Dalhousie University, Halifax, Canada*
- IVO FINSEL • *Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany*
- ANTJE FLIEGER • *Division of Bacterial Infections (FG11), Robert Koch-Institute, Wernigerode, Germany*
- GAD FRANKEL • *Division of Cell and Molecular Biology, Centre for Molecular Microbiology and Infection, Imperial College London, London, UK*
- NORMAN K. FRY • *Respiratory and Systemic Infection Laboratory, Health Protection Agency, London, UK*
- RAFAEL A. GARDUÑO • *Centre for Clinical Research, GEII Health Centres, Halifax, NS, Canada*
- CHRISTOPHE GINEVRA • *National Reference Center of Legionella, Centre de Biologie Est, Hospices Civils de Lyon, Lyon, France; Université de Lyon, Lyon, France*
- TIMOTHY G. HARRISON • *Respiratory and Systemic Infection Laboratory, Health Protection Agency, London, UK*
- ELIZABETH L. HARTLAND • *Department of Microbiology and Immunology, University of Melbourne, Melbourne, VIC, Australia*
- THOMAS R. HAWN • *Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA*
- YANN HÉCHARD • *Laboratoire de Chimie et Microbiologie de l'Eau, Université de Poitiers, Poitiers, France*
- JÜRGEN H. HELBIG • *Institute for Medical Microbiology and Hygiene, University of Technology, Dresden, Germany*
- KLAUS HEUNER • *Robert Koch-Institut, Working group "Cellular Interactions of Bacterial Pathogens", Berlin, Germany*
- HUBERT HILBI • *Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany*
- CHRISTINE HOFFMANN • *Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany*
- PAUL S. HOFFMAN • *Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, VA, USA*
- JULIANA I. HORI • *Department of Cell Biology, University of São Paulo, Ribeirao Preto Sao Paulo, Brazil*
- RALPH R. ISBERG • *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*
- JENS JÄGER • *Institut für Mikrobiologie, Technische Universität Braunschweig, Braunschweig, Germany*
- SOPHIE JARRAUD • *National Reference Center of Legionella, Centre de Biologie Est, Hospices Civils de Lyon, Lyon, France; Université de Lyon, Lyon, France*
- NICOLE JOLLER • *Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*
- CAROL JOSEPH • *Independent Consultant, Formerly of the Health Intection Agency, 61 Colindate Avenue, London NW9 SEQ, UK*

- ALINE KESSLER • *Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany*
- NATALIA A. KOZAK • *Centers for Disease Control and Prevention, Atlanta, GA, USA*
- TOMOKO KUBORI • *Research Institute for Microbial Diseases, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan*
- JOHN LEE • *Leegionella Ltd, Ruislip, UK*
- SUSANNE LEE • *Leegionella Ltd, Ruislip, UK*
- GERARD LINA • *National Reference Center of Legionella, Centre de Biologie Est, Hospices Civils de Lyon, Lyon, France; Université de Lyon, Lyon, France*
- CLARESSA E. LUCAS • *Centers for Disease Control and Prevention, Atlanta, GA, USA*
- CHRISTIAN LÜCK • *Institute for Medical Microbiology and Hygiene, University of Technology, Dresden, Germany*
- ZHAO-QING LUO • *Department of Biological Sciences, Purdue University, West Lafayette, IN, USA*
- AURELIE MOUSNIER • *Division of Cell and Molecular Biology, Centre for Molecular Microbiology and Infection, Imperial College of London, London, UK*
- HIROKI NAGAI • *Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
- TAMARA J. O'CONNOR • *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*
- ANNETTE OXENIUS • *Institute of Microbiology, ETH Zürich, Zürich, Switzerland*
- SOPHIE PÉCASTAINGS • *Faculté de Pharmacie, Laboratoire de Génie Chimique, BioSym Department, Université de Toulouse, Toulouse, France*
- BARBARA PERACINO • *Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy*
- EMILIE PORTIER • *Faculté de Pharmacie, Laboratoire de Génie Chimique, BioSym Department, Université de Toulouse, Toulouse, France*
- RODNEY M. RATCLIFF • *Microbiology and Infectious Diseases, Institute of Medical and Veterinary Science, SA Pathology, Adelaide, Australia; School of Molecular and Biomedical Science, University of Adelaide, Adelaide, Australia*
- CHRISTINE ROQUES • *Faculté de Pharmacie, Laboratoire de Génie Chimique, BioSym Department, Université de Toulouse, Toulouse, France*
- CRAIG R. ROY • *Section of Microbial Pathogenesis, Yale University School of Medicine, Boyer Center for Molecular Medicine, New Haven, CT, USA*
- TOBIAS SAHR • *Biologie des Bactéries Intracellulaires, Institut Pasteur, Paris, France; Paris, France*
- GIL SEGAL • *George S. Wise Faculty of Life Sciences, Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Ramat-Aviv, Israel*
- KATHLEEN SEIPEL • *Division of Bacterial Infections (FG11), Robert Koch-Institut, Wernigerode, Germany*
- HOWARD A. SHUMAN • *Department of Microbiology, Cummings Life Sciences Center, University of Chicago, Chicago, IL, USA*
- COSTI D. SIFRI • *Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia Health System, Charlottesville, VA, USA*

MICHAEL STEINERT • *Institut für Mikrobiologie, Technische Universität Braunschweig, Braunschweig, Germany*

MICHELE S. SWANSON • *Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA*

ANDRÉ N. TIADEN • *Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland*

ADAM J. VOGRIN • *Department of Microbiology and Immunology, University of Melbourne, Melbourne, VIC, Australia*

STEFAN S. WEBER • *Institute of Microbiology, ETH Zürich, Zürich, Switzerland*

STEPHEN WEBER • *Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany*

JONAS M. WINCHELL • *Centers for Disease Control and Prevention, Atlanta, GA, USA*

DARIO S. ZAMBONI • *Department of Cell Biology, School of Medicine Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Brazil*

WENHAN ZHU • *Department of Biological Sciences, Purdue University, West Lafayette, IN, USA*