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Susceptibility of Chemostat-Grown *Yersinia enterocolitica* and *Klebsiella pneumoniae* to Chlorine Dioxide

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The resistance of bacteria to antimicrobial agents could be influenced by growth environment. The susceptibility of two enteric bacteria, *Yersinia enterocolitica* and *Klebsiella pneumoniae*, to chlorine dioxide was investigated. These organisms were grown in a defined medium in a chemostat and the influence of growth rate, temperature, and cell density on the susceptibility was studied. All inactivation experiments were conducted with a dose of 0.25 mg of chlorine dioxide per liter in phosphate-buffered saline at pH 7.0 and 23°C. The results indicated that populations grown under conditions that more closely approximate natural aquatic environments, e.g., low temperatures and growth at submaximal rates caused by nutrient limitation, were most resistant. The conclusion from this study is that antecedent growth conditions have a profound effect on the susceptibility of bacteria to disinfectants, and it is more appropriate to use the chemostat-grown bacteria as test organisms to evaluate the efficacy of a certain disinfectant.

Growth conditions can profoundly influence the susceptibility of microorganisms to disinfectants (4, 6). We have previously shown that growth of *Escherichia coli* at submaximal rates due to nutrient limitation and at relatively low temperatures greatly increases the resistance of this bacterium to chlorine dioxide (3) and phenylphenol (1). Although coliforms are generally regarded as a suitable indicator organism, there is reason to doubt this premise (13), and we therefore decided to determine whether other organisms responded to nutrient limitation in the same manner as *E. coli*. Two pathogens of considerable current public health concern, *Yersinia enterocolitica* and *Klebsiella pneumoniae*, were chosen for these studies.

If antecedent growth conditions do influence resistance of bacteria in general, then the current practice for environmental management of microorganisms will need to be reexamined. This practice relies exclusively on batch culture-grown bacteria for determining the efficacy of various disinfectants. However, it is very unlikely that the nutrient excess conditions present in batch cultures are similar to those present either in the environment or in the human body, since in most natural environments bacteria grow at submaximal rates owing to severe nutrient limitation.

MATERIALS AND METHODS

Organisms. *K. pneumoniae* was obtained from Lynn Berg (Department of Biology, San Jose State University, San Jose, Calif.). *Y. enterocolitica* (8081) was obtained from D. Portnoy (Department of Medical Microbiology, Stanford University, Stanford, Calif.).

Growth media and growth conditions. *K. pneumoniae* was grown in a glucose-mineral salt medium containing (in milligrams per liter): Na_2HPO_4, 60; KH_2PO_4, 30; NaCl, 5; NH_4Cl, 10; $CaCl_2 \cdot 2H_2O$, 1.47; $MgSO_4 \cdot 7H_2O$, 0.39; and trace salt solution, 0.5 ml/liter, as described elsewhere (14). The last three were sterilized separately and added aseptically. *Y. enterocolitica* was grown in a mineral salt medium containing (in milligrams per liter): $MgSO_4 \cdot 7H_2O$, 1.5; $K_2HPO_4 \cdot 3H_2O$, 40; KH_2PO_4, 20; $(NH_4)_2SO_4$, 10; and glucose, 2 or 4 g/liter.

Populations were grown in a chemostat (Bio Flo; New Brunswick Scientific Co., Inc., Edison, N.J.). The growth rates were fixed by adjusting the dilution rate, which ranged between 0.025 and 0.150 h^{-1}.

Temperatures ranged from 15 to 29°C in the case of *Y. enterocolitica* and from 15 to 37°C in the case of *K. pneumoniae*. *Y. enterocolitica* did not grow at 37°C. A constant pH of 7.0 ± 0.2 was maintained by the automatic addition of 5% Na_2CO_3. Dilution rate, temperature, aeration rate, agitation, and pH were checked at least once daily and often more frequently. Populations were harvested after a minimum of five volume changes.

Disinfectant. Stock solutions of chlorine dioxide were prepared as described elsewhere (2). Dose-response experiments indicated that 0.25 mg of ClO_2 per liter resulted in a significant extent of kill while providing a measurable concentration of residual disinfectant and surviving organisms. Residuals were measured by the DPD method (12).

Inactivation procedure. Disinfection experiments were conducted in a well-mixed 1-liter Pyrex flask, held at 23 ± 1°C. Bacteria were harvested from the chemostat, centrifuged at 6,000 rpm, and suspended in 20 ml of phosphate buffer. These cells were used as a seeding stock. Cells were suspended in 700 ml of chlorine dioxide demand-free buffered basal salts solution at a concentration of approximately 10^7/ml. The mixture was stirred by a magnetic stirrer and the first sample was collected after 5 min of mixing. This presented the input at the start of the experiment. The mixture was then dosed with chlorine dioxide, and samples were withdrawn at 2-, 5-, and 15-min intervals. All samples were treated with $Na_2S_2O_3$ to neutralize any residual ClO_2.

Recovery conditions. Samples were diluted to yield 20 to 200 colonies ultimately and were recovered on membrane filters (Gelman GN-6) in triplicate. Filters were placed on M.Endo medium (Difco Laboratories, Detroit, Mich.) and incubated at 25°C for 72 h in the case of *Y. enterocolitica* and at 37°C for 24 h in the case of *K. pneumoniae*.

Presentation of data. We have presented resistance in terms of survival ratio (N_t/N_0) after t min of contact with

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chlorine dioxide, t being 15 min. Thus, the ratio N_{15}/N_0 is used as the criterion for comparing the effects of various antecedent growth conditions on the susceptibility of the populations to chlorine dioxide.

RESULTS

Reproducibility of the experiments. The t-test analysis of data indicated that variation among triplicate trials in a given experiment was not significant at the 0.05 level. A biphasic mode of inactivation was noted in all experiments. To investigate whether this biphasic mode was due to a resistant fraction of bacteria or the absence of a disinfectant residual, the chlorine dioxide residual was measured. At a dose of 0.25 mg/liter, a residual of 0.12 mg/liter with a standard deviation of 0.005 was detected at the end of the experiment. Thus, it can be concluded that the biphasic mode of inactivation was due to a resistant fraction, as we have suggested earlier (3).

Effect of D and growth temperature. The effect of dilution rate (D) on susceptibility was tested at two different temperatures (15 and 29°C) in the case of *Y. enterocolitica* and at three temperatures in the case of *K. pneumoniae* (15, 25, and 37°C); *Y. enterocolitica* did not grow at 37°C in the medium used. These temperatures were selected because they represent the range of temperatures that may be encountered by the organism in the aquatic environment and the human body. A composite of the results, based on the fraction of cells surviving after 15 min, is shown in Fig. 1 and 2 for *K. pneumoniae* and *Y. enterocolitica*, respectively: the plots show the resistant fraction (N_{15}/N_0) as a function of D and growth temperature. At a given temperature, the culture resistance to chlorine dioxide increased with decreasing D.

At a given dilution rate, temperature determined the extent of susceptibility of *K. pneumoniae*, and an increase in resistance occurred at low temperatures in the case of *K. pneumoniae*. Temperature had only a marginal effect on the susceptibility of *Y. enterocolitica* to chlorine dioxide (Fig. 2).

Comparison of the susceptibility of two strains of *Y. enterocolitica* 8081. We were also interested in investigating the difference in behavior of two strains of Y8081, a pathogenic strain and a nonpathogenic strain (Y8081 and Y8081C). Previous reports have suggested that a 40 × 10^6- to 41 × 10^6-dalton plasmid was involved in the pathogenesis of *Y. enterocolitica* (7, 8). A plasmid-bearing strain of *Y. enterocolitica* and an isogenic cured strain were grown at a fixed dilution rate and at the same temperature (D = 0.1; S_R [see below] = 0.2%; 29°C). The results (Fig. 3) indicated that the pathogenic strain was less resistant than the nonpathogenic one.

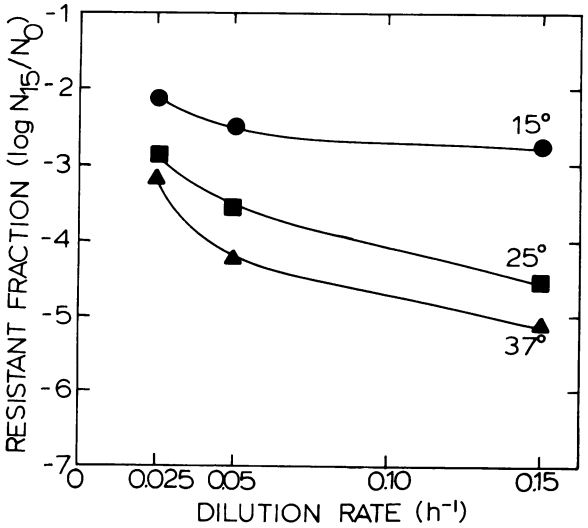

FIG. 1. Effect of dilution rate on susceptibility of *K. pneumoniae* to chlorine dioxide grown under glucose limitation, S_R = 0.4%, at three temperatures, 15°C (●), 25°C (■), and 37°C (▲).

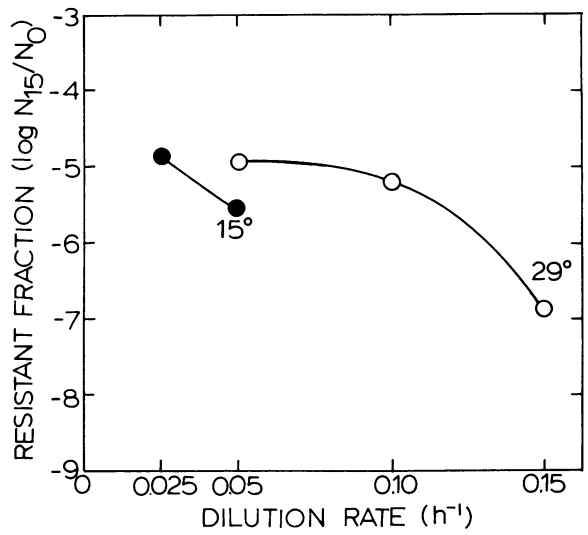

FIG. 2. Effect of dilution rate on susceptibility of *Y. enterocolitica* to chlorine dioxide grown under glucose limitation, S_R = 0.2%, at two temperatures, 15°C (●) and 29°C (○).

Effect of cell density. We have shown previously that culture cell density influences the susceptibility of *E. coli* to chlorine dioxide, a higher culture cell density during growth being accompanied by greater resistance (3). Studying the effects of cell density on the susceptibility is important because organisms in most aquatic environments grow at low cell densities compared with laboratory batch cultures. The effect of cell density on cell parameters can be conveniently studied with a chemostat since by simply changing the concentration of the limiting nutrient in the inflow medium (S_R), one can change the culture cell density without altering other parameters. *Y. enterocolitica* cultivated to a steady state in the chemostat at two S_R glucose concentrations did exhibit differences in susceptibility, the lower-density populations being more resistant (Table 1). *K. pneumoniae*, on the other hand, exhibited the same susceptibility after growth at two different culture densities. It should be emphasized that the cell density during growth is the determining factor here rather than the density of the populations subjected to disinfection. The latter was kept constant in all experiments as stated in Materials and Methods.

DISCUSSION

The results presented here demonstrate that slowly growing populations of *Y. enterocolitica* and *K. pneumoniae* grown under nutrient limitation are considerably more resistant to disinfection by chlorine dioxide than populations grown at more rapid rates. We have previously shown the same phenomenon for populations of *E. coli* and *Legionella pneumophila* with respect to their susceptibility to chlorine dioxide (3; J. D. Berg, A. Matin, and P. V. Roberts, *in* R. L.

TABLE 1. Effect of cell density on susceptibility of *Y. enterocolitica* and *K. pneumoniae* to chlorine dioxide

Organism	% Glucose in inflow medium	Temp (°C)	D (h^{-1})	Cell density[a]	Log N_{15}/N_0[b]
K. pneumoniae	0.2	37	0.05	0.8	−4.22 ± 0.061
	0.4	37	0.05	1.8	−4.29 ± 0.045
Y. enterocolitica	0.2	29	0.05	1.0	−4.98 ± 0.001
	0.4	29	0.05	1.6	−5.79 ± 0.017

[a] Average optical density at 660 nm.
[b] Survival ratio after 15-min contact with a dose of 0.25 mg of ClO_2 per liter at 23°C.

Jolley, ed., *Fifth Conference on Water Chlorination: Environmental Impact and Health Effects*, in press) and another disinfectant agent, phenylphenol (1). Thus, it may generally be stated that slowly growing populations of bacteria tend to be more resistant to disinfectant agents than their counterparts grown more rapidly.

Bacteria in nature as a rule grow much more slowly owing to severe limitation of nutrients compared with their counterparts cultivated in a laboratory batch culture (11). It follows that the natural populations are likely to be more resistant to disinfection than their batch culture-grown counterparts. Thus, a complete reliance on batch culture-grown organisms in the evaluation of disinfectant agents can lead to an overestimation of their effectiveness in situ.

It is not known what accounts for the increase in resistance to chlorine dioxide in slowly growing bacteria. Any speculation on this must await more definite information on the mode of action of chlorine dioxide; indeed, a comparison of bacteria grown at different dilution rates with respect to suitable traits can be an effective way of investigating the mode of action of this disinfectant. It is not surprising that growth under nutrient limitation affects bacterial susceptibility to disinfectants. Previous studies from this and other laboratories have established that this aspect of the growth environment profoundly affects several structural and physiological aspects of microorganisms (10, 11), which in turn must influence the bacterial response to these agents.

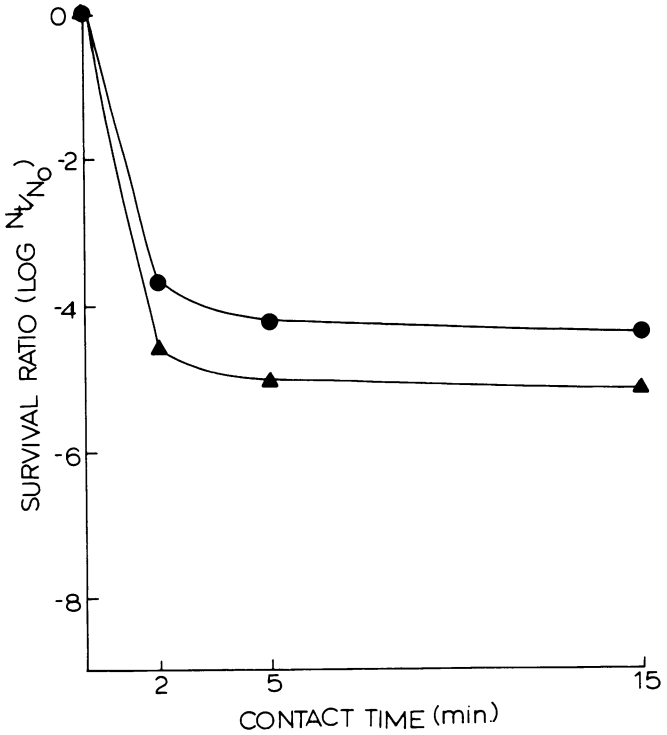

FIG. 3. Response of two strains of *Y. enterocolitica* grown at 29°C, $D = 0.1\ h^{-1}$, and $S_R = 0.2\%$ glucose. Cells were inactivated at 23°C by 0.25 mg of chlorine dioxide per liter. Symbols: ●, strain Y8081C; ▲, strain Y-8081.

The effect of growth temperature on susceptibility was more variable than that of growth rate. Temperature had only a marginal effect in the case of *Y. enterocolitica*, but in the case of *K. pneumoniae* an increase in susceptibility was associated with increasing temperature. It may be assumed that the fluidity of the lipid bilayer, and hence the permeability of the outer membrane to small molecules, is an important factor in the mechanism of action of chlorine dioxide. The results indicate that the nonpathogenic strain was less susceptible than the pathogenic strain. This could be due to the change in the outer membrane proteins coded for by the plasmid, found only in the pathogenic strain.

A comparison of the susceptibility patterns of the two microorganisms indicates that *Y. enterocolitica* was more susceptible to chlorine dioxide than *K. pneumoniae* was. For instance, at 15°C and $D = 0.025\ h^{-1}$, treatment of *Y. enterocolitica* with chlorine dioxide produced a 5-log reduction, whereas under the same experimental conditions *K. pneumoniae* showed a 2-log reduction. This finding emphasizes that different microorganisms respond differently to the same disinfectant.

In conclusion, populations grown under conditions that more closely approximate the natural environment, e.g., low temperatures and growth at submaximal rates, exhibit enhanced resistance to disinfectants.

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