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Identification of Microorganisms Isolated from Jet Fuel Systems

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Seventy-two samples from jet aircraft fuel systems were examined for microbial contamination. Ten contaminated samples yielded 43 microorganisms which were classified into nine genera of bacteria and three genera of fungi. The predominant types, comprising about 37% of the isolated cultures, were identified as *Bacillus* spp. The remaining cultures were distributed among 11 genera, each of which represented 2 to 9% of the total isolates. Four cultures could not be assigned to a genus on the basis of the diagnostic criteria used. Only five isolates, in the genera *Pseudomonas* and *Hormodendrum* (*Cladosporium*), grew abundantly in a mineral salts solution with JP-4 fuel as the sole source of carbon. The presence of fuel utilizers in a fuel system may be a better index to potential problems that have been correlated with microbial contamination than the presence of aerobic sporeforming bacilli.

In recent years, a great deal of attention has been attracted to the widespread occurrence of microorganisms in fuel systems. Fungal growth was extensive in Australian aviation fuel systems (10), and the microflora of oil fields in Japan included representatives of nine genera (13, 14, 15). In similar studies (3, 7, 16), a variety of microorganisms were isolated from jet fuel systems in the United States.

Undesirable effects, such as aluminum corrosion (5, 24) and malfunction of fuel gauges (3), have been correlated with microbial contamination, but a cause-effect relationship between types of microorganisms and specific problems has not been demonstrated. Therefore, we identified organisms isolated from fuel systems in our laboratory and tested each isolate for its ability to utilize jet fuel as a sole organic carbon source.

MATERIALS AND METHODS

Seventy-two samples from jet fuel systems were examined. Fuel or water (2 ml), or both, from each sample was inoculated into 40 ml of each medium: Fluid Thioglycollate Medium (Difco), Sabouraud Dextrose Broth (Difco), and a mineral salts solution (6) overlaid with sterile JP-4 fuel. Cultures in Fluid Thioglycollate Medium were incubated at 37 C, the others, at 26 C. Cultures which showed growth were streaked on Trypticase Soy Agar (TSA; BBL) and on Sabouraud Dextrose Agar (SDA) to obtain pure cultures of bacteria and fungi, respectively. Standard diagnostic methods (2, 21) were used for differentiating bacteria. Ability to oxidize gluconate was tested according to procedures of Wahba and Darrel (23).

Yeast cultures were identified on the basis of cellular morphology, failure to produce aerial mycelium, and pigment production (1). Filamentous fungi were differentiated on the basis of morphological arrangement of cells, type of mycelium, appearance of reproductive structures on slide cultures, pigment production, and dimorphic ability (1, 4).

Subsequently, each isolate was tested for its ability to grow in a mineral salts solution (6) with JP-4 jet fuel as sole organic carbon source. The *p*H of the mineral solution was adjusted to 5.6 (fungi) or 7.2 (bacteria). The solution was sterilized by autoclaving prior to overlaying with filter-sterilized fuel. Each flask, containing 40 ml of salts solution and 10 ml of fuel, was inoculated with 0.1 ml of a suspension of bacterial cells or with 1 ml of a suspension of fungal spores and mycelium. Growth of bacteria was assayed by the plate count method, and growth of fungi, by dry weight determination.

RESULTS

Of 72 samples examined for microbial contamination, 10 were contaminated. They yielded 43 isolates which were placed in four microbial groups on the basis of staining reactions and cellular morphology: gram-positive cocci, gram-positive bacilli, gram-negative bacilli, and fungi.

Gram-positive cocci. Six cultures were included in this group. All grew aerobically and were catalase-positive. Four of them (UD-2, -29, -33, and -34) had cells arranged in tetrad when grown on TSA and produced yellow, water-insoluble pigments. Each culture failed to coagulate litmus milk, produce H_2S, or reduce nitrates. Glucose,

TABLE 1. *Properties of nonsporeforming gram-positive rods*[a]

Characteristic	Fuel isolates			
	UD-6	UD-7	UD-4	UD-8
Catalase	+	+	+	+
Motility	+	+	−	−
Pigment	Yellow	Yellow	Salmon	Salmon
Nitrate reduction	−	−	+	+
Glucose	±	±	±	±
Lactose	−	−	−	−
Sucrose	−	−	±	±
Gelatin liquefication	+	+	−	−
Indole	−	−	−	−
Cellulose digestion	−	−	−	−
Acid-fast stain	−	−	−	−
Metachromatic granules	−	−	−	−
Growth at 10 C	−	−	−	−
Growth at 37 C	+	+	+	+
Litmus milk	NC	NC	Alk	Alk

[a] Symbols: +, positive reaction; −, negative reaction; ±, slightly positive; NC, no change; Alk, alkaline reaction.

sucrose, and lactose were not fermented. They were identified as *Sarcina flava*.

Cells of the two remaining cultures (UD-1 and -28) were arranged in irregular clusters. Both organisms reduced nitrates, fermented glycerol, and grew well in 6.5 and 10% NaCl broth. Both cultures were coagulase-negative. Mannitol was fermented by one (UD-1) but not by the other (UD-28). They were identified as *Staphylococcus aureus* and *S. epidermidis*, respectively.

Gram-positive bacilli. Twenty-two isolates, with rod-shaped cells of varied sizes, were placed in this group. All grew aerobically. Each culture was stained for spores over a period of 5 to 8 days, and each was examined for ability to survive for 10 min at 85 C. Sixteen cultures were positive for endospores and were identified as *Bacillus* spp. on the basis of this criterion. Two other cultures (UD-16 and -17) produced branching filaments with chains of conidia near the apex. Colonial growth on TSA appeared powdery and dry. These cultures were identified as *Streptomyces* spp. (20).

Characteristics of the remaining gram-positive rods are summarized in Table 1. Cells of two (UD-6 and -7) were short and motile. They produced yellow, water-insoluble pigments on TSA. They were not acid-fast, but they liquefied gelatin in 48 hr. Catalase was produced and glucose was fermented weakly. Tests for cellulose digestion, indole production, and nitrate reduction were negative. Both organisms grew in litmus milk, but produced no significant change. They were identified as *Brevibacterium* spp.

The other cultures (UD-4 and -8) consisted of large, pleomorphic and branching cells. They were nonmotile and produced salmon-colored, water-insoluble pigments on TSA. They were not acid-fast, and metachromatic granules were not observed. Organisms with these characteristics are present in four genera: *Arthrobacter*, *Mycobacterium*, *Nocardia*, and *Corynebacterium*. Neither isolate could be assigned to a genus with certainty. Cells of *Arthrobacter* are rod-shaped when young and coccoid in older cultures. When transferred to fresh medium, coccoid cells germinate and produce rod-shaped cells, completing the cycle (20). Neither culture exhibited a definite cycle of development. Lack of acid fastness excluded them from the genus *Mycobacterium*, and inability to produce a definite mycelium in early stages of growth excluded them from *Nocardia*. These cultures may be of an intermediate nature, similar to strains of *Mycobacterium rhodochrous* which Gordon (9) encountered under 11 different generic designations. She emphasized the need for a clearer delineation of the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Arthrobacter*.

Gram-negative bacilli. Nine cultures comprised this group. Three (UD-11, -18, and -19) were actively motile and oxidase-positive, and they produced pyocyanin pigments and slime. They oxidized gluconate to 2-keto-gluconate, grew well at 42 C, and liquefied gelatin in 24 hr. These characteristics were in agreement with criteria proposed by others (22, 23) for *Pseudomonas aeruginosa*.

The remaining cultures were catalase-positive and oxidase-negative. Three (UD-3, -9, and -14) were nonmotile and grew well at 37 and at 42 C.

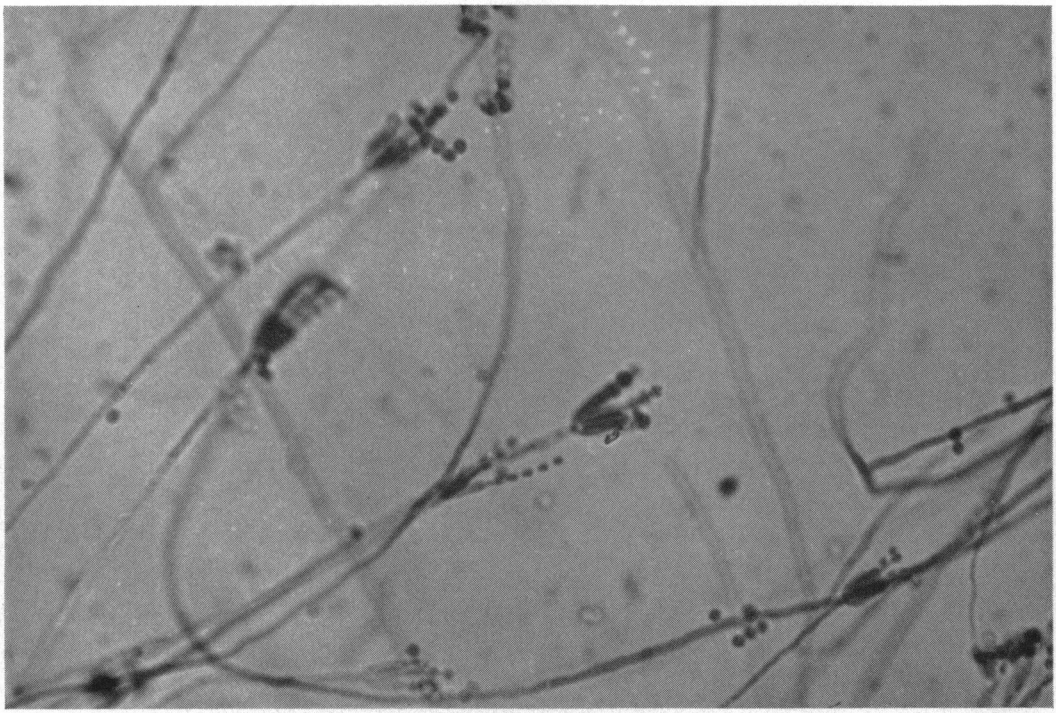

FIG. 1. *Photomicrograph of a Penicillium sp. (UD-35) from a 4-day slide culture, stained with lactophenol cotton blue, showing conidiophores and conidia.*

Each culture utilized citrate and produced bright-blue colonies on Levine's Eosin Methylene Blue Agar. They failed to ferment a 1% concentration of either dextrose, sucrose, or lactose in broth, but fermented 10% lactose in agar slants. Nelson and Shelton (18) described organisms with these characteristics as *Herellea* spp. The genus is not described in *Bergey's Manual of Determinative Bacteriology*.

One gram-negative culture (UD-12) was motile, reduced nitrates, and produced a yellow, non-diffusible pigment on TSA. It fermented dextrose weakly, but sucrose and lactose were not fermented. It failed to grow at 42 C, and did not utilize citrate as a sole carbon source. It was assigned to the genus *Flavobacterium*.

Two other gram-negative rods (UD-39 and -40) grew poorly on TSA and well on SDA at 26 C. They were motile and produced orange, water-insoluble pigments. They failed to grow in all differential media or gave negative reactions for tests used in this study. Spectrophotometric analysis did not show an absorption band at 590 mμ, which is indicative of bacteriochlorophyll (20), but their absorption spectra were characteristic of carotenoids. We were unable to assign these cultures to a genus.

Fungi. Six cultures (UD-35, -36, -37, -41, -42, and -43) were included in this group. Two (UD-37 and -41) were identified as *Rhodotorula* spp. on the basis of cellular morphology, failure to produce aerial mycelia, and the production of red pigments on SDA. Two of the remaining cultures (UD-35 and -36) were identified as *Penicillium* spp. (Fig. 1), and two (UD-42 and -43) as *Hormodendrum* spp. (Fig. 2). The genus *Hormodendrum* is very similar to *Cladosporium*, and the terms are often used synonymously (1). No attempt was made to speciate the fungi.

Identifications of the 43 isolates are summarized in Table 2. Nine genera of bacteria and three genera of fungi are included. *Bacillus* spp. comprised 37% of the isolates. Only 5 of the 43 isolates grew well in a mineral medium with JP-4 fuel as the sole carbon source. Three isolates, identified as *Pseudomonas aeruginosa*, yielded maximal viable populations of 1.2×10^8 to 1.7×10^8 cells per milliliter after 168 hr of incubation. The remaining 34 bacterial isolates showed a decrease in viable counts initially, or after the first 24 hr of incubation. *Hormodendrum* (*Cladosporium*) isolates developed thick mycelial mats (52 and 67 mg, dry weight, respectively) at the fuel-water interface (8). Cultures of *Penicillium*

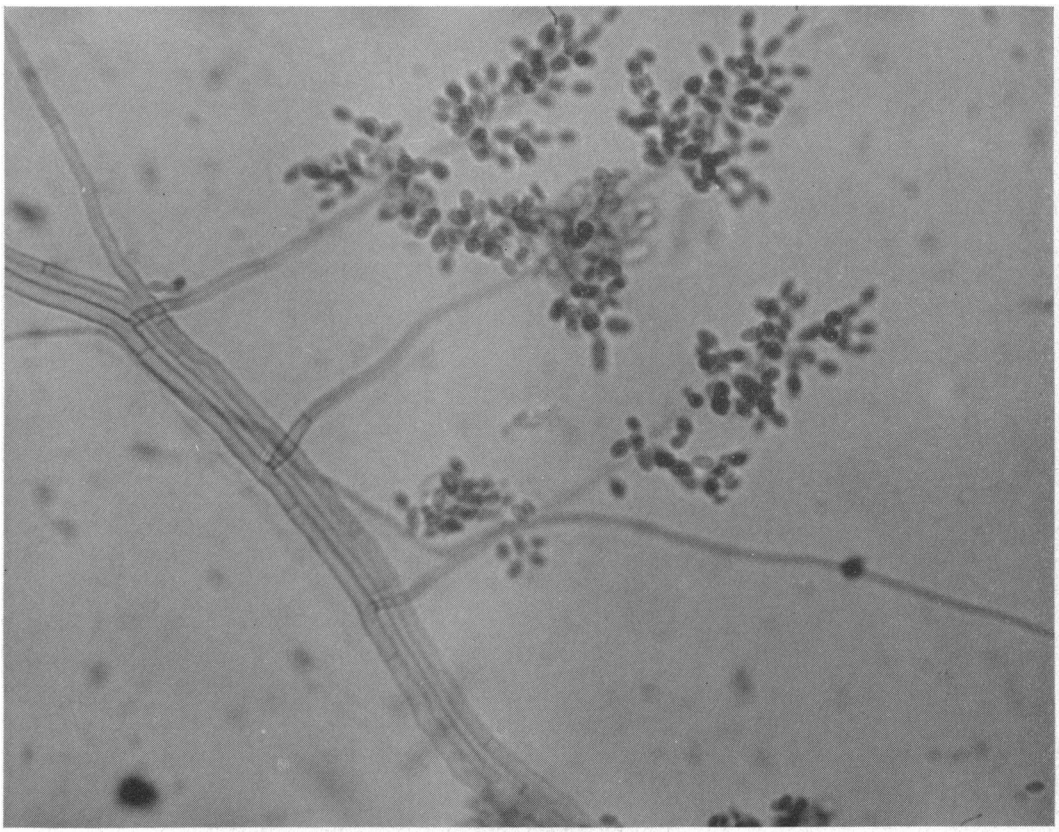

FIG. 2. *Photomicrograph of a Hormodendrum sp. (UD-42) from a 4-day slide culture stained with lactophenol cotton blue, showing conidiophores and conidia.*

TABLE 2. *Identification of fuel isolates*

Identification	No. of isolates	Utilize JP-4 fuel[a]	Stock culture no.
Staphylococcus aureus	1	−	UD-1
S. epidermidis	1	−	UD-28
Sarcina flava	4	−	UD-2, -29, -33, -34
Bacillus spp.	16	−	UD-5, -10, -13, -15, -20 through -27, -30 through -32, -38
Pseudomonas aeruginosa	3	+	UD-11, -18, -19
Brevibacterium spp.	2	−	UD-6, -7
Penicillium spp.	2	−	UD-35, -36
Streptomyces spp.	2	−	UD-16, -17
Rhodotorula spp.	2	−	UD-37, -41
Hormodendrum (*Cladosporium*) spp.	2	+	UD-42, -43
Unidentified (gram-negative bacilli)	2	−	UD-39, -40
Herellea spp.	3	−	UD-3, -9, -14
Flavobacterium spp.	1	−	UD-12
Unidentified (gram-positive bacilli)	2	−	UD-4, -8

[a] Symbols: +, growth in mineral medium with JP-4 as sole source of carbon; −, no growth in mineral medium with JP-4 as sole source of carbon.

and *Rhodotorula* did not grow in this system. The five fuel-utilizing isolates have now been maintained for longer than 2 years, by serial subculture, in the system described, which contains JP-4 jet fuel as the sole source of organic carbon.

DISCUSSION

London, Finefrock, and Killian (17) reviewed the problem of microbial contamination in jet fuel systems, and questioned the correlation of microorganisms with malfunctions. However, in view of the production of corrosive compounds by fuel-utilizing microorganisms (5), their abundant growth in fuel-water systems (8), and their implication in corrosion of integral wing tanks (24), it is conceivable that certain types of organisms in the genera *Pseudomonas* and *Hormodendrum* (*Cladosporium*) may be contributing to problems in fuel systems that appear to be nonbiological. In another study (7), an ecological succession of bacteria in the water bottoms of fuel storage tanks was proposed.

Representatives of 12 genera of microorganisms comprised the bulk of jet fuel isolates from other laboratories (3, 7, 11, 16), of which the predominant fuel-utilizing types were in the genera *Pseudomonas* and *Hormodendrum* (*Cladosporium*). All of the fuel-utilizing organisms found in this study were in these genera. However, hydrocarbon-utilizing bacteria isolated from petroleum and natural gas fields in Japan included representatives of *Corynebacterium*, *Flavobacterium*, *Brevibacterium*, *Pseudomonas*, and *Alcaligenes* (12).

Although *Bacillus* spp. represented over one-third of the organisms isolated in this study, none of them used fuel as a sole carbon source. The isolation of high numbers of aerobic sporeformers may reflect increased survival of bacterial spores in fuel systems. Hedrick et al. (11) reported that *Bacillus cereus* and *B. subtilis* survived up to 17 weeks in a fuel system, but they did not indicate whether the inocula or survivors represented spores or vegetative cells.

Isolation of microorganisms that are capable of fuel utilization may be a better index to potential problems of microbial origin than the isolation of organisms that are transient contaminants or which merely survive in a fuel system. Chemical or physical methods for the control of microorganisms in jet fuel systems should also be evaluated against active fuel-utilizing cultures. Iizuka and Komagata (12) reported that *P. maltophila* and other pseudomonads lost their abilities to assimilate hydrocarbons when cultured on Nutrient Agar slants. The five fuel-utilizing microorganisms isolated in the present study have been maintained for longer than 2 years by serial subculture with JP-4 jet fuel as the sole organic carbon source.

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