Morphological, Biochemical, and Growth Characteristics of *Pseudomonas cepacia* from Distilled Water

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Studies were conducted on three strains of *Pseudomonas cepacia* isolated and maintained in distilled water and on a laboratory-subcultured strain transferred to distilled water. Optimum growth rates and maximum population yields of the four strains in distilled water were obtained at 37 C, although high population levels (10^5-10^7/ml) were reached and maintained over extended incubation periods at temperatures from 18 C to 42 C. Two strains were able to grow in distilled water at temperatures ranging from 12 C to 48 C and to survive 48 h and 21 days at 50 C and 10 C, respectively. Cells from distilled water cultures inoculated into Trypticase soy broth showed an immediate two- to three-log drop at upper and lower temperature limits; survivors were able to initiate logarithmic growth. Results obtained in morphological, biochemical, and antibiotic tests affirmed the strain differences noted in growth studies.

*Pseudomonas cepacia* has been isolated from hospital water supplies (1, 11), physiological saline, and disinfectant solutions (1, 3, 13, 16) and from chemically disinfected equipment associated with infections of the urinary tract, respiratory tract, and wounds (1, 5, 8, 9). Phillips et al. (14) described the contamination of hospital solutions during autoclave cooling cycles by an organism with characteristics similar to *P. cepacia*, and traced the source of contamination to purified water held at 40 C. Burdon and Whitby (3) reported that strains of *P. multivorans* survived exposure to 70 C for 5 min. Bassett et al. (1) showed that *P. multivorans* detected in stock solutions of 1:30 Savlon (chlorhexidine 0.05%, cetrimide 0.5%) could multiply even after repeated transfers in Savlon : distilled water (1:30). However, cells subcultured onto nutrient media were unable to survive in 1:30 Savlon and showed sensitivity in nutrient agar containing 1:300 Savlon. We have shown (4, 7) that cells of *P. aeruginosa* isolated in distilled water from the reservoirs of mist therapy units differed from cells subcultured on conventional laboratory media both in growth characteristics and response to disinfectants. This study reports the growth and survival patterns at various temperatures of strains of *P. cepacia* isolated in distilled water and compares some characteristics of naturally occurring and subcultured cells.

MATERIALS AND METHODS

Growth studies. Samples of contaminated distilled water (10^6-10^9 microorganisms per ml) from the reservoirs of mist therapy units in a local hospital were pooled (MTUW) and sterilized by membrane filtration (Millipore Corp., 0.22-μm pore size, GA type). Liter bottles of commercially supplied sterile distilled water (CDW) were checked for sterility and used without further treatment. Resistivity measurements (Barnstead Conductivity Bridge, model PM-70 CB, Sybron Corp.) of MTUW and CDW at 24 C were 146,000 and 647,000 ohms, respectively; specific carbon and nitrogen analyses were not performed. Stock flasks of Trypticase soy broth (TSB; BBL) were prepared from dehydrated media. All media were stored at room temperature (25 C) and dispensed for growth studies in 50-ml amounts in 125-ml sterile Erlenmeyer flasks covered with aluminum foil. In preliminary studies, flasks were soaked overnight in 1% Haemo-Sol (Scientific Products) or in potassium dichromate-sulfuric acid solutions, rinsed 20 times in hot tap water and 15 times in distilled water, air-dried, and autoclaved using a standard dry cycle. Results showed no apparent differences in growth response due to cleaning procedures, and Haemo-Sol was routinely used in cleaning all glassware. Cells from distilled water cultures maintained at 25 C were diluted in CDW and used in inocula for all media. Flasks were incubated aerobically in stationary positions. At temperatures from 37 to 50 C, water baths equipped with Bronwill (Scien-
tific Products) constant temperature circulators (±0.02 C) were used; at temperatures of 25 C or below, biochemical oxygen demand cabinets were employed (±0.5 C). Samples were withdrawn at selected intervals and plated in duplicate into Trypticase soy agar (TSA; BBL) pour plates. Growth curves were plotted from mean values of colony counts after incubation at 37 C for 48 h; figures show typical results obtained in multiple experiments at each growth temperature studied.

**Strains.** Strains of *P. cepacia* isolated by the extinction-dilution technique previously described (7) included strains 1 and 3 from mist therapy units and strain 4 isolated in the source water from the Gamma Irradiation Facility at Sandia Laboratories, Albuquerque, N. M. The three isolates, referred to as naturally occurring strains, were maintained as stock cultures by monthly transfers in CDW. Strain 1064, maintained on heart infusion agar, was obtained from R. Weaver, Center for Disease Control, Atlanta, Ga. Strain 1054 was subcultured on TSA at 25 C for 24 h, washed three times in MTUW, and resuspended in MTUW to approximately 5 x 10^4 cells per ml. After 48 h at 25 C, the population had increased to 5 x 10^5 cells per ml. Two successive 48-h transfers were made in CDW, and transfers were made at monthly intervals thereafter.

**Morphological and biochemical characterization.** Motility was examined by hanging drop preparations from distilled water cultures and from TSA slants incubated at 25 and 37 C for 24 h, using methods previously described (22). Cells of strain 1 were also grown in nutrient broth at 37 C for 24 h, negatively stained with 2% sodium phosphotungstate (pH 7.0), and examined by electron microscopy (Zeiss EM 9S-2). One drop of 10% H2O2 (Fisher Scientific Co.) was mixed with cells from an 18-h TSA slant and observed for rapid evolution of gas bubbles as evidence of catalase activity. Colonies from 18-h TSA plates were streaked across strips of filter paper moistened with 1% tetramethyl-p-phenylenediamine dihydrochloride (Sigma Chemical Co.) to determine oxidase activity. Hydrolysis of fat (TWEEN-20, -40, and -80; Hilltop Laboratories) was determined by the method of Sierra (15). Starch and gelatin hydrolysis tests were performed using the combination medium of Oxborrow and Favero (12); gelatin hydrolysis was also tested by the tube method described by Edwards and Ewing (6). Nitrate respiration was tested by the method of Smith, Gordon, and Clark (15). Production of fluorescent or phenazine pigments was determined in asparagine broth by the method of Favero et al. (7) and on Flo and Tech Agars (BBL). Arginine dihydrolase activity was determined by the method of Sutter (18). Intracellular reserves of poly-β-hydroxybutyrate (PHB) were detected by the method described by Stanier et al. (17) using a Sudan Black stain. Ability to oxidize glucose to 2-ketogluconate was examined by the method of Haynes (15). The single disc method of Bauer et al. (2) was used to determine antibiotic sensitivity. Other tests were performed by the methods described in Edwards and Ewing (6); Moeller decarboxylase medium, Carquist Ninhydrin test, and lysine iron agar were used in determining lysine decarboxylase activity. Carbohydrate reactions were determined in OF basal medium (OFBM; Difco).

**RESULTS**

**Morphological and biochemical characterization.** Microscope examination of naturally occurring cells of the four strains of *P. cepacia* grown at 25 and 37 C, and of strains 1 and 3 grown at 44.5 C in CDW showed typical gram-negative rods occurring singly or in pairs. Slight pleomorphism was noted with TSA-subcultured cells of all strains grown at 37 C and with cells of strains 1 and 3 grown at 44.5 C. Strains 4 and 1054 showed no growth in CDW or on TSA at 44.5 C. Naturally occurring cells were consistently smaller (approximately 1/10 to 1/25 in width and length) than TSA-subcultured cells, regardless of the temperature or phase of growth. Cells grown in CDW at 25 and 37 C showed no motility in hanging drop preparations, and flagella were not observed in stained preparations. Motility was observed after a single subculture on TSA slants at both temperatures; flagella stains of strains 4 and 1054 showed cells with typical multirichous (3 to 4) polar arrangements, whereas strains 1 and 3 showed one to two long wavy polar flagella. Electron micrographs of strain 1 (Fig. 1A and B) showed some cells with three to four flagella and confirmed the unusual length of many of the flagella. Fimbriae were also demonstrated (Fig. 1C), but flagella and fimbriae were not observed on the same cell. A small zone which might be interpreted as capsular material was seen on some cells (Fig. 1D).

Colonies of all strains on TSA plates were circular, entire, and convex, averaging 1 to 2 mm in diameter after 48 h at 37 C, with a smooth, opaque, glistening appearance. No pigmentation was observed on TSA, but strains 4 and 1054 produced slight, insoluble yellow pigments on triple sugar iron agar slants in 48 to 72 h. Good growth of all strains was obtained both on MacConkey agar and on Simmons citrate slants; no growth was obtained on SS Agar. On cetrimide agar (Pseudosel; BBL) strain 1054 showed moderate growth, strain 4 showed only scanty growth, and strains 1 and 3 failed to grow.

In biochemical tests, all strains were positive for catalase and oxidase activities, oxidative metabolism of carbohydrates, hydrolysis of fat, accumulation of PHB as a cellular reserve material, utilization of malonate, asparagine, and acetamide, and growth at 41 C. All strains were negative in tests for production of indol,
H₂S, or fluorescent pigments, growth at 4 C, starch hydrolysis, denitrification, and arginine dihydrolase. Strain differences were observed and, as shown in Table 1, strains 1 and 3 were nutritionally less versatile than strains 4 and 1054. Of interest was the additional finding that strains 1 and 3 showed lysine decarboxylase activity after repeated subculture on TSA; naturally occurring cells of all strains inoculated directly into lysine test media showed no activity.

In antibiotic disc-sensitivity tests, all strains showed resistance to ampicillin (10 μg), polymyxin-B (50 μg), colistin (10 μg), gentamicin (10 μg), and cephalothin or cephaloridine (30 μg), showed moderate sensitivity to chloramphenicol (30 μg), and were sensitive to sulfonamide (50 μg) and nalidixic acid (30 μg). Strains 1 and 3 were also sensitive to tetracycline (30 μg) and kanamycin (30 μg); strains 4 and 1054 were resistant.

**Growth studies.** Growth patterns of the four strains were initially examined in MTUW and CDW. At temperatures of 15, 25, and 35 C, all strains showed essentially identical growth rates in both media; at 42 C slight strain differences were observed. Figure 2 shows typical results obtained with strains 1, 4, and 1054 in CDW. At 15 C (Fig. 2A), logarithmic growth was initiated slowly after a lag of 24 h, with generation times (Gt) of approximately 8 h. At 25 C (Fig. 2B), a short lag phase was observed, and cells increased from 10^2 to almost 10^7/ml in 48 h (Gt = 3 h). At 35 C (Fig. 2C) the lag phase was virtually eliminated and levels of 10^7 cells per ml were reached in less than 24 h (Gt = 1.2 h). At 42 C (Fig. 2D) generation times were 1.2, 1.8, and 2.3 h for strains 1, 1054, and 4, respectively; strain 3 (not shown) had a generation time of 1.4 h. Samplings of CDW cultures at all four temperatures were made at monthly intervals, with sufficient CDW added to maintain fluid levels at 50 ml per flask. Maximum stationary phases remained at levels of 10^7 to 10^8 cells per ml, and no apparent phases of decline and death were detected in monthly samplings up to one year.

At 44.5 C, strains 4 and 1054 showed a gradual decline in viable numbers in MTUW and CDW, and no survivors were detected after 72 h; in TSB no visible turbidity was observed up to 96 h. Strains 1 and 3 grew rapidly in MTUW and CDW (Gt = 0.9 h) and produced visible turbidity in TSB in 24 h. Strain 1 was selected for further study to determine upper and lower temperature limits of growth or survival, or both. Figure 3 shows results obtained in CDW and TSB at 46, 47, 48, and 50 C. In CDW (Fig. 3A), growth rates at 46, 47, and 48 C declined slightly (Gt = 1.3, 1.6, and 2.0 h, respectively), and, after reaching maximum levels averaging 5 x 10^6 cells per ml, stationary populations underwent typical phases of decline. At 50 C no growth was obtained, although survivors were detected up to 48 h. Naturally occurring cells inoculated into TBS showed a small but surprising loss of viability at 46 C within the first 4 h of incubation. Survivors were able to initiate logarithmic growth (Gt = 1.0 h), attaining levels of 10^6 per ml with visible turbidity within 24 h. At 47 and 48 C, two- and three-log drops in viable counts in TSB were seen during the first hours of incubation. Although survivors were able to initiate growth even at 48 C, the growth rate showed a marked decline (Gt = 5 h), and final population levels attained were 2 to 3 logs lower than in TSB at 46 or 47 C, or in CDW at 46, 47, and 48 C. It was not determined whether the ability of these small surviving populations to initiate growth was due to the selection of resistant mutants or to repair of injured cells; results of preliminary experiments have demonstrated a

| Carbohydrates (OFBM) | Test | P. cepacia strain* |
|----------------------|------|--------------------|
|                      |      | 1054 4 1 3         |
| Cellobiose           | +    | +     +     +     |
| Fructose             | +    | +     +     +     |
| Galactose            | +    | +     +     +     |
| Glucose              | +    | +     +     +     |
| Inositol             | +    | +     +     +     |
| Inulin               | +    | +     +     +     |
| Lactose              | +    | +     +     +     |
| Maltose              | +    | +     +     +     |
| Mannitol             | +    | +     +     +     |
| Rhamnose             | -    | -     -     -     |
| Sucrose              | +    | +     +     +     |
| Xylose               | +    | +     +     +     |
| Lysine decarboxylase | +    | +     -     -     |
| Nitrate reduction    | +    | +     -     -     |
| Urease               | -    | -     +     +     |
| Gelatin hydrolysis   | +    | (+) -  | -     -     |

* All strains were maintained as stock cultures in distilled water (CDW) and were subcultured once on TSA slants at 37 C prior to inoculation of test media.

**Parentheses indicate weak reaction.

Table 1. Characteristics of strains of Pseudomonas cepacia

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Fig. 1A and B. P. cepacia strain 1 after growth in nutrient broth at 37 C for 24 h; negatively stained with 2% sodium phosphotungstate (pH 7.0). A and B, multirichous polar arrangements and unusual length of flagella, x18,000.
Fig. 2. Comparative growth of *P. cepacia* strains 1, 4, and 1054 in distilled water (CDW). A, 15 °C; B, 25 °C; C, 35 °C; D, 42 °C.

Fig. 3. Growth of *P. cepacia* strain 1 at 46 °C, 47 °C, 48 °C, and 50 °C. A, In distilled water (CDW); B, in Trypticase soy broth (TSB).

Fig. 1C and D. *P. cepacia* strain 1 after growth in nutrient broth at 37 °C for 24 h; negatively stained with 2% sodium phosphotungstate (pH 7.0). C, Peritrichous distribution of fimbriae, ×112,000; D, cells surrounded by apparent capsular material, ×18,000.

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relationship between the phosphate content of TSB and its apparent lethality.

Growth patterns of strain 1 in TSB were also determined at 44.5, 42, 37, and 25°C. At all four temperatures, cells promptly initiated logarithmic growth with no initial viability loss. Optimum growth rates were found in the range of 42 to 44.5°C (Gt = 0.6 h); maximum population yields (10^4–10^5 cells per ml) were obtained at 25°C. In contrast to results obtained with cells grown in CDW or MTUW at these temperatures, cultures in TSB showed typical phases of decline and death.

Figure 4 shows results obtained with strain 1 at 18 and 12°C in CDW and TSB. In CDW, naturally occurring cells grown at 18°C showed an increased lag period and longer generation time (Gt = 3.3 h) than previously noted at higher temperatures. At 12°C only negligible increases in numbers were obtained up to 14 days. In TSB, cells at 18°C showed a slight initial loss in viability. The generation time was increased (Gt = 3.6 h), and final population levels were somewhat lower (10^7 cells per ml) than those attained at 25°C. At 12°C, naturally occurring cells in TSB rapidly declined; no survivors were detected at sampling times up to 14 days. In other experiments at 10°C, cells of strains 1 and 3 in CDW neither increased nor decreased over a period of 3 weeks; strains 4 and 1054 showed a gradual decline in viable numbers.

DISCUSSION

The characteristics of naturally occurring organisms described in these studies have a number of implications in the quality of care provided hospitalized patients. Distilled water, either as supplied to or used in hospitals, is not devoid of nutrients. Trace amounts of volatile organic materials, salts, and metals may be added from the distilling system itself, through absorption or leaching in storage containers or from improper or inadequate cleaning of reservoirs and stock bottles used in the preparation of working solutions. However limited in nutrients, hospital-distilled water supplies are capable of supporting the growth of contaminating organisms.

Cells of *P. cepacia* isolated in distilled water appear to differ both morphologically and physiologically from their laboratory-subcultured (TSA) counterparts. Rates of growth of naturally occurring strains of *P. cepacia* in distilled water over a temperature range of 25 to 42°C have been shown here to approach those obtained in a nutrient rich medium (TSB). Cells grown in TSB appear to be more sensitive to temperature extremes than do cells grown in distilled water. Whether this reflects a basic difference in enzyme constituents or altered cell permeability is not yet known. Maximum population levels (10^5 cells per ml) attained in distilled water produce no visible turbidity, presumably due to the extremely small cell size, so that high levels of contaminants may be present in what is assumed to be good-quality distilled water. Moreover, the wide temperature range of growth or survival of strains of this potential pathogen, as well as the ability to maintain high population levels even at temperature extremes, allows its persistence in the environment over long periods of time.

Chronically ill or severely debilitated patients, as well as those on antibiotic or immunosuppressive regimens, constitute the most susceptible populations in clinical manifestations involving *P. cepacia* as the specific etiologic agent. Both equipment and solutions used in urinary tract, cardiac, and respiratory instrumentation therapy have been implicated as primary sources of infection. Control measures employed must include not only effective cleaning, disinfecting, and drying of equipment but also must consider almost any source of water as a potential reservoir for this adaptable pathogen.
CHARACTERISTICS OF P. CEPACIA

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