Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Effects of chlorination and heat disinfection on long-term starved *Legionella pneumophila* in warm water

C-W. Chang[1,2], Y-H. Hwang[2,3], W-Y. Cheng[3] and C-P. Chang[4]

1 Institute of Environmental Health, College of Public Health, National Taiwan University, Taipei, Taiwan
2 Department of Public Health, College of Public Health, National Taiwan University, Taipei, Taiwan
3 Institute of Occupational Medicine and Industrial Hygiene, College of Public Health, National Taiwan University, Taipei, Taiwan
4 Institute of Occupational Safety and Health, Council of Labor Affairs, Taipei, Taiwan

Keywords:
chlorination, disinfection, heat, *Legionella pneumophila*, starvation.

Correspondence
Ching-Wen Chang, Institute of Environmental Health, College of Public Health, National Taiwan University, Rm 740, 7F, No. 17, Xuzhou Rd, Taipei 100, Taiwan.
E-mail: cchang@ha.mc.ntu.edu.tw

2005/1228: received 16 October 2005, revised 5 July 2006 and accepted 1 September 2006

doi:10.1111/j.1365-2672.2006.03195.x

Abstract

Aims: To characterize the efficacy of widely accepted heat and chlorination on culturable and non-culturable *Legionella pneumophila* in starved and warm water.
Methods and Results: For *L. pneumophila* starved for 1 day (S1), heating at 60°C or more for 30 min or chlorination at 0·5–20 mg l^{-1} for 60 min, a loss of 6–8 log culturability was observed, whereas only 17–47% of cells had membrane damage. Non-culturability was also observed after heating or chlorinating the cells starved for 14 days (S14). The effect of heating on membrane deterioration was reduced for S14 cells while the chlorination effect remained. *Legionella pneumophila* entered a non-culturable phase after being starved for 33–40 days. The disinfection effects of both heating and chlorination on non-culturable N4 and N35 cells (which were collected on the fourth and the 35th days of the non-culturability phase respectively) decreased, indicating the development of disinfection resistance among non-culturable cells that had been subjected to starvation for 1–2 months.
Conclusions: Heating and chlorination significantly reduce the culturability of starved *L. pneumophila*, and damage cell membrane to a much less extent.
Significance and Impact of the Study: This study shows the ability of long-term starved *L. pneumophila* to resist against disinfection treatments, which has implications in terms of public health.

Introduction

Legionella pneumophila is widely distributed in the natural (Fliermans *et al.* 1981) and man-made (States *et al.* 1987) aquatic environments. This bacterium can survive extended periods of starvation in tap water (Schofield 1985; Lee and West 1991) and multiply in high numbers as it enters warm water systems (Yee and Wadowsky 1982). Isolation of *L. pneumophila* from hot spring water (Lee and West 1991; Ohno *et al.* 2003) indicates this bacterium has adapted itself to aquatic environments with high temperature and low availability of nutrients.

In response to long-term starvation in 37°C warm water, the morphology of *L. pneumophila* may change to a short-rod shape with loss of culturability on selective medium, but may maintain membrane integrity and viability. This phenomenon is characterized as a viable but non-culturable (VBNC) state. VBNC cells induced in warm water and starved conditions could be resuscitated, demonstrating their pathogenicity in chick embryos (Hussong *et al.* 1987) and implying a potential health risk to humans. In previous studies, *L. pneumophila* was reported to cause nosocomial (Perola *et al.* 2002), community-acquired (Stout *et al.* 1992) and travel-associated (National Institute of Infectious Diseases 2003; Centers for Disease Control and Prevention 2005) infections via contamination of potable water distribution systems with elevated temperature.

© 2006 The Authors

Superheating and chlorination are the most commonly used (Knirsch *et al.* 2000; Perola *et al.* 2002, 2005; Centers for Disease Control and Prevention 2005) and officially suggested (Environmental Protection Agency 1999; Occupational Safety and Health Administration 1999; Tablan *et al.* 2003) methods for control of *Legionella* growth in potable water. Several studies demonstrate no detection of culturable *L. pneumophila* at 60°C or higher for 3- to 10-min exposure (Stout *et al.* 1986; Steinert *et al.* 1998; Miyamoto *et al.* 2000) or in aqueous solution containing chlorine at 0·4–2 mg l^{-1} within 5 min (Kuchta *et al.* 1983; Domingue *et al.* 1988; Miyamoto *et al.* 2000). However, *L. pneumophila* cells used to test the susceptibility to superheat and chlorination (Kuchta *et al.* 1983; Stout *et al.* 1986; Domingue *et al.* 1988; Steinert *et al.* 1998; Miyamoto *et al.* 2000) were derived from the enriched medium, which may respond differently from the cells under starvation in nature. By challenging *L. pneumophila* to low level, 0·25 mg l^{-1} of free chlorine for 2 h at 20°C, Kuchta *et al.* (1985) demonstrated an increased chlorine resistance of cells grown in low-nutrient tap water compared with those grown on agar medium, implying that starvation may induce a change of cellular response to free chlorine. Starvation is considered as one of the parameters that affect the susceptibility of non-*Legionella* bacteria to heat (Jenkins *et al.* 1988; Spector *et al.* 1999) and free chlorine (Lisle *et al.* 1998; Saby *et al.* 1999). However, no information has been available about the efficacy of widely accepted heat and chlorine disinfection on starved *L. pneumophila* cultured in warm water at 37°C for a long period of time. There is also lack of data regarding the responses of non-culturable *L. pneumophila* to superheat and free chlorine set at effective disinfection levels.

In this study, culturable and starvation-induced non-culturable *L. pneumophila* were both collected during a *c.* 70-day starvation period to investigate their responses to superheat and chlorination. The culturability of starved *L. pneumophila* at 50–70°C heat treatment and chlorination at 0·5–20 mg l^{-1} was determined. In addition to the culture assay, fluorogenic *Bac*Light stains were used to assess whether the membrane integrity of starved *L. pneumophila* was damaged by heat and chlorine challenges.

Materials and methods

Bacterial strain and starved condition

Legionella pneumophila serogroup 1 ATCC 33152 was cultured on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYEα agar) (Sigma Chemical Co., St Louis, MO, USA) at 37°C in an atmosphere of 5% CO_2. After incubation for 2 days, the cells were harvested and inoculated in 800 ml of buffered yeast extract broth supplemented with α-ketoglutarate (BYEα broth). Cells were incubated, with 100 rev min^{-1} shaking, in 5% CO_2 at 37°C for 2 days to reach a concentration of *c.* 8 log CFU ml^{-1}.

For starvation, the cells were harvested from BYEα broth, resuspended in 800 ml of sterile ultrapure water (Milli-Q Gradient; Millipore, Billerica, MA, USA), and incubated at 37°C without CO_2. Based on the culturability loss of cells in starved culture, *L. pneumophila* cells at four different days of incubation were selected for heat and chlorine challenge, respectively, to represent the cells at early starvation status (i.e. the first day in starved culture, S1), culturability–decline status (i.e. 14th day in starved culture, S14), early non-culturable status (i.e. fourth day of non-culturability, N4) and late non-culturable status (i.e. 35th day of non-culturability, N35). In experiments, we revealed the duration that cells became non-culturable differed in batches that prepared for chlorination and superheat tests. For cultures prepared for thermal disinfection the cells became non-culturable after starvation from 33 ± 3 days and afterwards, whereas *L. pneumophila* became non-culturable from 40 ± 3 days and afterwards in cultures prepared for chlorination testing. Thus, as shown in Table 1, the S1, S14, N4 and N35 cells subjected to the thermal challenge were obtained on starvation days 1, 14, 36 ± 2 and 69 ± 3, respectively, whereas the S1, S14, N4 and N35 cells in the chlorination test were collected on starvation days 1, 14, 43 ± 3 and 74 ± 3 respectively.

Thermal treatment

The flasks containing 25 ml of sterile ultrapure water were placed in a water bath (TKS Shaking Bath; SB 301, Taiwan, tolerance ± 0·5°C) and preheated at 50, 60 and

Table 1 Duration of *Legionella pneumophila* in starved cultures collected for heat and chlorine challenge

	Duration of cells in starvation (days, mean ± SD)	
Cell status	Heat treatment	Chlorination
Culturable phase		
S1 cells	1	1
S14 cells	14	14
Non-culturable phase*		
N4 cells†	36 ± 2	43 ± 3
N35 cells‡	69 ± 3	74 ± 3

*Cells lost the culturability on BCYEα agar as a result of long-term starvation. *Legionella pneumophila* became non-culturable after starvation from 33 ± 3 and 40 ± 3 days in cultures prepared for heat treatment and chlorination testing respectively.
†Cells collected on the fourth day of the non-culturability phase.
‡Cells collected on the 35th day of the non-culturability phase.

© 2006 The Authors

70°C. Samples (25 ml) of starved cells were harvested by centrifugation at 3320 ***g*** for 30 min at 4°C, and cell pellets were, respectively, inoculated into flasks in which sterile water had reached the required temperature. The cells, at *c.* 8 log cell ml^{-1}, were thermally exposed for 30 min with 100 rev min^{-1} shaking. The cells from the four phases were also simultaneously inoculated into 25 ml sterile ultrapure water for 30 min at room temperature as controls. After 5- and 30-min thermal exposure the culturability and membrane integrity of *L. pneumophila* was determined for the heated samples and controls, respectively, at the same time interval. The controls and heated samples were further incubated at 37°C for 7 days without CO_2, and the culturability was measured again. All the experiments were performed in triplicate.

Chlorination

Free chlorine solutions were freshly prepared by dilution of sodium hypochlorite (Sigma Chemical Co.) with sterile ultrapure water to obtain 0·5, 2 and 20 mg l^{-1}. Concentrations of free chlorine were determined using a pocket colorimeter analysis system (Hach Test Kit; HACH Company, Loveland, CO, USA), for which the relative error of free chlorine determination was determined within 5% when compared with the dialkyl-*p*-phenylene-diamine method.

Samples of starved cells at the four phases described were harvested by centrifugation at 3220 ***g*** at 4°C for 30 min. The free chlorine solution was adjusted to 0·5, 2 and 20 mg l^{-1}, and starved cells were added and exposed for 60 min at room temperature with gentle shaking at 100 rev min^{-1}. Concentrations of chlorine-exposed cells were adjusted to *c.* 6 log cell ml^{-1} for challenge of free chlorine at 0·5 mg l^{-1}, and 7 log cell ml^{-1} for free chlorine at 2 and 20 mg l^{-1} to ascertain that free chlorine was always available for *L. pneumophila* during the 60-min experiment. The mean (SD) percentage of free chlorine residual in the suspensions of *L. pneumophila* at the four phases was determined to be 52% (6%), 57% (4%) and 87% (2%), respectively, at the end of 60-min experiment relative to the initial concentration of 0·5, 2 and 20 mg l^{-1}. Low SD values indicate similar amount of free chlorine has been taken by cells of four phases.

Chlorinated samples were taken after 5 and 60 min of exposure, neutralized with sterile sodium thiosulfate, and analysed for cell culturability and membrane integrity. Sterile ultrapure water inoculated with starved cells of *c.* 6 and 7 log cell ml^{-1} was used as control (denoted as Cl-L-control and Cl-H-control respectively) and analysed along with the chlorine-treated samples. Additionally, the neutralized samples and controls were further incubated at 37°C without CO_2 for 7 days and the culturability and membrane integrity were measured again. All the experiments were conducted in triplicate.

Culturability

After exposure of cells to thermal and chlorine challenges, the samples and controls were serially diluted and spread on BCYEα agar. Plates were incubated at 37°C for 7 days with 5% CO_2 and the results were expressed as log CFU ml^{-1}.

Cell membrane integrity

Membrane integrity of heat-treated *L. pneumophila* cells was determined by fluorogenic stains, LIVE/DEAD *Bac*Light kit (Molecular Probe, Eugene, OR, USA). The sample (1 ml) was mixed with 3 μl of *Bac*Light stains containing SYTO 9 and propidium iodide in 1 : 1. After incubation in the dark for 15 min at room temperature, the stained sample was filtered through a 0·2 μm black polycarbonate filter (Millipore) and examined with a fluorescent microscope (model DMR; Leica, Wetzlar, Germany). Cells with an intact membrane fluorescence green whereas injured cells with a compromised membrane exhibit orange to red fluorescence. The images were acquired using a CCD (Lecia DC 300F; Leica) and further analysed using software of LUCIA Measurement v. 4·81 (Laboratory Imaging, Prague, Czech Republic). The number of total cells was calculated as the sum of intact and injured cells, and the percentage of intact or damaged cells was also quantified.

Membrane integrity of chlorine-treated cells was determined as described previously in heat experiments except for the volume of *Bac*Light stain used. The stain volume was increased to 10 μl for the samples exposed to 0·5 and 2 mg l^{-1} of free chlorine for 1 h and for those exposed to 20 mg l^{-1} for 5 min; the volume was further increased to 100 μl for the samples exposed to 20 mg l^{-1} of free chlorine for 1 h in order to optimize the stain quality.

To ascertain the reliability of fluorescent microscopic enumeration, heated S1 cells were repeatedly counted seven times microscopically to determine the percentage of membrane-intact cells after being stained with *Bac*Light kit. The relative SD (i.e. 100 × SD/average) of determined percentage was 6% for the samples containing 7% of membrane-intact cells, and 17% for those containing 2% membrane-intact cells. Moreover, by using the percentage determined with 100 microscopic fields as reference, the percentage of membrane-intact cells determined with 36 random fields of the same sample were found only 0·1–0·7% away from the reference value. The above data verified the precision and accuracy of cell counts by

© 2006 The Authors

fluorescent microscopic enumeration. Consequently, a strategy of counting in 36 randomly selected fields was applied for the enumeration of the heat-treated samples, and that of 64 fields was used for chlorinated samples because of their relatively low percentage of membrane-intact cells shown in pilot tests.

Statistical analysis

The difference in the percentage of membrane-intact cells between the controls and the samples exposed to 30-min heat or 60-min free chlorine was determined to illustrate the level of membrane damage contributed by disinfection challenges. Linear regression model was performed by using SPSS software (SPSS Inc., Chicago, IL, USA) to examine the effects of starvation duration and heat or chlorine challenges on membrane integrity of *L. pneumophila*. The influence of chlorination on membrane integrity of starved cells was also statistically assessed by a two-tailed Student's *t*-test. Statistical significance was referred to as $P < 0{\cdot}01$.

Results

Response of starved *L. pneumophila* to heat disinfection

The average log total cell ml^{-1} during starvation periods was 8·37 with a SD of 0·04 for non-heated controls and 8·36 with a SD of 0·06 for heat-treated cells, indicating no evidence of significant cell lysis caused by thermal treatment. As for culturability (Table 2), the average number of culturable *L. pneumophila* was 8·08 log CFU ml^{-1} at day 1 (S1) and 4·8 log CFU ml^{-1} at day 14 (S14). After exposure at 50°C for 5 and 30 min, respectively, the number of culturable cells decreased to 8·06 and 7·99 for S1, and to 4·15 and 3·67 for S14, representing 95% and 81% of S1 cells remaining culturable and only 22% and 7% of S14 remaining culturable after heat treatment. Continuous decline of culturability was revealed for both controls and 50°C-treated S1 and S14 samples postincubated at 37°C for 7 days (control-PI and 50°C-PI in Table 2). When exposed to 60 and 70°C, neither S1 nor S14 cells remained culturable after 5- and 30-min exposure or after 7-day postincubation (PI). Non-culturable N4 and N35 cells continuously remained non-culturable after all the thermal treatments.

The results of superheating on membrane integrity are presented in Fig. 1. Exposure to 70°C for 30 min significantly damaged cell membrane of S1 cells, resulting in only 5% intact cells compared with 58% of cells being treated with 50°C for 5 min. Figure 1 also shows that the elevation of water temperature from 50 to 70°C increased the membrane damage of S1 cells that were exposed to heat for 5 min. Moreover, an increase in exposure time from 5 to 30 min resulted in an evident reduction of intact cells, with the percentage of intact S1 cells declining from 58% to 34%, from 45% to 35% and from 27% to 5%, respectively, when exposed to 50, 60 and 70°C.

Table 2 Effects of heat treatment on culturability of *Legionella pneumophila* starved at 37°C for 1 day (S1) and 14 days (S14) in sterile ultrapure water

	Cell status in starvation			
	S1		S14	
Treatment	Total cell (log cell ml^{-1})*	Culturable cell (log CFU ml^{-1})†	Total cell (log cell ml^{-1})	Culturable cell (log CFU ml^{-1})
Control‡	8·43 (0·05)§	8·08 (0·06)	8·35 (0·13)	4·80 (0·91)
50°C, 5 min	8·47 (0·05)	8·06 (0·06)	8·40 (0·08)	4·15 (0·50)
50°C, 30 min	8·44 (0·04)	7·99 (0·06)	8·38 (0·14)	3·67 (0·55)
60°C, 5 min	8·46 (0·06)	ND¶	8·41 (0·12)	ND
60°C, 30 min	8·45 (0·06)	ND	8·37 (0·16)	ND
70°C, 5 min	8·43 (0·05)	ND	8·38 (0·11)	ND
70°C, 30 min	8·44 (0·07)	ND	8·36 (0·11)	ND
Control-PI**	8·36 (0·11)	7·99 (0·04)	8·35 (0·10)	3·50 (0·55)
50°C-PI††	8·38 (0·11)	7·89 (0·07)	8·35 (0·09)	3·06 (0·59)
60°C-PI††	8·40 (0·08)	ND	8·32 (0·10)	ND
70°C-PI††	8·35 (0·13)	ND	8·32 (0·05)	ND

*The sum of intact and injured cells as determined by the *Bac*Light staining method with a fluorescence microscope.
†Spread plate assay on BCYEα agar and incubation of plates at 37°C for 7 days with 5% CO_2.
‡No thermal exposure.
§Standard deviations of the mean values in parentheses.
¶Non-detected (limit of detection = 1 CFU ml^{-1}).
**Postincubation of the controls for 7 days at 37°C without CO_2.
††Postincubation for 7 days at 37°C of samples having been heated at specified temperature for 30 min.

© 2006 The Authors

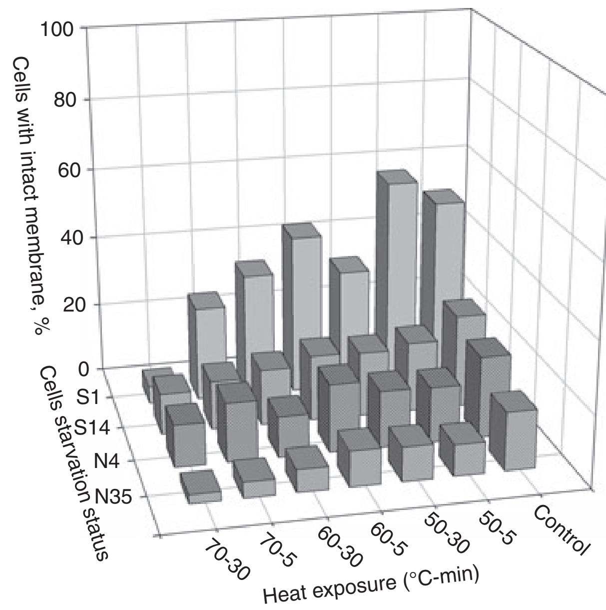

Figure 1 Average percentages of starved *Legionella pneumophila* cells with intact cellular membranes after exposure to thermal treatment. S1, S14, N4, and N35 cells represented *L. pneumophila* at early starvation phase (i.e. the first day in starved culture), culturability–decline phase (i.e. 14th day in starved culture), early non-culturable phase (i.e. fourth day of non-culturability) and late non-culturable phase (i.e. 35th day of non-culturability) respectively.

Similarly, increased temperature and contact time positively reduced the percentage of intact S14 *L. pneumophila*, although not as significant as shown in S1 cells. Heat treatment at 50°C and higher also showed adverse effects on the membrane integrity of non-culturable N4 and N35 cells after starvation for 1–2 months, when compared with their respective controls. Although thermal challenge damaged the cellular membrane, 12% of S14 and N4 *L. pneumophila* remained intact after being heated at 70°C for 30 min.

The statistical results of the stepwise linear regression model indicate that the duration of *L. pneumophila* in starvation is the most statistically significant parameter that adversely affects the percentage of membrane-intact *L. pneumophila*, followed by heating temperature and exposure time. All these three factors are statistically significant ($P < 0{\cdot}005$).

Responses of starved *L. pneumophila* to chlorination

The average (SD) of log total cell ml^{-1} measured at the four phases of the non-chlorinated controls was 6·24 (0·11) for Cl-L-control and 7·31 (0·12) for Cl-H-control. The mean values (SD) of log total cell ml^{-1} for cells exposed to free chlorine levels of 0·5, 2 and 20 mg l^{-1} were, respectively, 6·34 (0·2), 7·24 (0·18) and 7·26 (0·1) after 60-min exposure, and 6·24 (0·25), 7·27 (0·17) and 7·18 (0·1) after chlorination and PI of 7 days. A comparison of the concentrations of total cells with and without chlorination indicates that chlorination at 0·5–20 mg l^{-1} for 60 min did not cause significant lysis of *L. pneumophila*. However, as shown in Table 3, the culturability of S1 and S14 cells with an initial averaged log CFU ml^{-1} of 5·83 and 3·92, respectively, was not detectable following exposure to free chlorine at 0·5 mg l^{-1} for 5 min. In fact, non-culturability was revealed at all the chlorine concentrations tested regardless of cell status and presence of PI.

In addition to culturability, the significant impact of chlorination on membrane integrity of *L. pneumophila* is presented in Fig. 2: 11–31% of the cells without chlorination had intact membranes, while only 0·2–5·1% of the cells had intact membranes after chlorination ($P < 0{\cdot}001$). However, neither duration of starvation, free chlorine concentration and exposure time shows statistically significant effects on cell membrane integrity. PI of chlorinated cells resulted in 0·4–5·3% of intact cells, which was very close to the range (0·2–5·1%) determined immediately after 60-min chlorination.

Disinfection efficacy of heat and chlorination

The efficacy of heat disinfection and chlorination was characterized as the decrease in percentage of membrane-intact cells, exclusive of that contributed by starvation alone. Table 4 illustrates that, when heated at 50°C or higher, the greatest efficacy was observed with *L. pneumophila* starved for 1 day (S1). The efficacy decreased as cells stayed in starvation for 14 days or longer. On the other hand, chlorination of culturable S1 and S14 cells with 0·5 and 2 mg l^{-1} free chlorine for 1 h caused membrane damage to 27–31% of *L. pneumophila*, but to <12% of non-culturable N4 and N35 cells. Similar patterns in membrane deterioration were revealed for culturable S1 (27%) and non-culturable N4 (9%) cells exposed to the highest level of 20 mg l^{-1} free chlorine.

Discussion

This is the first study to quantitatively characterize the responses of long-term starved *L. pneumophila* to heat and free chlorine at the recommended effective concentrations, using both culture and non-culture assays. This study demonstrates that superheating at 60°C or higher for 5 and 30 min completely eliminated the culturability of both S1 and S14 *L. pneumophila* with initial concentration of 8 and 4·8 log CFU ml^{-1} respectively. Our findings were in agreement with previous studies applied to non-starved *L. pneumophila*. Stout *et al.* (1986) tested

© 2006 The Authors

Table 3 Effects of chlorination on culturability of *Legionella pneumophila* starved at 37°C for 1 day (S1) and 14 days (S14) in sterile ultrapure water

Treatment	Cell status in starvation			
	S1		S14	
	Total cell (log cell ml^{-1})*	Culturable cell (log CFU ml^{-1})†	Total cell (log cell ml^{-1})	Culturable cell (log CFU ml^{-1})
Cl-L-control‡	6·14 (0·03)§	5·83 (0·14)	6·37 (0·17)	3·92 (0·32)
0·5 mg l^{-1}, 5 min	6·23 (0·17)	ND¶	6·40 (0·28)	ND
0·5 mg l^{-1}, 60 min	6·28 (0·24)	ND	6·38 (0·27)	ND
2 mg l^{-1}, 5 min	7·15 (0·19)	ND	7·43 (0·27)	ND
2 mg l^{-1}, 60 min	7·15 (0·21)	ND	7·34 (0·19)	ND
20 mg l^{-1}, 5 min	7·17 (0·21)	ND	NA‡‡	ND
20 mg l^{-1}, 60 min	7·23 (0·15)	ND	NA	ND
Cl-L-control-PI**	6·08 (0·07)	5·08 (0·15)	6·33 (0·28)	2·75 (0·15)
0·5 mg l^{-1}-PI††	6·29 (0·24)	ND	6·16 (0·27)	ND
2 mg l^{-1}-PI††	7·14 (0·18)	ND	7·20 (0·22)	ND
20 mg l^{-1}-PI††	7·15 (0·10)	ND	NA	ND

*The sum of intact and injured cells as determined by the *Bac*Light staining method with a fluorescence microscope.
†Spread plate assay on BCYEα agar and incubation of plates at 37°C for 7 days with 5% CO_2.
‡Starved cell culture at *c.* 6 log cells ml^{-1} without chlorination.
§Standard deviations of the mean values in parentheses.
¶Non-detected (limit of detection = 1 CFU ml^{-1}).
**Postincubation of the controls for 7 days at 37°C without CO_2.
††Postincubation for 7 days at 37°C of samples having been chlorinated at specified concentration for 60 min.
‡‡NA, not available.

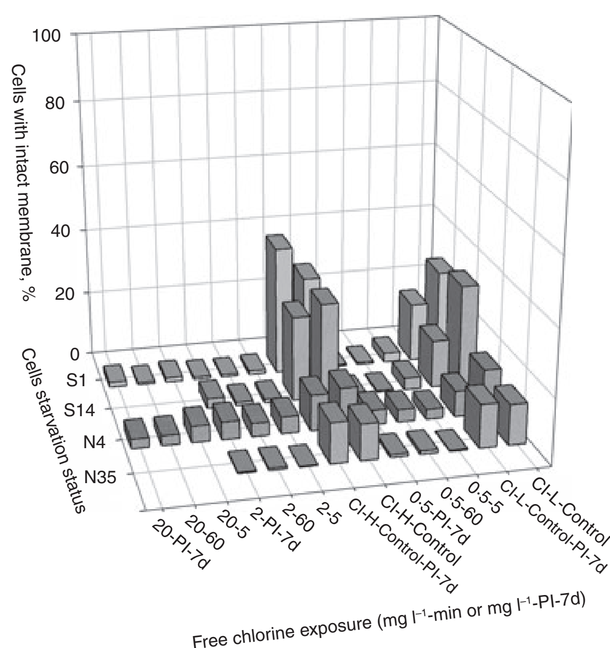

Figure 2 Average percentages of starved *Legionella pneumophila* cells with intact cellular membranes after exposure to chlorination. S1, S14, N4, and N35 cells represented *L. pneumophila* at early starvation phase (i.e. the first day in starved culture), culturability–decline phase (i.e. 14th day in starved culture), early non-culturable phase (i.e. fourth day of non-culturability) and late non-culturable phase (i.e. 35th day of non-culturability), respectively.

Table 4 Decrease in membrane-intact *Legionella pneumophila* (%) by 30-min heat treatment or 60-min chlorination

Treatment	Decrease in membrane-intact cells (%, mean ± SD)*			
	Culturable phase		Non-culturable phase	
	S1	S14	N4	N35
Heat (°C)				
50	18 ± 16	8 ± 6	7 ± 10	7 ± 4
60	17 ± 13	11 ± 4	10 ± 13	10 ± 3
70	47 ± 5	15 ± 4	11 ± 7	14 ± 3
Free chlorine (mg l^{-1})				
0·5	27 ± 9	31 ± 5	10 ± 4	11 ± 2
2	27 ± 7	28 ± 6	8 ± 2	10 ± 5
20	27 ± 8	NA†	9 ± 3	NA

*All percentages are relative to the total cells.
†NA, not available.

L. pneumophila in broth with 60–80°C heat treatment and reported an 8-log reduction of culturability within 25 min. Five and six log reductions were reported after exposure of *L. pneumophila* at 60 or 70°C for 3 min (Miyamoto *et al.* 2000) and at 60°C for 10 min respectively (Steinert *et al.* 1998). On the other hand, chlorination with 0·5 and 2 mg l^{-1} for 5 min resulted in complete loss of culturability for S1 and S14 *L. pneumophila*. This loss of culturability was also consistent with

© 2006 The Authors

the results obtained from non-starved *L. pneumophila* exposed to free chlorine of 0·5 mg l^{-1} (Kuchta *et al.* 1983), 2 mg l^{-1} (Miyamoto *et al.* 2000) and 3·3 mg l^{-1} (Skaliy *et al.* 1980) respectively. Although the decrease of culturability differed in magnitude among the present and previous studies, probably due to various strains and disinfection doses tested, the consistence in significant reduction of culturability suggests the response of starved *L. pneumophila* is similar to that of non-starved cells in terms of culturability loss when challenged with heat or chlorination. In addition to heat and chlorination, the present study revealed that starvation also has detrimental effects on culturability of *L. pneumophila*. As observed, superheating at 50°C for 5 and 30 min resulted in 95% and 81%, respectively, of S1 cells remaining culturable whereas only 22% and 7% of S14 cells showed culturability. It is suggested that, following superheating, a greater loss of culturability was apparent in cells that were starved longer.

In addition to the culture assay, *Bac*Light stains were applied to determine the disinfection efficacy. Among the diverse applications in bacteriological research (Ericsson *et al.* 2000; Auty *et al.* 2001), the *Bac*Light kit has been used specifically for evaluation of the efficacy of heat and chlorine disinfection methods through the effect on cell membrane (Arana *et al.* 1999; Boulos *et al.* 1999; Ramirez *et al.* 2000; Gunasekera *et al.* 2002). The current data demonstrates that the membrane of starved *L. pneumophila* was impaired by superheating, resulting in injuries to 7–47% of the cells (Table 4). Statistical analysis demonstrates that an increase of heating temperature and contact time significantly ($P < 0{\cdot}005$) increases membrane damage of *L. pneumophila* starved at 37°C, providing further evidence of the dose–response relationship between superheating treatment and damage to membrane integrity.

Furthermore, the degree of susceptibility to heat tends to relate to the duration of cells in starved culture. Table 4 shows, after starvation for 1 day, S1 cells demonstrated a greater susceptibility to heat treatment than S14, N4 and N35 cells starved for 14 days or longer. These findings suggest that starvation probably activates intracellular genetic, physiological or structural changes, which not only represent the responses of *L. pneumophila* to an unfavourable environment but also an increased cellular resistance to superheat challenge. Starvation of *L. pneumophila* at room temperature for 1 day causes a reduction in cell size with fairly smooth outer membrane observed by transmission electron microscopy, but becomes poor in uptake of ruthenium red (Harley *et al.* 1997). The present study also demonstrated that there was 11–16% of *L. pneumophila* cells still remaining membrane integrity after being starved at 37°C for over 2 months (N35 controls in Figs 1 and 2). Stabilization of cell membrane enables long-term stability and persistence of cells in the environment (McDougald *et al.* 1998). Additionally, starvation may induce proteins and other gene products that cross-protect cells against heat stress, such as has been shown in *Escherichia coli* (Jenkins *et al.* 1988) and *Salmonella typhimurium* (Spector *et al.* 1999). However, the exact intracellular response of starved and superheat-challenged *L. pneumophila* remains unclear, and warrants further investigation.

As for chlorination challenge, the majority of starved *L. pneumophila* treated at 0·5 mg l^{-1} or higher for 60 min exhibited significant compromised membranes (Fig. 2), which was statistically different from the responses of non-chlorinated cells. However, this detrimental effect on non-culturable N4 and N35 cells was approximately one-third of that shown in culturable S1 and S14 cells (Table 4), indicating *L. pneumophila* starved for 43 and 74 days are more resistant to chlorination than the cells starved for 14 days or less. Using fluorogenic stains, we demonstrated that long-term starvation remarkably increases chlorine resistance of *L. pneumophila* up to a high chlorine concentration of 20 mg l^{-1}.

A biphase process for chlorine resistance was proposed by Lisle *et al.* (1998), in which the extrinsic components (e.g. capsule layers and outer membrane) of bacteria provide a disinfection demand and effectively decrease the concentration of chlorine presented to a cell's membrane and other intracellular targets (e.g. nucleic acids). At the end of 60-min chlorination we observed low SD values of free chlorine residuals in the suspensions of *L. pneumophila* starved for various days (1–74 days), which might indicate that the amount of free chlorine entering *L. pneumophila* at each of four phases was similar, given the same concentration of free chlorine in the beginning. Under this situation, the more free chlorine is taken by the extrinsic components of a cell, the less free chlorine remains to interact with the cell membrane and thus to cause membrane damage. Based on our finding that the damage to membrane integrity was reduced for non-culturable *L. pneumophila* after long-term starvation, it is suggested that the constituents outside the cell membrane were modified during long-term starvation in ways that allowed them to interact with free chlorine increasingly, resulting in less remaining free chlorine to react with cell membranes. The thickening of the cell wall, observed in non-culturable cells during starvation (McDougald *et al.* 1998), could be one of the explanations for the increase of chlorine uptake outside the cell membrane. In addition to the alternations in cellular structure and composition, induction of starvation proteins may also play a role in regulation of cellular resistance to oxidative agents (Martin and Harakeh 1990).

© 2006 The Authors

As starvation commonly occurs in nature and in tap water, *L. pneumophila* may have developed a phenotype that is resistant to superheating and chlorination. Although the culturability of *L. pneumophila* could be completely inhibited, the ability of *L. pneumophila* to resist against disinfection treatments has implications of concern in public health, and may explain, in part, the presence and persistence of legionellae in potable as well as recirculating water systems.

In conclusion, superheating and chlorination significantly inhibit the culturability of starved *L. pneumophila* in warm water, and damage the cellular membrane to a much lesser extent compared with culturability loss. The membrane damage caused by superheating and chlorination was reduced along with increased duration of *L. pneumophila* in starvation, indicating the occurrence of resistance of starved cells to superheat and chlorine stresses. Long-term starvation at 37°C accelerates culturability loss and damages cell membrane, but plays a role in promoting *L. pneumophila* to develop resistance to stresses such as superheating and chlorination. The abovementioned characteristics should be taken into account in evaluation of disinfection efficacy and prevention of Legionnaires' disease as low nutrient is common in nature and in tap water where *L. pneumophila* survive.

Acknowledgements

This study was supported by the grants from Institute of Occupational Safety and Health, grant no. IOSH93-H301, and from National Science Council, grant no. NSC-94-2321-B-002-025.

References

Arana, I., Santorum, P., Muela, A. and Barcian, I. (1999) Chlorination and ozonation of waste-water: comparative analysis of efficacy through the effect on *Escherichia coli* membranes. *J Appl Microbiol* **86**, 883–888.

Auty, M.A.E., Gardiner, G.E., McBrearty, S.J., O'sullivan, E.O., Mulvihill, D.M., Collins, J.K., Fitzgerald, G.F., Stanton, C. *et al.* (2001) Direct *in situ* viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. *Appl Environ Microbiol* **67**, 420–425.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J. and Desjardins, R. (1999) LIVE/DEAD *Bac*Light: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* **37**, 77–86.

Centers for Disease Control and Prevention (2005) Legionnaires' disease associated with potable water in a hotel – Ocean City, Maryland, October 2003-February 2004. *MMWR* **54**, 165–168.

Domingue, E.L., Tyndall, R.L., Mayberry, W.R. and Pancorbo, O.C. (1988) Effects of three oxidizing biocides on *Legionella pneumophila* serogroup 1. *Appl Environ Microbiol* **54**, 741–747.

Environmental Protection Agency (1999) *Legionella*: *Human Health Criteria Document*. US Environmental Protection Agency EPA-822-R-99-001, Washington, DC.

Ericsson, M., Hanstorp, D., Hagberg, P., Enger, J. and Nystrom, T. (2000) Sorting out bacterial viability with optical tweezers. *J Bacteriol* **182**, 5551–5555.

Fliermans, C.B., Cherry, W.B., Orrison, L.H., Smith, S.J., Tison, D.L. and Pope, D.H. (1981) Ecological distribution of *Legionella pneumophila*. *Appl Environ Microbiol* **41**, 9–16.

Gunasekera, T.M., Sorenser, A., Attfield, P.V., Sorensen, S.J. and Veal, D.A. (2002) Inducible gene expression by nonculturable bacteria in milk after pasteurization. *Appl Environ Microbiol* **68**, 1988–1993.

Harley, V.S., Brasar, B.S. and Tovey, G. (1997) The ultrastructure of stressed *Legionella pneumophila*. *Microbios* **91**, 73–78.

Hussong, D., Colwell, R.R., O'Brien, M., Weiss, E., Pearson, A.D., Weiner, R.M. and Burge, W.D. (1987) Viable *Legionella pneumophila* not detectable by culturable on agar media. *Biotechnology* **5**, 947–950.

Jenkins, D.E., Schultz, J.E. and Martin, A. (1988) Starvation-induced cross protection against heat and H_2O_2 challenge in *Escherichia coli*. *J Bacteriol* **170**, 3910–3914.

Knirsch, C.A., Jakob, K., Schoonmaker, D., Kiehlbauch, J.A., Wong, S.J., Della-Latta, P., Whittier, S., Layton, M. *et al.* (2000) An outbreak of *Legionella micdadei* pneumonia in transplant patients: evaluation, molecular epidemiology, and control. *Am J Med* **108**, 290–295.

Kuchta, J.M., States, S.J., McNamara, A.M., Wadowsky, R.M. and Yee, R.B. (1983) Susceptibility of *Legionella pneumophila* to chlorine in tap water. *Appl Environ Microbiol* **46**, 1134–1139.

Kuchta, J.M., States, S.J., McGlaughlin, J.E., Overmeyer, J.H., Wadowsky, R.M., McNamara, A.M., Wolford, R.S. and Yee, R.B. (1985) Enhanced chlorine resistance of tap water-adapted *Legionella pneumophila* as compared with agar medium-passed strains. *Appl Environ Microbiol* **50**, 21–26.

Lee, J.V. and West, A.A. (1991) Survival and growth of *Legionella* species in the environment. *J Appl Bacteriol* **70**(Suppl.), 121S–129S.

Lisle, J.T., Broadaway, S.C., Prescott, A.M., Pyle, B.H., Fricker, C. and McFeters, G.A. (1998) Effects of starvation on physiological activity and chlorine disinfection resistance in *Escherichia coli* O157:H7. *Appl Environ Microbiol* **64**, 4658–4662.

Martin, A. and Harakeh, S. (1990) Effect of starvation on bacterial resistance to disinfectants. In *Drinking Water Microbiology* ed. McFeters, G.A. pp. 88–103. New York, NY: Springer-Verlag.

© 2006 The Authors

McDougald, D., Rice, S.A., Weichart, D. and Kjelleberg, S. (1998) Nonculturability: adaptation or debilitation? *FEMS Microbiol Ecol* **25**, 1–9.

Miyamoto, M., Yamaguchi, Y. and Sasatsu, M. (2000) Disinfectant effects of hot water, ultraviolet light, silver ions and chlorine on strains of *Legionella* and nontuberculous mycobacteria. *Microbios* **101**, 7–13.

National Institute of Infectious Diseases (2003) Legionellosis, April 1999-December 2002, Japan. *Infect Agents Surveill Rep* **24**, 27–28.

Occupational Safety and Health Administration (1999) Legionnaires' disease. In *In OSHA Technical Manual (OTM)*, TED 01-00-015 [TED 1-0·15A]. ed. Jeffress, C.N. pp. 15–25. Washington, DC: Occupational Safety and Health Administration.

Ohno, A., Kato, N., Yamada, N. and Yamaguchi, K. (2003) Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. *Appl Environ Microbiol* **69**, 2540–2547.

Perola, O., Kauppinen, J., Kusnetsov, J., Heikkinen, J., Jokinen, C. and Katila, M.L. (2002) Nosocomial *Legionella pneumophila* serogroup 5 outbreak associated with persistent colonization of a hospital water system. *APMIS* **110**, 863–868.

Perola, O., Kauppinen, J., Kusnetsov, J., Karkkainen, J.U.-M., Luck, P.C. and Katila, M.-L. (2005) Persistent *Legionella pneumophila* colonization of a hospital water supply: efficacy of control methods and a molecular epidemiological analysis. *APMIS* **113**, 45–53.

Ramirez, G.W., Alonso, J.L., Villanueva, A., Guardino, R., Basiero, J.A., Bernecer, I. and Morenilla, J.J. (2000) A rapid, direct method for assessing chlorine effect on filamentous bacteria in activated sludge. *Water Res* **34**, 3894–3898.

Saby, S., Leroy, P. and Block, J.-C. (1999) *Escherichia coli* resistance to chlorine and glutathione synthesis in response to oxygenation and starvation. *Appl Environ Microbiol* **65**, 5600–5603.

Schofield, G.M. (1985) A note on the survival of *Legionella pneumophila* in stagnant tap water. *J Appl Bacteriol* **59**, 333–335.

Skaliy, P., Thompson, T.A., Gorman, G.W., Morris, G.K., McEachern, H.V. and Mackel, D.C. (1980) Laboratory studies of disinfectants against *Legionella pneumophila*. *Appl Environ Microbiol* **40**, 697–700.

Spector, M.P., del Portillo, F.G., Bearson, S.M., Mahmud, A., Magut, M., Finlay, B.B., Dougan, G. and Foster, J.W. (1999) The rpoS-dependent starvation-stress response locus *stiA* encodes a nitrate reductase (*narZYWV*) required for carbon-starvation-inducible thermotolerance and acid tolerance in *Salmonella typhimurium*. *Microbiology* **145**, 3035–3045.

States, S.J., Conley, L.F., Kuchta, J.M., Oleck, B.M., Lipovich, M.J., Wolford, R.S., Wadowsky, R.M., McNamara, A.M. *et al.* (1987) Survival and multiplication of *Legionella pneumophila* in municipal drinking water systems. *Appl Environ Microbiol* **53**, 979–986.

Steinert, M., Ockert, G., Luck, C. and Hacker, J. (1998) Regrowth of *Legionella pneumophila* in a heat-disinfected plumbing system. *Zentralbl Bakteriol* **288**, 331–342.

Stout, J.E., Best, M.G. and Yu, V.L. (1986) Susceptibility of members of the family Legionellaceae to thermal stress: implications for heat eradication methods in water distribution systems. *Appl Environ Microbiol* **52**, 396–399.

Stout, J.E., Yu, V.L., Muraca, P., Joly, J., Troup, N. and Tompkins, L.S. (1992) Potable water as a cause of sporadic cases of community-acquired Legionnaires' disease. *N Engl J Med* **326**, 151–155.

Tablan, O.C., Anderson, L.J., Besser, R., Bridges, C. and Hajjeh, R. (2003) *Guidelines for Preventing Health-Care-Associated Pneumonia, 2003*. Atlanta, GA: US National Centers for Infectious Disease, MMWR 53/RR-3, pp. 1–37.

Yee, R.B. and Wadowsky, R.M. (1982) Multiplication of *Legionella pneumophila* in unsterilized tap water. *Appl Environ Microbiol* **43**, 1330–1334.

© 2006 The Authors
Journal compilation © 2006 The Society for Applied Microbiology,