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Enhanced Chlorine Resistance of Tap Water-Adapted *Legionella pneumophila* as Compared with Agar Medium-Passaged Strains

JOHN M. KUCHTA,[1]* STANLEY J. STATES,[1] JANE E. McGLAUGHLIN,[1] JEAN H. OVERMEYER,[1] ROBERT M. WADOWSKY,[2] ANN M. McNAMARA,[2] RANDY S. WOLFORD,[2] AND ROBERT B. YEE[2]

Department of Water, Pittsburgh, Pennsylvania 15215,[1] and Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261[2]

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Previous studies have shown that bacteria maintained in a low-nutrient "natural" environment such as swimming pool water are much more resistant to disinfection by various chemical agents than strains maintained on rich media. In the present study a comparison was made of the chlorine (Cl_2) susceptibility of hot-water tank isolates of *Legionella pneumophila* maintained in tap water and strains passaged on either nonselective buffered charcoal-yeast extract or selective differential glycine-vancomycin-polymyxin agar medium. Our earlier work has shown that environmental and clinical isolates of *L. pneumophila* maintained on agar medium are much more resistant to Cl_2 than coliforms are. Under the present experimental conditions (21°C, pH 7.6 to 8.0, and 0.25 mg of free residual Cl_2 per liter, we found the tap water-maintained *L. pneumophila* strains to be even more resistant than the agar-passaged isolates. Under these conditions, 99% kill of tap water-maintained strains of *L. pneumophila* was usually achieved within 60 to 90 min compared with 10 min for agar-passaged strains. Samples from plumbing fixtures in a hospital yielded legionellae which were "super"-chorine resistant when assayed under natural conditions. After one agar passage their resistance dropped to levels of comparable strains which had not been previously exposed to additional chlorination. These studies more closely approximate natural conditions than our previous work and show that tap water-maintained *L. pneumophila* is even more resistant to Cl_2 than its already resistant agar medium-passaged counterpart.

Legionella pneumophila has been isolated from a wide variety of environmental niches (9, 12, 13, 19, 22). It has been shown to be present and to grow in samples obtained from the hot-water tanks of hospitals which are routinely kept at low temperatures (e.g., 43 to 55°C) and may contain elevated concentrations of certain metals due to corrosion (16, 26; S. J. States, J. M. Kuchta, M. Ceraso, T. E. Stephenson, R. M. Wadowsky, A. M. McNamara, R. S. Wolford, and R. B. Yee, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q5, p. 205). The organism is also capable of increasing 2 to 3 logs in membrane filter-sterilized tap water (28).

We have shown that clinical and environmental strains of *L. pneumophila* are much more resistant to chlorine disinfection than are organisms comprising the coliform group, the commonly used indicators of the potability of municipal water supplies (17). We could routinely isolate *L. pneumophila* from the Allegheny River, the source of the potable supply for most of the City of Pittsburgh, and also isolate the organism at the end of the water distribution system, i.e., the hospital hot-water tanks. Our current hypothesis is that small numbers of the bacterium may occasionally pass through the water treatment plant and seed the hot-water tanks of hospitals. Whether or not this hypothesis is correct, measures may need to be taken to control the number of legionellae in internal recirculating water systems typically found in hospitals and large public buildings.

Although we have shown the agar-passed strains of legionellae to be much more resistant to chlorine than other bacteria tested, we wanted to more closely approximate a more natural environmental situation. This could be accomplished by inoculating a hot-water tank sample containing legionellae into filter-sterilized tap water, passing the organism into freshly dechlorinated tap water after several log increases had occurred, and subsequently "pooling" a number of these tap water-maintained samples. Since growth of this organism in tap water has already been reported (28), chlorine resistance studies with an organism never exposed to artificial media were now possible.

We thought that chlorine resistance of agar-passed organisms should now be compared with the resistance of those organisms never exposed to an artificial medium. Dramatic changes in sensitivity to various chemical agents have been reported for several organisms under different growth conditions (1, 5, 6, 10, 18). Carson et al. (5) found *Pseudomonas aeruginosa* and atypical mycobacteria (6) grown in deionized water to be much more resistant to chlorine dioxide and chlorine, respectively, than agar medium-grown strains. Favero and Drake (10) found that an analogous situation existed for swimming pool isolates which were much more resistant to iodine when maintained in "pool water" rather than Trypticase soy agar (BBL Microbiology Systems). Recently, it has been reported that certain components of the cell envelope of *Escherichia coli* could be altered, and this change may be related to whether the organism had been grown on rich media or bay water (7). This organism grown in filtered bay water was less sensitive to bacteriophage and colicins but more sensitive to heavy metals and detergents as compared with the same strains grown on rich media.

We wondered if an analogous situation existed with legionellae and whether we might see significant differences in resistance to chlorine between tap water-grown legionellae and those subcultured on agar medium. Another concern of this study was to determine whether or not a hospital which practiced "in-house" chlorination would inadvertently select for organisms which are even more resistant to

* Corresponding author.

TABLE 1. Comparison of chlorine demand of boiled and inoculated[a] tap water with demand of deionized, distilled water[b]

Sample	mg/liter[c]	
	Total chlorine	Free chlorine
Boiled and inoculated tap water	0.10	0.05
	0.20	0.15
	0.45	0.40
Deionized, distilled water	0.10	0.10
	0.20	0.15
	0.50	0.45

[a] A 30-ml portion of tap water-grown cells was heat killed and added to 570 ml of boiled tap water.
[b] Essentially chlorine demand-free.
[c] Determined by the amperometric method.

chlorine than those not previously exposed to this disinfectant. Further, if these organisms have acquired greater resistance to chlorine, would they also lose it on passage to agar medium?

MATERIALS AND METHODS

Water cultures and bacterial isolates. A suspension of *L. pneumophila* and other bacteria which had never been grown on artificial media was derived from a water sample obtained from the bottom drain valve of a peripherally heated hot-water tank. *L. pneumophila* serogroup 1 determined by direct fluorescent-antibody analysis (8) was the most prevalent organism in this hot-water tank, outnumbering a yellow-pigmented organism, the next more prevalent species, by about 5 to 1. This ratio was determined by comparing the number of *L. pneumophila* recovered on differential glycine-vancomycin-polymixin B agar (DGVP) (24) with the number of yellow-pigmented organisms recovered on unsupplemented buffered charcoal-yeast extract agar (UNBYCE) (26). The pigmented organism was presumptively identified as a *Flavobacterium* sp. (27) by the following characteristics: yellow to orange pigmented, smooth, entire, slightly mucoid, colonies on UNBCYE, and no growth observed on MacConkey agar. The bacterium was a gram-negative bacillus, asporogenous, and nonmotile. It was oxidase and catalase positive. The tank was also inhabited by even smaller numbers of two other unidentified bacteria. The naturally occurring bacteria were maintained in the laboratory by periodic transfer to membrane filter-sterilized chlorine-free tap water and incubated at 35°C as previously described (R. M. Wadowsky, Sc.D. thesis, University of Pittsburgh, Pittsburgh, Pa., 1984). Samples were periodically diluted and plated on DGVP (24), buffered charcoal-yeast extract agar (BCYE) (20), and UNBCYE (26), and legionellae were identified as described previously on the basis of colonial morphology, the inability to grow on UNBCYE, and the direct immunofluorescence test (8, 24). The number of non-legionellae was determined by plating a sample on UNBCYE.

Agar medium-passaged cultures were obtained by plating the above water-maintained strains on BCYE. The surfaces of 10 colonies were picked and either restreaked on BCYE if additional passages were desired or immediately suspended in distilled water and treated as previously described (17). "Super"-chlorine-resistant strains were obtained from a sink faucet spout of a hospital plumbing system which had had a hyperchlorinator installed 2 months previously. The concentration of total residual Cl_2 had been adjusted to 2 ppm (mg/liter) for a period of 3 weeks. Control isolates were obtained from the same fixture before installation of the hyperchlorinator.

Experimental procedure and chlorine determination. The chlorine sensitivity of the bacteria was tested as previously described (17) with some modifications. The cells were subjected to a "standard" initial free Cl_2 concentration of 0.25 mg/liter since preliminary experiments indicated that this concentration effected a more rapid kill rate in agar-grown cells. The tap water used in the experiments was found to remain between pH 7.7 and 7.8, and therefore no additional adjustment was made with phosphate buffer as we had previously done (17). All experiments were carried out at 20°C.

Tap water was "aged" several days to remove any residual chlorine. It was subsequently boiled before use to kill any indigenous organisms. Sporeformers were not indigenous to this tap water since we never recovered them on UNBCYE or BCYE medium. After cooling to room temperature, 30 to 40 ml of tap water-maintained legionellae was added to a 1-liter Erlenmeyer flask to give a final test volume of 600 ml.

In a control flask, an identical portion of water-maintained culture was heated to 60°C for 30 min to kill legionellae and other organisms and added to boiled tap water to give a volume of 594 ml. This step was included to control for any effect attributable to any indigenous organic compounds in the sample. Legionellae derived from the water-maintained sample were grown on BCYE agar plates, washed two times with distilled water, and diluted so that a 6-ml sample would give a final cell concentration of 3,000 CFU/ml when added to the test vessel already containing 594 ml. Each flask was then ready for chlorine addition.

As in the previous study (17), chlorine demand of the test system was compared with essentially demand-free distilled deionized water. Calcium hypochlorite was added to each type of water, and free and total chlorine residuals were determined for each. Boiled tap water with added tap water-maintained bacteria was essentially demand-free (Table 1).

The test system was inoculated as previously described (17).

Viability of centrifuged organisms. To determine if centrifugation may have contributed to the decrease of chlorine resistance of the agar medium-passaged isolates, we centrifuged water-maintained cells at 3,000 × *g* for 20 min, plated the cells on a modified DGVP medium containing low charcoal (J. H. Overmeyer, J. M. Patti, S. J. States, and J. M. Kuchta, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q39, p. 264), and tested them with chlorinated filter paper disks (23) as previously described. The chlorine sensitivity was then compared with that of cells which had not been centrifuged. Three separate trials gave nearly identical zones for inhibition. In a separate experiment, damage to cells was ascertained by centrifuging the cells before diluting them into sterile tap water for growth. Again, cells which had been centrifuged had the same ability to initiate 2- to 3-log growth as cells which had not been centrifuged.

RESULTS

We initially transferred tap water-grown *L. pneumophila* to BCYE agar and compared the chlorine susceptibility of this and subsequently agar medium-passaged strains with those which had never been grown on agar. Figure 1 shows that the greatest decrease in chlorine resistance occurs when

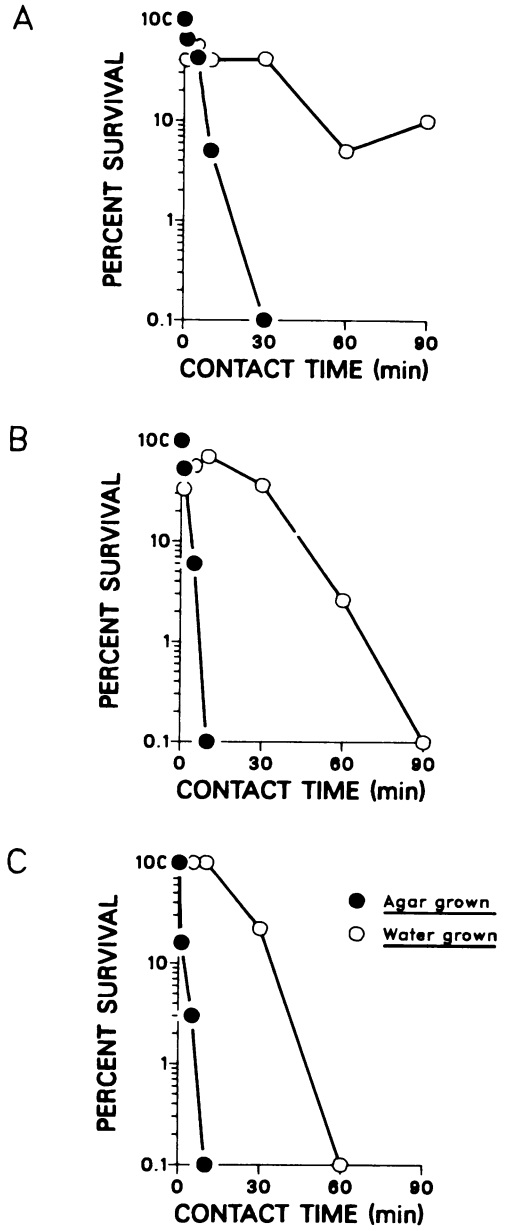

FIG. 1. Effect of BCYE agar medium passages on bactericidal activity of Cl_2 under standard conditions (see text). (A) One agar medium passage; (B) two agar passages; (C) six agar passages.

the organism is initially subcultured on artificial medium. Subsequent subcultures on BCYE medium, as many as six, had little effect on further decreasing the organisms' resistance to chlorine. The absolute difference in kill rate between agar-passed and water-maintained cells was nearly the same whether the organisms were plated on BCYE or DGVP medium (Fig. 1B and 2). However, BCYE medium routinely provided 10 to 30% better recovery of chlorine-damaged organisms.

We next attempted to determine if the difference in resistance patterns between agar medium-passed and tap water-maintained cells was unique to the organisms obtained from one particular hot-water tank. We therefore obtained a sample from another hot-water tank in a different building. These organisms were passed in tap water or on artificial

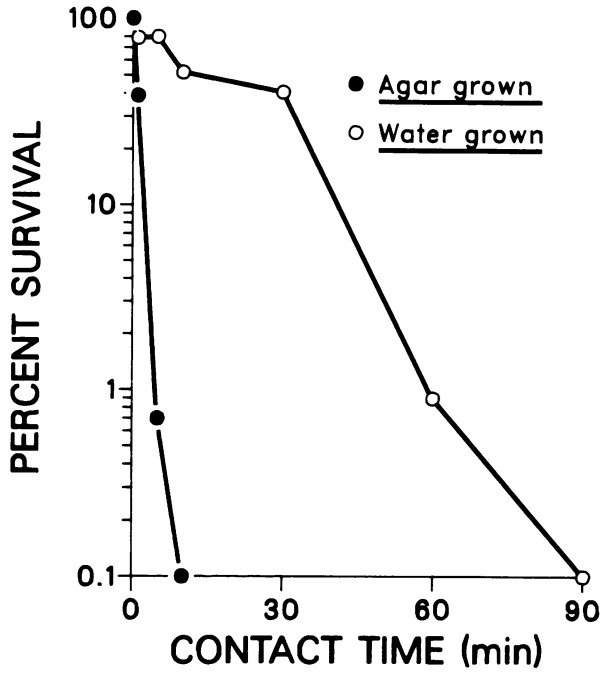

FIG. 2. Survival of legionellae after DGVP agar medium passages when treated with Cl_2 and plated on selective medium (see text).

media as described above. Figure 3 shows that one agar passage decreases chlorine resistance significantly in this strain, whereas the tap water-maintained culture was very resistant to chlorine. These results substantiated our findings with the first hot-water tank.

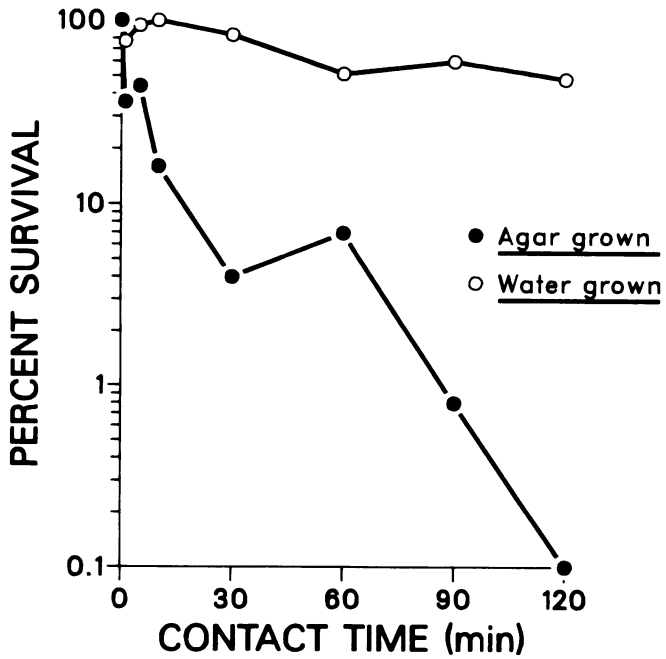

FIG. 3. Effect of one BCYE agar medium passage on Cl_2 activity of a legionella strain from another hot-water tank in a different building (see text).

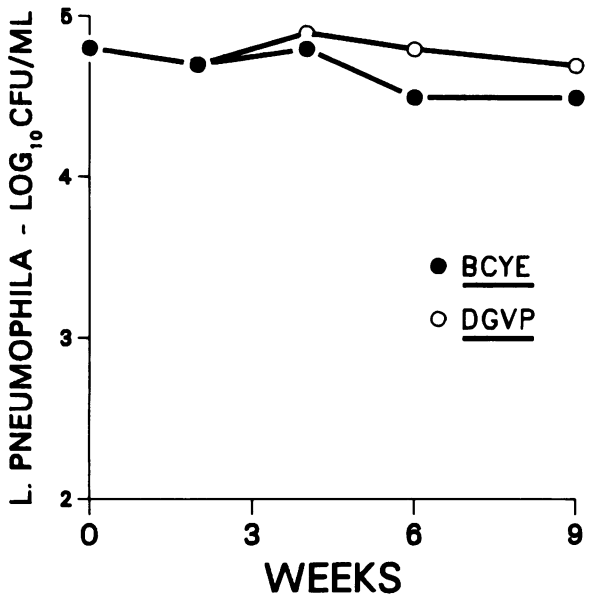

FIG. 4. Survival of legionellae in the "pooled" sample over the course of these experiments. Samples were diluted and plated on both selective (DGVP) and nonselective (BCYE) media.

During the course of these experiments the number of legionellae present in the pooled sample remained fairly constant (Fig. 4). No significant difference was seen in cells recovered whether they were plated on DGVP or BCYE. This indicates not only that the tap culture bottles maintained constant numbers of cells during the course of the experiments, but also that the cells showed no additional stress over this period since they grew as well on DGVP as on BCYE. Also, *Flavobacterium* sp. had no observable effect on legionella growth since similar numbers of legionellae were recovered on BCYE in the presence of this organism.

In another series of experiments, we wanted to see if high chlorine doses in a hospital might select for organisms more resistant to chlorine and, if that were the case, whether the organism would maintain its resistance when cultured on artificial media. Figure 5 shows that legionellae which survive exposure to 2 ppm of total chlorine residual in a hospital hot-water system appear to be even more resistant to chlorine than the tap water-maintained culture used in our previous experiments. Also, when each of these strains was passaged twice on artificial media and the experiment was repeated, both strains became susceptible to chlorine to the same extent.

Finally, once-passaged strains isolated from a respiratory therapy faucet in a hospital were compared before and after the system had been chlorinated for 2 weeks. The cultured strains were both susceptible to chlorine to the same extent whether they had previously been exposed to chlorine or not (data not shown).

DISCUSSION

We have previously shown *L. pneumophila* to be much more resistant to chlorine disinfection than a number of organisms, especially members of the coliform group commonly used as indicators of potability of public water supplies (17). The present study shows that more "natural" populations of legionellae which had never been cultured on artificial media are even more resistant to chlorine than their agar medium-passaged counterparts. The phenomenon of altered sensitivity to various chemical or microbiological agents accompanying growth on agar is not unique to the present study. Carson et al. (5, 6) showed not only that *P. aeruginosa*, and atypical mycobacteria, could grow in deionized water, but also that a single agar medium passage drastically reduced the resistance of these organisms to a variety of disinfectants. An analogous situation exists in legionellae as far as chlorine resistance is concerned. Yee and Wadowsky have shown legionellae to multiply in nutrient-deficient media such as tap water (28). The greatest increase in chlorine sensitivity was seen here and in other studies after one agar passage. This might be attributed to differences in growth rates under the two conditions, changes in the cellular envelope, changes in anticedent cell density, or some combination of the above or other subtle changes. All of the above factors affect the sensitivity of *E. coli* to chlorine dioxide (ClO_2) (3). Berg et al. (2) have reported legionellae grown in continuous culture to be more resistant to chlorine dioxide disinfection than *E. coli*. Also, the more slowly growing chemostat cultures of legionellae were 2 orders of magnitude more resistant to ClO_2 than batch-grown cultures. Perhaps an analogous situation occurs here since we have shown that the cell numbers remained relatively constant in the tap water-maintained culture over a period of weeks in stationary phase, whereas those on plates would be growing at a relatively faster rate with diverse nutrient availability.

If *Flavobacterium* sp. affected the resistance of legionellae in tap cultures, we would expect the survival rates to be different after Cl_2 addition if we plated the samples on BCYE where viable *Flavobacterium* sp. could grow or DGVP

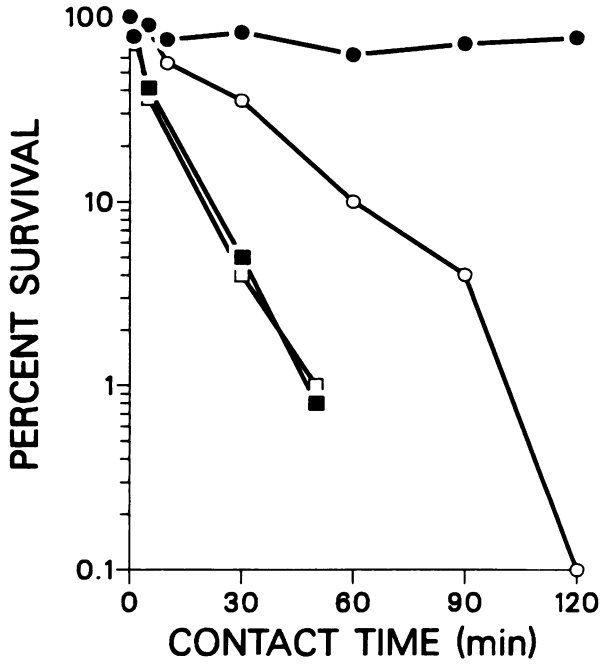

FIG. 5. Comparison of bactericidal activity of Cl_2 on a super-Cl_2-resistant, tap water-maintained strain isolated after in-house chlorination at 2 ppm of free residual Cl_2 for 3 weeks (●). Other symbols: (■) Cl_2 sensitivity of the super Cl_2-resistant strain after two BCYE agar medium passages; (○) control strain, never subjected to additional in-house chlorination; (□) Cl_2 sensitivity of control strain after two BCYE agar medium passages. (See text.)

where only viable legionellae would survive. Survival rates were nearly identical on either medium. Also, in our earlier work (17) isolated legionellae were much more resistant to Cl_2 than were other organisms we tested. In preliminary studies to these experiments we found nearly identical survival rates of legionellae whether or not we added back isolated *Flavobacterium* sp. to the pure culture of legionellae and subsequently checked for chlorine resistance. Although little interaction of *Flavobacterium* sp. with legionellae occurred in our chlorine resistance work, members of our group (25) have demonstrated that *Flavobacterium breve* could cause satellite growth of legionellae, presumably by supplying some essential nutrient such as cysteine. Numerous attempts were made here to obtain legionellae in pure culture by extinction dilution of the tap water cultures so that we could test the pure isolates for Cl_2 resistance. Although legionellae free of other species could be obtained in this way, they failed to multiply during subsequent passages in sterile tap water without the other bacteria.

Another point we considered was that a physical aggregation of cells may account for the resistance observed in the tap water-maintained strains. That this was not the case was proven by direct fluorescent-antibody analysis (8) of both tap water-grown and agar medium-grown legionellae. In the tap water-grown culture the legionellae appeared as single cells, whereas the cells in the agar-passaged strain existed mostly as short filaments. The mechanisms of resistance in the tap water-grown culture is not a result of protection by aggregation. Actual cell density during growth was relatively low in tap water cells, approximately 10^5 CFU/ml, compared with the dense populations achieved on plates. Berg et al. (3) reported that low cell density during growth contributed to enhanced resistance of *E. coli* to ClO_2. The reason for this enhanced resistance at lower cell density was unexpected and unclear.

Perhaps the composition of the outer membrane of this organism contributes to its resistance to chlorine. Several investigators (11, 15) have reported a high concentration of phosphatidyl choline in the cell envelope of *L. pneumophila*. This phospholipid is common in eucaryotes but rare in bacterial membranes (14). Several reports have suggested that *L. pneumophila* maintains a hydrophobic cell surface (4, 21). Agar-passaged legionellae has already been shown to be a great deal more Cl_2 resistant than coliforms or other indicator organisms. Maybe the composition of the membrane has something to do with the organisms' resistance. The membrane may change under different growth conditions which could change the resistance of legionellae to Cl_2.

It is important to note that observed resistance was not unique to a particular tap water culture but was also found in a culture from another source. This suggests that the resistance patterns we see here may represent a more general phenomenon of natural strains.

We wanted to address another issue: that is, could strains in a plumbing system exposed to chlorine become even more resistant to chlorine and how would they respond to agar passage? After a 3-week exposure to 2 ppm, a plumbing system which previously had high levels of legionellae was sampled at a tap previously reported to be positive. The tap still contained high levels of the organism and, further, this strain proved to be even more resistant to chlorine disinfection than strains not previously exposed to chlorine. However, as has been shown previously, when this high-level chlorine-resistant organism is agar passed, its resistance falls to levels comparable to those of strains not previously exposed to high levels of chlorine.

As has been shown in other studies with other organisms, more natural populations of legionellae are much more resistant to Cl_2 than their agar-passaged counterparts. The difference in resistance appears to be caused by physiological rather than genetic changes since even strains which have acquired greater Cl_2 resistance by in-house chlorination lose resistance to levels comparable to organisms not previously exposed to Cl_2. Changes in growth rate, cell density, differences in amounts or kinds of carbon and nitrogen sources, and degree of nutrient limitation, in combination or with other factors, probably contribute to the differences described here. Evaluations of the effectiveness of chlorine in disinfecting cooling towers, hospitals, and other buildings with recirculating hot-water systems should be determined empirically. It is likely that in actual practice higher dosages of Cl_2 for longer contact times may be required to achieve disinfection of natural populations compared with agar medium-adapted strains.

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