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The Lly Protein Protects *Legionella pneumophila* from Light but Does Not Directly Influence Its Intracellular Survival in *Hartmannella vermiformis*

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The *lly* locus (legiolysin) mediates the browning of the culture medium of *Legionella pneumophila* in the late stationary growth phase, presumably as a result of synthesis of homogentisic acid. Mutagenesis of the *lly* gene of the *L. pneumophila* Philadelphia I derivative JR32 did not affect intracellular replication in the natural host *Hartmannella vermiformis*. The Lly-negative mutant, however, showed a markedly decreased resistance to ordinary light. The cloned *lly* gene conferred an increased resistance to light in recombinant *L. pneumophila* and *Escherichia coli* K-12, indicating a contribution of the Lly protein to ecological adaptation of *Legionella* species.

Legionella pneumophila inhabits aquatic biotopes and hot water systems (20) and is known to survive intracellularly in protozoan hosts or in association with cyanobacteria (1, 24). When transmitted by aerosol inhalation, *L. pneumophila* may cause a severe, atypical bronchopneumonia termed Legionnaires' disease and a flulike illness called Pontiac fever (31). The epidemiology of the agent of Legionnaires' disease and its ecological needs to multiply to high infectious concentrations are closely related. Oxygen concentration, pH, and even the quality of the pipe systems are known to influence the colonization of water systems by legionellae (19). However, the knowledge about factors that contribute to the survival of *L. pneumophila* in its natural environment is very limited. *Hartmannella*, *Acanthamoeba*, *Naegleria*, and *Tetrahymena* species are the most frequent hosts for legionellae (8, 17, 18, 29), and their interaction is known to be affected by temperature, the nutritional condition of the host, and the concentration of iron ions (12, 16, 21). Recent studies demonstrated that the macrophage infectivity potentiator (Mip) protein is involved in the infection of *Hartmannella* and *Tetrahymena* species (4, 8).

The lethal activity of solar radiation, in particular UV radiation at 290 to 320 nm, to legionellae in surface water has been demonstrated (27). A cryptic conjugative plasmid of 69 kb from a virulent strain of *L. pneumophila* has been shown to confer low-level, error-prone resistance to UV light (26). We recently described the nucleotide sequence and the mutational analysis of the *lly* locus of *L. pneumophila*, which encodes a 39-kDa protein with striking homology to the enzyme 4-hydroxyphenyl-pyruvate dioxygenase (32). The Lly protein confers browning of GC-FC culture medium (32) in the late stationary growth phase, fluorescence, and hemolytic activity of human erythrocytes. The oxygen-dependent browning is significantly enhanced if the culture medium is supplemented with L-tyrosine (32). It can be observed in a wide range of temperatures (tested from 27 to 40°C) and is obviously not influenced by light (9a). To determine the function of the brown pigment of *L. pneumophila* strains carrying the *lly* gene, we investigated its possible role in protecting the bacteria against the lethal effect of light. In particular systems, pigments play a role in promoting intracellular survival (9). In *Hartmannella vermiformis*, the pigment might exert its protective effect either by serving as a scavenger molecule for oxygen radicals or by damaging the host and thereby eliminating its antimicrobial activities.

Bacterial strains and plasmids used in this study are described elsewhere (3, 15, 22, 32, 33). For mutagenesis and complementation analysis, the restriction-deficient, streptomycin-resistant *L. pneumophila* Philadelphia I derivative JR32 was used (15). The avirulent strain *L. pneumophila* Philadelphia I (XXXV) was obtained by prolonged passage of the wild-type strain on Mueller-Hinton agar (3). All *Legionella* strains were grown on buffered charcoal yeast extract agar (BCYE) obtained from Oxoid, Wesel, Germany, or in GC-FC broth. The plates were incubated at 37°C with a 5% CO_2 atmosphere for 3 days.

Complementation in *trans* of the legiolysin-negative mutant JR32-1 was performed with plasmid pEWB34-113, a derivative of the vector pMMB34 (10). pEWB34-113 was constructed by insertion of the chloramphenicol resistance marker (*cat*) from Tn*1725* (28) and a 2.4-kb *Eco*RI-*Sma*I fragment containing the *lly* gene (pEWL113) (33). For expression of the Lly protein in *E. coli*, plasmid pEWL2 was introduced into *E. coli* K-12 (33). *E. coli* K-12 strains were cultivated at 37°C in YT broth (32).

DNA manipulations and transformations were performed as described elsewhere (22) unless specified otherwise. Mating procedures were performed as described by Wintermeyer et al. (32). *H. vermiformis* OS-101 Koblenz was obtained from the Ernst Rodenwaldt Institut, Koblenz, Germany. Axenic cultures of *H. vermiformis* were prepared in PYNFH medium (30) (ATCC 1034) at 30°C. Subculture was carried out at intervals of 8 days (30). Growth kinetics were performed by the spread plate technique on BCYE agar plates as described previously (23). To determine whether legiolysin plays a role in intracellular survival of *Legionella* strains, the *lly*-negative mutant JR32-1, the wild-type strain JR32, the complemented *lly*-positive strain JR32-1(pEWB34-113), and the avirulent derivative XXXV of *L. pneumophila* Philadelphia I were tested for their

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TABLE 1. Analysis of the influence of pigmentation of *L. pneumophila* and *E. coli* strains on survival in *H. vermiformis* and on resistance to light

Strain	Lly	Pigmentation	Survival in *H. vermiformis*[a]	Resistance to light
L. pneumophila JR32	+	+	+	+
L. pneumophila JR32-1	−	−	+	−
L. pneumophila JR32-1 (pEWB34-113)	+	+	+	+
L. pneumophila Philadelphia avirulent XXXV	+	+	−	ND
E. coli K-12(pUC18)	−	−	ND	−
E. coli K-12(pEWL2)	+	+	ND	+

[a] Intracellular growth behavior of *L. pneumophila* in *H. vermiformis*. The formation of colonies (CFU per milliliter) was determined at 0 and 24 h postinfection in three independent experiments. Intracellular multiplication is indicated by +, while − indicates that at 24-h postinfection, fewer bacteria were detected than at the beginning of the infection (0-h value).

ability to multiply intracellularly in axenic cultures of the *H. vermiformis* OS-101 Koblenz. The host organisms were kept in the dark during the whole experiment except for the time when they were inoculated with *Legionella* cells. At time zero postinfection, the bacterial counts were approximately 4×10^2 to 8×10^2 CFU/ml. Intracellular values of 5.8×10^3 to 3.6×10^4 CFU/ml were obtained 24 h postinfection for all *Legionella* strains and clones. In contrast, the avirulent strain XXXV did not multiply detectably. These data indicate that the Lly protein is not involved in intracellular multiplication of legionellae in *H. vermiformis* (Table 1).

To determine if legiolysin affects the viability of *L. pneumophila* during light exposure, stationary-phase cultures of *L. pneumophila* were exposed to light for 8 days. *L. pneumophila* strains were first grown on BCYE agar plates for 2 days and then used to inoculate 20 ml of GC-FC broth. Cells were grown until stationary phase for 48 h at 37°C and diluted to a final concentration of 10^{10} CFU/ml. Then 3 ml of the culture was pipetted into a nine-well plate (Nunclon, Wiesbaden, Germany), and the plates were sealed with Parafilm to prevent evaporation. *L. pneumophila* JR32 (Lly^+), *L. pneumophila* JR32-1 (Lly^-), and *L. pneumophila* JR32(pEWB34-113) (Lly^+) were exposed to a 75-W, 12-V halogen lamp (Osram, Munich, Germany) with a spectrum reaching from 290 to 780 nm and a maximum at 635 nm. The distance between the light source and the culture plate was 40 cm, and the temperature was adjusted to 18°C. As a control experiment, the same strains were kept in the dark. Serial dilutions were plated on BCYE agar plates every other day for a total of 8 days. The protective capacity of the brown pigment as a result of the activity of the Lly protein can be seen in Fig. 1. The Lly-negative mutant cannot be cultured after 8 days, whereas the wild-type strain and the complemented strain exhibit 2.2×10^7 and 1.5×10^8 CFU/ml, respectively. In the dark, no decrease in the bacterial counts was observed (data not shown). If the Lly-negative mutant JR32-1 was resuspended in the supernatant of the wild-type strain JR32 and vice versa, only bacteria in the brown-pigmented supernatant survived. The analog experiment with the *E. coli* K-12 strains carrying the *lly* gene on plasmid pEWL2 and its isogenic *lly*-negative counterpart carrying pUC18 also revealed the protective capacity of the pigment (Fig. 2), although the *E. coli* strains were less affected by light than was *L. pneumophila. E. coli* K-12 clones expressing the pigment survived 100-fold better after 8 days of light exposure in comparison with the nonpigmented strains.

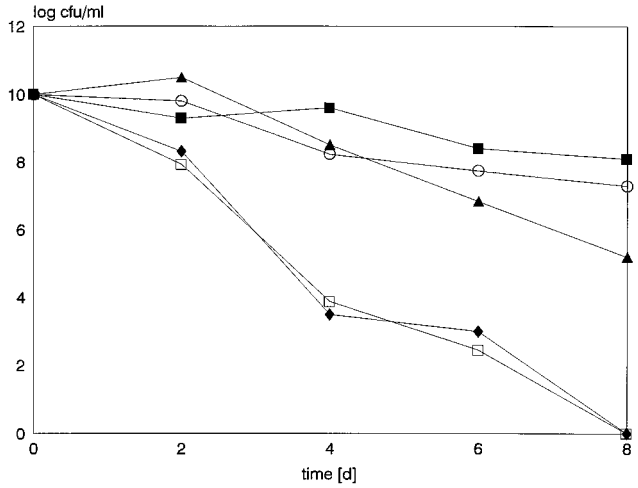

FIG. 1. Effect of light (halogen lamp: 12 V, 75 W) on the survival of *L. pneumophila* in GC-FC broth. The formation of colonies (CFU per milliliter) was determined at 0, 2, 4, 6, and 8 days for three independent experiments. Symbols: ○, JR32 (Lly^+); □, JR32-1 (Lly^-); ■, JR32-1(pEWB34-113) (Lly^+, plasmid encoded); ◆, JR32 (Lly^+) in the supernatant of JR32-1 (Lly^-); ▲, JR32-1 (Lly^-) in the supernatant of JR32 (Lly^+). The experiment was performed three times, and values represent the means of the three experiments. The standard derivation never exceeded half a log phase.

Natural freshwater biotopes are the sources for dissemination of legionellae to man-made water systems, where a habitat of intensive amplification is often provided. The lethal activity of sunlight is absent in plumbing systems, but UV light has been used to prevent outbreaks of legionellosis (7). The aim of this study was to evaluate whether the Lly-mediated pigmentation protects *L. pneumophila* from light or influences the survival in the amoeba-legionella relationship. Pigmentation is a common protector against light and has been shown to be responsible to some extent for the indigestibility of *Pseudomonas* and *Chromobacterium* species by amoebae (9). Several

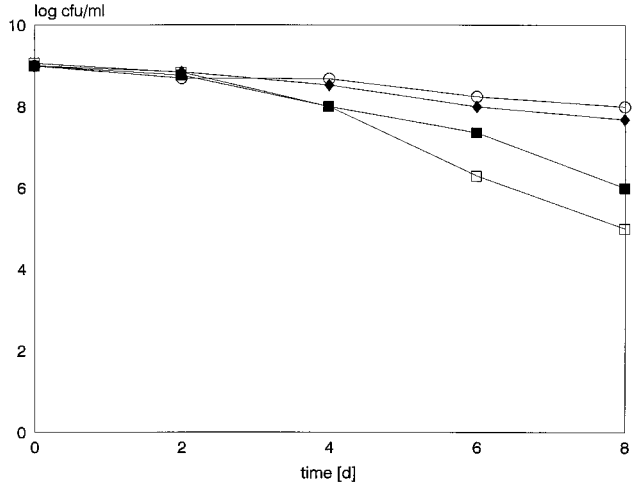

FIG. 2. Effect of light (halogen lamp: 12 V, 75 W) on the survival of recombinant *E. coli* strains in YT broth. The formation of colonies (CFU per milliliter) was determined at 0, 2, 4, 6, and 8 days for three independent experiments. Symbols: ○, *E. coli*(pEWL 2) (Lly^+); □, *E. coli*(pUC18) (Lly^-); ■, *E. coli*-(pEWL2) (Lly^+) in the supernatant of *E. coli*(pUC18) (Lly^-); ◆, *E. coli*(pUC18) (Lly^-) in the supernatant of *E. coli*(pEWL2) (Lly^+). The experiment was performed three times, and values represent the means. The standard derivation never exceeded half a log phase.

Bacillus (2), *Serratia* (25), *Pseudomonas* (34), *Streptomyces* (14), and *Vibrio* (13) species produce melanin-like pigments. The MelA protein from the aquatic bacterium *Shewanella colwelliana* shows a significant homology (81.3% on the amino acid level) with the Lly protein of *L. pneumophila* and is apparently responsible for melanin synthesis (11, 13). Recently, it was reported that homogentisic acid is not only the product of the MelA enzyme activity (5) but also a product of the 4-hydroxyphenylpyruvic acid dioxygenase-like protein enzyme activity in *Streptomyces avermitilis* (6).

The pigmentation of *L. pneumophila* seems to be important for the survival of *L. pneumophila* cells stressed by light but does not have any influence on the virulence of *L. pneumophila* cells in guinea pigs or the infection of U937 macrophage-like cells (32). Furthermore, the intracellular growth of *L. pneumophila* JR32 in *Acanthamoeba castellanii* (32) or *H. vermiformis* is not affected by the Lly protein.

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REFERENCES

1. **Barbaree, J. M., B. S. Fields, J. C. Feeley, G. W. Gorman, and W. T. Martin.** 1986. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. Appl. Environ. Microbiol. **51:**422–424.
2. **Barnett, T. A., and J. H. Hageman.** 1983. Characterization of a brown pigment from *Bacillus subtilis* cultures. Can. J. Microbiol. **29:**309–315.
3. **Bender, L., M. Ott, R. Marre, and J. Hacker.** 1990. Genome analysis of Legionella pneumophila spp. by orthogonal field alteration gel electrophoresis (OFAGE). FEMS Microbiol. Lett. **72:**253–258.
4. **Cianciotto, N. P., and B. S. Fields.** 1992. *Legionella pneumophila* mip gene potentiates intracellular infection of protozoa and human macrophages. Proc. Natl. Acad. Sci. USA **89:**5188–5191.
5. **Coon, S. L., S. Kotob, B. B. Jarvis, S. Wang, W. C. Fuqua, and R. M. Weiner.** 1994. Homogentisic acid is the product of MelA, which mediates melanogenesis in the marine bacterium *Shewanella colwelliana* D. Appl. Environ. Microbiol. **60:**3006–3010.
6. **Denoya, C. D., D. D. Skinner, and M. R. Morgenstern.** 1994. A *Streptomyces avermitilis* gene encoding a 4-hydroxyphenylpyruvic acid dioxygenase-like protein that directs the production of homogentisic acid and an ochronotic pigment in *Escherichia coli*. J. Bacteriol. **176:**5312–5319.
7. **Farr, B. M., J. C. Gratz, J. C. Tartaglino, S. I. Getchell-White, and D. H. M. Groschell.** 1988. Evaluation of ultra violet light for disinfection of hospital water contamination with *Legionella*. Lancet **ii:**669–672.
8. **Fields, B. S., E. B. Shotts, Jr., J. C. Feeley, G. W. Gorman, and W. T. Martin.** 1984. Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. Appl. Environ. Microbiol. **47:**467–471.
9. **Flemming, E., and R. Ronn.** 1994. Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. FEMS Microbiol. Rev. **15:**321–353.

9a. **Flügel, M.** Unpublished data.

10. **Frey, J., M. Bagdasarian, M. D. Feiss, F. C. H. Franklin, and J. Deshusses.** 1983. Stable cosmid vectors that enable the introduction of cloned fragments into a wide range of gram negative bacteria. Gene **24:**299–308.
11. **Fuqua, W. C., V. E. Coyne, D. C. Stein, C. M. Lin, and R. M. Weiner.** 1991. Characterization of *mel* A: a gene encoding melanin biosynthesis from the marine bacterium *Shewanella colwelliana*. Gene **109:**131–136.
12. **Gebran, S. J., C. Newton, Y. Yamamoto, R. Widen, T. W. Klein, and H. Friedman.** 1994. Macrophage permissiveness for *Legionella pneumophila* growth modulated by iron. Infect. Immun. **62:**564–568.
13. **Ivins, B. E., and R. K. Holmes.** 1981. Factors affecting phaeomelanin production by melanin producing (*mel*) mutant *Vibrio cholerae*. Infect. Immun. **34:**895–899.
14. **Katz, J., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129:**2703–2714.
15. **Marra, A., and H. A. Shuman.** 1989. Isolation of a *Legionella pneumophila* restriction mutant with increased ability to act as recipient in heterospecific matings. J. Bacteriol. **171:**2238–2240.
16. **Maurelli, A. T.** 1989. Temperature regulation of virulence genes in pathogenic bacteria: a general strategy for human pathogens? Microb. Pathog. **7:**1–10.
17. **Moffat, J. F., and L. S. Tompkins.** 1992. A quantitative model of intracellular growth of *Legionella pneumophila*. Infect. Immun. **60:**296–301.
18. **Newsome, A. L., R. L. Baker, R. D. Miller, and R. R. Arnold.** 1985. Interactions between *Naegleria fowleri* and *Legionella pneumophila*. Infect. Immun. **50:**449–452.
19. **Ott, M., L. Bender, P. C. Lück, P. Meyer, and J. Hacker.** 1992. Distribution of legionellae in a hospital water system: prevalence of immunologically related *Legionella pneumophila* serogroup 6 isolates. FEMS Microbiol. Lett. **95:**201–206.
20. **Ott, M., P. Messner, J. Heesemann, R. Marre, and J. Hacker.** 1991. Temperature-dependent expression of flagella in *Legionella*. J. Gen. Microbiol. **137:**1955–1961.
21. **Ott, M., M. Steinert, L. Bender, B. Ludwig, P. C. Lück, and J. Hacker.** 1993. Temperature-dependent replication of virulent and avirulent *Legionella pneumophila* isolates in *Acanthamoeba castellanii*, p. 149–151. *In* J. M. Barbaree, R. F. Breiman, and P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Steinert, M., M. Ott, P. C. Lück, E. Tannich, and J. Hacker.** 1994. Studies on the uptake and intracellular replication of *Legionella pneumophila* in protozoa and in macrophage like cells. FEMS Microbiol. Ecol. **15:**299–308.
24. **Tison, D. L., D. H. Pope, W. B. Cherry, and C. B. Fliermanns.** 1980. Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). Appl. Environ. Microbiol. **39:**456–459.
25. **Trias, J., M. Vinas, J. Guinea, and J. G. Loren.** 1989. Brown pigmentation in *Serratia marcescens* cultures associated with tyrosine metabolism. Can. J. Microbiol. **35:**1037–1042.
26. **Tully, M.** 1991. A plasmid from a virulent strain of *Legionella pneumophila* is conjugative and confers resistance to ultra violet light. FEMS Microbiol. Lett. **90:**43–48.
27. **Tully, M.** 1993. A *Legionella* plasmid mediates resistance to UV light but not to solar radiation, p. 120–124. *In* J. M. Barabaree, R. F. Breimann, and P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
28. **Ubben, D., and R. Schmitt.** 1986. Tn 1721 derivates for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41:**145–152.
29. **Wadowsky, R. M., T. W. Wilson, N. J. Kapp, A. J. West, J. M. Kuchta, S. J. States, J. N. Dowling, and R. B. Yee.** 1991. Multiplication of *Legionella* spp. in tap water containing *Hartmannella vermiformis*. Appl. Environ. Microbiol. **57:**1950–1955.
30. **Weekers, H. H., R. J. Gast, and T. J. Byers.** 1993. Small subunit ribosomal RNA analysis of three *Hartmannella vermiformis* strains. Sequences, secondary structure and phylogenetic implications. *In Acanthamoeba* and *Hartmannella*. Ecophysiological, biochemical and molecular biological differences. Ph.D. thesis. University of Leiden, Leiden, The Netherlands.
31. **Winn, W. C., Jr.** 1988. Legionnaires' disease: a historical perspective. Clin. Microbiol. Rev. **1:**60–81.
32. **Wintermeyer, E., M. Flügel, M. Ott, M. Steinert, U. Rdest, K. H. Mann, and J. Hacker.** 1994. Sequence determination and mutational analysis of the *lly* locus of *Legionella pneumophila*. Infect. Immun. **62:**1109–1117.
33. **Wintermeyer, E., U. Rdest, B. Ludwig, A. Debes, and J. Hacker.** 1991. Characterization of legiolysin (lly), responsible for hemolytic activity, colour production and fluorescence of *Legionella pneumophila*. Mol. Microbiol. **5:**1135–1143.
34. **Yabuuchi, E., and A. Ohyama.** 1972. Characterization of pyomelanin-producing strains of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **22:**53–64.