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Legionella

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INTERACTION OF *LEGIONELLA PNEUMOPHILA* WITH *DICTYOSTELIUM DISCOIDEUM*

M. Steinert, S. Hägele, C. Skriwan, D. Grimm, M. Fajardo, K. Heuner, M. Schleicher, U. Hentschel, W. Ludwig, R. Marre, and J. Hacker

28

Several pathogens exhibit a considerable host range. *Legionella pneumophila*, for example, can infect various protozoa species, experimentally inoculated guinea pigs, and human macrophages, as well as epithelial cells (7, 9, 20). This suggests that there are common infection strategies regardless of the host (8). In addition, it has become apparent that certain aspects of the host defense are highly conserved during evolution (13, 17). Therefore important insights into *Legionella*-host interactions are expected from the use of well-characterized host models (3). One such model system is the haploid amoeba *Dictyostelium discoideum*. Vegetative cells of *Dictyostelium* feed on bacteria and upon starvation aggregate and differentiate into pluricellular fruiting bodies (16). Beside its amenability to genetic manipulation, *D. discoideum* expresses highly conserved cellular markers, and cell signaling pathways are well characterized. Moreover, the complete genome sequence will be available in the year 2002.

ESTABLISHMENT OF THE *DICTYOSTELIUM* MODEL SYSTEM

To evaluate whether *D. discoideum* is a suitable model system for studying *Legionella* pathogenicity, we compared the intracellular growth of different *Legionella* species in *Dictyostelium* with the established host model system *Acanthamoeba castellanii* (Table 1). We found that virulent *Legionella* species including *L. pneumophila* Corby, LLAP10, and *Sarcobium lyticum* are able to grow intracellularly in single-cell stages of *D. discoideum* and that infection results in host cell lysis. After 96 h of coculture, the inoculum of 10^3 cells/ml of these strains increased 150- to 1,500-fold, as measured by CFU. The increasing numbers of bacteria were the result of intracellular replication, since they were unable to grow in the cell culture medium. The avirulent strain *Legionella erythra* exhibited decreasing counts in *D. discoideum*. These results showed that the infection process parallels the infection of freshwater amoebae and macrophages (11).

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TABLE 1 Intracellular growth of *Legionella* in *Dictyostelium discoideum* and *Acanthamoeba castellanii*[a]

Strain (reference)	Phenotype	Intracellular growth in:	
		D. discoideum	*A. castellanii*
L. pneumophila Corby (21)	Patient isolate	Yes	Yes
L. pneumophila Corby KH3 (14)	FlaA-negative mutant	Attenuated	Attenuated
L. pneumophila Corby-1 (21)	Mip-negative mutant	Attenuated	Attenuated
L. pneumophila (ligA-) (6)	*lig*A-negative mutant	Avirulent	Avirulent
L. erythra (11)	Avirulent	Avirulent	Avirulent
LLAP10 (11)	Legionella-like amoebal pathogen	Yes	Yes
Sarcobium lyticum (11)	Obligate intracellular parasite of amoebae	Yes	Yes

[a] Abbreviations: FlaA, flagellin major subunit; Mip, macrophage infectivity potentiator; *ligA*, *Legionella pneumophila* infectivity gene A. For infection of *D. discoideum* and *A. castellanii* 5×10^5 host cells/ml were infected with 10^3 legionellae. After 0, 24, 48, 72, and 96 h of incubation, the bacterial numbers of CFU were determined by plating.

SUBCELLULAR ANALYSIS OF INFECTION AND TESTING OF MUTANTS

The subcellular analysis of the infection indicates that *Legionella* grows within membrane-bound vesicles of *Dictyostelium* (Fig. 1). In addition, the bacteria inhibit the fusion of phagosomes and lysosomes in this particular host system. Colocalization studies with green fluorescent protein (GFP)-tagged bacteria and antibodies directed against specific lysosomal markers (DdLIMP) revealed that the bacteria inhibit the phagolysosome fusion. These data suggest that the replicative phagosome in *Dictyostelium* exhibits important features characteristc for *Legionella* infections (11, 18).

Testing of various well-established *Legionella* mutants and their corresponding complementants in infection assays showed that *Dictyostelium* is a representative model system. *L. pneumophila* mutants that are unable to grow in amoebae and macrophages are also unable to grow in *Dictyostelium* (Table 1). The FlaA- and the Mip-negative mutant of *L. pneumophila* Corby revealed moderate growth defects and the *lig*A-negative mutant was severely impaired to grow intracellularly (5, 6, 12, 14, 15, 21). To examine host functions required for growth we also investigated defined *Dictyostelium* mutants. The infection of mutated host cells revealed that the profilin-minus phenotype had a slight positive effect on bacterial growth when compared with *Dictyostelium* wild-type cells. This observation is consistent with the finding that profilin-minus cells have a higher rate of phagocytosis (11).

DETECTION OF *DICTYOSTELIUM* IN THE ENVIRONMENT

Due to the occurrence of *Legionella* in wet soils and the fact that *Dictyostelium* feeds on bacteria by phagocytosis, it is conceivable that *Dictyostelium* represents a natural reservoir of *Legionella*. Therefore we surveyed the occurrence of *Dictyostelium* and other well-established host organisms in *Legionella*-positive environmental samples by culture and in situ hybridization with a fluorescence-labeled 16S rRNA probe that specifically detects *L. pneumophila* and two eukaryotic 18S rRNA probes that specifically detect *Dictyostelium* (DICT2) and *Hartmannella* (HART498) (Table 2) (10). Isolation and morphological characterization of potential host protozoa revealed that the genera *Acanthamoeba, Echinamoeba, Hartmannella, Platyamoeba, Saccamoeba, Thecamoeba*, and *Vexillifera* were present in various *Legionella*-positive water habitats. In situ hybridization confirmed the results of the morphological identification of environmental *Hartmannella* isolates. In addition, we were able to use amoeba-specific 18S rRNA probes and *Legionella*-specific 16S probes simultaneously to monitor the infection of *Hartmannella vermiformis* with *L. pneumophila* in vitro. They hybridized with the target strains and no cross-reactions with other strains were observed. The natural interaction of *Legionella* and *Dictyostelium* in the environment, however, remains to be confirmed. Since we were able to detect *Dictyostelium* in soil samples, future studies may show a colocalization with *Legionella* in these habitats.

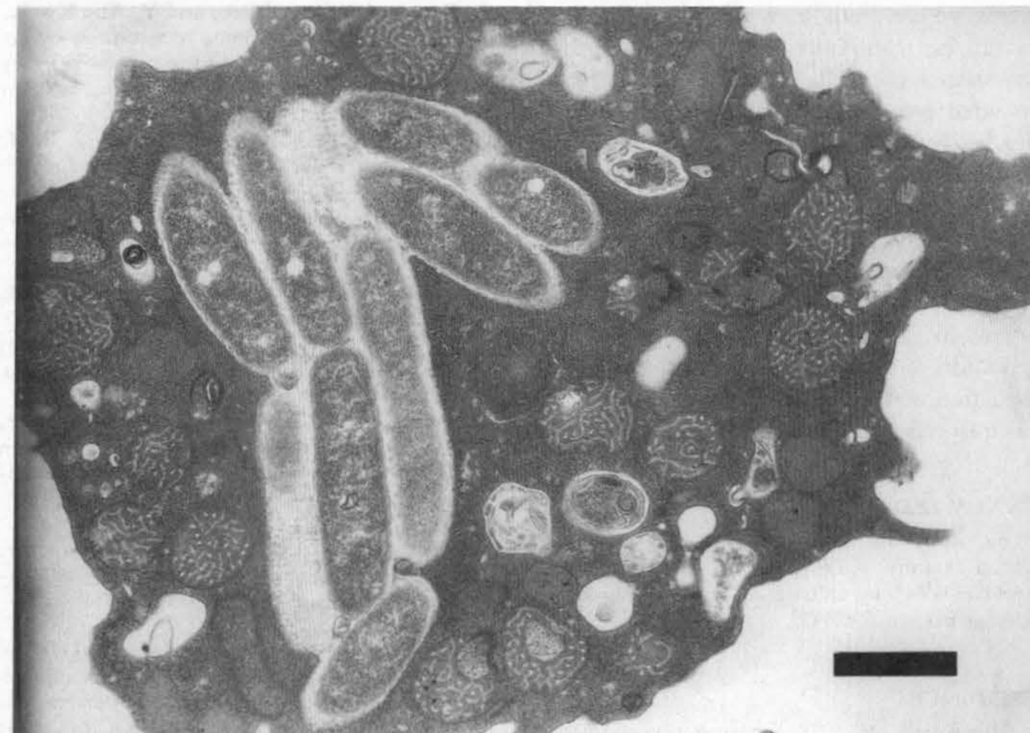

FIGURE 1 Transmission electron micrograph of *L. pneumophila* within a single vacuole of *D. discoideum* after 48 h of coincubation. Bar, 1 μm.

CONCLUSION

New methods to limit or prevent growth of *Legionella* within protozoan or human host cells will be based on the understanding of the factors that promote intracellular survival and growth (1, 2, 4). Insights are expected from

TABLE 2 Identification of *Legionella* spp., *Dictyostelium* spp., and *Hartmannella* spp. by fluorescence-labeled rRNA probes[a]

Samples	Hybridization with rRNA probe		
	LEG705[b]	DICT2[c]	HART498[c]
D. discoideum-Legionella coculture	+	+	−
Hartmannella spp.-*Legionella* coculture	+	−	+
Water samples[d]	+	−	+
Soil sample	−	+	n.d.

[a] The genus-specific probes have been developed on the basis of a comparative sequence analysis (ARB software environment for sequence data).
[b] Genus-specific 16S rRNA probe.
[c] Genus-specific 18S rRNA probe. n.d., not done.
[d] River and fountain water.

systems where both bacterial and host factors can be manipulated (11, 19). Since the *Dictyostelium-Legionella* interaction allows a two-sided genetic approach, our future strategies will rely on genetic mutational analysis of the pathogen and the host. Available molecular tools to manipulate the host are transformation with integrating and nonintegrating eukaryotic vectors, homologous recombination, antisense techniques, and restriction enzyme mediated integration (REMI). The application of these methods should allow the elucidation of the interaction of bacterial virulence factors with specific host targets.

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CHARACTERIZATION OF A 16-KILODALTON SPECIES-SPECIFIC PROTEIN OF *LEGIONELLA PNEUMOPHILA* PROMOTING UPTAKE IN AMOEBAE

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29

Legionella pneumophila is the major agent responsible for Legionnaires' disease. To identify proteins that participate in the interaction of *Legionella* and its host, we screened a genomic library of *L. pneumophila* strain Corby with anti-Corby antiserum. Briefly, rabbits were immunized with heat inactivated (15 min at 70°C) *L. pneumophila* strain Corby grown on solid medium. Immunization was done by intravenous (i.v.) injections, each 3×10^8 CFU without adjunvants, on days 1, 4, 7, 10, and 45, and serum was collected at day 55. To reduce cross-reactivity, the collected serum was absorbed to total cells of *Escherichia coli* DH5α harboring plasmid pUC19 either inactivated by heat or by formalin treatment. Construction of the genomic library was done according to Heuner et al. (5) with minor modifications. Chromosomal DNA of *L. pneumophila* strain Corby was partially digested with *Sau*3AI. Fragments of 1.0 to 4.0 kb were ligated into the *Bam*HI restriction site of vector pUC19 and transformed into *E. coli* DH5α. Replicates of recombinant clones were screened for reactivity with anti-Corby antiserum by immuno colony dot assays and reactive clones were further analyzed by Western blotting.

One recombinant clone expressed a protein with an apparent molecular mass of 16 kDa. This protein was designated protein P16. By sequence analysis, the corresponding open reading frame of 411 bp encoding a protein of 136 amino acids was identified (AC Z97066). The predicted molecular mass of 15.7 kDa was in good agreement with the size of protein P16 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Databank searches revealed no significant homology to previously published bacterial or eukaryotic genes and proteins.

To facilitate protein isolation, an N-terminal 6xhisTag protein of P16 was constructed. The gene encoding P16 was amplified by PCR. Primers (P16P/5′ GCG GGC CTG CAG CAT ATT CTT TTT GTA TTG TGA 3′; P16B/5′ CGA CCG GAT CCA GTA AAA AAT CTA TCT T '3) were chosen to amplify the open reading frame without a start and stop codon and additional restriction sites for *Bam*HI and *Pst*I. After cloning in vector pQE30 (QIAGEN GmbH, Hilden, Germany), the resulting

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