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Physiological Studies of Chloramine Resistance Developed by *Klebsiella pneumoniae* under Low-Nutrient Growth Conditions

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This study investigated the physiological mechanisms of resistance to chloramines developed by *Klebsiella pneumoniae* grown in a nutrient-limited environment. Growth under these conditions resulted in cells that were smaller than cells grown under high-nutrient conditions and extensively aggregated. Cellular aggregates ranged from 10 to more than 10,000 cells per aggregate, with a mean population aggregate size of 90 cells. This aggregation may have been facilitated by the presence of extracellular polymer material. By using glucose as a reference of capsule content, it was determined that growth under low-nutrient conditions produced cells with 8×10^{-14} to 41×10^{-14} g of carbohydrate per cell, with a mean ± standard deviation of $27 \times 10^{-14} \pm 16 \times 10^{-14}$ g of carbohydrate per cell. In comparison, growth under high-nutrient conditions resulted in 2.7×10^{-14} to 5.9×10^{-14} g of carbohydrate per cell, with a mean and standard deviation of $4.3 \times 10^{-14} \pm 1.2 \times 10^{-14}$ g of carbohydrate per cell. Cell wall and cell membrane lipids also varied with growth conditions. The ratio of saturated to unsaturated fatty acids in cells grown under low-nutrient conditions was approximately five times greater than that in cells grown under high-nutrient conditions, suggesting possible differences in membrane permeability. An analysis of sulfhydryl (-SH) groups revealed no quantitative difference with respect to growth conditions. However, upon exposure to chloramines, only 33% of the -SH groups of cells grown under low-nutrient conditions were oxidized, compared with 80% oxidization of -SH groups in cells grown under high-nutrient conditions. The reduced effectiveness of chloramine oxidization of -SH groups in cells grown under low-nutrient conditions may be due to restricted penetration of chloramines into the cells, conformational changes of enzymes, or a combination of both factors. The results of this study suggest that chloramine resistance developed under low-nutrient growth conditions may be a function of multiple physiological factors, including cellular aggregation and protection of sulfhydryl groups within the cell.

The presence of microorganisms in treated water distribution systems can be problematic, especially when these organisms are primary or opportunistic pathogens, support the growth of pathogens, interfere with the detection of coliforms, or result in undesirable aesthetic problems. The application of disinfectants, such as chlorine or chloramines, before distribution is generally the most important step in the control of these organisms and in many cases is the only form of treatment. Even though the use of chlorine and chloramines has proven effective, numerous investigators have reported that coliforms and other organisms can be isolated from treated-water distribution systems containing a disinfectant residual (28, 35, 36). The survival of microorganisms in the presence of disinfectant agents may be due to non-cell-mediated or cell-mediated factors or a combination of both. Non-cell-mediated events are physicochemical in nature and include the presence of disinfectant-demand compounds (31), microbial association with particles (25, 31, 36), growth within protective microhabitats promoted by corrosion processes within the distribution system (29), and association with invertebrate or protozoan vectors (19, 26).

Microbial resistance to disinfectants may also be due to cell-mediated physiological alterations of the organism. This process is poorly understood but presumably is due to phenotypic changes of the organism in response to the prevailing growth environment. In studies conducted by Wolfe and Olson (46), a naturally occurring isolate of *Flavobacterium* was determined to be approximately 200 times more resistant to chlorine than the same strain following subculturing in R2A broth. Other investigators have also demonstrated that naturally occurring organisms or those grown under low-nutrient conditions have greater resistance to disinfectant agents (2, 8, 23). Collectively, these studies suggest that physiological adaptations of the cell to adjust to reduced nutrient availability may confer additional survival characteristics to the organism.

Several physiological traits of the cell, including aggregation, capsule production, and alteration of envelope properties or sites within the cell, may enhance resistance to disinfectants. In early work by Wei and Chang (45), it was observed that protozoans capable of aggregating were more resistant to disinfectant agents than protozoans incapable of aggregating. In work with aggregating and nonaggregating isogenic strains of *Acinetobacter*, Olson and Stewart (34) have demonstrated that resistance to various potable water disinfectants could be increased 2- to 30-fold by bacterial aggregation.

The cellular capsule has also been implicated in enhancing resistance to various antimicrobial agents, including both clinical and potable water disinfectants (21, 33, 36). Some investigators (24, 44) believe that biofilms occurring on pipe walls in distribution systems create a protective environment and can provide a continuous source of encapsulated coliforms into the potable water supply.

Previous studies focusing on mechanisms of inactivation

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by disinfectants provide important information concerning possible disinfectant resistance due to alterations of cellular components. For example, the primary sites of cellular inactivation by chlorine and chloramines are sulfhydryl groups of proteins located in the cell membrane and the cytoplasmic region of the cell (3, 14, 15). Work by Jacangelo et al. (15) has indicated that chloramines inactivate bacterial cells by oxidization of sulfhydryl groups to disulfides or to higher oxidized states, resulting in impairment of enzyme function and eventually cellular death. Presumably, physiological alterations or reduced accessibility of this primary target site may limit oxidation and thus enhance cellular resistance to chlorine-based disinfectants.

Previous work in our laboratory with a *Klebsiella pneumoniae* strain indicated that growth under low-nutrient conditions similar to those in the potable water environment increased resistance to chloramines (43). Accordingly, the objective of this study was to characterize physiological components in *K. pneumoniae* responsible for this enhanced resistance. Specifically, chloramine-sensitive and chloramine-resistant cells were examined for aggregation potential, capsule production, lipid composition, and differences in sulfhydryl content and reactivity to chloramines.

MATERIALS AND METHODS

Organism and culture conditions. The *K. pneumoniae* strain used in this study was provided by Mark LeChevallier of the American Water Works Service Company (Belleville, Ill.). Growth conditions promoting chloramine-resistant and chloramine-sensitive strains were described in a previous article (43). Briefly, *K. pneumoniae* grown in a 1:1,000 dilution of a yeast extract-based medium described by Ristroph et al. (37) was determined to be approximately five times more resistant to chloramines than *K. pneumoniae* grown in an undiluted solution of the same medium. Before the assay, chloramine-sensitive cells were prepared by growing them in full-strength Ristroph's medium to the stationary phase at 23°C, harvesting them by centrifugation at 10,000 × *g* for 10 min at 4°C (model J2-21; Beckman Corp., Palo Alto, Calif.), and washing them two times in 10 mM phosphate buffer (pH 7.0). Growth conditions and cell preparation procedures for chloramine-resistant cells are described below.

Inorganic monochloramine solution and disinfection conditions. The preparation of inorganic monochloramine solution, the cleaning regimen for glassware used in disinfection studies, and the disinfectant assay conditions were described in detail by Stewart and Olson (43). In general, a stock 1-g/liter chlorine solution was prepared from a concentrated 5% sodium hypochlorite solution (J. T. Baker Chemical Co., Phillipsburg, N.J.), stored at 4°C, and monitored weekly with an amperometric titrator (Fisher & Porter, Warminster, Pa.) to ascertain any chlorine decay. A stock 1-g/liter ammonium chloride (Mallinckrodt Inc., St. Louis, Mo.) solution was filter sterilized (0.2-μm-pore-size filter; Nalge Co., Rochester, N.Y.) and stored at 4°C. Inorganic monochloramine was formed by initially adding ammonium chloride to 500 ml of chlorine demand-free 10 mM phosphate buffer (pH 8.0), mixing for approximately 10 min, and then adding stock chlorine solution. This solution was mixed for an additional 20 min to ensure complete residual formation. The residual chloramine concentration of this solution was twice the desired final assay concentration. The Cl_2-to-N ratio was 3:1 for all experiments. All glassware used for disinfection studies was critically cleaned, soaked in chlorine overnight, rinsed with deionized, glass-distilled water, and heated overnight at 160°C in a dry-air oven. This cleaning procedure produced chlorine demand-free glassware. All chloramine inactivation studies were conducted at 23°C in sterile 1-liter glass beakers. The concentration of monochloramine was determined before and immediately after each inactivation experiment with a DR 1A colorimeter (Hach Co., Loveland, Colo.). The disinfectant demand was ≤0.2 mg/liter for all assays, and the final pH for all experiments ranged from 7.8 to 8.0.

Aggregation assay. Cellular aggregation of chloramine-sensitive and chloramine-resistant *K. pneumoniae* was assayed with a Coulter Counter (Coulter Multisizer, model 663394; Coulter Electronics, Hialeah, Fla.). Chloramine-sensitive cells (grown in full-strength Ristroph's medium) were grown to the stationary phase and harvested as described above. Microscopic observation at a magnification of ×1,250 (model BH-2; Olympus Corp., Tokyo, Japan) confirmed that harvesting conditions did not alter cellular aggregation for chloramine-sensitive cells. Chloramine-resistant cells were grown to the stationary phase in a 1:1,000 dilution of Ristroph's medium. Because of the minimal amount of chlorine demand compounds present in the 1:1,000 dilution of Ristroph's medium, no harvesting or washing procedures were used for chloramine resistant cells. In addition, control experiments indicated that harvesting of cells by centrifugation did not affect the inactivation kinetics of the chloramine-resistant cells. Aliquots (approximately 5 ml) of cells from each growth condition were added to 15 ml of particle-free isotonic solution (Isoton II; Coulter), mixed in a 25-ml vessel, and placed in the Coulter Counter. Cell suspensions ranging from 5,000 to 50,000 particles were then counted by using a 20-μm orifice (for detection of particles with diameters of 0 to 20 μm) or a 100-μm orifice (for detection of particles with diameters of 0 to 100 μm). The Coulter Counter was calibrated with latex beads (Coulter) ranging from 2.02 to 44 μm in diameter. The number of cells per aggregate was determined by dividing the summed volume of cells occurring in all aggregate sizes in the total cell population (percent totalized cell volume) by the volume of a single cell for either chloramine-sensitive or chloramine-resistant cells.

Experiments were also conducted to determine the effect of chloramine exposure on aggregated cells. In these experiments, chloramine-resistant cells were grown to the stationary phase and dispensed into 500-ml volumes into three sterile 1-liter beakers. To the first beaker, 500 ml of phosphate buffer (pH 8.0) was added; the second and third beakers received 500 ml of chloramine disinfectant solution to produce final residual chloramine concentrations of 1.5 and 15.0 mg/liter, respectively. The cells were mixed for 20 min at 100 rpm with a paddle stirrer (Phipps and Bird, Richmond, Va.). After 20 min of mixing, 1 ml of 10% sodium thiosulfate (J. T. Baker Chemical Co.) was added to each beaker and mixed for an additional 5 min. The cells were then immediately analyzed for aggregation.

Microscopic analysis was also used to confirm cellular aggregation and size of individual cells. Cells were prepared as described above and examined at a magnification of ×1,250 with an Olympus BH-2 microscope equipped with differential interfering contrast optics. The level of aggregation was divided into the following categories: 1 to 5, 6 to 10, 11 to 15, 16 to 20, and 21 or more cells. A minimum of 12 fields were scored, and the mean was determined for each category on the basis of the total cell count. Four replicates

were used for both chloramine-sensitive and chloramine-resistant cells.

Extracellular polymer analysis. Extracellular polymer material of chloramine-sensitive and chloramine-resistant cells was isolated by high-speed centrifugation (4). Initially, the cells were grown in Ristroph's broth solution as described above and concentrated by filtration (0.45-μm-pore size filters; Gelman Sciences, Ann Arbor, Mich.); they were then washed twice with 50 ml of 10 mM phosphate buffer (pH 7.0) to remove any carbohydrate material from the growth medium. The filter was then placed in a sterile 50-ml centrifuge tube containing 10 ml of 10 mM phosphate buffer (pH 7.0) and vortexed for approximately 2 min to release cells from the filter. The filter was discarded, and an additional 30 ml of phosphate buffer was added to the centrifuge tube containing the cells. The contents of the tube were mixed, and a 1-ml volume was removed, diluted in phosphate buffer, and plated in triplicate on R2A agar (Difco, Detroit, Mich.) to determine cell density. The densities of chloramine-sensitive and chloramine-resistant cells were approximately 3.7×10^8 and 1.4×10^7 CFU/ml, respectively. Replicate analysis with chloramine-sensitive cells indicated that concentrating the cells by filtration did not result in levels of extracellular material different from those obtained by the low-speed centrifugation procedure used to harvest cells for disinfection assays. After filtration and resuspension in phosphate buffer, cells were centrifuged at $48,000 \times g$ for 15 min at 4°C. To enhance recovery of extracellular polymer material, the pellet was vigorously resuspended by vortexing for 3 min and centrifuged again at $48,000 \times g$ for an additional 15 min. The supernatant was then analyzed for extracellular polymer content by the method described by Dubois et al. (9a), with glucose as a reference. Glucose concentrations in cell samples were determined by using a standard curve of absorbance versus moles of glucose. Samples were analyzed with a Beckman DU 5 spectrophotometer set to measure the A_{490}. Results were based on triplicate samples.

Lipid assay. Chloramine-sensitive and chloramine-resistant cells were analyzed for alterations of lipid composition before and after exposure to chloramines. Chloramine-sensitive cells were grown and harvested as described above. To produce a sufficient quantity of cells, chloramine-resistant cells were grown on agar plates of Ristroph's medium diluted 1:1,000. Cells were harvested by adding approximately 3 ml of sterile phosphate buffer (pH 7.0) to each plate and scraping the plate with a sterile glass rod. The contents of each plate were poured into a sterile 1-liter beaker, dispensed into 50-ml centrifuge tubes, and harvested by centrifugation. After being harvested, these cells were dispensed into three equal volumes into sterile 1-liter chlorine demand-free beakers containing 500 ml of phosphate buffer (pH 8.0). In the first beaker, an additional 500 ml of phosphate buffer was added. In the second and third beakers, 500 ml of disinfectant solution was added to produce final chloramine residual concentrations of 2.0 and 20.0 mg/liter, respectively. The cells were mixed for 20 min at 100 rpm with a paddle stirrer. At the end of this period, 1 ml of 10% sodium thiosulfate was added and allowed to mix for approximately 5 min. The exposure conditions for the chloramine-resistant cells were the same as those described above for the chloramine-sensitive cells. Following disinfection, chloramine-sensitive and chloramine-resistant cells were harvested and concentrated by centrifugation to a final volume of approximately 2 ml. The final cell concentrations were approximately 3×10^8 and 3×10^{10} CFU/ml for chloramine-sensitive and chloramine-resistant cells, respectively. Cells were frozen at −20°C and sent by overnight delivery to Microbial ID, Inc. (Newark, Del.), for lipid analysis. Fatty acids were analyzed by the method described by Miller and Berger (30a). Briefly, fatty acids were released from the cells by the addition of 1.0 ml of 15% NaOH in 50% methanol with heating for 30 min in a boiling water bath. The fatty acids were methylated by addition of 2 ml of 3 N HCl in 50% methanol in an 80°C water bath. The aqueous extract was then washed with 1.25 ml of hexane-ether (1:1, vol/vol) by tumbling on a hemocytology mixer for 10 min. Finally, the aqueous wash of the organic phase was performed by addition of 3 ml of 1.2% NaOH and tumbling for 5 min. The organic phase was then analyzed with a gas chromatograph (model HP 5890A; Hewlett-Packard Co., Palo Alto, Calif.) equipped with HP 5898A Microbial Identification System software (Hewlett-Packard). Replicate analysis of the chloramine-resistant cell condition confirmed that the results were highly reproducible. The mean variability ± standard deviation for each lipid constituent was 3% ± 3.5%.

Sulfhydryl analysis. Whole cells and cell extracts of chloramine-sensitive and chloramine-resistant cells were analyzed for sulfhydryl group content and sulfhydryl reactivity to chloramines. Chloramine-sensitive and chloramine-resistant cells at concentrations of approximately 7×10^9 and 4×10^9 CFU/ml, respectively, were prepared as described in "Lipid assay" above. Chloramine exposure conditions were identical to those described in "Lipid assay," with the exception that final chloramine residual concentrations were 2.5 and 25.0 mg/liter for chloramine-sensitive and chloramine-resistant cells, respectively. Cell extracts were prepared by suspending the cells in 10 mM phosphate buffer (pH 7.0) in 1.5-ml microcentrifuge tubes containing approximately 0.3 g of zirconium beads (Ultrasonics, Inc., Farmingdale, N.Y.) to enhance cell disruption. The samples were chilled in an ice bath for 20 min prior to sonication. The cells were sonicated for 10 min in a Heat Systems sonicator (Ultrasonics) set at full power. The sonicator horn was cooled to 4°C by continuous recirculation of polyethylene glycol contained in an ice bath. This procedure produced a final cell extract temperature of 23°C or less after sonication. The efficiency of sonication was confirmed by direct microscopic observation of cells before and after sonication. In general, more than 90% of all cells were lysed after sonication.

Cysteine sulfhydryl group (-SH) concentrations were determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma Chemical Co., St. Louis, Mo.) by the method described by Jacangelo et al. (15). DTNB reagent was prepared before each assay by dissolving 39.6 mg of DTNB in 10 ml of 0.1 M phosphate buffer (pH 7.0). A 0.1-ml sample was added to 2.0 ml of 0.1 M phosphate buffer (pH 8.0), and 0.1 ml of DTNB was added immediately thereafter. Samples were analyzed after 2 min with a Beckman DU 5 spectrophotometer set at 412 nm. Determinations of -SH content were made in reference to a standard curve of absorbance versus moles of sulfhydryl group. Disulfide (S-S) concentrations were determined with dithioerythritol (Sigma) as described by Zahler and Cleland (47). A 0.2-ml volume of sample was added to a mixture of 0.1 ml of 0.5 M Tris buffer (pH 9.0) and 0.1 ml of 3.0×10^{-3} M dithioerythritol. After 20 min, 1.0 ml of 1.0 M Tris (pH 8.1) and 1.5 ml of 5.0×10^{-3} M sodium arsenite were added. After 2 min, 0.1 ml of DTNB was added, and the A_{412} was recorded for 5 min. The monothiol sulfhydryl group content was determined by extrapolating the linear portion of the curve to the time of DTNB addition. S-S content was determined in reference to a standard curve as described above.

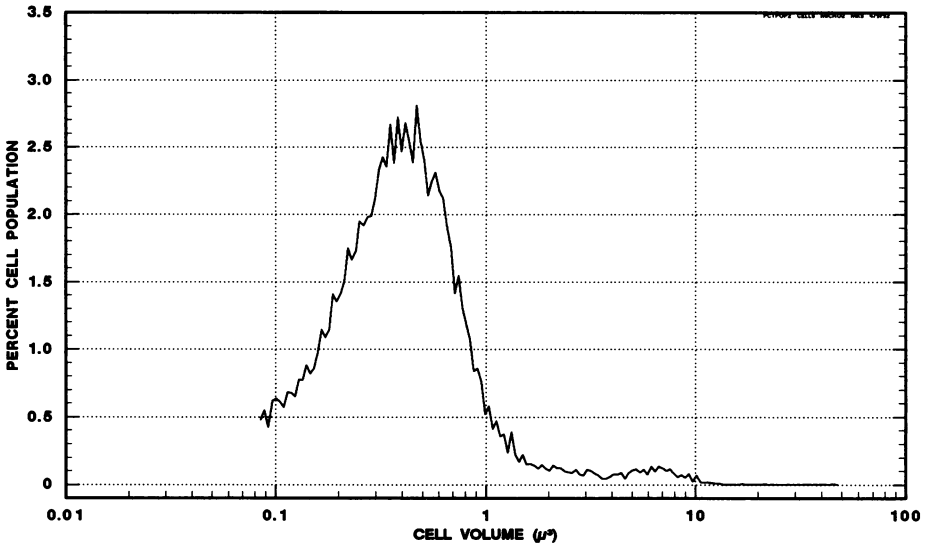

FIG. 1. Distribution of volumes of individual chloramine-resistant *K. pneumoniae* cells.

Statistical analyses. Statistical analyses using a two-tailed Student's *t* test were conducted with RS/1 software (version 3.0; BBN Software Products Co., Cambridge, Mass.) on a minicomputer (VAX 8200; Digital Equipment Corp., Maynard, Mass.). A minimum of three replicates were used for each experiment.

RESULTS

Aggregation profile of chloramine-sensitive and chloramine-resistant cells. Chloramine-sensitive and chloramine-resistant cells were initially analyzed to determine the average size of the individual cells for each growth condition. Chloramine-resistant cells (grown in Ristroph's medium diluted 1:1,000) ranged in volume from 0.1 to 1 μm^3, with a mean cell volume of 0.41 μm^3 (Fig. 1) and were coccobacillar in morphology with dimensions of approximately 0.5 μm in width and 1.0 μm in length. Chloramine-sensitive cells (grown in undiluted Ristroph's medium) were considerably larger than the chloramine-resistant cells and ranged in volume from approximately 1 to 10 μm^3, with a mean cellular volume of 6.3 μm^3 (Fig. 2), and were rod shaped with dimensions of 1 μm in width and 3 to 3.5 μm in length.

The level of cellular aggregation in the chloramine-sensitive and chloramine-resistant cell populations was determined by plotting cell volume versus percent totalized cell volume (Fig. 3). These results indicated that the total volume of chloramine-sensitive cells was equivalent to the summed volume of single cells, indicating that chloramine-sensitive cells did not form aggregates. This was confirmed by microscopic observation, which revealed that 100% of the chloramine-sensitive cells occurred in aggregates of one to five, with most cells occurring individually or in pairs (data not shown). In contrast, a significant amount of the total cellular population for chloramine-resistant cells was greater than that represented by the summed volume of single cells,

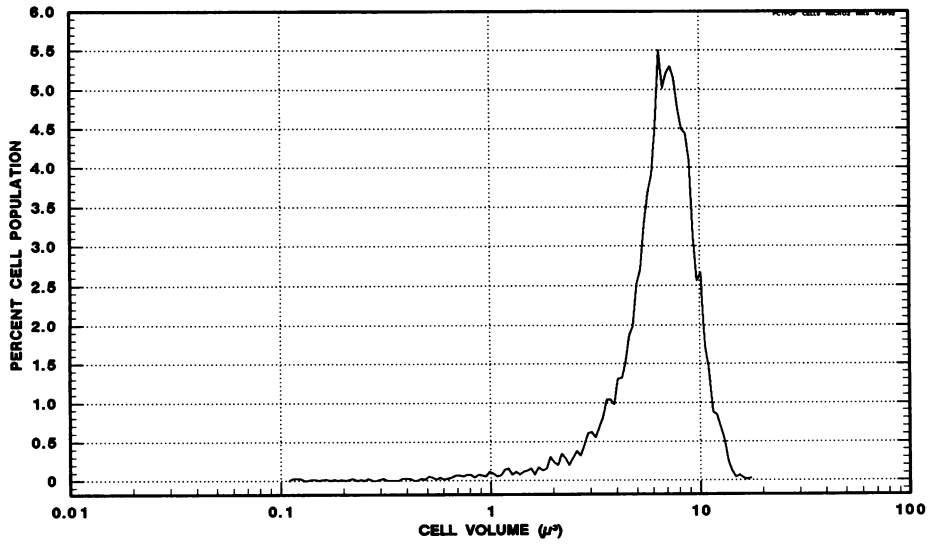

FIG. 2. Distribution of volumes of individual chloramine-sensitive *K. pneumoniae* cells.

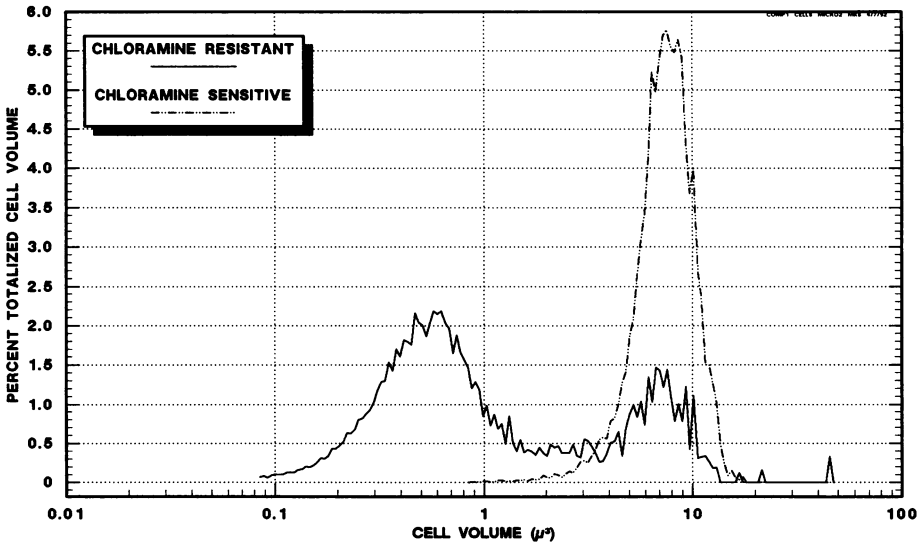

FIG. 3. Percent totalized cell volume of chloramine-resistant and chloramine-sensitive *K. pneumoniae* cells.

indicating cellular aggregation (Fig. 3). Replicate microscopic analysis (data not shown) indicated that chloramine-resistant cells occurred in large aggregates that could not be accurately quantified by microscopic methods. When analyzed with the Coulter Counter, chloramine-resistant cells ranged from less than 10 to more than 10,000 cells per aggregate, with a mean population aggregate size of 90 cells (Fig. 4).

Chloramine-resistant cells were exposed for 20 min to 1.5 or 15.0 mg of residual chloramines per liter to evaluate the effect of chloramine exposure on cellular aggregation. Even at a level of 15 mg of residual chloramine per liter, the aggregation profile of the chloramine-resistant cells was not altered (Fig. 5). This suggests that chloramine exposure did not promote disaggregation of the cells.

Comparison of extracellular polymer in chloramine-sensitive and chloramine-resistant cells. *K. pneumoniae* grown under conditions promoting chloramine resistance produced approximately six times more capsule material per cell than it did when grown under conditions resulting in chloramine sensitivity. Chloramine resistant cells produced 8×10^{-14} to 41×10^{-14} g of carbohydrate per cell, with a mean ± standard deviation of $27 \times 10^{-14} \pm 16 \times 10^{-14}$ g of carbohydrate per cell. However, chloramine-sensitive cells produced only 2.7×10^{-14} to 5.9×10^{-14} g of carbohydrate per cell, with a mean ± standard deviation of $4.3 \times 10^{-14} \pm 1.2 \times 10^{-14}$ g of carbohydrate per cell. These differences were found to be statistically significant by a two-tailed Student's t test ($P < 0.05$).

Analysis of lipid composition in chloramine-sensitive and chloramine-resistant cells. Chloramine-sensitive cells demonstrated a lipid profile different from that of chloramine-resistant cells (Table 1). There was a notable difference in the ratio of saturated to unsaturated fatty acids between

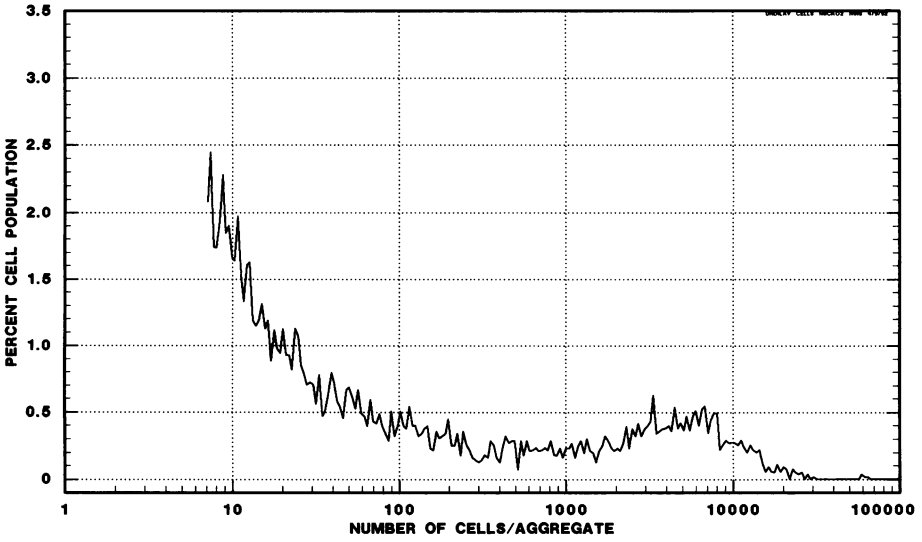

FIG. 4. Distribution of cell aggregation sizes for chloramine-resistant *K. pneumoniae* cells.

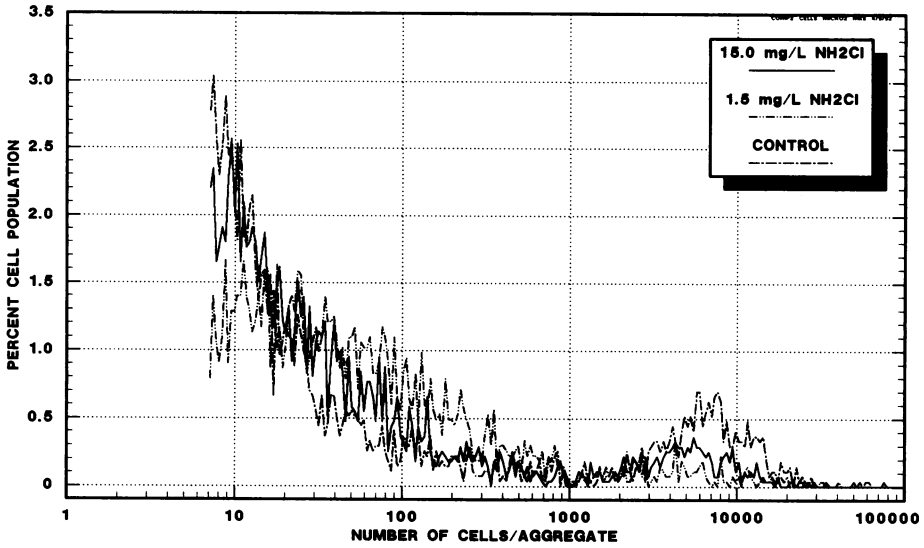

FIG. 5. Effect of chloramine exposure on aggregation profile of chloramine-resistant *K. pneumoniae* cells.

chloramine-sensitive and chloramine-resistant cells. Chloramine-sensitive cells had approximately equal amounts of saturated and unsaturated fatty acids (i.e., a saturated-to-unsaturated fatty acid ratio of 1.4). In contrast, chloramine-resistant cells had higher levels of saturated fatty acids than of unsaturated fatty acids (ratio of 7).

Chloramine-sensitive and chloramine-resistant cells also demonstrated differences in the relative percentages of cell wall and cell membrane fatty acids (Table 1). In the cell wall (i.e., fatty acid carbon chain lengths of 15 or less), the only difference between chloramine-sensitive and chloramine-resistant cells was in the amount of lipids with carbon chain lengths of 14; chloramine-resistant cells had approximately twice the amount that chloramine-sensitive cells had (14 versus 7%). However, in the cell membrane (i.e., fatty acids with carbon chain lengths of 16 or greater), there were more distinct lipid composition differences between the two strains. The most notable differences occurred in lipid fractions 16:1 cis 9 and 17:0 and the mixed lipid fraction 18:1, in which the differences between the strains were 7.4, 17.2, and 21.2 percentage points, respectively.

Chloramine exposure studies were conducted to determine whether cell wall or cell membrane lipids, especially those lipids that differed between chloramine-sensitive and chloramine-resistant cells, were reactive to chloramines. When chloramine-sensitive cells were exposed to chloramines at concentrations of up to 20 mg/liter, there was no change in cellular lipid composition, suggesting that lipids were not altered by chloramine exposure (Table 2). Similarly, when chloramine-resistant cells were exposed to chloramines, there was no change in cell wall or cell membrane lipid composition (Table 3). These results indicate that

TABLE 1. Comparison of lipid contents in chloramine-sensitive and chloramine-resistant *K. pneumoniae*

Lipid	% of total lipids		Difference (percentage points)
	Chloramine sensitive[a]	Chloramine resistant[b]	
12:0	3.0	2.4	0.6
14:0	7.4	14.1	6.7
15:0	0	0.7	0.7
—[c]	8.0	11.3	3.3
16:1 cis 9	13.0	5.6	7.4
16:0	30.6	28.7	1.9
17:0 cyclo	6.2	23.4	17.2
—[d]	28.0	6.8	21.2
19:0 cyclo c11-12	2.1	6.1	4.0
Saturated fatty acids	57.3	86.7	29.4
Unsaturated fatty acids	41	12.4	28.6
Ratio[e]	1.4	7.0	

[a] Grown in undiluted Ristroph's medium.
[b] Grown in Ristroph's medium diluted 1:1,000.
[c] Represents sum of 12:0 Alde, 16:1 iso I/14:0 3OH, and 14:0 3OH/16:1 iso I.
[d] Represents sum of 18:1 cis 11/t 9t6, 18:1 trans 9/t6 /c11, and 18:1 trans 6/t9 /c11.
[e] Ratio of saturated to unsaturated fatty acids.

TABLE 2. Analysis of lipid composition in chloramine-sensitive *K. pneumoniae* after exposure to chloramines

Lipid	% of total lipids		
	Control	After exposure to NH_2Cl at:	
		2.0 mg/liter	20 mg/liter
12:0	3.0	5.2	5.1
14:0	7.4	9.5	8.7
—[a]	8.0	13.1	14.0
16:1 cis 9	13.0	12.2	10.4
16:0	30.6	27.0	26.0
17:0 cyclo	6.2	6.6	5.2
—[b]	28.0	26.5	30.3
19:0 cyclo c11-12	2.1	0	0
Saturated fatty acids	57.3	61.4	59
Unsaturated fatty acids	41	38.7	40.7
Ratio[c]	1.4	1.6	1.4

[a] Represents sum of 12:0 Alde, 16:1 iso I/14:0 3OH, and 14:0 3OH/16:1 iso I.
[b] Represents sum of 18:1 cis 11/t 9t6, 18:1 trans 9/t6 /c11, and 18:1 trans 6/t9 /c11.
[c] Ratio of saturated to unsaturated fatty acids.

TABLE 3. Analysis of lipid composition in chloramine-resistant *K. pneumoniae* after exposure to chloramines

Lipid	% of total lipid		
	Control	After exposure to NH_2Cl at:	
		2.0 mg/liter	20 mg/liter
12:0	2.4	2.1	2.1
14:0	14.0	11.0	10.4
15:0	0.7	1.1	1.0
—[a]	11.3	10.5	10.5
16:1 cis 9	5.6	4.2	4.3
16:0	28.7	29.9	27.7
17:0 cyclo	23.4	18.5	18.7
—[b]	6.8	8.5	8.9
19:0 cyclo c11-12	6.1	12.5	13.3
Saturated fatty acids	86.7	85.6	83.7
Unsaturated fatty acids	12.4	12.7	13.2
Ratio[c]	7.0	6.7	6.3

[a] Represents sum of 12:0 Alde, 16:1 iso I/14:0 3OH, and 14:0 3OH/16:1 iso I.

[b] Represents sum of 18:1 cis 11/t 9t6, 18:1 trans 9/t6 /c11, and 18:1 trans 6/t9 /c11.

[c] Ratio of saturated to unsaturated fatty acids.

chloramines did not oxidize lipid components of the cell wall or cell membrane of chloramine-sensitive or chloramine-resistant cells.

Sulfhydryl group content and reactivity in chloramine-sensitive and chloramine-resistant cells. Chloramine-sensitive and chloramine-resistant cells were grown to equivalent concentrations and compared for sulfhydryl group content (Table 4). Detection of -SH and S-S groups in nonlysed chloramine-sensitive cells revealed $<1.8 \times 10^{-10}$ μmol of -SH per cell and 1.6×10^{-9} μmol of S-S per cell. Chloramine-resistant cells and chloramine-sensitive cells contained similar amounts of -SH and S-S groups. For example, chloramine-resistant cells had $<2.8 \times 10^{-10}$ μmol of -SH per cell and 7.6×10^{-10} μmol of S-S per cell. In general, in nonlysed chloramine-sensitive and chloramine-resistant cells, amounts of -SH and S-S groups were at or below the limit of detection. However, the levels of -SH and S-S groups increased one- to twofold in sonicated cells, suggesting that most -SH and S-S groups were associated with proteins and other cellular constituents within the cells. In sonicated chloramine-sensitive cells, there were 1×10^{-8} μmol of -SH per cell and 4.9×10^{-9} μmol of S-S per cell. In comparison, chloramine-resistant cells had 5.5×10^{-9} μmol of -SH per cell and 5.0×10^{-9} μmol of S-S per cell. Statistical analysis by Student's *t* test indicated that there was no statistical difference ($P < 0.05$) in the levels of -SH or S-S groups between chloramine-sensitive and chloramine-resistant cells. To evaluate the reactivity of -SH groups, chloramine-sensitive and chloramine-resistant cells were exposed to increasing levels of chloramines (Table 5). After exposure of chloramine-sensitive cells to 2.5 and 25 mg of chloramines per liter, the levels of -SH groups decreased by 46 and 80%, respectively. However, when chloramine-resistant cells were exposed under the same conditions, only 28 and 33% of -SH groups were oxidized, suggesting that the -SH groups in these cells were subjected to less chloramine oxidization. The difference in -SH reactivity between chloramine-sensitive cells and chloramine-resistant cells was determined to be statistically significant by Student's *t* test ($P < 0.05$). There was no detectable increase in the amount of S-S groups in chloramine-resistant cells following chloramine exposure, confirming the lack of reactivity of SH groups within these cells. In contrast, there was an increase in the amount of S-S groups in chloramine-sensitive cells, suggesting -SH group oxidation. However, there was no statistical difference between the S-S content in chloramine-sensitive cells and that in chloramine-resistant cells following exposure to increasing levels of chloramines. This may have been due to the high level of variation of measurable S-S content in the experimental assay.

DISCUSSION

The *K. pneumoniae* strain used in this study was previously determined to develop increased resistance to chloramines when grown under low-nutrient conditions (43). Importantly, nutrient levels promoting the greatest resistance were equivalent to or lower than those found in potable water environments. Other investigators have also reported that antecedent growth conditions could stimulate microbial resistance to disinfectant agents (2, 8, 23, 46). To adapt to low-nutrient conditions commonly occurring in potable water, an organism may undergo physiological changes that can render it phenotypically distinct from the same organism grown in nutrient-rich environments (20, 41). The present study examined physiological traits of *K. pneumoniae* that were altered under low-nutrient growth conditions in relation to enhanced resistance to chloramines.

Chloramine resistance promoted by aggregation. When *K. pneumoniae* was grown in a 1:1,000 dilution of the yeast extract-based medium (37), the cells were smaller, were coccobacillar in morphology, aggregated extensively, and were more chloramine resistant. When the same strain was grown in an undiluted broth of the same medium, the cells were much larger, were rod shaped, did not aggregate, and

TABLE 4. Comparison of sulfhydryl contents in chloramine-sensitive and chloramine-resistant *K. pneumoniae*[a]

Type of bacteria	Bacterial concn (CFU/ml)	Content (μmol/cell)			
		Whole cells[b]		Cell extract	
		-SH	S-S	-SH	S-S
Chloramine sensitive[c]	$(6.8 \pm 3.3) \times 10^9$	$(<1.8 \pm 0.8) \times 10^{-10}$	$(1.6 \pm 1.5) \times 10^{-9}$	$(1.0 \pm 0.5) \times 10^{-8}$	$(4.9 \pm 1.4) \times 10^{-9}$
Chloramine resistant[d]	$(4.1 \pm 1.7) \times 10^9$	$(<2.8 \pm 1.6) \times 10^{-10}$	$(7.6 \pm 9.8) \times 10^{-10}$	$(5.5 \pm 1.1) \times 10^{-9}$	$(5.0 \pm 0.1) \times 10^{-9}$

[a] Data represent the means ± standard deviations from at least three experiments. There was no statistically significant difference between chloramine-sensitive and chloramine-resistant *K. pneumoniae* in bacterial concentration or -SH or S-S content ($\alpha = 0.05$); the statistical significance in -SH content of whole cells was not determined because such a determination was not appropriate.

[b] In most cases, levels of -SH and S-S groups in whole cells were at or below the limit of detection.

[c] Grown in undiluted Ristroph's medium at 23°C.

[d] Grown in Ristroph's medium diluted 1:1,000 at 23°C.

TABLE 5. Effect of chloramine exposure on the amount of sulfhydryl groups contained in chloramine-sensitive and chloramine-resistant *K. pneumoniae*[a]

Chloramine residual concn (mg/liter)[b]	% of -SH remaining in:		Statistical difference[c]	Net % S-S increase in:		Statistical difference
	Sensitive cells[d]	Resistant cells[e]		Sensitive cells[d]	Resistant cells[e]	
2.5	54 ± 17	72 ± 36	No	46 ± 111	0	No
25.0	20 ± 20	67 ± 6	Yes	13 ± 44	0	No

[a] Data are means ± standard deviations from at least three experiments. All results are based on analyses of cell extracts.
[b] Cl_2-to-N ratio = 3:1, pH 8.0, temperature = 23°C, contact time = 20 min.
[c] Statistical significance at the $\alpha = 0.05$ level, two-tailed Student's t test, $n = 3$.
[d] Grown in undiluted Ristroph's medium, $n = 5$.
[e] Grown in Ristroph's medium diluted 1:1,000, $n = 3$.

were more sensitive to chloramines. Cell size reduction and changes in cellular morphology have been noted by other researchers for bacteria growing under low-nutrient conditions (1, 22). In addition, environmental conditions including nutrient type and availability, the ionic state of the surrounding solution, pH, and temperature have been reported to modify the potential of cells to aggregate (7). This cellular aggregation is due to the presence of fimbriae, polymer material, or proteins located on the surface of the cell (7).

In this study, chloramine-resistant *Klebsiella* cells formed aggregates of more than 10,000 cells in some cases, with a mean population aggregate size of 90 cells. Clearly, aggregates of this size should provide substantial protection of internally located cells. Exposure to chloramine residuals of up to 15 mg/liter did not disaggregate the chloramine-resistant *Klebsiella* strain; however, inactivation did occur after sufficient contact time. This suggests that while the aggregates may remain stable, chloramines will eventually penetrate and inactivate internally located cells. In related studies done in our laboratory, it was observed that an aggregating strain of *Acinetobacter* was more resistant to chlorine, chlorine dioxide, and chloramines than the nonaggregating isogenic strain (34). Importantly, when the aggregating strain was treated with a surfactant to disassociate the cells, the aggregating strain was readily inactivated and demonstrated inactivation kinetics similar to those of the nonaggregating strain (42). These previous studies suggest that aggregation acts as the primary variable promoting chloramine resistance.

Documentation in the literature by other investigators concerning disinfectant resistance due to microbial aggregation is limited but does suggest that this phenomenon is common to several different types of organisms. For example, Wei and Chang (45) found that the amoeba *Naegleria gruberi* could form aggregates of various sizes. When *N. gruberi* was exposed to iodine, it was observed that as the clump size increased, there was a concomitant decrease in the rate of inactivation. In addition, Scarpino (38) reported that aggregated polioviruses were 1.7 times more resistant to chloramines than monodispersed viruses, and Katzenelson et al. (18) found that aggregated polioviruses were 4 times more resistant to ozone than nonaggregated polioviruses.

Role of capsular material in chloramine resistance. The chloramine-resistant strain of *K. pneumoniae* produced approximately six times the amount of capsule per cell produced by the chloramine-sensitive strain. Some investigators have indicated that capsule production by *K. pneumoniae* grown under low-nutrient conditions may stimulate resistance to chlorine or chloramines (23, 33). Olivieri et al. (33) observed that disinfectant resistance was enhanced when cells were grown under low-nutrient conditions and allowed to form a biofilm on glass microscope slides. Using the same *K. pneumoniae* strain as in the present study, LeChevallier et al. (23) also found that growth under low-nutrient conditions could significantly increase the capsule content of this organism; the increase in capsule content resulted in a threefold increase in resistance to free chlorine. However, increased capsule content did not increase cellular resistance to chloramines. Like Olivieri et al., these investigators observed that attachment to glass microscope slides significantly increased resistance to chlorine and to a lesser extent provided resistance to chloramines. These previous studies suggest that disinfection protection mediated by the presence of capsular material is primarily due to the indirect action of capsular polymer material in biofilm formation. Similarly, the enhanced amount of capsular material present on the chloramine-resistant *K. pneumoniae* cells in the present study, while aiding in cellular aggregation (7), may also not have been directly responsible for chloramine resistance.

Role of cell envelope lipid composition in chloramine resistance. Chloramine-sensitive and chloramine-resistant *Klebsiella* cells differed in cell wall and cell membrane lipid composition. There were only minor differences in the composition of cell wall lipids, but there were distinct differences in the composition of lipids contained in the cell membrane. It is well known that growth conditions, including temperature and nutrient availability, can alter the lipid composition of cells (6, 11, 13, 30). These alterations can result in changes in membrane permeability (11) which may affect an organism's resistance to a variety of antimicrobial agents (5). Indeed, other researchers have observed that bacteria grown at lower temperatures (e.g., 10 to 25°C) were more resistant to antimicrobial agents such as acetic acid (8) and chlorine dioxide (2) than bacteria grown at higher temperatures. Harakeh et al. (12) found that the resistance of *K. pneumoniae* to chlorine dioxide was 2.5-fold greater when the organisms were grown at 15°C than when they were grown at 37°C. These authors hypothesized that decreases in membrane fluidity resulted in decreased permeability, thus restricting the movement of chlorine dioxide into the cell.

In the present study, it was observed that the proportion of saturated to unsaturated fatty acids was five times greater in chloramine resistant *K. pneumoniae* cells than in chloramine-sensitive cells. This may be significant, because higher proportions of saturated fatty acids decrease membrane fluidity (10, 27) and may have limited the movement of chloramines into the cell. This restriction of movement most likely would occur in the cell membrane, as the cell wall is generally considered a poor barrier to molecules of 650 Da or less (32). Analysis of lipid components after exposure to

increasing levels of chloramines indicated no changes in lipid composition, although direct halogenation of lipids was not determined. These results suggest that lipids are not reactive with chloramines but may act as a physical barrier reducing the rate of penetration and subsequent oxidization of internal cellular sites. It should be noted, however, that other researchers have stated that chloramine penetration into *Escherichia coli* cells is not impeded by the membrane (3, 16), because enzymes in whole cells and cell extracts had equivalent sensitivities to chloramines. However, these studies did not compare lipid compositions of chloramine-sensitive and chloramine-resistant cells.

Reactivity of sulfhydryl groups to chloramine oxidation. The mode of bacterial inactivation by chloramines has been studied by a number of investigators (3, 14–16). In general, these studies have suggested that sulfhydryl groups of amino acids are the primary target sites of bacterial inactivation by chloramines through oxidization of sulfhydryl groups to disulfides or to higher oxidation states, resulting in irreversibly impaired protein function and subsequently cellular inactivation. The results of the present study suggest that chloramines could not penetrate into the cell or that sulfhydryl groups contained in chloramine-resistant cells were physically protected from the presence of chloramines. These results support the observation of decreased permeability due to the higher percentage of saturated fatty acids in chloramine-resistant cells. Alternatively, the increased resistance may have been due to conformational changes of target sulfhydryl groups.

It is not uncommon for cells grown under low-nutrient conditions to have an altered protein composition (9, 39). These protein alterations have been associated with enhanced resistance to compounds such as hydrogen peroxide (17) and heavy metals (9). Stelmaszynska and Zgliczynski (40) observed that cystine groups located within the internal protein structure of the insulin molecule were protected from the oxidizing action of chlorine. Possibly, the growth conditions that enhanced the resistance of *K. pneumoniae* to chloramines in this study also resulted in conformational changes of enzymes previously susceptible to oxidation by chloramines. Further work, including protein analysis and radioisotopic uptake studies with chloramines, will be necessary to resolve this issue.

Mechanism of chloramine resistance. The results from this study indicate that growth under low-nutrient conditions can alter physiological structures of the cell, resulting in increased resistance to chloramines. These physiological changes include increased aggregation potential, increased capsule production, alteration of membrane lipids, and decreased sulfhydryl group oxidation. These changes most likely act in a concerted manner to enhance the resistance of *K. pneumoniae* to chloramines.

Some investigators believe that the presence of a biofilm in a drinking water distribution system is the primary source for the protection and release of coliforms (24, 44). Regardless of the presence of a protective biofilm, it is well documented that coliforms and other organisms can be isolated from distribution systems containing a disinfectant residual. In the low-nutrient environment of the distribution system, these organisms may be under selective pressure to modify physiological attributes to maximize nutrient acquisition. Inadvertently, these cellular modifications may also provide protection against stressor compounds such as disinfectants. This report delineates chloramine resistance mechanisms developed by *K. pneumoniae* under low-nutrient growth conditions that may explain the occurrence of this organism and possibly other coliforms in disinfected water distribution systems.

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