CURRENT MICROBIOLOGY Vol. 21 (1990), pp. 139–143

Current Microbiology

Inhibition of Growth of *Legionella* Species by Heterotrophic Plate Count Bacteria Isolated from Chlorinated Drinking Water

Simon Toze, Lindsay I. Sly, Ian C. MacRae, and John A. Fuerst

Department of Microbiology, University of Queensland, Queensland, Australia

Abstract. The ability of heterotrophic plate count bacterial strains isolated from chlorinated drinking water on low-nutrient media to inhibit the growth of *Legionella* species was examined. Between 16% and 32% of these strains were able to inhibit the growth of *Legionella* species when tested on buffered charcoal yeast extract agar. The exact proportion of inhibiting strains varied with the individual *Legionella* species. Two strains that inhibited the growth of several *Legionella* species could also stimulate the growth of the same species when both the test strain and the *Legionella* species were grown on buffered charcoal yeast extract agar that lacked the essential amino acid L-cysteine.

Legionella is a nutritionally fastidious bacterium requiring amino acids as the source of energy [8]. A suggested mode of survival in the environment is by the interaction of the *Legionella* cells with non-*Legionella* bacteria [16]. There have been several reports of microorganisms sharing the same environment as *Legionella* being able to support the growth of *Legionella*. Microorganisms supporting the growth of *Legionella* include algae such as *Fischerella* [2, 3], green algae [12], and cyanobacteria [18]; amoebae [14, 19, 22]; and non-*Legionella* heterotrophic bacteria [16, 17, 20, 21]. Such heterotrophic bacteria have been shown to support the growth of *L. pneumophila* on media lacking the amino acid L-cysteine [16, 17, 20, 21]. It has been suggested that the phenomena involving the stimulation of *Legionella* growth by non-*Legionella* bacteria are important to the survival and growth of *Legionella* in aquatic ecosystems [17, 20].

Very little has been documented on non-*Legionella* bacteria inhibiting the growth of *Legionella*. Carrington and Emmerling and Sticht-Groh found that several respiratory bacterial strains were capable of inhibiting the growth of *L. pneumophila* [4, 6]. Chandler noted that *L. pneumophila* was inhibited in vitro by *Aspergillus* sp. [5]. Makin noted inhibition of *L. pneumophila* growth by 48% of non-*Legionella* bacterial strains isolated from water samples [10]. This study was undertaken to determine whether bacterial strains isolated from heterotrophic plate counts of chlorinated drinking water were capable of inhibiting the growth of *Legionella* species on solid media.

Methods and Materials

***Legionella* strains and growth conditions.** The *Legionella* strains used were the following: *L. pneumophila* (Philadelphia 1) 87A-2120 (= ATCC 33152); *L. bozemanii* NCTC 11368 (= ATCC 33217); *L. dumoffii* NCTC 11370 (= ATCC 33279); *L. gormanii* NCTC 11401 (= ATCC 33297); *L. longbeachae* NCTC 11477 (= ATCC 33462); *L. micdadei* NCTC 11371 (= ATCC 33218); *L. oakridgensis* NCTC 11531 (= ATCC 33761); and *L. wadsworthii* NCTC 11532 (= ATCC 33877). All the *Legionella* species were derived from the National Collection of Type Cultures (NCTC) except for the *L. pneumophila* strain, which was from the California State Health Service.

All *Legionella* species were grown aerobically at 35°C for 3 days, on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (αBCYEA).

Isolation and identification of heterotrophic plate count (HPC) bacteria from chlorinated drinking water. All strains were isolated from the water distribution systems of the Brisbane City Council and the Gold Coast City Council (Queensland, Australia) and the Mornington Peninsular Water Board (Victoria, Australia) by use of the heterotrophic plate count media R_2A [13], Standard Methods Agar (SMA) [9], Casein Peptone Starch Agar (CPS) [9], or Dilute Peptone Agar (DPA) [9]. All isolated strains were maintained on R_2A at 20°C. R_2A is a low-nutrient medium specifically designed to yield maximum viable counts of bacteria from drinking water [13]. The disinfection of the water differs among the three water authorities. The Brisbane City Council uses chloramination, the Gold Coast City Council uses a chlorine dioxide and

Address reprint requests to: Simon Toze, Department of Microbiology, University of Queensland, Queensland, Australia 4072.

Table 1. Proportion of HPC strains from drinking water able to inhibit *Legionella* growth

Legionella species	Percent strains inhibiting *Legionella* growth[a]
L. pneumophila	16
L. bozemanii	23
L. dumoffii	29
L. gormanii	20
L. longbeachae	26
L. micdadei	23
L. oakridgensis	32
L. wadsworthii	24

[a] 100 strains were tested.

chlorine mixture, and the Mornington Peninsular Water Board uses free chlorine.

In total, 100 HPC strains were isolated from the different water distribution systems and used to test for the ability to inhibit *Legionella* growth.

Identification of the 100 HPC strains was attempted by either the API20NE rapid identification system (API), or by the identification protocol outlined in an EPA report on the identification of heterotrophic bacteria from drinking water [15] and the scheme for the identification of Gram-negative nonfermentative bacteria devised by Ward et al [23]. Where possible, an identity was given to each HPC strain. Strains for which a definite identity could not be given but that were Gram-negative rods that were oxidase and catalase positive and oxidative in the glucose oxidation/fermentation test were grouped as *Pseudomonas*-like strains. Strains that could not be placed in the *Pseudomonas*-like group owing to the lack of one or more of the characters of the *Pseudomonas*-like group but were Gram-negative rods that did not ferment glucose were grouped as Gram-negative, nonfermentative (GNNF) bacteria [23]. If the strain was classified as unidentifiable by the API20NE identification system or did not fit into either of these groups by the classification methods from the EPA report and the identification scheme of Ward et al, it was characterized as unidentifiable.

Culture media. The full growth medium used in this study to assay for the inhibition of *Legionella* growth and to maintain the *Legionella* species was buffered charcoal yeast extract agar supplemented with α-ketoglutarate (αBCYEA). This medium contains a final concentration of 0.04% of L-cysteine and 0.025% of ferric pyrophosphate [7].

The medium used to assay for the stimulation of *Legionella* growth was the unsupplemented buffered charcoal yeast extract agar (UNBCYEA). This medium is the same as the αBCYEA medium except that no L-cysteine or α-ketoglutarate is added, and only 0.001% (final concentration) of ferric pyrophosphate is added [20].

Assay of ability to inhibit *Legionella* growth. Each strain of *Legionella* was suspended in 5 ml of sterile distilled water to an absorbance of 1 Mcfarland standard [11]. A sterile swab was dipped into the suspension of each *Legionella* species and used to inoculate the entire surface of a number of αBCYEA plates with a 'Mast Laboratory' rotary inoculator. A small amount of growth of each HPC strain from chlorinated drinking water was patched onto the surface of the inoculated αBCYEA plates with a nichrome wire loop. One or two strains were patched onto each αBCYEA plate. Inoculated plates were incubated aerobically at 28°C for 3–4 days.

After incubation the plates were examined with a stereo plate microscope for zones of inhibition of *Legionella* growth around each of the HPC drinking water strains. The inhibition zones were measured from the edge of the HPC growth to the edge of the inhibition zone.

Table 2. Extent of inhibition of *Legionella* growth around HPC isolates from drinking water

	HPC bacterial isolates			
Legionella sp.	M101[a]	7A-1[b]	M66[c]	M37[d]
L. pneumophila	1[e]	0	5	0
L. bozemanii	7	1	4	17
L. dumoffii	19	8	12	16
L. gormanii	6	0	3	3
L. longbeachae	8	1	5	3
L. micdadei	7	0	6	22
L. oakridgensis	NG[f]	6	NG	NG
L. wadsworthii	10	0	5	15

[a] M101 was identified as an *Aeromonas* sp.
[b] 7A-1 was a strain that was unidentifiable.
[c] M66 was a strain that was unidentifiable.
[d] M37 was identified as an *Aeromonas* sp.
[e] Inhibition zone around the HPC strain, in mm.
[f] NG = no growth of *Legionella* owing to complete inhibition.

Assay of ability to stimulate *Legionella* growth. This method, a modification of the method used by Wadowsky and Yee [20], is exactly the same as that described above for the assay of inhibition of *Legionella* growth except that the unsupplemented medium UNBCYEA was used instead of the fully supplemented medium αBCYEA. A positive result was recorded when satellite growth of *Legionella* colonies was observed around colonies of the HPC test strain with the aid of a stereo plate microscope.

Results

Preliminary identification of the HPC strains used in this study assigned ten strains to the genera *Aeromonas*, six strains to *Pseudomonas vesicularis*, two to *Ps. paucimobilis*, one to the genus *Pseudomonas*, one to *Vibrio fluvialis*, and one strain to *Ps. maltophilia*. Of the remaining 63 strains, 16 were characterized as being *Pseudomonas*-like, 22 were characterized as being GNNF bacteria, and 29 strains could not be identified. No identification was attempted for the remaining 12 strains.

The proportion of HPC strains able to inhibit the growth of *Legionella* varied with the *Legionella* species (Table 1). Where inhibition was observed, for each *Legionella* species the size of the inhibition zone depended on the HPC strain tested. An example of this can be seen in Table 2. This pattern was

Table 3. Spectrum of inhibition and stimulation of *Legionella* by two HPC strains with both stimulating and inhibiting ability

	HPC strains			
	4-1[a]		7C-3[a]	
Legionella species	Stimulation	Inhibition	Stimulation	Inhibition
L. pneumophila	+	–	+	–
L. bozemanii	+	–	+	–
L. dumoffii	+	–	+	+
L. gormanii	+	+	+	–
L. longbeachae	+	+	+	–
L. micdadei	+	–	+	–
L. oakridgensis	+	+	+	+
L. wadsworthii	+	–	+	–

[a] Both strains were unidentifiable.

confirmed over a wider data set for the 100 HPC strains tested. Although some of the HPC strains were not capable of completely inhibiting the growth of a particular *Legionella* species, it was noted that some of these strains were, however, able to reduce the amount of *Legionella* growth.

Aeromonas strains were identified as inhibitors of each of the *Legionella* species listed in Table 1. One strain of *Pseudomonas vesicularis* inhibited all of the *Legionella* species. The single *Vibrio fluvialis* strain inhibited the growth of all the *Legionella* species except for *L. pneumophila*. The remaining strains were either *Pseudomonas*-like, unable to be identified, or in the group for which no identification was attempted. Neither the *Pseudomonas paucimobilis* strains nor any of the GNNF bacteria not attributed were able to inhibit the growth of any of the *Legionella* species tested.

One of the interesting phenomena observed was that two of the HPC strains, capable in some trials of inhibiting the growth of a particular *Legionella* species on the fully supplemented αBCYEA medium, were also able to stimulate the growth of the same *Legionella* species on the unsupplemented growth medium that lacked L-cysteine and contained only minimal iron (UNBCYEA). The spectrum of stimulatory or inhibitory activity of these two strains against different *Legionella* species is given in Table 3. Strain 4-1 displayed this phenomenon when tested against *L. gormanii*, *L. longbeachae*, and *L. oakridgensis*, whereas strain 7C-3 displayed this phenomenon only when tested against *L. dumoffii* and *L. oakridgensis*. The ability of these two strains to stimulate the growth of *Legionella* was consistent over all tests performed. However, it was noted that the ability to inhibit the growth of *Legionella* occurred in some tests but failed to occur in some replicate tests.

Discussion

It has been suggested that water distribution systems are the original source of the *Legionella* cells that cause the *Legionella* outbreaks in artificial aquatic environments such as cooling towers and hot water tanks [17]. Water distribution systems are generally nutrient-poor environments and *Legionella*, a nutritionally fastidious microorganism, must survive in these environments. It has been suggested that *Legionella* is able to interact with other microorganisms to obtain the nutrients it requires to survive [16, 20, 22]. It has been shown that *Legionella* strains are able to interact positively with a variety of non-*Legionella* microorganisms present in the same environment [16, 18–20].

Although much work has been conducted on the stimulation of *Legionella* growth by other microorganisms, little work has been done on the negative interactions that occur between *Legionella* cells and other microorganisms. This study has shown that up to a third of a collection of heterotrophic plate count bacteria isolated from water distribution systems of typical subtropical Australian cities are capable of inhibiting *Legionella* growth, with the exact proportion of inhibition depending on the *Legionella* species. The study also showed that there are some strains that are capable of both stimulating and inhibiting *Legionella* growth, depending on the medium composition.

The results suggest that up to 32% of heterotrophic plate count bacteria isolated from chlorinated drinking water are capable of inhibiting the growth of *Legionella* species. This is comparable to the results of Makin, who found that 48% of the non-*Legionella* strains isolated from water samples with a selective medium were able to inhibit the growth of *L. pneumophila* on solid media [10].

Preliminary identification of the HPC strains tested in this study indicated that the strains able to inhibit the growth of the *Legionella* species tested were taxonomically diverse and included *Aeromonas* species, *Vibrio fluvialis*, *Pseudomonas vesicularis* and *Pseudomonas*-like strains.

A large number of the HPC strains could not be identified. Carrington found that five normal respiratory bacterial species, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumon-*

iae, *Bacillus* sp., and *Streptococcus* viridans group were capable of inhibiting the growth of *L. pneumophila* [4]. In a similar study, Emmerling and Sticht-Groh showed that a number of strains of the *Streptococcus* viridans group, pneumococci, *Pseudomonas aeruginosa*, *Staphylococcus*, and *Bacillus* sp. inhibited *L. pneumophila* growth in vitro [6]. The results of our study showed that, of the 32 HPC strains that were capable of inhibiting *Legionella* growth, only 6 were Gram-positive. This contrasts with the results obtained in the two studies conducted by Carrington and Emmerling and Sticht-Groh, in which all the strains inhibiting *Legionella* growth were Gram-positive organisms except for *K. pneumoniae* in Carrington's study [4] and *Pseudomonas aeruginosa* in Emmerling and Sticht-Groh's study [6].

Makin suggested that the presence of bacterial strains capable of inhibiting *Legionella* growth in the water sample has the potential for causing a false negative result [10]. Thus, although *Legionella* is present in a water sample, it may not be isolated on solid media owing to masking or inhibition by non-*Legionella* strains growing on the isolation medium. We support this view because of the high precentage of inhibiting bacterial strains we detected. It is conceivable that if there are a large number of non-*Legionella* strains present in a water sample capable of growth on the selective isolation medium and able to inhibit *Legionella* growth, this would reduce the chance of isolating *Legionella* from the water sample. Further work is being carried out to assess the number of inhibiting bacterial strains present in a water sample by a quantitative plate assay.

At present little is known about the characteristics or mode of action of the inhibiting substances released by the HPC strains. Initial studies have shown that the inhibiting substances differ among different HPC strains, with some of the inhibiting substances having bactericidal action and some possessing a bacteriostatic action. Further work is being conducted to further characterize the inhibiting substances and to study their modes of action.

The action of the two strains capable of both stimulating and inhibiting *Legionella* is yet to be determined. The mechanisms underlying the phenomenon, whereby an HPC strain inhibits the growth of *Legionella* on the fully supplemented medium (αBCYEA) and also stimulates the growth of the same *Legionella* strain on the unsupplemented medium which contains no L-cysteine and only a marginal amount of ferric pyrophosphate, has not been determined. It is possible that production of inhibitory substances by HPC bacteria is, in some cases, dependent on L-cysteine in the medium.

The in situ ability of the HPC isolates from drinking water to inhibit the growth of *Legionella* in the natural aquatic environment needs to be examined. The results gained in this and future experiments may lead to an understanding of why some *Legionella* species are more prevalent in the environment than others. For example, *L. pneumophila* is the most commonly detected species of *Legionella* [1]. As can be seen in Table 1, the *L. pneumophila* strain used in this study was the species that was inhibited by the smallest proportion of the HPC strains tested. This could be at least a partial explanation as to why *L. pneumophila* is the most prevalent *Legionella* species detected in environmental samples.

Demonstration that HPC drinking water bacteria often possess the ability to inhibit *Legionella* growth may lead to a better understanding of the interaction of *Legionella* with other bacteria in the environment. This understanding could lead to improved control measures in which the presence of inhibiting non-*Legionella* strains in the water source is encouraged and efforts made to preserve or increase their numbers.

ACKNOWLEDGMENTS

This project was funded by Catoleum Pty. Ltd., Marford Feedwater Pty. Ltd., University of Queensland Building and Grounds Section, and a Special Projects Grant from the University of Queensland. We thank Dr. J. Michael Janda and Dr. T.F. Midura of the California State Health Service for providing us with the *L. pneumophila* strain (Philadelphia 1).

Literature Cited

1. Bartlett CLR, Kurtz JB, Hutchison JGP, Turner GC, Wright AE (1983) *Legionella* in hospital and hotel water supplies. Lancet II:1315
2. Bohach GA, Snyder IS (1983) Cyanobacterial stimulation of growth and oxygen uptake by *Legionella pneumophila*. Appl Environ Microbiol 46:528–531
3. Bohach GA, Snyder IS (1983) Characterisation of surfaces involved in adherence of *Legionella pneumophila* to *Fischerella* species. Infect Immun 42:318–325
4. Carrington GO (1979) Legionnaires' disease bacillus: inhibition by normal flora. Clin Microbiol Newslett 1:7–8
5. Chandler FW (1981) In vivo inhibition of *Legionella pneumophila* by *Aspergillus*. Lancet I:994
6. Emmerling P, Sticht-Groh V (1983) In vitro growth inhibition of *Legionella pneumophila* by different grampositive and gramnegative organisms. Abstracts of the 39th meeting of the Section Medical Microbiology and Immunology, Deutsche

Gesellschaft für Hygiene und Mikrobiologie 1983. Zbl. Bakt. Hyg. A. 256:413

7. Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB (1979) Charcoal–yeast extract agar: primary isolation medium for *Legionella pneumophila*. J Clin Microbiol 10:437–441
8. George JR, Pine L, Reeves MW, Harrell WK (1980) Amino acid requirements of *Legionella pneumophila*. J Clin Microbiol 11:286–291
9. Maki JS, LaCroix SJ, Hopkins BS, Staley JT (1986) Recovery of heterotrophic bacteria from chlorinated drinking water. Appl Environ Microbiol 51:1047–1055
10. Makin T (1986) Inhibition of *Legionella* by other organisms. Med Lab Sci 43:854
11. Paik G (1980) Reagents, stains and miscellaneous test procedures. In: Lenette EH, Balows A, Hauslew WJ, Truant JB (eds) Manual of clinical microbiology, 3rd edn. Washington: American Society for Microbiology, pp 1000–1024
12. Pope DH, Soracco RJ, Gill HK, Fliermans CB (1982) Growth of *Legionella pneumophila* in two-membered cultures with green algae and cyanobacteria. Curr Microbiol 7:319–322
13. Reasoner DJ, Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49:1–7
14. Rowbotham TJ (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol 33:1179–1183
15. Staley JT (1985) Enumeration and identification of heterotrophic bacteria from drinking water. Project summary. United States Environmental Protection Agency Report EPA/600/S2-85/061
16. Stout JE, Yu VL, Best MG (1985) Ecology of *Legionella pneumophila* within water distribution systems. Appl Environ Microbiol 49:221–228
17. Stout JE, Best MG, Yu VL, Rihs JD (1986) A note on the symbiosis of *Legionella pneumophila* and *Tatlockia micdadei* with human respiratory flora. J Appl Bacteriol 60:297–299
18. Tison DL, Pope DH, Cherry WB, Fliermans CB (1980) Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). Appl Environ Microbiol 39:456–459
19. Tyndall RL, Domingue EL (1982) Cocultivation of *Legionella pneumophila* and free-living amoebae. Appl Environ Microbiol 44:954–959
20. Wadowsky RM, Yee RB (1983) Satellite growth of *Legionella pneumophila* with an environmental isolate of *Flavobacterium breve*. Appl Environ Microbiol 46:1447–1449
21. Wadowsky RM, Yee RB (1985) Effect of non-*Legionella* bacteria on the multiplication of *Legionella pneumophila* in potable water. Appl Environ Microbiol 49:1206–1210
22. Wadowsky RM, Butler LJ, Cook MK, Verma SM, Paul MA, Fields BS, Keletii G, Sykora JL, Yee RB (1988) Growth-supporting activity for *Legionella pneumophila* in tap water cultures and implication of Hartmannellid amoebae as growth factors. Appl Environ Microbiol 54:2677–2682
23. Ward NR, Wolfe RL, Justice CA, Olson BH (1986) The identification of gram negative nonfermentative bacteria from water: problems and alternative approaches to identification. Adv Appl Microbiol 31:293–365