Note

Expression of *gyrB* and 16S Ribosomal RNA Genes as Indicators of Growth and Physiological Activities of *Legionella pneumophila*

TOSHIHIRO OKUNO¹, KATSUJI TANI^{1,2*}, NOBUYASU YAMAGUCHI¹,
AND MASAO NASU¹

¹Environmental Science and Microbiology, Graduate School of pharmaceutical Sciences,
Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan

²Environmental Science and Microbiology, Faculty of Pharmacy, Osaka Ohtani University,
3-11-1 Nishikiori-kita, Tondabayashi, Osaka 584-8540, Japan

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To determine whether the DNA gyrase (*gyrB*) and 16S ribosomal RNA (16S rRNA) genes can be used as indicators of the biological activities of *Legionella pneumophila*, the expression levels were estimated. The ratio of mRNA/DNA in *gyrB* was 0.7 in mid log phase and decreased drastically after the log phase. For 16S rRNA, the ratio was highest in mid log phase (7.0×10^3), and the value that was about 10% of that in the log phase was maintained for six days. The rRNA may be vital in the resting or active but nonculturable cells that are not growing but physiologically active. The expression levels of *gyrB* mRNA and 16S rRNA can be used as indicators of the growth activity and the physiological activity of *L. pneumophila*, respectively. Therefore, by measurement of these indicators, we can evaluate the activities of *Legionella* cells in various environments.

Key words : *Legionella pneumophila* / Growth activity / Physiological activity / Gene expression.

Legionella spp. are ubiquitous in aquatic environments, and cause legionellosis (Legionnaires' disease and Pontiac fever) when people inhale contaminated aerosols or spray particles (File, 2003). There are many sources of spray particles throughout the town; fountains, and cooling towers. Therefore, it is important to understand the ecological dynamics of *Legionella* spp. in order to effectively prevent legionellosis.

The culture method has been mainly used to understand the ecology of *Legionella* spp. (Guerrieri et al., 2005; Koide et al., 2001; Murga et al., 2001; Türetgen et al., 2001; Wright et al., 1989). However, this method cannot detect cells that are in the active but nonculturable (ANC) state, and is time consuming (Bej et al., 1991; Steinert et al., 1997). As a consequence, it is difficult to evaluate the physiological activity of *Legionella* spp. in water environments.

The *gyrB* gene encodes the B subunit of DNA gyrase that is indispensable for DNA replication. Regulation of *gyrB* transcription is associated with the cell cycle in each cell (Robert and Shapiro, 1997). Therefore, in *Escherichia coli*, the expression level of *gyrB* mRNA can be used as an indicator of the growth activity (Tani et al., 2012). 16S rRNA is indispensable for translation. It is detected from not only culturable cells but from ANC, and has been used as an indicator of physiologically active cells (Coutard et al., 2005). To estimate the expression levels of *gyrB* mRNA and 16S rRNA, we used real-time PCR and real-time RT PCR to quantify the copy number of DNA and RNA, and calculated the ratio of the RNA number to DNA number in *Legionella pneumophila* at various growth phases.

L. pneumophila JCM7571 was cultivated in AYET medium [1% yeast extract, 1% N-(2-acetamido)-2-aminoethanesulfonic acid (ACES; pH6.9), 3.3 mM L-cysteine, 0.33 mM Fe(NO₃)₃, 100 µg/ml thymidine] at 37°C with shaking (Roy and Isberg, 1997; Sexton and

*Corresponding author. Tel & Fax: +81-721-24-9742, E-mail :
tanika(a)osaka-ohtani.ac.jp

Vogel, 2004). Bacterial DNA was extracted by the freeze-thaw-based method (Tsai and Olson, 1991). First, *Legionella* cells were collected from 5 ml of culture and washed by phosphate buffered saline (pH 8.0). The pellet was resuspended in 2 ml of lysis solution (0.15 M NaCl, 0.1 M Na₂EDTA [pH 8.0]) containing 15 mg of lysozyme/ml and 2×10^9 copies of the internal standard (DNA fragment of firefly luciferase gene), and incubated at 37°C for 2 h. Then, 2 ml of 0.1 M NaCl-0.5 M Tris-HCl (pH 8.0)-10% sodium dodecyl sulfate (SDS) was added. Cycles of freezing in liquid nitrogen and thawing at 65°C were repeated three times to release DNA from cells.

After the freeze-thaw cycles, an equal volume of PCI (phenol:chloroform:isoamyl alcohol = 25:24:1) was added. After being shaken gently, the mixture was centrifuged at $10,000 \times g$ for 20 min. The aqueous layer was transferred to the new tube and then purified once again by PCI. Finally, the aqueous layer was purified by CIAA (chloroform:isoamyl alcohol = 49:1) and DNA was precipitated by isopropanol. After precipitation, DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at -20°C until use.

RNA was extracted by bead-beating method (Stahl et al., 1988). Cells of *L. pneumophila* from 5 ml of culture were collected by centrifugation, resuspended in 800 µl of TE buffer, and transferred to a 2 ml vial with 1.3 ml of baked 0.1 mm glass beads, 350 µl of phenol equilibrated with pH 5.1 buffer, 60 µl of 10% SDS and 2×10^9 copies of the internal standard (transcript of firefly luciferase gene). Then, cells were crushed by beating at 4,800 rpm for 40 s by using MINI-BEADBEATER (Biospec products). After centrifugation, the superna-

tant was recovered and purified by PCI. After precipitation, DNA was degraded by DNase I (Roche) and stored at -20°C until use.

The primers and probes used in this study are listed in TABLE 1. For quantification of the DNA copy number, LightCycler FastStart DNA Master Hybridization Probe was used. PCR for quantification of *gyrB* gene was performed under the following conditions: 95°C for 10 min (hot start) and 50 subsequent cycles of 10 s at 95°C, 10 s at 56°C, and 10 s at 72°C. The conditions for quantification of the 16S rRNA gene were 95°C for 10 min and 50 subsequent cycles of 10 s at 95°C, 10 s at 57°C, and 15 s at 72°C, and that for luciferase gene was 95°C for 10 min and 40 subsequent cycles of 10 s at 95°C, 10 s at 60°C, and 15 s at 72°C, as in previous reports (Tani et al.; 2012, Wellinghausen et al., 2001). For quantification of the RNA copy number, LightCycler RNA Master Hybridization Probe was used. Instead of a hot start at the quantification of the DNA copy number, reverse transcription (20 min at 61°C) and denaturation (30 s at 95°C) were performed. The conditions of the PCR cycle were the same to those for the quantification of the DNA copy number except for time: 1 s at 95°C, 10 s at the annealing temperature, and 8 s at 72°C.

To monitor the change in the expression level during cultivation, we measured the ratio of the copy number of *gyrB* mRNA and 16S rRNA to the copy number of *gyrB* gene and 16S rRNA gene, respectively. *L. pneumophila* was inoculated into AYET medium (initial conc. = 1.4×10^7 cells/ml), and grown at 37°C. After 12, 24, 28, 32, 36, 40, 44, and 60 hours cultivation, the cells in the culture were enumerated by the total direct count method using 4',6-diamidino-2-phenylindole (DAPI)

TABLE 1. Primers and probes used in this study.

Primer or probe	Target gene	Sequence (5' -3')	Reference
Primer			
LeggyrBf4	<i>gyrB</i> of <i>L. pneumophila</i>	acgtttcactacgaaggcg	This study
LeggyrBr4	<i>gyrB</i> of <i>L. pneumophila</i>	gtcttttccgatgtcattgag	This study
JFP	16S rRNA gene of <i>Legionella</i>	agggttgataggttaagtgc	Wellinghausen and Marre, R. (2001)
JRP	16S rRNA gene of <i>Legionella</i>	ccaacagctagttgacatcg	Wellinghausen, N., Frost, C. and Marre, R. (2001)
pgL1908f	Luciferase gene	aggaagctttccatggaaga	Tani et al. (2012)
Luc175r	Luciferase gene	cagcgtaagtgtatgccacc	Tani et al. (2012)
Probe			
LeggyrB-4a-LCRed a	<i>gyrB</i> of <i>L. pneumophila</i>	ttatttttatttaagtgtcaacaaagg	This study
LeggyrB-4b-FITC b	<i>gyrB</i> of <i>L. pneumophila</i>	aaacaacggggtggactgggtgt	This study
LegFL b	16S rRNA gene of <i>Legionella</i>	agtggcgaaggcggtacct	Wellinghausen, N., Frost, C. and Marre, R. (2001)
LegLC a	16S rRNA gene of <i>Legionella</i>	tactgacactgaggcacgaaagcgt	Wellinghausen, N., Frost, C. and Marre, R. (2001)
n-LucHP1 b	Luciferase gene	tgaagagatacgccctgggtcc	Tani et al. (2012)
n-LucHP2 a	Luciferase gene	ggaacaattgctttacagatgcacata	Tani et al. (2012)

a: 5' LCred640 labeled. b: 3' FITC labeled.

FIG. 1. Growth curve of *L. pneumophila*. Strain JCM7571 was incubated in AYET medium, and cells were enumerated by epifluorescent microscopy using DAPI stain. DNA or RNA was extracted after 24 (early log phase), 28 (log phase), 40 (post log phase), 60 (stationary phase) hours incubation.

staining. Total cells were counted by epifluorescence microscopy (FIG. 1). Simultaneously, five ml of cultures in the early log phase (24 h), mid log phase (28 h), log-stationary phase (40 h), and stationary phase (60 h) were collected for DNA and RNA extraction. After extraction, recovery rates of DNA and RNA were estimated based on the concentration of the internal standard (DNA or RNA of firefly luciferase gene) quantified by real-time (RT) PCR. Then, concentrations of the *gyrB* gene, 16S rRNA gene, *gyrB* mRNA, and 16S rRNA in the culture at each cultivation time were estimated by results of RT PCR and recovery rates of DNA or RNA extraction.

We showed the changes in copy numbers of *gyrB*

FIG. 2. Change in the copy number of *gyrB* mRNA, 16S rRNA and DNA of these genes. White; *gyrB* gene; Light gray; *gyrB* mRNA; Dark gray; 16S rRNA gene; Black; 16S rRNA. Values obtained were means of triplicate samples.

mRNA, 16S rRNA and DNA of these genes (FIG. 2). In mid log phase, the ratios of RNA to DNA of both genes were highest (*gyrB* mRNA to *gyrB* gene = 0.7; 16S rRNA to 16S rRNA gene = 7.0×10^3). The value of *L. pneumophila gyrB* in mid log phase was about less than one-sixth that of *E. coli*. This reflected the difference in the growth rate between *L. pneumophila* and *E. coli* (Tani et al, 2012). The ratio of mRNA/DNA in *gyrB* decreased drastically after the mid log phase. The values in the log-stationary and stationary phases were less than one-tenth and one-hundredth that in mid log phase, respectively. Thus, *gyrB* can be used to determine growth activity of *L. pneumophila*.

In contrast, the expression levels of 16S rRNA in the log-stationary phase and the stationary phase were kept at about 10% of the level in the mid log phase. This being so, we measured the expression level of 16S rRNA for 6 days (FIG. 3). After 6 days cultivation, the ratio of 16S rRNA to 16S rRNA gene was 8.0×10^2 , and it was maintained constantly for 6 days at about 10% of that of mid log phase culture. This may be because of the need to maintain a certain amount of rRNA for *Legionella* cells to continue to live, while half-life of mRNA is very short (Bej et al., 1991; Coutard et al.; 2005, Ingle and Kushner, 1996). It thus became clear that the ratio of 16S rRNA gene could be used to monitor the change in the physiological activity of *L. pneumophila*. In addition, the detection limit of this method was 10 copies of 16S rRNA/reaction. In other words, this technique is useful for the detection of *Legionella* spp. The DNA gyrase (*gyrB*) and 16S ribosomal RNA (16S rRNA) genes can be used as indicators of the biological activities of *Legionella pneumophila*. In this study we collected all cells including dead cells. Thus the ratio may become more accurate,

FIG. 3. Gene expression level as an indicator of physiological activity. Values of the DNA or RNA copy number obtained were means of triplicate samples.

if we can remove the dead cells from the samples or use EMA-PCR (Nogva et al., 2003).

The rate of incorporation of specific labeled compounds is used as an indicator of bacterial growth activity (Fuhrman and Azam, 1980; 1982). However, these approaches require samples to be incubated with the appropriate substance in a closed environment, where the bacterial growth activity could change during incubation. Gene expression can be measured without incubation; hence, it can provide a more accurate indicator of *in situ* bacterial biological activity. Thus, measurement of the expression level of *gyrB* mRNA and 16S rRNA could show us the environments where *L. pneumophila* is growing at present and the habitats of physiologically active or active but nonculturable *Legionella* spp.

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