covered by filtration. The inhibitors were partitioned into diethyl ether and taken to near dryness on a rotary evaporator. The residue was then taken up in a small volume of ether, spotted onto preparative (0.5 mm) thin-layer plates of silica gel, and chromatographed in a solvent consisting of benzene and ether (80 : 20 by volume). After extraction from silica gel with ether, the preparation was dried and sublimed under vacuum in a short-path (20 mm) apparatus onto a condenser cooled with Dry Ice in acetone.

The chromatographic mobility (R_F) of the zone which contained the inhibitor was found in preliminary tests to be about 0.5 and coincided with an ultraviolet-fluorescent band about 1 cm wide at that R_F. To isolate the inhibitor without exposing it to ultraviolet light, we located the fluorescent zone by guide spots while the remainder of the plate was kept darkened. The silica gel containing the zone was scraped from the plate, extracted, and bioassayed. This zone contained as much inhibitor as the original ether solution applied to the plate, and tests of 1-cm bands of the plate showed very little activity elsewhere. The potency of the inhibitor was assayed at each step in the purification process by similar procedures.

The mass spectrum of a sample of the sublimate showed the following diagnostic peaks [mass to charge ratio (*m/e*); parent peak (P)]: 208 (P, base peak); 193 ($P - CH_3$); 177 ($P - OCH_3$); and 149 ($P - COOCH_3$). In addition, there were fragments at *m/e* 145, 117, 105, 91, and 77 indicative of aromaticity. The infrared spectrum had absorption peaks at 3430 cm^{-1} (hydroxyl), 1702 and 1718 cm^{-1} (ester carbonyl), 1598 and 1634 cm^{-1} (a pair of peaks of equal intensity), 1512 cm^{-1} (aromatic), and 1264 cm^{-1} (aryl ether). The ultraviolet absorption spectrum of the inhibitor dissolved in absolute methanol had absorption maximums at 325, 295, 235, and 217 nm. The spectra suggested that the inhibitor was the methyl ester of ferulic acid. The infrared and ultraviolet spectra of methyl isoferulate were not compatible with the spectra of the inhibitor.

The masses and relative abundance of the various fragments in the mass spectrum of synthetic methyl *trans*-ferulate (*3*) were identical to those of the unknown. The infrared spectrum of the inhibitor was consistent with that of a mixture of the *cis* and *trans* isomers of methyl ferulate. The *cis* isomer was prepared from the *trans* isomer by irradiation in methanol for 3 hours with ultraviolet light at 254 nm. The mixture of isomers was then resolved and purified by thin-layer chromatography on cellulose plates irrigated with water. Identity of the synthetic and natural *cis* and *trans* isomers was verified by infrared spectroscopy and by chromatography on thin-layer plates of cellulose (R_F 0.6 and 0.3, respectively). We conclude that the inhibitor from wheat rust uredospores is a mixture of methyl *cis*-ferulate and methyl *trans*-ferulate. Further chromatographic and infrared studies showed that a fresh sample of this inhibitor is composed predominantly of the *cis* isomer.

The extinction coefficient of a 1 percent solution of methyl *trans*-ferulate measured through a 1-cm light path at 325 nm was 750 in absolute methanol. With this value, it was determined that approximately 0.5 μg of native inhibitor can be extracted from 1 g of spores by our procedures. The ED_{50} of the unresolved mixture of isomers as determined from a plot of the probit of percent inhibition of germination against the logarithm of concentration was 8×10^{-9} mole/liter. The native inhibitor and the synthetic esters were equally toxic to spore germination, since their ED_{50} values were identical.

The self-inhibitors of uredospores of three other species of rust fungi—bean (*2*) and snapdragon and sunflower (*4*)—were each shown recently to be methyl 3,4-dimethoxycinnamate. Our finding that methyl ferulate is the self-inhibitor of wheat stem rust uredospores suggests that self-inhibitors in rust uredospores are a family of cinnamic acid derivatives.

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Pseudomonas aeruginosa: Growth in Distilled Water from Hospitals

Abstract. Pseudomonas aeruginosa *can grow relatively fast in distilled water obtained in hospitals and achieve high cell contaminations which remain stable for long periods of time. Cells grown in distilled water react quite differently to chemical and physical stresses than cells grown in standard laboratory culture media.*

Pseudomonas aeruginosa is recognized as a major causative agent of hospital-acquired infections. It is commonly associated with urinary tract infections and many generalized and subsequently fatal infections in infants and weakened patients. This organism can utilize a broad range of compounds as carbon and energy sources and may therefore survive and multiply in many liquids (*1*), including fuels (*2*). In 1962, Leifson detected this organism and several other Gram-negative bacterial species in 36 samples of distilled water from ten sources (*3*). When the organism is found in distilled water, however, it is often assumed that it is simply surviving or else growing very slowly. We found that *P. aeruginosa* will grow at a rapid rate in distilled water and that cells grown in distilled water react to various chemical stresses in a manner significantly different from that of cells grown in conventional laboratory culture media.

During a survey of environmental microbial contamination in a hospital pediatric ward, relatively high levels of Gram-negative bacteria, including *P. aeruginosa* and fecal coliform bacteria, were detected in the distilled water reservoirs of several mist therapy units.

Table 1. Bacterial contamination in distilled water from mist therapy unit reservoirs in two hospitals.

Hospital	Samples (No.)	*Pseudomonas aeruginosa*			Viable count	
		Positive samples (No.)	Most probable number per milliliter			
			Mean	Range	Mean	Range
A	25	7	7.5×10^3	1.5×10^0–4.3×10^4	2.4×10^6	1.7×10^1–2.7×10^7
B	25	13	4.9×10^2	2.3×10^0–4.3×10^3	1.4×10^7	3.5×10^3–3.0×10^8

A special effort was made to enumerate *P. aeruginosa* in the reservoir water of mist therapy units (MTU) of this and another hospital. Total bacterial counts were obtained by use of the pour-plate technique with trypticase soy agar [TSA; Baltimore Biological Laboratories (*4*)]. Colonies were counted after 48 hours of incubation at 37°C. Specific enumeration of *P. aeruginosa* was done by use of a modification of the multiple tube dilution system (three tube series) described by Drake (*5*). Each sample was diluted serially (10^{-1} to 10^{-7}) in sterile buffered distilled water (BDW) (*6*). One milliliter of each dilution was then added to each of three tubes of asparagine broth (0.1 percent K_2HPO_4, 0.05 percent $MgSO_4$, and 0.3 percent asparagine in distilled water). The tubes were incubated at 37°C and observed for fluorescence under ultraviolet light (long-wave) after 24 and 48 hours. Tubes from the two highest dilutions showing fluorescence were selected, and 0.1 ml from each tube was inoculated into acetamide broth (0.5 percent NaCl, 0.14 percent K_2HPO_4, 0.07 percent KH_2PO_4, 0.05 percent $MgSO_4$, 1.0 percent acetamide, and 0.001 percent of phenol red in distilled water). The presence of *P. aeruginosa* was confirmed by a strong alkaline reaction in acetamide broth in 24 hours. An additional confirmatory step, which we consider optional, was made by inoculating 0.1 ml of broth from each positive asparagine broth into Pseudomonas-P broth (2.0 percent Bacto-peptone, 0.2 percent DL-alanine, 0.8 percent KCl, 0.28 percent $MgSO_4$, and 1.0 percent sodium citrate in distilled water) and, after 24 hours of incubation at 37°C, demonstrating the presence of pyocyanin by a blue color in a chloroform extract of the broth. Standard most probable number tables were used to estimate the number of *P. aeruginosa* per milliliter.

Total viable counts were high, as were levels of *P. aeruginosa* (Table 1). We subsequently found that this Gram-negative flora, consisting primarily of pseudomonads, and *P. aeruginosa* grew relatively well in distilled water. On one occasion at 25°C, the population increased from 4,300 to 1,100,000 cells per milliliter in a 24-hour period.

A sample of contaminated water from a mist therapy unit reservoir (MTU water) was diluted serially (tenfold) in MTU water sterilized by means of a membrane filter. The suspensions were incubated at 39°C in order to produce a selective pressure favoring the growth of *P. aeruginosa*. The highest dilutions of tubes positive for this microorganism were rediluted serially and reincubated. A pure culture of *P. aeruginosa* was obtained after three dilution sequences. Since these cells had never been exposed to artificial culture media and were in their natural habitat, or ecological niche, we refer to them as "naturally occurring."

To study growth kinetics, the naturally occurring cells were inoculated into sterile MTU water and portions were withdrawn at various time intervals, diluted in BDW, and plated in duplicate with TSA supplemented with 0.5 percent KNO_3 to enhance subsurface growth. Colonies were counted after 48 hours of incubation at 37°C. Growth was relatively rapid (that is, an increase from 100 to 1×10^7 cells per milliliter in 48 hours) and the maximum cell population remained high (10^6 to 10^7 per milliliter) and fairly constant up to 42 days. We assume that the organisms were utilizing organic compounds that had been absorbed in the distilled water during storage. This hypothesis is supported by our observation that naturally occurring *P. aeruginosa* grows at a faster rate and reaches a higher maximum cell population in distilled water of poor quality or water that has been allowed to stand for a few days, or in both, than in "fresh," high-quality, distilled water.

We compared the survival rate of the naturally occurring *P. aeruginosa*, in water, with washed cell suspensions of the organism after a single subculture on TSA. Both filter-sterilized MTU water (*p*H 6.4) and BDW (*p*H 7.2) were used. Results are shown in Fig. 1. The growth curves of the naturally occurring *P. aeruginosa* at 25°C essentially paralleled one another in MTU and BDW. The generation times (time required for population to double) were 4.6 and 4.8 hours in MTU and BDW, respectively, and the cells attained a higher maximum population in the MTU water. Subcultured cells, on the other hand, showed a decline in both waters initially. At 72 hours, no viable *P. aeruginosa* were detected in the BDW. The initial decline in concentrations of subcultured *P. aeruginosa* in MTU water was followed by logarithmic growth (generation time, 4.6 hours). At the end of 168 hours of incubation in MTU water, the naturally occurring cells and those subcultured in TSA were at the same level.

Additional tests, which will be reported in detail later, showed that the naturally occurring *P. aeruginosa* are markedly more resistant to inactivation by chlorine dioxide, a quaternary ammonium compound, 0.25 percent acetic acid, and activated glutaraldehyde than cells subcultured one time on TSA.

These results have two important implications. First, the ability of *P. aeruginosa* to grow and maintain high numbers of viable cells in distilled water constitutes a hazardous environmental situation. At these numbers, *P. aeruginosa* cells in distilled water do not produce visible turbidity, so that a seemingly crystal clear drop of distilled water can literally contain 100,000 viable cells. Consequently, a dangerous source of this microorganism can exist, virtually without detection, and act as a focus of contamination that can be amplified in the immediate environment and possibly throughout the hospital. Transferred to other locations in the hospital via the airborne route, fomites, or personnel, the naturally occurring *P. aeruginosa* cells are physiologically capable of initiating growth immediately with essentially no lag phase. In addition to *Pseudomonas*, several other genera of Gram-negative bacteria, such as *Achromobacter* and *Flavobacterium*, have species capable of growing in distilled water. Although most of these

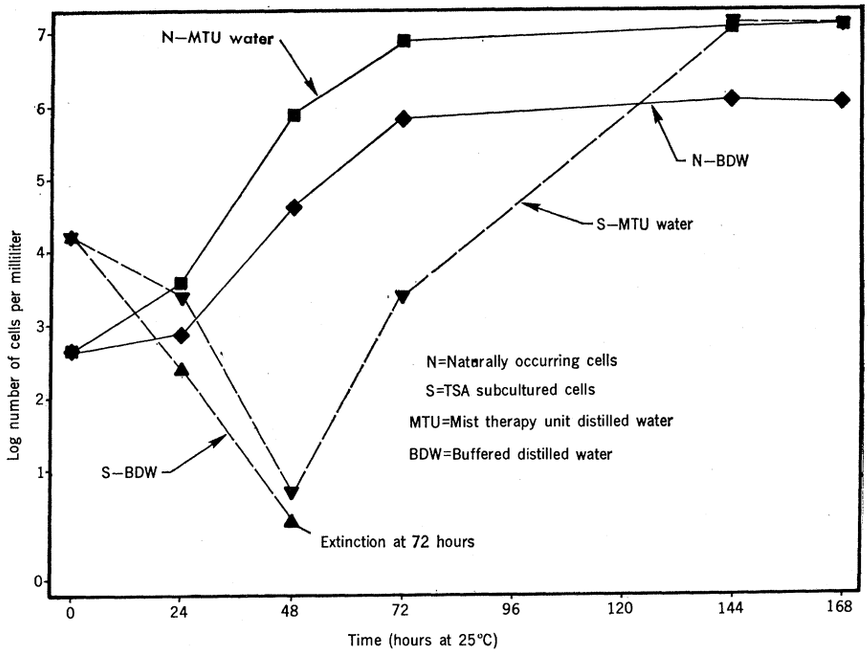

Fig. 1. Effect of subculturing naturally occurring *Pseudomonas aeruginosa* on growth and on survival in distilled water.

organisms are saprophytic and nonpathogenic, they can cause disease when introduced in large numbers into debilitated patients or patients with open wounds. Foley and co-workers (*7*) described six deaths among premature infants caused by *Achromobacter* sp. The organism was found in high numbers in incubators and in water used for washing infants' eyes and could grow well in distilled water. In a more recent report involving several hospitals in seven states, 405 cases of septicemia with nine deaths were traced to intravenous fluids manufactured by a particular pharmaceutical company (*8–12*). Subsequently, it was found that the elastomer plastic cap liners were contaminated with *Erwinia* sp. (lathyri-herbicola group) and *Enterobacter cloacae*, apparently introduced from the intramural environment at the manufacturing plant. Both organisms can grow rapidly in saline and Ringer solutions, reaching levels of 10^6 to 10^7 per milliliter (*11*). Similarly, these organisms, normally considered nonpathogenic, were apparently able to reproduce in the intravenous fluids and attain invasive population levels that ultimately led to illness and, in some cases, death. Since microbiological monitoring of intravenous fluid is not done routinely in hospitals, these high levels of contaminants normally would not be detected.

Second, naturally occurring *P. aeruginosa* cells appear to survive physical and chemical stresses much better than cells of the same microorganism which have been subcultured on TSA. Standard protocols of disinfectant testing by necessity employ subcultured and washed bacterial cells. Although microbiologists realize that the results of such tests should never be used as an absolute criterion for the use-dilution of a disinfectant, these data are often very influential and may create a false sense of confidence in a particular disinfectant use-formulation. For example, Favero and Drake (*13*) showed that naturally occurring *P. alcaligenes* grown in iodinated water from swimming pools were significantly more resistant to free iodine than subcultured cells of the same organism. In fact, standard laboratory tests with subcultured cells suggested that *P. alcaligenes* should never be found in pools, even those minimally disinfected with iodine. Additional tests, however, showed that the naturally occurring cells were extremely iodine-resistant. This explained why high concentrations of *P. alcaligenes* could accumulate in pool waters that had been iodinated for several weeks. The organisms grew well during periods of low or zero levels of iodine and were able to survive high levels of iodine, the net result being an increase in the number of viable cells in the pool waters. Although there are obvious differences between this situation and the hospital environment, the principle of differing physiological capabilities and characteristics of the naturally occurring cells appears to be the same. Another example of this principle is the recent work of Bassett *et al.* (*14*), who isolated *P. multivorans* from infected surgical wounds of nine hospitalized patients. The source of these organisms was found to be Savlon (chlorohexidine 0.25 percent and cetrimide 0.5 percent), used for preoperative skin preparation. When tests were done on broth cultures of the strains that had been isolated from 1 : 30 dilution of Savlon in the hospital, the minimum inhibitory concentration of Savlon was 1 : 320, and the organisms did not survive in a 1 : 30 dilution. On the other hand, the "naturally occurring" *P. multivorans* could actually grow in a 1 : 30 dilution of Savlon.

Control of contaminated hospital equipment and solutions is relatively simple, provided routine precautions are observed. We found that frequent cleaning, disinfecting, drying of glassware, and the use of sterile distilled water and aseptic technique in filling water reservoirs can virtually eliminate the *P. aeruginosa* contamination of inhalation therapy equipment. Similar diligence coupled with a program of bacteriological monitoring in other hospital areas could further reduce potential sources of Gram-negative infections.

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