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Changes in *Escherichia coli* Associated with Acquired Tolerance for Quaternary Ammonium Compounds[1]

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Acquired tolerance for a quaternary ammonium compound produced a tolerance for a similar compound. Tolerance was associated with the structure and the extent of adsorption of the compound. Morphological changes and resistance to disruption by pressure and by sonic treatment accompanied the development of tolerance. An otherwise weakened culture evolved with the acquisition of tolerance. The maximum obtainable viable population density of tolerant cells in growth medium was approximately 5% of that obtained in the parent culture. Tolerant cultures died off more rapidly in the original growth medium as well as when washed cell suspensions were stored at 5 C. Since acquired tolerance was associated with an otherwise weakened culture, the occurrence of the tolerant cells to limit the efficacy of quaternary ammonium compounds in sanitation operations is highly unlikely.

Although quaternary ammonium compounds (quats) have wide use in cleaning and sanitizing operations, the mechanism of their antibacterial activity is not completely understood. Materials for sanitization often contain a quat (9). Quats are bacteriostatic in low concentrations and bactericidal in high concentrations. The degree of antibacterial activity depends on the nature of the organic fraction which contributes the lipophilic properties (2). Explanations of the mechanism of the antibacterial activity have been sought in terms of inhibition of a key enzyme in the general metabolic process (8) and in cell wall differences (5, 6). Sensitivity of bacteria to these compounds varies greatly. Antibacterial activity of the quats is associated with adsorption by the bacterial cells (13) and subsequent leakage of cellular constituents (1, 13). Adsorption and antibacterial activity, however, are not limited to the cationic surface-active agents as fatty acids may act similarly on certain bacteria (12).

Bacteria vary greatly in susceptibility to quats. Gram-negative bacteria, when compared to gram-positive bacteria, are slightly more resistant to quats in bactericidal concentration and markedly more tolerant in bacteriostatic concentrations. Certain species of bacteria have been shown to acquire tolerance for progressive concentrations of quat in serial transfers at progressive challenging levels of quat (3, 4, 10, 16). Some morphological and physiological changes have been observed to accompany the acquisition of tolerance for a quat (4, 16).

Although quats have desirable features in cleaning, sanitizing, and disinfecting processes, the ability of bacteria to acquire tolerance to these compounds under laboratory conditions has restrained their use in industrial processes (4).

Since there are physiological changes in cells during the process of acquiring tolerance, it was reasoned that these changes might be instrumental in the cellular defense against quats. Further work was therefore undertaken to determine the relationship between adsorption, adaptation, and tolerance of bacteria to quats.

MATERIALS AND METHODS

Cultures and media. *Escherichia coli* from the departmental stock culture collection was carried in nutrient broth (Difco), which also served as the basal medium to which the quat was added for observations. The quat for producing adaptation was a 10% active-ingredient commercial preparation, Sterbac, containing *n*-alkyl dimethyl benzyl ammonium and *n*-alkyl methyl ethyl benzyl ammonium chloride (Klenzade Products Division of Economics Laboratores, Inc., St. Paul, Minn.). Quantitative expressions were in terms of concentration of quats.

Adaptation of cultures to quats was achieved by a previously described process (16), which involved daily challenge in progressive increments of 2 μg of quat per ml. The plateau of essentially maximum tolerance for *E. coli* was 28 μg/ml and was obtained

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in 12 to 14 daily transfers. This culture was then identified as *E. coli* T.

Preparation of cell suspensions. Cultures were grown for 24 hr at 32 C and centrifuged at 5,000 × *g* for 10 min. Harvested cells were washed twice with 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.4) and resuspended in 0.1 M Tris buffer (*p*H 7.4) to the desired concentration.

Determination of population density. Total viable counts of the cultures and cell suspensions were determined with Plate Count Agar (PCA; Difco). The selective medium was Violet Red Bile Agar (VRBA; Difco) to detect injured cells (11). After suitable dilution, optical density readings were taken at 500 nm, with a Bausch & Lomb Spectronic-20 colorimeter.

Disruption of cells. For breaking of cells by pressure differential, a Ribi cell fractionator (Ivan Sorvall, Inc., Norwalk, Conn.) was operated at 20,000 to 50,000 psi. A Biosonic III sonicator (Bronwill Scientific, Rochester, N.Y.) was used with successive 30-sec treatments at maximum power and intermittent cooling of the cell suspension in an ice bath.

Radioactivity and materials. Two radioactive quats, each with a carbon-14 label, were used. One was Amprolium [1-(4-amino-2-*n*-propyl-5-pyrimidinyl-methyl)-2-picolinium chloride hydrochloride] from Merck & Co., Rahway, N.J., with a specific activity of 1.18 μCi/mg. The other was BDMAC (benzyl-*1*-^{14}C dimethymyristyl ammonium chloride) from New England Nuclear Corp., Boston, Mass., with a specific activity of 1.0 μCi/mmole. These quats were substituted for the Sterbac in the medium of adaptation when adsorption observations were to be made.

The radioactivity measurements were made with a Baird Atomic general purpose multiscaler with a thin-window Geiger gas flow counter. Counting conditions were established for a mean counting error ≦ 2%. Periodically, the counting rate was checked by using a standard carbon-14 source.

Adsorption and filtration. The ^{14}C-labeled quats were used to study accumulation of the compounds by bacterial cells. Bacterial cultures were inoculated into nutrient broth and incubated for 24 hr at 30 C. The growth was harvested by centrifugation, washed three times with sterile nutrient broth, and suspended in sterile nutrient broth to which the radioactive quat had been added. The concentration of quat was 28 μg/ml, and the density of bacteria was approximately 10^6 cells/ml. The suspension in 5-ml portions was filtered with a membrane having a mean pore size of 0.45 μm (Millipore Co., Bedford, Mass.). The membranes were rinsed with sterile nutrient broth, and the radioactivity was determined after drying.

RESULTS

Comparative tolerance of different cultures to inhibition by quats. The data in Table 1 show the extreme differences in susceptibility of bacteria to inhibition by different quats. A culture having tolerance for one member of the class of quats may or may not have a parallel tolerance for another member. The data presented in Table 1 indicated that *E. coli* had more than a 56-fold greater tolerance for the heterocyclic compound Amprolium than for the other two quats. Each of these compounds has proven bactericidal efficacy. Acquired tolerance by *E. coli* for one member of the class of quats (Sterbac), however, gave a similar increased tolerance for another quat (BDMAC).

TABLE 1. *Comparative tolerance of E. coli and E. coli T to quats in nutrient broth*

Bacterial culture	Maximum concn (μg/ml) allowing growth		
	Amprolium	Sterbac	BDMAC[a]
E. coli	>280	5	5
E. coli T	>280	28	28

[a] Benzyl-*1*-^{14}C dimethylmyristyl ammonium chloride.

TABLE 2. *Affinity of carbon-14-labeled quats for bacterial cells*

Bacterial culture	Per cent removal of quat by rinsing	
	BDMAC[a]	Amprolium
E. coli	1	86
E. coli T	28	90

[a] Benzyl-*1*-^{14}C dimethylmyristyl ammonium chloride.

TABLE 3. *Comparative susceptibility of E. coli and E. coli T to destruction by sonic treatment*

Duration of sonic treatment (min)	*E. coli*		*E. coli* T	
	Optical density	Plate count per ml	Optical density	Plate count per ml
0	110	1.0×10^{11}	105	1.2×10^8
2	5	4.5×10^6	80	3.6×10^3
5	5	2.2×10^5	75	<100
7.5	5	1.2×10^4	70	<100

Adsorption and elution of quats. The comparative affinity of the quats for the cells was determined by adsorption, filtration, and subsequent rinsing for elution of quats. Average results of five individual trials are given in Table 2. The figures were corrected for control mixtures without bacterial cells. Only 1% of BDMAC was removed from *E. coli*, whereas a comparable treatment removed 28% of this compound from *E. coli* T. In general, there was less affinity for the Amprolium than for BDMAC. The tenacity of adsorption of Amprolium by sensitive cells, however, was greater than by resistant cells (significant at the

1% level). These data indicate that a major difference in effectiveness of the two classes of quats is associated with the physicochemical condition at the cell wall-menstruum interface.

Disruption of cells. Cell suspensions from tolerant and sensitive strains were prepared with comparable optical density. There were only approximately 0.1% as many viable tolerant cells. The tolerant cells were more resistant to disruption by sonic treatment and by pressure in the Ribi cell fractionator as shown by a change in optical density. The destructive effect of sonic treatment as judged by ability to grow on PCA, however, was at least as much in the tolerant strain as in the sensitive strain. The data representing an average of three trials with sonic treatment are shown in Table 3. It is apparent that the resistance to cell wall disruption does not provide a similar resistance to the killing effect.

Survival characteristics as influenced by previous growth and storage in the presence of quats. With the acquisition of tolerance, there was an alteration in the growth pattern. The maximum attainable viable population density of *E. coli* T in 25 μg/ml quat medium was less than 5% of the maximum population attained with *E. coli* grown in nutrient broth. The viable population was 3.0×10^7/ml compared to 9.8×10^8/ml.

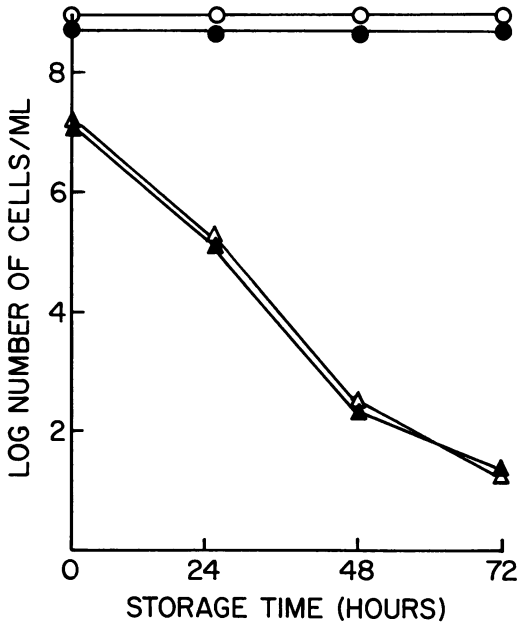

FIG. 1. *Comparative survival at 5 C of E. coli in nutrient broth and E. coli T in nutrient broth containing added quat. Sumbols: ○, E. coli count on Plate Count Agar (PCA); ●, E. coli count on Violet Red Bile Agar (VRBA); △, E. coli T count on PCA; ▲, E. coli T count on VRBA.*

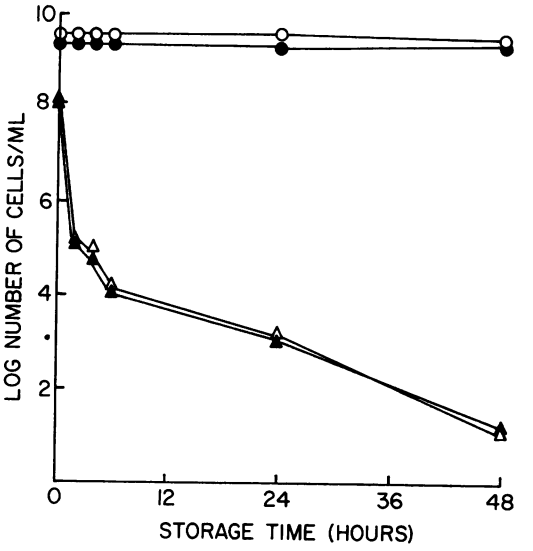

FIG. 2. *Survival of E. coli and E. coli T at 5 C in Tris buffer, pH 7.4. Symbols: ○, E. coli count on Plate Count Agar (PCA); ●, E. coli count on Violet Red Bile Agar (VRBA); △, E. coli T count on PCA; ▲, E. coli T count on VRBA.*

When *E. coli* T was grown for 24 hr in the presence of 25 μg of quat per ml and then stored at 5 C, the death rate was rapid, as shown in Fig. 1. Comparative data on *E. coli* grown in nutrient broth and subjected to similar storages are also given in Fig. 1. The population density of the latter was essentially stable for the 72-hr test period.

Since it has been shown that many forms of cell injury of *E. coli* reduce its ability to recover on selective media (11, 14), VRBA counts were run on the preceding cultures to explore for potential cell injury. *E. coli* showed fewer living cells when evaluated by VRBA than by PCA. *E. coli* T, on the other hand, showed essentially the same count on each of the media. The colonies of *E. coli* on VRBA, however, were about one-fourth the size of colonies of *E. coli*, which indicated an altered physiology in *E. coli* T as reported earlier by Crocker (4). Morphological changes were apparent as bizarre forms of cells when examined microscopically. A recovery of *E. coli* T on VRBA comparable to that on PCA indicates that the altered physiology is not involved per se in the injury phenomena reported earlier (11).

Comparative survival of E. coli and E. coli T. The washed cell suspensions in Tris buffer (*p*H 7.4) were stored at 5 C, and the viable counts were determined at periodic intervals. *E. coli* T died off much more rapidly than *E. coli* (Fig. 2). Injury with failure to recover on selective media was slight with each culture. Thus, the acquisition

of tolerance by a culture is accompanied by a loss in ability to survive.

DISCUSSION

Since physiological changes resulting from adaptation are associated with cell wall characteristics, the site of activity of quats would appear to be cell wall. The physiochemical condition of *E. coli* T permitted less tenacious adsorption of quats, therefore allowing less interference with the cellular activity. Acquired tolerance is also associated with an increased resistance of the cell wall to distintegration by sonic treatment. Changes in tolerance result in morphological changes apparent as pleomorphic cell structure and as small colonies on VRBA.

Presently available data indicate that inhibition is not related to a single, specific chemical process, e.g., the inhibition of a key enzyme. Cell injury in *E. coli* resulted in a weakened tolerance for selective media such as VRBA and Brilliant Green-lactose-bile broth (11), and the primary contributor for the inhibition of injured cells was the surface activity. More recently, Scheusner et al. (14, 15) have shown that *E. coli*, when injured by exposure to quats, was sensitive to VRBA and they too attributed the primary inhibition to the bile salts. On the other hand, our work showed that *E. coli* T did not exhibit a similar sensitivity to VRBA, indicating that adaptation to quats confers a resistance to surface-active agents. In addition, Hill (7) has shown the constituents of *E. coli* cell wall to be sensitive to bile salts. Therefore, the results indicate the relationship between adaptation to quats and bile salts and the cell wall characteristics.

This work substantiates an earlier report (16) questioning the significance of adaptation and acquired tolerance for quats under commerical conditions of cleaning, sanitization, and disinfection. The acquired tolerance is well below a commonly recommended use concentration of 200 μg/ml (17). *E. coli* T grew less rapidly than *E. coli* and failed to reach a comparable population density even under the most favorable conditions. *E. coli* T died off more rapidly than the parent culture after a full growth cycle when stored in the original growth medium as well as when stored as a washed cell suspension. If further tolerance were developed under yet unstudied conditions, the resultant culture would be expected to show even more drastic morphological and physiological changes than those thus far reported. These changes would be expected to impart a further weakening of the strain for competitive survival.

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