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Fourteen-Year Survival of *Pseudomonas cepacia* in a Salts Solution Preserved with Benzalkonium Chloride

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A strain of *Pseudomonas cepacia* that survived for 14 years (1963 to 1977) as a contaminant in an inorganic salt solution which contained commercial 0.05% benzalkonium chloride (CBC) as an antimicrobial preservative, was compared to a recent clinical isolate of *P. cepacia*. Ammonium acetate was present in the concentrated stock CBC solution, and served as a carbon and nitrogen source for growth when carried over into the salts solution with the CBC. The isolate's resistance to pure benzalkonium chloride was increased step-wise to a concentration of 16%. Plate counts showed 4×10^3 colony-forming units per ml in the salts solution. Comparison of growth rates, mouse virulence, antibiotic resistance spectra, and substrate requirements disclosed no differences between the contaminant and a recently isolated clinical strain of *P. cepacia*. The results indicate that it is critical that pharmaceutical solutions containing benzalkonium chloride as an antimicrobial preservative be formulated without extraneous carbon and nitrogen sources or be preserved with additional antimicrobial agents.

Pseudomonas cepacia has a wide distribution in nature. In the hospital environment, it has been recovered from many diversified sources, including equipment, antiseptic, and disinfectant solutions (3, 15, 17, 21, 23) and commercially packaged urinary catheter kits (11, 14) in which the detergent-germicide, 0.15% *N*-alkyl, dimethyl benzyl ammonium chloride (benzalkonium chloride) in water and polyethoxyethanol was contaminated. The organism is also evolving in importance as an agent causing severe nosocomial infections (3, 7, 8, 14, 19, 22) and in a number of cases of death (5, 20).

The survival and multiplication of *P. cepacia* in quaternary ammonium compounds have been reported by Hardy et al. (11) and other investigators (1, 13). This organism was also isolated from benzalkonium chloride solutions by Gilardi (10). That benzalkonium chloride solutions can support pseudomonas contaminants is well established by the studies of Adair et al. (1). In 1975, Mathews et al. (R. J. Mathews, G. M. Ederer, L. V. Cunningham, and J. M. Matsen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, C86, p. 41) reported the maintenance of a *P. cepacia* isolate over a period of 6 years. The organism was originally recovered from 0.15% *N*-alkyl dimethyl benzyl ammonium chloride solution that functioned as a cleansing germicidal agent in commercial catheters.

The present study describes the survival of a *P. cepacia* strain for 14 years in an inorganic salts solution preserved with commercial 0.05% benzalkonium chloride (CBC). This contaminant was compared with a recent clinical isolate in regard to nutritional, biochemical, virulence, and antibiotic resistance characteristics. A stepwise increase of the resistance of the isolate to extraordinary high levels of benzalkonium chloride was also carried out. The practical importance of these findings is discussed.

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MATERIALS AND METHODS

Isolation. In 1963, we prepared 10 liters of an inorganic minimal salts solution composed of ingredients shown in Table 1. The solution was preserved with an aqueous solution of CBC at a final concentration of 0.05%. CBC refers to a commercial benzalkonium chloride preparation that contained ammonium acetate (38% of the final concentration of CBC) as a buffer and antioxidant. This type of preparation has been employed as an antimicrobial preservative in pharmaceutical products. It also has been utilized as a disinfecting agent for surgical instruments and hospital equipment. Pure benzalkonium chloride (PBC) contains no ammonium acetate. The preserved inorganic minimal salts solution was tightly capped, covered, and stored at room temperature in a dark closet.

Enriched salts medium and growth. The enriched medium facilitated the growth and maintenance of the $OC_{(9/63)}$ culture and in the development of the isolate's resistance to higher levels of PBC (Table 1). The pH of the medium was adjusted to 7.2 with NaOH. PBC was filtered (Millipore Corp., 0.22 μm porosity) and added, as required, at a final concen-

tration of 500 μg/ml (0.05% wt/vol).

Identification. The criteria used to identify the $OC_{(9/63)}$ isolate and to compare it to a known *P. cepacia* strain 153 were based on the schemes proposed by Gilardi (10) and by Weaver (26). The tests and methods followed were selected from both schemes (Table 2).

Antimicrobial susceptibility spectrum. The in vitro antimicrobial susceptibility of the two strains was determined by the standardized single disk method of Bauer et al. (4) on Mueller-Hinton agar (Becton, Dickinson & Co.). A 10^{-4} dilution of an overnight growth of cells was used for seeding the agar. Single, high-potency Sensi-Discs (BBL) of the antibiotics and antimicrobials, as detailed in Table 3, were placed on the seeded agar surface. Zone diameters were measured, and activities were designated as susceptible, intermediate, or resistant.

Nutritional requirements. To ascertain how the $OC_{(9/63)}$ strain maintained itself in minimal salts solution, preserved with CBC, cells from the contaminated salts solution were centrifuged, washed three times with sterile minimal salts solution, and then resuspended in fresh salts solution. Samples (0.1 ml; about 10^7 cells) were inoculated in duplicate into Bausch & Lomb Spectronic test tubes (20 mm inside diameter), as follows: (i) Series I, 9.9 ml of minimal salts solution, containing 0.05% CBC; (ii) Series II, 9.9 ml of minimal salts solution, containing 0.05% PBC (no ammonium acetate); (iii) Series III, 9.9 ml of minimal salts solution, containing 0.05% PBC and ammonium acetate added in the same concentration (38%) found in CBC.

Tubes were incubated without shaking at 30°C. Growth was measured turbidimetrically in a Bausch & Lomb Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, N. Y.), set at 545 nm.

Growth studies. To compare the growth rates of the $OC_{(9/63)}$ strain and the clinical isolate, *P. cepacia* 153, both strains were inoculated into enriched salts medium and incubated at 30°C without shaking for 24 h. The growths were then diluted 1:10 in sterile, triple-distilled water. Samples (0.1 ml) of the dilution were inoculated in duplicate into Bausch & Lomb Spectronic tubes (20 mm inside diameter) that contained 9.9 ml of enriched salts medium. The seeded tubes were incubated at 30°C without shaking. Optical density readings were taken, after mixing, at 0, 4, 7, 14, 28, 32, and 48 h of incubation in a Bausch & Lomb Spectronic 70 set at 545 nm.

TABLE 1. *Composition of salt solutions*

Ingredients	Amt	Benzalkonium chloride (final concn)
Minimal salts medium		
NaCl	3.31 g	CBC, 0.05% (vol/vol) added or PBC, 0.05% (vol/vol)
Na_2HPO_4	2.58 g	
KCl	2.23 g	
KH_2PO_4	7.42 g	
Distilled H_2O	1,000 ml	
Enriched salts medium		
Minimal salts medium	1,000 ml	PBC added as needed (0.05 to 16% vol/vol)
Casamino Acids (Difco)	0.5% (wt/vol)	
Glucose	0.5% (wt/vol)	
$CaCl_2 \cdot 2H_2O$	0.005 g	
$FeSO_4 \cdot 7H_2O$	0.005 g	
$MgSO_4 \cdot 7H_2O$	0.1 g	
$(NH_4)_2SO_4$	1.0 g	

Mouse virulence studies. A study of the comparative pathogenicity of the two strains of *P. cepacia* was performed with inocula prepared from 24-h growth at 30°C in enriched salts medium. Tenfold dilutions were made in sterile 0.9% physiological saline. The number of bacteria at each dilution was determined by plate counts on Trypticase soy agar (BBL). Groups of six CF1 male mice, weighing between 17 and 20 gm each, were injected intraperitoneally with 0.5 ml of each dilution. The numbers of organisms injected per mouse, day of death, and the numbers and percent of survivors were recorded for 14 days, at which time the study was terminated.

Development of resistance to 16% PBC. The $OC_{(9/63)}$ isolate was found to grow consistently in 0.05% PBC. Higher levels of resistance were attained by inoculating enriched salts medium containing increasing concentrations of PBC with cells (at least 10^8/ml) from growth obtained in the presence of lower levels of PBC. The concentration of PBC was increased in increments of 0.5%. The organism that grew in 16% PBC was designated as strain $PC_{R\text{-}16\%\ BZK\text{-}Cl}$.

RESULTS

Growth and nutritional requirements. After several months of storage, the inorganic minimal salts solution preserved with 0.05% CBC was found to be contaminated with a gram-negative rod. The contaminant was isolated and maintained in salts solution to which was added CBC. This isolate was designated as $OC_{(9/63)}$ strain and was suspected of being *P. cepacia*. After a 14-year period of storage at room temperature, strain $OC_{(9/63)}$ was reisolated from the original solution and compared to *P. cepacia* 153, a recent clinical isolate obtained from G. L. Gilardi (Hospital for Joint Diseases and Medical Center, New York, N. Y.). A wet-mount preparation of the $OC_{(9/63)}$ strain, examined with phase microscopy, revealed the presence of dividing cells and aggregates of cells varying in numbers from 10 to 300 cells per aggregate. Plate count determinations on Trypticase soy agar (BBL) confirmed the presence of 4×10^3 colony-forming units per ml of solution. Both the $OC_{(9/63)}$ and *P. cepacia* 153 strains grew abundantly on Trypticase soy agar and brain heart infusion agar (Difco). Microscopic examination of hanging-drop preparations from 24-h growths at 30°C of the two organisms in enriched salts medium divulged the presence of actively motile rods.

Figure 1 shows the results of the study of the

TABLE 2. *Characteristics of the strains of P. cepacia*

Test	Test result[a]		
	$OC_{(9/63)}$[b]	153[b]	$PC_{R\text{-}16^{s}\ BZK\text{-}C1}$[b]
Pigment			
Olive (dark, non-diffusible, Trypticase soy agar)	−	+	−
Pyocyanine (Pseudomonas agar P)	−	−	−
Oxidase	+	+	+
$NO_3 \rightarrow NO_2$	+	+	+
Triple sugar iron	No change	No change	No change
MacConkey	+	+	+
Acetamide	+	+	−
Esculin	−	+	−
Nicotinamide	+	−	−
OF dextrose	O	O	O
OF adonitol	−	−	−
OF lactose	+	+	+
Growth at 37°C	+	+	−
Growth at 30°C	+	+	+
Gelatin	+	−	+
Cetrimide	−	−	+
Indole	−	−	−
Deoxyribonuclease	−	−	−
Lysine decarboxylase	+	+	+
Arginine dihydrolase	−	−	−
Ornithine decarboxylase	±	±	−
Sellers agar	No fluorescence	No fluorescence	No fluorescence

[a] Symbols: +, positive growth; −, negative growth; ±, doubtful reaction; O, oxidative.
[b] *P. cepacia* strain.

nutritional requirements for the $OC_{(9/63)}$ strain to maintain itself in the minimal inorganic salts solution preserved with either CBC or PBC. The CBC minimal inorganic salts solution that contained ammonium acetate at a final concentration of 0.019% (wt/vol) supported the growth of the $OC_{(9/63)}$ isolate, whereas no growth occurred on replacement of CBC by PBC. When ammonium acetate was added to the PBC-salts solution at a final concentration of 0.019% (wt/vol), the $OC_{(9/63)}$ strain had a growth pattern comparable to that seen in CBC-salts solution. In addition, ammonium acetate (0.019% wt/vol) was used by the organism as a sole carbon, nitrogen, and energy source. However, the level of growth attained (as measured by optical density) was less when only ammonium acetate was present. Evidently, some portion of the benzalkonium chloride molecule was also used as a substrate for growth when ammonium acetate was added. We did not investigate the exact contribution of the benzalkonium chloride molecule to the nutrition of the $OC_{(9/63)}$ strain.

Growth rates. It was found that both the $OC_{(9/63)}$ and *P. cepacia* 153 strains growing in enriched salts medium containing 500 μg of 0.05% PBC per ml had a generation time of about 240 min.

Identification. Although some slight differences in the biochemical test results seem to exist between the two strains (Table 2) the essential similarities in their characteristics definitely identifies the $OC_{(9/63)}$ isolate as a *P. cepacia* strain. Even though this organism remained viable for a period of 14 years in a hostile environment of limited nutrients (minimal inorganic salts plus 0.05% CBC), it still retained all the characteristics of *P. cepacia*. Significantly, neither strain grew on Cetrimide agar; this lack of growth of *P. cepacia* on Cetrimide agar was also reported by Bassett et al. (3). The $OC_{(9/63)}$ isolate produced no pigment on any medium, whereas the 153 strain produced a non-diffusible, dark-olive pigment in Trypticase soy agar. Neither of the strains produced pyocyanin on Pseudomonas agar P (Difco).

Apparently, the $OC_{(9/63)}$ strain is resistant to higher levels of several of the antibiotics tested (kanamycin, neomycin, colimycin) than the 153 strain (Table 3). Both strains are resistant to chloramphenicol and demonstrate degrees of sensitivity to the sulfonamides—triple sulfa, sulfadiazine, and sulfamerazine. Of interest, however, is the finding that both organisms are sensitive to rifampin. Their susceptibility to the combination of trimethoprim-sulfamethoxazole is similar to the results of Moody and Young (16) and other workers (18, 22, 24).

TABLE 3. *Antimicrobial and antibiotic susceptibility of P. cepacia strains $OC_{(9/63)}$, 153, and $PC_{R\text{-}16\%\ BZK\text{-}Cl}$*

Antibiotic	Concn (μg/disk)	Susceptibility[a]		
		$OC_{(9/63)}$[b]	153[b]	$PC_{R\text{-}16\%\ BZK\text{-}Cl}$[b]
Ampicillin	10	R	R	R
Cephacetrile	30	R	R	R
Chloramphenicol	30	R	R	R
Erythromycin	15	R	R	R
Furadantin	100	R	R	R
Gentamicin	10	R	R	R
Kanamycin	30	R	S	R
Methicillin	5	R	R	R
Neomycin	30	R	I	R
Penicillin	10[c]	R	R	R
Polymyxin B	300[c]	R	R	R
Streptomycin	10	R	R	R
Tetracycline	30	R	R	R
Cephalothin	30	R	R	R
Triple sulfa	250	S	I	R
Sulfadiazine	0.25	S	S	R
Sulfamerazine	1	S	I	R
Colimycin	10	R	S	R
Rifampin	30	S	S	I
Trimethoprim + Sulfamethoxazole	1.25, 23.75	S	S	S

[a] Symbols: S, sensitive; I, intermediate; R, resistant.
[b] *P. cepacia* strain.
[c] Measured in units.

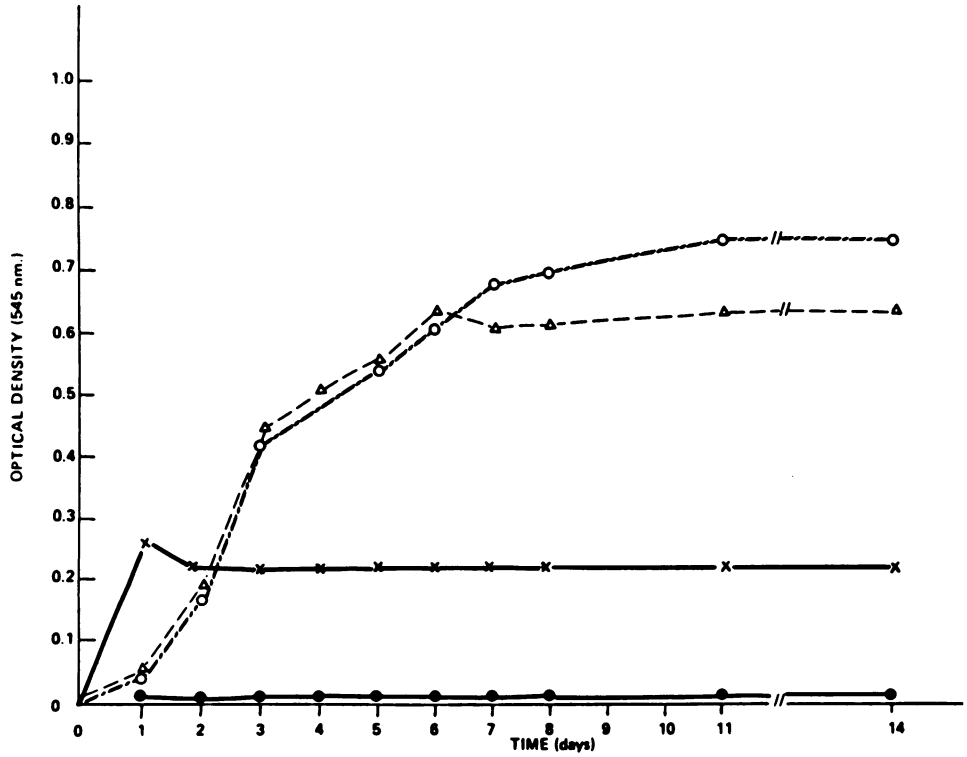

FIG. 1. *Growth of P. cepacia strain $OC_{(9/63)}$ in minimal inorganic salts solution containing either PBC or CBC. The final concentration of PBC and CBC was 0.05% (vol/vol); ammonium acetate was at a concentration of 0.019% (wt/vol). Symbols: ○, commercial benzalkonium chloride containing ammonium acetate; △, pure benzalkonium chloride containing added ammonium acetate; ●, pure benzalkonium chloride containing no ammonium acetate; ×, ammonium acetate and no PBC.*

Mouse virulence studies. Both strains of *P. cepacia*, $OC_{(9/63)}$ and 153, required a minimum infecting dose of 10^9 organisms to cause death. Strain 153 killed all six mice, whereas strain $OC_{(9/63)}$ killed five of six animals. Death ensued within 48 h postinfection. Doses of 10^8 organisms and less did not kill any of the mice. No clinical symptoms were observed in any of the groups of mice injected with diluted cultures.

Development of resistance to PBC. The $OC_{(9/63)}$ organism acquired resistance to 16% PBC, as indicated by growth at this concentration after 10 days of incubation. This strain ($PC_{R\text{-}16\%\ BZK\text{-}Cl}$) grew slowly, reaching an optical density at 545 nm of 0.41 after 15 days of incubation at 30°C in enriched salts medium containing 16% PBC. The parent strain, $OC_{(9/63)}$, attained an optical density of 1.6 after 48 h of incubation under the same conditions (0.05% PBC present). The $PC_{R\text{-}16\%\ BZK\text{-}Cl}$ strain differed from the parent strain, $OC_{(9/63)}$, in the following characteristics: did not grow at 37°C; grew on Cetrimide medium; was slightly sensitive to rifampin but resistant to all other antibiotic and antimicrobial agents (Table 3). This strain caused no deaths in mice, even when they were injected with concentrations as high as 10^{12} cells.

DISCUSSION

P. cepacia $OC_{(9/63)}$, which had been isolated from an inorganic salts solution preserved with 0.05% CBC, grew and survived in this nutritionally-deficient environment for a period of 14 years. The organism obtained carbon and nitrogen from ammonium acetate that was present as an antioxidant and buffer in CBC. We found the $OC_{(9/63)}$ organism adapted itself to nutritionally rich conditions within a short period of time. Moreover, when the $OC_{(9/63)}$ isolate and the clinical strain, *P. cepacia* 153, were compared with regard to their nutritional, biochemical, virulence, and antibiotic sensitivity characteristics, no apparent changes had occurred in any of the salient taxonomic attributes of this organism.

When resistance to 16% PBC was induced in the $OC_{(9/63)}$ isolate, its progeny, $PC_{R\text{-}16\%\ BZK\text{-}Cl}$, retained most of the characteristics of the $OC_{(9/63)}$ parent strain (Table 2). Of interest is the inability of the organism to infect animals. Apparently, as the organism's resistance increased from 0.05% [$OC_{(9/63}$ isolate] to 16% ($PC_{R\text{-}16\%\ BZK\text{-}Cl}$) PBC, its virulence and infectivity diminished. Death occurred in the mice when 10^9 organisms of the $OC_{(9/63)}$ strain were injected per mouse. These results are in agreement with those of Jonsson (12). No death resulted when 10^{12} organisms of the more resistant $PC_{R\text{-}16\%\ BZK\text{-}Cl}$ mutant were injected. Adair and co-workers (2) reported the differences between benzalkonium chloride-sensitive and -resistant strains of *P. aeruginosa*. Their findings demonstrated that the resistant strains of *P. aeruginosa*, grown in the presence of PBC, possessed significantly less capacity than the sensitive parent strain to infect mice and rabbits.

Although the virulence of benzalkonium chloride-resistant *P. cepacia* may be low, an accidental infection of humans by an organism such as the $PC_{R\text{-}16\%\ BZK\text{-}Cl}$ strain, that was resistant to all antimicrobials tested with the exception of rifampin, might prove to be highly resilient to any chemotherapeutic treatment. Taplin et al. (24) raised concern regarding the possible transference of the resistance determinants of *P. cepacia* to other gram-negative organisms. Such transference of resistance factors to organisms previously susceptible to these antibiotics could lead to serious problems.

The ability of *P. cepacia* to thrive and remain viable for long periods of time in benzalkonium chloride solutions admonishes that caution should be exerted if this antimicrobial agent is to be used for disinfection and preservative purposes. Contaminated solutions may appear clear and may be visually indistinguishable from truly sterile solutions, because *P. cepacia* produces no turbidity at levels as high as 10^7 colony-forming units per ml (6). This level of bacteria was also found by Gelbart et al. (9) to be present in the water reservoir of unheated nebulizer units and was confirmed by Bassett et al. (3).

Any source of water must be considered as a reservoir for this adaptable microorganism. Therefore, water to be used for the preparation of pharmaceuticals, disinfectants, or antiseptics should be treated by heat or filtration to render it free of any *P. cepacia* contamination. Because *P. cepacia* is resistant to a wide variety of antimicrobial agents, including benzalkonium chloride, pharmaceutical products and other preparations containing benzalkonium chloride as an antimicrobial agent to prevent bacterial contamination should be formulated to exclude extraneous carbon and nitrogen sources, especially ammonium acetate. Pharmaceuticals and cosmetics must be preserved with antimicrobial agents that are effective against *P. cepacia* and other microorganisms. Therefore, *P. cepacia* should be included in the spectrum of microorganisms required to determine the antimicrobial efficacy of preservatives as described in the United States Pharmacopeia XIX (25).

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