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Temperature-Dependent Parasitic Relationship between *Legionella pneumophila* and a Free-Living Amoeba (*Acanthamoeba castellanii*)[▽]

Akira Ohno,[1]* Naoyuki Kato,[2] Ryota Sakamoto,[1,3] Soichiro Kimura,[1] and Keizo Yamaguchi[1]

Departments of Microbiology and Infectious Disease[1] and Chemistry,[2] Faculty of Medicine, Toho University, Tokyo, and Department of Public Health, Kyoto University School of Medicine, Kyoto,[3] Japan

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We analyzed the effects of temperature on the interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*. At <20°C, overexpression of type 1 metacaspase, a stimulator of *A. castellanii* encystation, was associated with a reduced number of bacteria within amoeba. At low temperatures, *A. castellanii* seems to eliminate *L. pneumophila* by encystation and digestion.

The intracellular pathogen *Legionella pneumophila* causes Legionnaires' disease and exploits aquatic protozoa for replication. *L. pneumophila* is more frequently isolated from man-made water systems with high water temperature (9, 17) than from relatively cold freshwater environments (4, 16). This fact suggests that thermal conditions affect the relationship between *L. pneumophila* and protozoa, a notion supported by some reports (1, 3, 7, 11). The trophozoite of protozoa transforms into a cyst under harmful environments such as starvation, cold, and certain chemicals used in medical treatment. The effect of protozoal encystation on *Legionella* infection is not well understood although it seems that encystation is enhanced in a freshwater environment at low temperature. With these considerations in mind, we investigated whether the host-parasite relationship between *L. pneumophila* and protozoa is temperature dependent.

Two strains of *L. pneumophila* serogroup 1, Suzuki and Lp01, and *Acanthamoeba castellanii* ATCC 30234 were used in the present study. Intracellular growth kinetics assays using *A. castellanii* were performed as described previously (15). Reverse transcription and quantitative reverse transcription-PCR analyses were performed to examine (i) the expression of virulence- and growth-related genes (*pmrA*, *cpxR*, *csrA*) following transfer of *L. pneumophila* Lp01 growing in the post-exponential phase in buffered yeast extract α (BYEα) broth to fresh medium and incubation at 15°C or 35°C for 24 h (as an in vitro model of intracellular growth of *L. pneumophila* in *A. castellanii* at 15 and 35°C [13]) and (ii) the expression of the type 1 metacaspase gene, which was recently implicated in the encystation of *A. castellanii* (20), in *A. castellanii* buffer at 15 and 35°C with and without infection of *L. pneumophila* Lp01. The comparative $\Delta\Delta CT$ approximation method was used to analyze relative changes in gene expression (10).

Total RNA was purified using an RNeasy minikit (Qiagen, Valencia, CA) according to the instructions provided by the manufacturer. Total RNA was reverse transcribed into cDNA using an RNA PCR kit (Takara Bio Inc., Shiga, Japan). Real-time PCR with Sybr greenER (Invitrogen Life Sciences) was performed using the ABI Prism 7000 system (Applied Biosystems, Foster City, CA). Table 1 lists the primer pairs for target and internal control genes. Data were analyzed by Student's *t* test, and a two-tailed *P* value of <0.01 was considered significant.

L. pneumophila showed rapid increase in growth in protozoa or in broth at temperatures above 35°C (data not shown).

TABLE 1. Primer sets used in the present study

Organism	Gene or rRNA[a]	Primer sequences (forward, reverse) (5′–3′)
L. pneumophila Lp01	*cpxR* (target gene; lpg1483)	CCTCTTTGGAAAATGCTTGC, AAAATCCCAACTCCCAAACC
	pmrA (target gene; lpg1292)	CCTGGCCACTGTTTTCAAGT, AGATAGCGGTGCTGACGATT
	16S rRNA (internal control; lpg0569)	ACGGCAGCATTGTCTAGCTT, TGAGTTTCCCCAAGTTGTCC
A. castellanii ATCC 30234	*mcasp1* (target gene)	CGCTCTTCCTTCATTTCTCG, GTAGACGTAGGGCAGGTCCA
	18S rRNA (internal control)	GCATCTGCCAAGGATGTTTT, TCACAAGCTGCTGCTAGGGGAGT

[a] GenBank accession numbers are as follows: *cpxR* and *pmrA*, AE017354; *mcasp1*, AF480890; and 18S rRNA, AF239162.

* Corresponding author. Mailing address: Department of Microbiology and Infectious Disease, Faculty of Medicine, Toho University, 5-21-16 Omori-Nishi, Ota-Ku, Tokyo 143-8540, Japan. Phone: 81 (3) 3762-4151. Fax: 81 (3) 5493-5415. E-mail: akira@med.toho-u.ac.jp.

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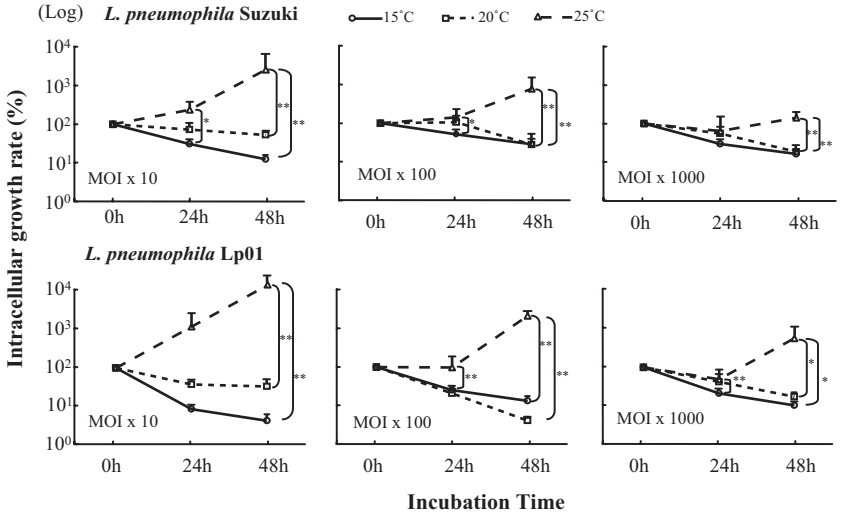

FIG. 1. Effects of temperature, inoculum size, and strain on multiplication of *L. pneumophila* in *A. castellanii* ATCC 30234. The numbers of *Legionella* cells in amoeba vacuoles after 24 and 48 h of incubation were expressed as percentages of that at 0 h (bacterial number in amoebas when *Legionella* and amoebas were coincubated for 3 h at 35°C and for a further 1 h with 50 μg/ml of gentamicin). Data are means ± SD. *, $P < 0.05$; **, $P < 0.01$. MOI, multiplicity of infection; numbers indicate bacterium/amoeba cell ratios.

However, an intracellular kinetics assay of *A. castellanii* using different strains and doses showed decreases of intracellular counts of both *L. pneumophila* Suzuki and Lp01 at temperatures below 20°C, with significant differences to 25°C (Fig. 1). *A. castellanii* in peptone-yeast extract-glucose medium (PYG) grew actively at 15°C but not at 35°C (Fig. 2). On the other hand, *L. pneumophila* did not multiply at 15°C in BCYEα broth (data not shown). These findings suggest that *A. castellanii* can kill *L. pneumophila* at low temperature.

The *pmrA* and *cprX* genes comprise a two-component system and play a major role in regulating the *icm* and *dot* genes, respectively, which are components of a major virulence system in *L. pneumophila* (6, 21), while CsrA is a global repressor of the *L. pneumophila* transmission phenotype and essential activator of intracellular replication (5, 14). Although the expression of *pmrA* and *cpxR* was not markedly upregulated at both 15 and 35°C shifts, the fact that *csrA* expression was significantly higher at 35°C than at 15°C explains the active proliferation of *L. pneumophila* within *Acanthamoeba*. Conversely, *csrA* expression was reduced by the shift to 15°C, highlighting the inability of *L. pneumophila* to resist digestion by *Acanthamoeba* at 15°C (Fig. 3).

Greub and Raoult (8) proposed that encystment is the main process by which an amoeba escapes *Legionella* infection. Figure 4 shows that the numbers of *L. pneumophila* cells decreased by more than 3 log units after incubation for 14 days at 15°C in *A. castellanii* buffer containing amoebas. Furthermore, the percentage of cysts to cells at day 17 of this experiment was 44.0 ± 1.1 (mean ± standard deviation [SD]) in the microcosm, and most trophozoites were dead, as determined by trypan blue staining. In addition, the infectivity of *L. pneumophila* for cysts in the population (75.4%) following encystment treatment was significantly lower than that for the trophozoite component (data not shown).

The expression level of the type 1 metacaspase gene in *A. castellanii* increased with incubation time at both 15 and

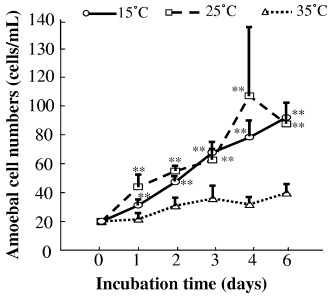

FIG. 2. Growth rate of *A. castellanii* ATCC 30234 in PYG at different temperatures. Data are means ± SD. **, $P < 0.01$, compared with the growth rate at 35°C.

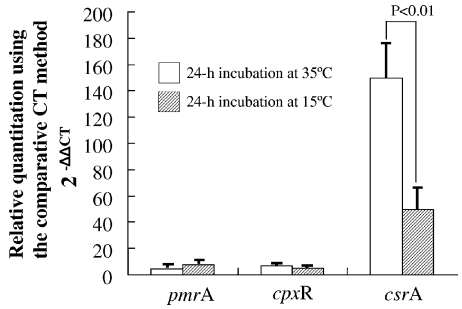

FIG. 3. Expression of virulence- and intracellular-growth-related genes (*pmrA*, *cpxR*, and *csrA*) using an in vitro model of intracellular growth of *L. pneumophila* Lp01 in *A. castellanii* ATCC 30234 at 15 and 35°C. *L. pneumophila* cells grown aerobically to post-exponential phase at 35°C in BYEα broth were transferred to a fresh broth at a final density of 1×10^7 CFU/ml and incubated aerobically at 15°C or 35°C for 24 h. Data are means ± SD. CT, threshold cycle.

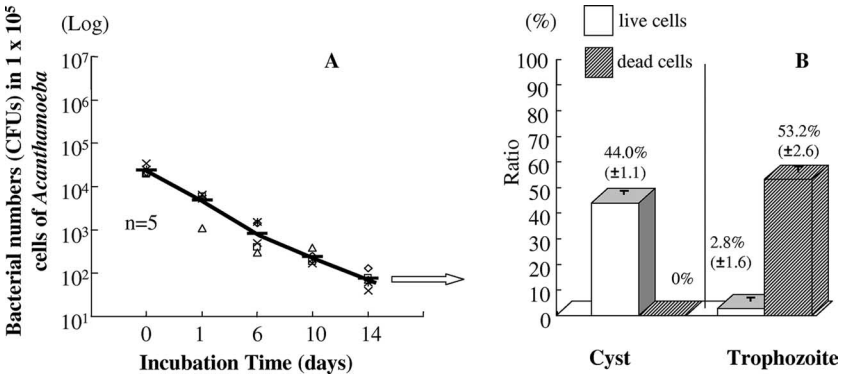

FIG. 4. Survival of *L. pneumophila* Suzuki in *A. castellanii* ATCC 30234 after 14 days of incubation at 15°C (A) and ratio of trophozoites to cysts at day 17 (B). Data are means ± SD.

35°C. In both amoebas alone and amoebas infected with *L. pneumophila* after 24 h of incubation, the increases in expression were nearly the same, although the expression levels in the bacterium-infected population were slightly higher than in noninfected amoebas. However, the difference was more marked after a 48-h incubation. In short, the expression level of the type 1 metacaspase gene at 15°C was approximately 12 times higher in amoebas with bacteria than in the amoebas at baseline (amoebas grown in PYG at 25°C; data not shown in figure), while the gene upregulation at 15°C was approximately eightfold in noninfected populations relative to infected populations. The significantly low expression in infected amoebas after 48 h at 35°C was probably due to *L. pneumophila*-induced cell disruption (Fig. 5). Our results suggest that encystation is prompted both by stimulation at 15°C and by *L. pneumophila* infection and that encystment serves to protect amoeba populations infected by *L. pneumophila*. However, the mechanism by which *L. pneumophila* induces type 1 metacaspase gene expression remains elusive; *L. pneumophila* could perhaps activate the Icm/IcDot type IV secretion system, which in turn triggers caspase-3 activation (12).

Berk et al. (2) demonstrated abundant release of vesicles from amoebas following ingestion of live *L. pneumophila*, with many vesicles containing bacteria. Most of the vesicles appeared in the medium just prior to encystment. This observation was supported by reports that *Acanthamoeba* spp. expel food vacuoles prior to encystment rather than retaining them within the cyst (18, 19). We also observed vesicles expelled from amoebas in the present study (data not shown).

In conclusion, *L. pneumophila* infects the trophozoite form of free-living amoebas and replicates intracellularly by activating the global regulator gene *csrA* at temperatures over 25°C. In contrast, at temperatures below 20°C, *L. pneumophila* is actively digested by *A. castellanii*, and the process of encystation eliminates *L. pneumophila* from the host, transmitting it to the extracellular environment.

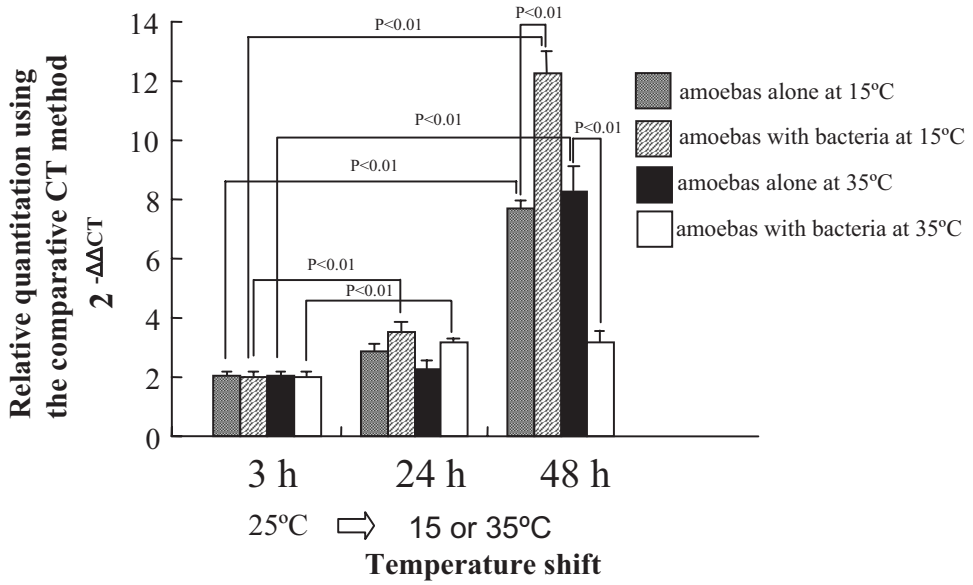

FIG. 5. Effects of incubation temperature on expression of type 1 metacaspase gene in *A. castellanii* ATCC 30234 with or without infection with *L. pneumophila* Lp01. Data are means ± SD. CT, threshold cycle.

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