Phylogenetic Characterization of Viable but-not-yet Cultured \textit{Legionella} Groups Grown in Amoebic Cocultures: A Case Study using Various Cooling Tower Water Samples

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\textit{Legionella} spp. exist naturally in association with amoeba in water environments and are known to be the etiological agent of a severe form of pneumonia. To detect diverse \textit{Legionella} populations in cooling tower water systems, amoebic coculturing was performed for 15 water samples obtained from five different kinds of facilities in six geographically different locations. The growth of \textit{Legionella} in coculture with \textit{Acanthamoeba} sp. cells was monitored by quantitative PCR targeting \textit{Legionella}-specific 16S rRNA genes. Seven out of the 15 samples were positive for \textit{Legionella} growth and subjected to clone library analysis. A total of 333 clones were classified into 14 operational taxonomic units composed of seven known species and seven previously undescribed groups. Four of the seven \textit{Legionella}-growth-positive samples harbored detectable levels of free-living amoeba and were predominated by either \textit{L. drozanskii} or \textit{L. lytica}, by both \textit{L. bozemanii} and \textit{L. longbeachae}, or by a not-yet-described group named OTU 4. The \textit{Legionella}-growth-positive samples contained higher ATP levels ($>980$ pM) than the growth-negative samples ($<160$ pM), suggesting that ATP content would be a good indicator of the presence of viable but nonculturable \textit{Legionella} populations able to grow with amoeba.

Key words: Adenosine triphosphate (ATP) / Amoebic coculture / Cooling tower / \textit{Legionella} / Viable but nonculturable (VBNC).

INTRODUCTION

The genus \textit{Legionella} in the class Gammaproteobacteria currently comprises more than 60 species (Euzéby, 2018) that are found in not only natural environments but also artificial water environments such as cooling towers. One of their unique characteristics is the ability to proliferate and survive as intracellular parasites in free-living protozoa (Fields, 1996; Rowbotham, 1980). \textit{Legionella pneumophila}, the best-characterized species of the genus, is an intracellular pathogen of humans and the etiological agent of a severe form of pneumonia called Legionnaires’ disease (Vogel and Isberg, 1999). This disease is caused by the inhalation of aerosols from pathogen-contaminated water, mainly from artificial water systems. Therefore, the control of \textit{Legionella} populations in water systems and monitoring for contamination by members of the \textit{Legionella} group are very important tasks in public health management.

The population levels of \textit{Legionella} groups in environmental water samples have been estimated by both culture-based plate counting and culture-independent gene detection methods. Quantitative PCR (qPCR) is a useful and rapid method to estimate \textit{Legionella} population densities but often gives results that are inconsistent with those of plate counts because it cannot distinguish viable cells from dead and viable but nonculturable (VBNC) cells (Yamamoto et al., 1993; Ng et al., 1997). Previously, we reported that the use of DNA-intercalating dyes such as ethidium monoazide (EMA) before PCR was effective for the specific amplification of DNA from \textit{Legionella} cells with intact membranes (Inoue et al., 2015a). The diversity of viable but not-yet-described \textit{Legionella} species in environmental water samples, especially in cooling tower waters, was reported by Wéry
et al. (2008) and our previous study (Inoue et al., 2015b). Recently, the amoebic coculture technique, which was developed to grow VBNC Legionella groups in vitro, has been applied for those living in bath water samples (Edagawa et al., 2015). In this study, we examined the applicability of the amoebic coculture technique to grow diverse VBNC Legionella populations present in cooling tower waters from various facilities in different geographic locations. To this end, we used quantitative PCR (qPCR) assays to monitor the growth of Legionella and phylogenetically characterized the growth-positive groups.

MATERIALS AND METHODS

Water samples
Water samples (CTW-A to CTW-O) were collected from a total of 15 cooling tower sites between May 2015 and December 2015: from buildings (number of sites, 5), commercial facilities (5), factories (3), hospital (1), and hotel (1) in Tokyo (6), Ibaraki (3), Kanagawa (2), Shizuoka (2), Chiba (1) and Gunma (1). The water quality management processes used at each site before water sampling are summarized in Table 1. Water samples were taken in sterile 500-mL polypropylene bottles that were kept in the dark at 4°C until microbiological analyses. Microbiological assays were carried out within 3 d after sample collection. ATP and pH of the water samples were measured with an ATP analyzer (Lumitester C-110; Kikkoman, Japan) and a pH meter (ORION 4-STAR pH/ion meter; Thermo Scientific, USA), respectively.

Plate counts of Legionella
Culturable Legionella populations were enumerated by the standard culture method (ISO11731, 1998). Collected water samples were concentrated 100-fold by centrifugation (6,400 x g, 30 min). A portion of the concentrated samples (0.5 mL) was pretreated with an equal volume of 0.2 M acid-phosphate buffer (Inoue et al., 2004) for 10 min, and 200 µL portions were inoculated onto GVPC selective agar plates (Nikkien Bio Medical Laboratory, Japan). The plates were incubated at 37°C for 8 d. The detection limit of this method was 10 CFU/100 mL.

Quantitative PCR (qPCR)
The copy number of Legionella-specific 16S rRNA genes was estimated by qPCR using a Cycleave PCR Legionella (16S rRNA) Detection Kit (Takara Bio Inc., Japan). Briefly, 2 mL of the 100-fold concentrated samples were further concentrated by centrifugation (14,000 x g, 10 min, 4°C) to a final volume of 40 µL and extracted by the alkaline-boil method of Beige et al. (1995). Subsequently, the extracts were purified using a NucleoSpin gDNA Clean-up kit (Macherey-Nagel, Germany) according to manufacturer’s instructions, and 50 µL of purified DNA solutions were prepared. PCR was performed on a Thermal Cycler Dice Real Time System II (Takara Bio Inc.) according to the manufacturer’s instructions.

Ameobic coculture
Acanthamoeba sp. AC3722-12, isolated in our laboratory from a cooling tower water, was used as the amoebic host and grown in 25 cm² tissue culture flasks containing PYGC broth (10 g of Bacto Proteose Peptone, 10 g of yeast extract, 10 g of glucose, 5 g of NaCl, 1.74 g of Na₂HPO₄, 1.36 g of KH₂PO₄, 0.95 g of L-cysteine·HCl in 1,000 mL of deionized water; pH 6.8) at 30°C for 4 d. Ameobic cocultures were performed by replacing the PYGC broth with 10 mL of Page’s saline (120 mg of NaCl, 4 mg of MgSO₄·7H₂O, 4 mg of CaCl₂·2H₂O, 142 mg of Na₂HPO₄, and 136 mg of KH₂PO₄ in 1,000 mL of deionized water) and adding 0.1 mL of 100-fold concentrated cooling tower water samples. The cocultures were incubated at 30°C for 15 d. The growth of Legionella cells was monitored by the standard plate culture method and qPCR assays as described above.

Detection of free-living amoebae (FLA) and plate counts of heterotrophic bacteria
Collected water samples were concentrated 50-fold by centrifugation (1,150 x g, 10 min). The concentrated samples (1 mL) were inoculated onto 1.5% (w/v) agar plates spread with heat-treated (60°C, 1 h) Escherichia coli IAM 12119 cell suspension, and incubated at 30°C for 2 wk. The amoebic plaques were enumerated, and amoebae were identified by microscopic observation. The detection limit of this method was 2 plaque-forming units (PFU)/100 mL. Heterotrophic bacteria were counted by the pour plate culture method using R2A medium (Nissui Pharmaceutical, Japan) incubated at

| TABLE 1. Water quality management before water sampling |
|-------------------------------------------------------|
| Biocide treatment | Samples |
|-------------------|---------|
| Continuously with 5-chloro-2-methyl-4-isotiazolin-3-one (CMI) | CTW-A, CTW-D, CTW-F, CTW-H, CTW-I, CTW-J, CTW-K |
| Intermittently with CMI | CTW-C, CTW-G |
| Continuously with stabilized chlorine | CTW-B, CTW-O |
| Intermittently with cation surfactant | CTW-E |
| None | CTW-L, CTW-M, CTW-N |
TABLE 2. Population densities of *Legionella*, free-living amoebae, heterotrophic bacteria, and ATP levels in the cooling tower water samples tested

| Sample | Copy no. of *Legionella*-specific 16S rRNA genes/100 mL | Free-living amoebae, PFU/100 mL | Plate counts of heterotrophic bacteria, CFU/mL | ATP, pM | pH |
|--------|--------------------------------------------------------|---------------------------------|-----------------------------------------------|---------|----|
| CTW-A  | 2.2×10³                                                   | 1.8×10⁸ (AC, VK, HT)³         | 2.6×10⁸                                       | 2,200   | 7.8|
| CTW-B  | 1.1×10⁵                                                   | <2                             | 10                                            | 15      | 8.1|
| CTW-C  | 9.9×10⁵                                                   | <2                             | 3.9×10⁶                                       | 32      | 8.1|
| CTW-D  | 1.0×10⁵                                                   | <2                             | 1.2×10³                                       | 33      | 8.4|
| CTW-E  | 1.6×10⁴                                                   | 2 (AC)                         | <10                                           | 42      | 7.9|
| CTW-F  | 4.5×10²                                                   | <2                             | <10                                           | 38      | 8.8|
| CTW-G  | 6.5×10⁴                                                   | <2                             | 1.6×10⁴                                       | 87      | 8.6|
| CTW-H  | 1.3×10³                                                   | <2                             | 1.1×10⁵                                       | 160     | 7.6|
| CTW-I  | 4.9×10⁴                                                   | 8.0×10³ (VN, VK)                | 1.1×10⁶                                       | 1,600   | 8.3|
| CTW-J  | 1.3×10³                                                   | <2                             | 3.7×10⁶                                       | 2,000   | 8.5|
| CTW-K  | 1.2×10⁴                                                   | 12 (AC)                        | 2.6×10⁶                                       | 2,100   | 8.0|
| CTW-L  | 3.0×10⁶                                                   | <2                             | 1.8×10⁶                                       | 1,300   | 8.2|
| CTW-M  | 1.5×10⁵                                                   | 6 (HT)                         | 1.3×10⁷                                       | 2,900   | 8.0|
| CTW-N  | 2.5×10⁴                                                   | 1.5×10⁸ (AC, HT, VK)           | 8.4×10⁵                                       | 980     | 8.4|
| CTW-O  | 7.8×10³                                                   | 6.0×10³ (VK, AC)               | 6.1×10⁴                                       | 1,500   | 8.4|

³The *Legionella* population levels of all the samples were less than 10 CFU/100 mL based on the plate culture method.

³Symbols in parentheses represent species of amoebae detected: AC, *Acanthamoeba*; VN, *Vannella*; VK, *Vahlkampfiidae*; HT, *Hartmannella*.

30°C for 7 d.

Clone library analysis

To construct clone libraries, PCR using primers LEG-225F (5’-AAG ATT AGC CTG CGT CCG AT-3’) and LEG-858R (5’-GTC AAC TTA TCG CGT TTG CT-3’) (Miyamoto et al., 1997) was carried out according to the protocol of Nishizawa et al. (2008) to minimize PCR bias: an initial denaturation step of 2 min at 95°C followed by each threshold cycle as determined by qPCR, denaturation for 15 s at 95°C, annealing for 30 s at 65°C, and extension for 60 s at 72°C. The reaction mixture (30 μL) was composed of 5 μL of template DNA, 1 μL each of the primers (10 μM), 0.15 μL of Ex Taq polymerase, 2.4 μL of dNTPs, and 3 μL of 10× Ex buffer (Takara Bio Inc.) in a Thermal Cycler SP (Takara Bio Inc.). The PCR products were purified by using a QIAquick PCR purification kit (Qiagen, USA), ligated with the vector pMD20-T using a Mighty TA-cloning kit (Takara Bio Inc.), and the ligation products were used to transform *E. coli* DH5α Competent Cells (Takara Bio Inc.) according to the manufacturer’s instructions. The nucleotide sequences of clones were determined with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) using M13 primer RV (5’-CAG GAA ACA GCT ATG ACC-3’) or M13 primer M4 (5’-GTT TTC CCA GTC ACG AC-3’) according to the manufacturer’s instructions and were read on an Applied Biosystems 3130xl genetic analyzer. Operational taxonomic units (OTUs) were defined as sequences with at least 98% identity for all clones based on an analysis using Mothur platform software (http://www.mothur.org). A phylogenetic tree was constructed by the neighbor-joining method using MAGA5 software. Good’s coverage was calculated on Mothur platform software at a cut-off level of 0.02 (98% sequence identity with gaps) in the average neighbor method.

Accession numbers of nucleotide sequences

The partial sequences of 16S rRNA genes obtained through this study were deposited in the DDBJ database under the accession numbers LC278607 to LC278939.

RESULTS

Growth of *Legionella* populations by amoebic coculturing

All 15 tested water samples were positive for *Legionella* based on qPCR, but negative for *Legionella* detection by the plate culture method (<10 CFU/100 mL). The copy numbers of *Legionella*-specific 16S rRNA genes in the original water samples ranged from 4.5×10² to 3.0×10⁶/100 mL (Table 2). The popula-
tions of heterotrophic bacteria ranged from $<10$ CFU/mL to $10^7$ CFU/mL and those of free-living amoebae (FLA) were between $<2$ PFU/100 mL and $1.8 \times 10^3$ PFU/100 mL (Table 2). Among the 15 samples, amoebic cocultures from seven samples (CTW-A, -I, -J, -L, -M, -N, and -O) were successful in growing Legionella cells and gave 40- to 660,000-fold increases in the copy numbers of Legionella-specific 16S rRNA genes (Table 3). The coculture from sample CTW-N resulted in the isolation of Legionella cells that were all identified as Legionella bozemanii based on 16S rRNA gene sequencing (data not shown).

With respect to FLA, five Legionella-growth-positive samples (CTW-A, -I, -M, -N, and -O) harbored detectable levels of FLA ($6$ PFU/100 mL to $1.8 \times 10^3$ PFU/100 mL).

### Table 3

| Sample | Copy numbers of Legionella-specific 16S rRNA genes (Log [copies/mL]) at 0 d | Increase (fold) |
|--------|---------------------------------|-----------------|
| CTW-A  | 2.12 6.35 6.35 17,000           |                 |
| CTW-I  | 2.25 7.69 8.07 660,000          |                 |
| CTW-J  | 2.00 2.00 5.80 6,300            |                 |
| CTW-L  | 3.95 6.46 6.87 830              |                 |
| CTW-M  | 3.00 4.42 4.60 40               |                 |
| CTW-N  | 1.65 4.16 4.56 810              |                 |
| CTW-O  | 0.70 2.12 2.30 40               |                 |

**FIG. 1.** Neighbor-joining phylogenetic tree based on the alignment of 16S rRNA gene sequences (616-bp) of the representative Legionella clones obtained from the amoebic cocultures at 0 d (○), CTW-A; and 15 d (●), CTW-A; ○, CTW-A; ▲, CTW-I; ▲, CTW-I; ▲, CTW-L; ▲, CTW-M; □, CTW-N; ●, CTW-O, the related Legionella spp. and known Legionella clones. Numbers at nodes are bootstrap percentages (based on 1000 resamplings); values above 60% are shown. After the representative clone, the accession number of the representative clone and the number of similar sequences (based on a 2% cut-off) are given in parentheses and square brackets, respectively.
mL) (Table 2). It was also noted that the samples of successful amoebic cocultures contained higher ATP levels (>980 pM) than those of Legionella-growth-negative samples (ATP, <160 pM), except for sample CTW-K (ATP, 2,100 pM).

**Identification of Legionella populations grown in amoebic cocultures**

A total of 333 clones were recovered from the Legionella-growth-positive amoebic cocultures after the 15-d incubation (275 clones) and one water sample at 0 d (CTW-A; 58 clones). Good’s coverage of these libraries was 96.9% to 100%. These clones were classified into 14 OTUs at a cut-off level of 0.02 (98% sequence identity) for their 16S rRNA gene sequences (616 bp). Figure 1 shows a phylogenetic tree constructed with the 14 representative OTUs and related known Legionella 16S rRNA gene sequences. Seven of the 14 OTUs clustered with the known Legionella species, Legionella bozemanii, Legionella drozanskii, Legionella longbeachae, Legionella lytica, Legionella maceachernii, Legionella massiliensis, and Legionella nautarum. Among the other seven OTUs (OTUs 1 to 7), which were distantly related to the known Legionella species, OTUs 1 and 4 grew in the amoebic cocultures of CTW-J, -K, -M, and -O (Fig. 1).

The compositions of Legionella populations grown in the amoebic cocultures are shown in Table 4. The coculture of CTW-A was analyzed for its clone libraries at 0 d and at 15 d to follow the change in Legionella populations. Based on our constructed libraries, one specific Legionella group, L. drozanskii, seemed to grow selectively and dominate the coculture: this species accounted for 67% of the Legionella population at 0 d and 100% at 15 d. The selective growth of L. lytica and OTU 4 also occurred in the cocultures of CTW-I and CTW-O, respectively. Results were similar for CTW-J, which was predominated by L. drozanskii, and CTW-L and CTW-M, which were predominated by OTU 1. In contrast, two Legionella groups, L. bozemanii and L. longbeachae, grew equally well and both were predominant in the amoebic coculture of CTW-N. L. bozemanii was isolated from this coculture as mentioned above.

**DISCUSSION**

We examined the applicability of amoebic coculturing for the detection of VBNC Legionella groups in various cooling tower water samples. Of the 15 tested samples, this method was successful for seven samples (CTW-A, -I, -J, -L, -M, -N, and -O). Considering the association of Legionella with FLA, the Legionella population was expected to be detectable in water samples harboring FLA. This was the case for the four FLA-positive samples, CTW-A, CTW-I, CTW-N, and CTW-O. L. drozanskii found in CTW-A and L. lytica found in CTW-I are known...
as *Legionella*-like amoebal pathogens and parasites of FLA (Adeleke et al., 2001; Birtles et al., 1996; Drożański, 1991; Hookey et al., 1996), suggesting that these two *Legionella* groups grew as parasites in the coculture.

In the case of *L. bozemanii* and *L. longbeachae* found in the coculture of CTW-N, these species have been isolated from home garden soil (Kubota et al., 2000) and potting soil (Velonakis et al., 2009). Interestingly, Conza et al. (2013) reported that the close association of *Legionella* spp. (including *L. pneumophila* and *L. bozemanii*) and FLA occurred in compost and 62.8% of the tested compost samples were positive for *Legionella* spp. and FLA contemporaneously. Moreover, the authors showed that *Legionella* spp. and FLA were never detected simultaneously in bioaerosols and thus assumed that compost could release bioaerosol containing FLA or *Legionella* spp. and represented a source of infection. These findings suggest that the *Legionella*-FLA association may be ubiquitous in soil and water environments. The water sample CTW-O contained a detectable level of FLA (600 PFU/100 mL), and a not-yet-described *Legionella* group, OTU 4, was found in the amoebic coculture (Fig. 1, Table 4). Based on the above discussion, OTU 4 might be characterized as an amoebal parasite.

The other *Legionella*-growth-positive samples (CTW-J, CTW-L, and CTW-M) harbored low (<6 PFU/100 mL) or undetectable (<2 PFU/100 mL) levels of FLA (Table 2). *L. massiliensis* was found at much lower levels than OTU 1 in the amoebic cocultures of CTW-L and CTW-M (Table 4). This species was isolated by an amoebic coculture and found on BCYE agar plates below 32°C under a 5% CO2 atmosphere (Campocasso, A. et al., 2012). It appears that this species was not outcompeted by the OTU 1 group in our amoebic coculture using *Acanthamoeba* sp. AC3722-12 as the host.

As for the eight *Legionella*-growth-negative samples (CTW-B, -C, -D, -E, -F, -G, -H, and -K), their ATP contents were clearly lower (<160 pM) than those of the *Legionella*-growth-positive samples (>980 pM), except for sample CTW-K (2,100 pM) (Table 2). As shown in Fig. 2, when the copy number of *Legionella* 16S rRNA genes of each original sample was plotted against the ATP content of the corresponding sample, it was evident that the occurrence of *Legionella* growth in the amoebic coculture was positively correlated with the sample ATP content but independent of the *Legionella* gene content. This result suggests that the ATP content, namely the viability of the total microbial population, can be useful for predicting the level of VBNV *Legionella* populations. The low viability of microbial populations was supported in part by the very low heterotrophic bacterial counts in the samples CTW-B, -E, and -F (<10 or 10 CFU/mL) (Table 2). One exception was found for sample CTW-K, which contained 2,100 pM ATP, 105 CFU/mL of heterotrophs, and *Acanthamoeba* (12 PFU/100 mL). At present, there is no satisfactory explanation for the absence of *Legionella* growth in this sample, but the simplest explanation is that the viability of the *Legionella* cells was very low.

In conclusion, although cooling tower water systems harbor diverse *Legionella* populations, amoebic coculturing is effective for the detection not only of *Legionella* cells that are difficult to culture, but also VBNV *Legionella* groups such as OTUs 1 and 4 in our classification. Moreover, our results showed that the sample ATP content would serve as a good indicator of the presence of VBNV *Legionella* populations able to grow with amoeba. The analysis of the correlation between ATP content and VBNV *Legionella* populations in the cooling tower water samples is now in progress.

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