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PART 1

MICROBIOLOGICAL INVESTIGATIONS

TECHNICAL DOCUMENTARY REPORT NO. RTD-TDR-63-4118, PART 1

November 1963

AF Aero Propulsion Laboratory
Research and Technology Division
Air Force Systems Command
Wright-Patterson Air Force Base, Ohio

Project No. 3048, Task No. 304801

(Prepared under Contract No. AF 33(657)-9175
by the University of Dayton Research Institute, Dayton, Ohio;
Dorothy Nunn, author)

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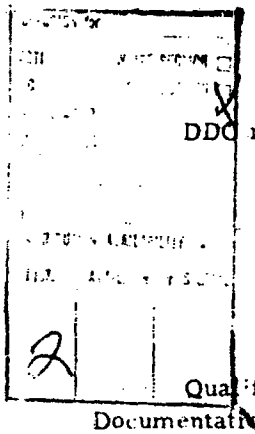

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~~PART I~~

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(1) Dorothy Nunn, author)

FOREWORD

This report was prepared by the University of Dayton Research Institute under USAF Contract No. AF 33(657)-9175. The contract was initiated under Project No. 3048, "Aviation Fuels," Task No. 304801, "Hydrocarbon Fuels." The work was administered under the direction of the Air Force Propulsion Laboratory, Research and Technology Division, Mr. A. V. Churchill, project engineer.

This report covers work from July, 1962 to July 1, 1963.

ABSTRACT

The effect of sodium dichromate, Mil-I-27686B Anti-icing additive (mixture of ethylene glycol monomethyl ether and glycerine), and 52 different fuel additives have been examined for their ability to inhibit microbial growth in jet fuels.

With the exception of six of the 52 additives tested, all of these agents demonstrated some degree of inhibitory activity against vegetative growth of the test organisms selected for the various studies. As a result of initial evaluation, 12 of the above 52 materials look promising and are being further evaluated.

Bacteria capable of forming spores appeared to be resistant to the effects of the sodium dichromate and the anti-icing additive. However, the data suggest that these organisms survive rather than grow and multiply in the test environment.

The maintenance of stock cultures of microorganisms isolated from jet fuels in various laboratories involved in the jet fuels project has been undertaken. To date 35 bacterial strains, 9 fungi, and 1 yeast are included in the stocks.

A simulated fuel tank has been designed and set up for long-term observation leading to data on the ecology of a representative mixed microbial flora in a fuel tank.

This technical documentary report has been reviewed and is approved.

Marc P. Dunnam

Marc P. Dunnam
Technical Support Division
Aero Propulsion Laboratory

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I. INTRODUCTION

The major objectives of the research reported here may be stated as follows:

1. To assess the microbicidal efficacy of 2% sodium dichromate for the microorganisms associated with the tubercles on corroded aluminum coupons in fuel samples.

2. To obtain data correlating the percent concentration of Mil-I-27686B icing additive in the water layer of jet fuel samples with inhibition of microbial growth.

3. To screen fuel additives for their ability to inhibit the growth of microorganisms in fuels, and to determine the minimal concentration of selected additives necessary for effective inhibition in fuels.

4. To associate certain types of microorganisms with particular ecological "niches" (i.e., water layer, fuel layer, or aluminum surface) in a simulated fuel tank.

II. EVALUATION OF 2% DICHROMATE AS A BACTERIOCIDE

It has been suggested that the organisms forming the tubercles on corroded aluminum surfaces exposed to jet fuels may be protected from the bacteriocidal action of dichromate by the outer layer of cells forming the tubercle, thus implying a penetration problem in the effective use of dichromate as a bacteriocide. Therefore, a procedure was outlined for assessing the efficacy of 2% sodium dichromate as a bacteriocide in jet fuel samples containing corroded aluminum coupons.

Five aircraft sump samples received from Wright-Patterson Air Force Base were assayed for numbers of organisms before and after treatment of the sample with dichromate. Types of cells in the tubercles and their viability after exposure to dichromate were also investigated. Results of the several methods employed to estimate numbers of organisms before and after the addition of 2% Na dichromate to the fluid phases (water, fuel) and sediment of the various samples are presented below. Representative data are shown in Table 1.

A. Before the addition of dichromate, 10-fold serial dilutions of a 1-ml aliquot of each sample were prepared in physiological saline and the various dilutions plated using trypticase-soy agar (TSA) medium and standard

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pour-plate techniques. One ml of each dilution was pipetted into a separate sterile petrie dish. TSA was added to each dish and the dish rotated to distribute the inoculum evenly throughout the agar. After the petrie plates had hardened, they were incubated at 37°C in an inverted position. After 48 hours incubation, the colonies were counted and recorded.

With the exception of #924, a few colonies were isolated from the water layer of each sample; however, all were surface colonies with no deep colonies being observed. The actual number of colonies counted ranged from 2 to 20. Their distribution was erratic and not correlated at all with the various dilutions. Colonies were observed from the fuel layer of #904, #926, #940; again, but few colonies were observed per plate and no correlation with dilution was obtained. No colonies grew out of the sediment.

B. In an effort to obtain more interpretable results, an alternate technique was employed, the "spread-plate." One-tenth (0.1) ml aliquots of the appropriate layers of each sample were transferred onto the surface of TSA plates from a sterile 21 gauge needle with a 2 cc syringe attached. An equal volume (0.1 ml) of sterile trypticase-soy broth was also added and the mixture spread evenly over the surface of the agar by means of sterile glass rods which had been bent to resemble hockey sticks. Growth of all organisms present in the aliquot were restricted to surface colonies using the spread-plate technique. Only the water layer of #904 and #924 showed growth before addition of 2.0% dichromate. Sediments and fuel layers showed negative growth.

C. One ml aliquots of each well-mixed sample (representing water, fuel, and sediment) were filtered through polypore membrane filters (Gilman) with a pore size of 0.25μ. Each polypore membrane was then transferred to a TSA plate.

Abundant growth was observed on the membrane after 24 hours incubation in samples #904, #926, and #940. Two colonies grew out of #924 and eight colonies in the case of #2287. The predominant organism in each case was a large gram-positive to gram-variable coccus appearing singly, in pairs and in tetrads or regular packets.

D. One drop (± 0.04 ml) of the various layers of each sample was streaked on TSA plates and incubated at 37°C for 24 hours. Growth was obtained from all water layers and from the fuel layer of #904 and #926. None of the samples yielded growth when streaked similarly on iron citrate agar.

E. Serum vials containing API anaerobic liquid medium (Difco) were inoculated with 1.0 ml of each well-mixed sample using a sterile needle (21 gauge) and syringe (2 ml). After one week incubation at room temperature,

a tan precipitate was observed. No black precipitate (indicative of Desulfovibrio)was apparent. The tan sediments were plated on TSA and incubated under nitrogen in Brewer anaerobic jars. No growth occurred.

The fluid phases of the samples tested showed no visible turbidity; thus, we may assume that abundant vegetative growth had not occurred (or, was not occurring) in the samples. It should be emphasized that actual numbers of colonies counted on the various dilution plates were too few to be statistically valid. Moreover, since assays for numbers of cells involved sampling each layer separately, i.e., water, fuel, and sediment, agitation of the sample during removal of an aliquot for culture was avoided. These two factors could contribute to the erratic distribution of numbers and to the lack of correlation between the various techniques used in gaining data on relative numbers.

Analogous experiments were performed to determine changes in numbers and/or types of bacteria in the jet-fuel samples after addition of 2% sodium dichromate (wt/vol). Aliquots were removed after 24 hours, 48 hours, 7 days, and 10 days exposure to the dichromate and examined as described above.

After 7 days exposure, no growth was observed in any of the plated aliquots with two exceptions: #940 showed abundant growth on spread plates when sampled at 24 hours, 48 hours and 7 days, but was negative for growth after 10 days. Number 2287 showed abundant growth at 48 hours, but was negative after 7 days. The growth in both instances was essentially a pure culture of the large gram-variable coccal form mentioned previously.

The tubercles formed on the aluminum coupons included in each of the samples were also assayed for amount of growth and types of organisms before and after the addition of dichromate to the gross specimen. Inocula were taken from the top surface of the tubercle and from the "protected" bottom surface, i.e., that part of the tubercle in direct contact with the aluminum coupon. It should be noted that, since once the tubercle was disturbed, the bottom surface was no longer protected from the fluid phase containing the dichromate, it was necessary to assay two different tubercles on the same coupon: one before and one after the addition of dichromate. The possible variations in viable numbers present in the undisturbed pairs of tubercles tested must be considered in interpreting the results. The corroded aluminum coupons are pictured in Figures 1 through 12.

Results of these experiments are tabulated in Table 2. In most cases a nominal number of colonies (1-18) were isolated from the tubercle before treatment of the sample with dichromate. Unexpectedly, there was an immediate trend (within 24-48 hours) toward increased numbers at the bottom of

the tubercle after the addition of dichromate except in those cases where there was abundant growth before dichromate treatment in which instances numbers were reduced after dichromate addition (re. sample #940). In the case of isolates from the tubercles, the organism involved was the large gram-variable coccus previously noted. These data are compatible with the suggestion that the significant bacterium involved is a sporeformer, and that after the addition of the dichromate, the spores germinate rapidly. That this may be the result of increased organic matter in the micro-environment of the tubercle due to the death and dissolution of the surface or vegetative cells is not an untenable hypothesis. Further, since after 7-10 days all cultures were negative for growth, it would appear that the spores germinated and the vegetative cells were subsequently killed by exposure to the dichromate. If this should prove to be the case, it would seem doubtful that metabolically active (vegetative) bacteria are present in numbers which would make the cells, per se or even their metabolic end-products significant in the corrosion process. Increased turbidity in the culture medium is a standard technique often employed for measuring growth of microorganisms. The lack of turbidity in the fluid phases of our samples, plus the relatively few isolates from the tubercles would give support to this contention.

To the author's knowledge, only one sporulating coccus, Sporosarcina ureae, has been described in the microbiological literature. The cellular and colonial morphology of our ubiquitous gram-variable coccal isolate is similar to the description of Sporosarcina. Special media are being employed in an effort to demonstrate sporulation and motility of this organism. A vacterium isolated from jet fuel in another laboratory and designated "aluminum corrosion No. 308, GD/FW #B-18," and which is reported to cause rapid corrosion has been acquired by this laboratory as an assay organism for corrosion studies in simulated fuel tank experiments. Since morphologically it appears to be identical to the gram-variable coccus (designated UD-1), it is also being examined for classification as a Sporosarcina species.

In all, 18 different bacteria were isolated from the samples examined in this study. These organisms are listed in Appendix II. Each has been given an identifying number prefixed by the letters UD (University of Dayton) and subcultures of each have been added to the stock culture collection of duel isolates.

Since the results of the study indicated 2% dichromate to be ultimately bacteriocidal, UD-1 was selected as the test organism in a further experiment in which the concentration of the dichromate was varied between 0.1% and 2% (wt/vol). The purpose of this experiment was to determine the lowest concentration of dichromate showing bacteriocidal activity in the test system.

Procedure:

(1) Two duplicate series of test tubes were set up, each containing 20 ml of sterile trypticase-soy broth (TSB) containing 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% sodium dichromate (wt/vol), respectively.

(2) 0.1 ml of an 18-hour broth culture (TSB) of the test bacterium, UD-1, was added to each tube.

(3) Each tube was assayed for growth after 24 hours and after 20 days incubation at room temperature by the "spread-plate" technique described above.

Results:

Incubation time:	% sodium dichromate added (wt/vol)					
	0%	0.1%	0.5%	1.0%	1.5%	2.0%
24 hours	+	+	0	0	0	0
20 days	+	+	0	0	0	0

Uninoculated controls all negative for growth

Growth = +
No growth = 0

The data indicates that 0.5%, but not 0.1%, sodium dichromate is bacteriocidal for the cells in the 18-hour inoculum. These results are not incompatible with the results of the former experiments, since, in this instance, a culture of young vegetative cells was used. If as suggested, UD-1 is a Sporosarcina species, it may be pointed out here that very adverse environmental (growth) conditions must attain before this organism will sporulate. TSB does not represent such a minimal environment.

III. EXAMINATION OF THE ROLE OF MICROORGANISMS IN CORROSION

That microorganisms play a significant role in the corrosion of aluminum has not been demonstrated conclusively. Many investigators have expressed doubt that organisms play any role at all in the corrosion process and that chemical compounds such as ferric oxide, surface-active agents, and substances found in sea water are the principal causes of the observed phenomenon. An experiment has been designed to gain information on the relative significance of these substances in the corrosion process. Additional variables in the experiment will be the presence and absence of Buna N

topcoating on aluminum coupons and stressed metal versus non-stressed metal. The design allows the effect of these variables to be evaluated singly and in combinations. Each environmental combination will be duplicated. One of each pair will remain sterile, while the other will be inoculated with an organism (UD-1) which has been found in association with the corrosion process.

Glass tanks measuring 6 1/2 in. x 10 in. x 7 in. deep will be used as the experimental containers in these experiments. Three by five inch aluminum (7075-T6) coupons of 0.040 inch gauge will be used as the major experimental surfaces. These aluminum coupons will be held upright in the tank by means of a holding rack. The coupons are bent to a 90-degree angle to create a stressed area in each coupon. Smaller coupons of the same metal will be placed flat on the bottom of each tank. All of the aluminum surfaces have been iridite treated. One-half of the coupons in each tank will be topcoated with Buna N type topcoating. There will be four topcoated and four non-topcoated coupons in each tank, thus allowing evaluation of corrosion at four intervals.

Twenty-four tanks will be included in the experiment. Based on liquid contents, there will be three groups of eight tanks each. One group will contain sterile JP-4 jet fuel sufficient to cover all the metal coupons (3500 ml). One group will contain a water phase of sterile distilled water (1000 ml), with a JP-4 fuel phase (2500 ml). The third group will be the same as the second, except that sterile sea water will replace the sterile distilled water. The eight tanks in each group will be modified as follows: two tanks will have no chemical additions to the fuel, or fuel and water; two will contain a surfactant at a concentration of 3 ppm; two will contain ferric oxide at a concentration of 2 mg per liter; two will contain both the surfactant and the ferric oxide, at a concentration of 3 ppm and 2 mg/L, respectively.

The upper rims of the tanks have been ground flat to allow glass lids to be sealed on with vacuum grease. A sterile, cotton-packed drying tube will be attached to an opening in the lid of each tank as an air vent. All tanks, metal pieces, and liquids will be sterilized prior to assembly. The twenty-four tanks will comprise twelve duplicate pairs. One of each pair will remain sterile. The second tank of each pair will be inoculated with a pure culture of the test bacterium.

All of the materials for this experiment are on hand and ready for assembly.

IV. EXAMINATION OF A SIMULATED FUEL TANK

Two of the tanks described in Item II were utilized in a simulated fuel tank study. The purpose of this experiment was to inoculate a tank with a

representative flora of fuel isolates and, subsequently, to attempt to associate certain of the isolates with a particular phase (ecological niche) in the tank, i.e., water phase, fuel phase, metal phase, etc.

To this end, two tanks were set up, each containing 1000 ml of 100% sterile sea water and 2500 ml of sterile JP-4 fuel. Sterilization was by Seitz filtration. Stressed and unstressed aluminum panels were inserted in such a manner that the metal was exposed to both fluid phases. One tank was inoculated with a mixture of 17 fuel isolates (Table 3) contained in 10 ml of sterile sea water. The second tank provided an uninoculated control.

Within 24-48 hours after the tank had been inoculated with the mixture of microorganisms, a white flocculent sediment could be observed. Patches of a white precipitate also were observed on the aluminum frame in the vicinity of an electrolytically welded area. The white sediment was covered with bubbles. However, no turbidity which might be associated with the growth of the microorganisms of the inoculum has been observed after four weeks. The uninoculated control tank showed the same sediment and white precipitate covered with bubbles, over the welded area of the metal frame. A control tank containing only JP-4 and aluminum (no sea water and no sea water phase) showed no precipitation. Again in an uninoculated tank in which distilled water replaced sea water, no sediment has been observed to date.

A series of 25 ml test tubes with screw-top caps each containing 10 ml of sea water, 10 ml of JP-4 and containing an aluminum coupon 1/2 in. x 4 in., were also set up. The aluminum strip in each test tube was inoculated with one colony of one of the 17 organisms included in the simulated tank inoculum. Since, to obviate contamination, it was desirable to disturb the large simulated tank as little as possible, the test tube series were set up so that any gross change in the large tank could be examined from a test tube showing a similar change, for example, development of a precipitate, etching of the metal strip, color change, etc.

A duplicate set of these test tubes was set up from which the inoculated aluminum strips were replaced after one week incubation at room temperature, by an uninoculated metal strip. The second metal strip was removed after 4 weeks incubation at room temperature. The metal strips were then examined for indications of corrosion. Since no reference was available for grading corrosion, the strips were graded from 1 to 3 plus depending on the size of the area of the metal strip showing etching or pitting as compared to an uninoculated control showing only discoloration. Results on inoculated strips after 1 week are shown in Table 4.

The significance of the results after up to four weeks incubation is questionable. Generally, some degree of deterioration was observed on all strips exposed to bacteria. Longer exposure may give more definitive results.

V. EVALUATION OF MIL-I-27686B ADDITIVE AS A MICROBICIDE

Mil-I-27686B anti-icing additive appears to inhibit microbial growth. A study of the effect of an anti-icing additive (98% cellosolve and 2% glycerol) was undertaken. Experiments designed to furnish data correlating the percent concentration of the additive in the water layer with inhibition of microbial growth were initiated.

MATERIALS AND METHODS

Materials:

A. 15% sea water, sterilized by autoclaving at 15# for 15 minutes.

B. Anti-icing additive (98% cellosolve, 2% glycerol), added to sterile sea water to yield 0%, 10%, 15%, 20% concentration of the additive.

C. JP-4 fuel, sterilized by Seitz filtration.

D. Inoculum: 0.5 ml of a 72 hour sea water culture of the appropriate test organisms prepared as follows:

1. One colony from TSA slant was emulsified in 5 ml of 15% sterile sea water and overlaid with 5 ml of sterile JP-4. Cultures were incubated at 25°C for 72 hours.

   0.5 ml of the sea water layer was used to inoculate the appropriate test tubes.

2. Organisms used: Bacteria: UD-2, -5, -8, -7, -10, B-3, -12, -22.
   Fungi: F-3, -4, -6, -9.
   Yeast: Y-1.

Method:

1. Duplicate series of four test tubes were set up for each of the 13 organisms used.

2. Each series included anti-icing additive at concentrations of 0%, 10%, 15%, and 20% in 10 ml of sterile sea water.

3. Each tube was inoculated with 0.5 ml sea water culture of the appropriate test organism.

4. Each tube was then overlayed with 5 ml sterile JP-4 fuel and incubated at 25°C.

5. After 24 hours, 48 hours, 72 hours, 7 days, and 25 days, each of the tubes were assayed for growth as follows:

   a. The experimental tube was mixed well and the JP-4 was permitted to separate out.

   b. 0.04 ml of the sea water layer was removed with a sterile pipette and streaked on a TSA plate.*

   c. The plates were read for the presence (+) or absence (-) of growth after 24 hours and 48 hours incubation at room temperature and the data recorded (Table 5).

CONCLUSIONS

This experiment was plagued with contaminants. First, the sea water, sterilized originally by Sietz filtration, was found to be contaminated. The experiment was repeated using autoclaved (15# for 15 minutes) sea water. However, this modification did not eradicate the contaminant. The source of the contamination is being sought and the experiment is being repeated. Albeit there is a contaminant present, from the data presented one might at least anticipate the following results:

A. UD-5 grows well in 15% sea water and is not inhibited by up to 20% of the anti-icing additive. It is of interest that this organism is a gram positive spore-forming rod. It could survive in spore-form and begetate on subculture to TSA, a complex medium.

B. B-3, -12, -22 also grow well in 15% sea water, but growth is inhibited by as little as 10% anti-icing additive. It might be noted that B-3 and B-12 are pseudomonads. They are not spore formers and the vegetative cells appear to be inhibited by the additive. B-22 is an anaerobic sporeformer.

C. F-3 and F-9 controls on these 2 organisms show no growth at 7 days and 25 days. Therefore, no conclusions can be drawn.

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* B-22 (Clostridium sporogenes) is anaerobic; therefore, it was subcultured into thioglycollate broth instead of on TSA plates. Growth was indicated by increased turbidity in this instance.

D. UD-7, Y-1, F-6 do not grow in sea water even in the absence of the anti-icing additive. Sea water might be considered toxic for F-6 since the inoculum was not reisolated; however, F-6 is a fungus and the plates should have been held at least six days before reporting as negative. Y-1 is a yeast; UD-7 a gram-positive bacterium.

E. In general, "time-growth" comparisons of the other organisms with the contaminated controls, make the results too ambiguous to be worth even an educated guess as to what the final results will show.

Indications of differential data based on the ability of some of the test organisms to form spores and thus survive under adverse environmental conditions are extremely significant. Indeed, if this proves to be the case, it would suggest that there is little or no vegetative growth occurring in the two fuels containing the anti-icing additive; that the positive test plates represent re-isolation of the original inocula which have survived in spore form.

VI. EVALUATION OF FUEL ADDITIVES FOR THEIR ABILITY TO INHIBIT MICROBIAL GROWTH

To date, 32 different fuel additives (Table 6) provided by WPAFB have been screened for their ability to inhibit microbial growth. The additives starred (*) are being examined further using dilution tube techniques.

Screening Procedure

Materials:

A. 130 mm diameter petrie plates containing Nutrient agar.

B. Sterile filter paper disks measuring 12.8 mm in diameter.

C. Sterile cotton swabs.

D. 24 hour Nutrient broth cultures of the following test bacteria: UD-1, -3, -9, -11; and a 5 day culture of F-2, a fungus.

Method:

A. Series of agar plates were inoculated with each of the test organisms using sterile swabs in order to obtain confluent growth on the agar surface.

B. Sterile filter paper disks were saturated with each of the various liquid additives and placed about 40 mm apart on the inoculated agar plates.

C. Solid additives were spotted directly on the plates.

D. The plates inoculated with test bacteria were incubated at 25°C for 2-3 days. Plates inoculated with the fungus, F-2, were incubated for 5-7 days.

E. Zones of inhibition of growth of the test organisms were reported in mm, measured from the outer edge of the filter paper disk to the outer edge of the clear zone surrounding the disk.

As shown by the results tabulated in Table 6, 12 materials look promising and are being tested further using tube-dilution techniques in which changes in turbidity will be read as the end-point.

VII. EXAMINATION OF FTD SAMPLES FOR THEIR ABILITY TO SUPPORT MICROBIAL GROWTH

To date, 13 FTD samples of unknown origin and composition have been received from WPAFB. These samples were examined firstly for the presence of microorganisms and secondly for their ability to support the growth of microorganisms inoculated into them.

A. Test for the presence of microbial growth in FTD samples:

(1) 0.04 ml of each well-mixed FTD sample was streaked on a separate TSA petrie plate.

(2) After 48 hours incubation at 25°C, the plates were examined for the presence of bacterial colonies. The plates were held for one week before being reported as negative for fungi.

B. Test for support of microbial growth in FTD samples:

(1) 10 ml aliquots of each well-mixed FTD sample were pipetted into separate sterile test tubes.

(2) Single colonies of the following organisms were used as the inocula:
Bacteria: UD-2, -5, B-12
Fungus: F-4.

(3) Single colonies of the four different test organisms were used to inoculate four of the test tubes in each series. The fifth test tube served as an uninoculated control.

(4) After 48 hours incubation at 25°C, each test tube was sub-cultured to a TSA plate.

(5) These TSA plates were incubated at 25°C for 48 hours and then examined for growth.

Results of the experiments are presented in Table 7.

The first three FTD samples examined, No. 22-63, No. 23-63, and No. 25-63, were cultured on trypticase-soy agar for growth. While no microorganisms were isolated from these samples when plated on TSA, inoculation of aliquots of each sample with a gram-negative rod (R-12) isolated from fuel by Leathen showed No. 22-63 to be capable of supporting bacterial growth. This sample yielded luxuriant growth of typical colonial and cellular morphology of the test organism after 24 hours incubation at 37°C. Nos. 23-63 and 25-63 did not support growth of this organism.

All subsequent FTD samples received were treated as described in Items A and B above.

Of the remaining 10 FTD samples tested, a bacterium was isolated only from sample No. 63-B-7. Moreover, as can be seen from the data (Table 7), this sample alone supported the growth of all of the test organisms used in this experiment.

UD-5 was re-isolated from 63-B-11, 63-B-12, 63-B-20 as well as from 63-B-7. F-4 was re-isolated from 63-B-11 and 63-B-7. Significantly, UD-5 is a gram-positive sporeformer. F-4 is a fungus and also forms spores. The other test organisms could not be re-isolated after 48 hours exposure to the FTD samples with the exception of No. 63-B-7 noted above, which supported the growth of all organisms used.

It is suggested that with the exception of FTD sample, No. 63-B-7, none of the other samples support growth; however, bacterial spores can survive in some of them, namely, 63-B-11, 63-B-12, and 63-B-20.

VIII. MAINTENANCE OF THE STOCK CULTURE COLLECTION OF FUEL ISOLATES

At the request of WPAFB, stock cultures of all microorganisms isolated from fuels in this laboratory and in other laboratories involved in the Jet-Fuels Project are being maintained at the University of Dayton.

Each bacterial strain is maintained in TSA slant cultures and in Bushnell-Haas mineral salts fluid cultures overlayed with sterile JP-4 fuel. The fungi are maintained on Sabouraud's agar slants. The fluid cultures have proven highly unsatisfactory. More often than not, the organisms cannot be subcultured from this medium. Lack of increased turbidity in these cultures suggests further that if the organism can be re-isolated, it is a result of the ability to survive rather than to grow in this medium. At present, we are substituting 15% sterile sea water for the B-H broth in an effort to obtain measurable growth in a medium containing JP-4 fuel as the major carbon source.

The TSA slants provide luxuriant growth of the aerobic bacteria. TSA slants of anaerobic bacteria are incubated under nitrogen; duplicate cultures are maintained in thioglycollate broth. Subcultures of all stocks are made at 60 day intervals. The cultures are incubated at 37°C for 18 hours and subsequently stored at 0°C.

Appendix II comprises a list of the 52 organisms presently in stock. The laboratory in which they were originally isolated is noted. Three requests for subcultures of these stock organisms have been filled to date. In one instance, 47 strains were subcultured to 3 different media.

IX. SUMMARY

Sodium dichromate, Mil-I-27686B anti-icing additive, and 12 different fuel additives have been shown to inhibit to varying degrees the growth of microorganisms isolated from jet fuels.

In general, an examination of the data presented indicates that 13 of the experimental compounds tested inhibit significantly vegetative microbial growth. Those test organisms which appeared to be less sensitive or completely insensitive to the dichromate and the anti-icing additive may be characterized, as a group, by their ability to form spores. The formation of spores by certain genera of bacteria endows them with the ability to survive in adverse environments. If bacterial spores are transplanted to more optimal environments, the spores germinate and the resultant cells multiply vegetatively as long as the environment can support them. It is suggested, therefore, that the additives added to jet fuels represent adverse environments for most bacteria. Some organisms survive in the fuels in spore form and vegetate only after removal to a nutritionally complete medium in the laboratory. Some of the non-sporeforming microorganisms, such as the physiologically versatile pseudomonads, which have adapted to growth in a fuel environment are killed by the addition of the various fuel additives examined.

Although a great deal more experimental evidence is necessary, the preferential survival of sporeformers, the lack of increased turbidity in the fuel samples which we have examined, the lack of measurable growth in media utilizing JP-4 fuel as a sole source of carbon, all suggest that little vegetative growth occurs in these fuels.

X. CONCLUSIONS

A. Seven to ten days exposure to 2% sodium dichromate is bacteriocidal for all microorganisms surviving in the jet fuels tested, including those organisms associated with the tubercles formed during the corrosion of aluminum. Eighteen different organisms were isolated from the five fuel samples used to assess the efficacy of dichromate as a microbicide. One of the 18, designated UD-1, was found consistently in association with the tubercles. Using UD-1 as the test organism, it was found that less than 0.5% dichromate is bacteriocidal for vegetative cells in a complex broth medium. Concentrations of dichromate between 0.1% and 0.5% will be examined in a fuel system containing corroded aluminum coupons and employing a mixed microbial flora.

It is felt that the organism UD-1 warrants further examination with respect to both its possible classification in the genus Sporosarcina and its significance in jet fuels.

B. Experiments assessing the inhibitory activity of the anti-icing additive against selected microorganisms are still in progress. Results to date indicate that organisms capable of forming spores are resistant to concentrations of 20% anti-icing additive whereas vegetative cells are sensitive to 10% of this fuel additive.

C. Thirteen of the 52 fuel additives tested were inhibitory for one or more of the test organisms when the additive was used undiluted. Twelve of these additives, showing significant inhibition of the test organisms in the screening procedures, were selected for further examination. These studies are in progress.

D. It must be concluded that a defined medium utilizing JP-4 fuel as the sole carbon source for measurable growth of fuel isolates has yet to be described.

SAMPLE NO. 904

Figure 1. Full View of Metal Strip Showing Corrosion Areas

Figure 2. Close Up View of a Deep Corrosion Pit with Tubercle Products Removed.

SAMPLE NO. 924

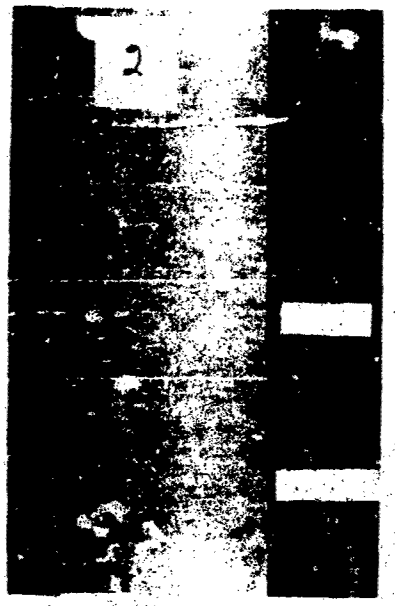

Figure 3. Full View of Metal Strip Showing Corrosion Area

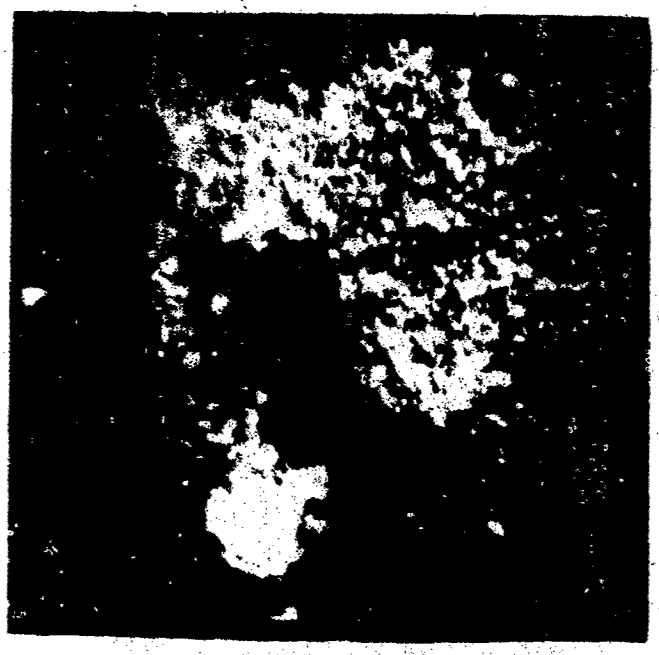

Figure 4. Close Up View of Typical Corrosion with Corrosion Products Removed.

SAMPLE NO. 926

Figure 5. Full View of Metal Strip Showing Corrosion Area.

Figure 6. Close Up View of Corrosion Showing Cracking of the Aluminum.

SAMPLE NO. 940

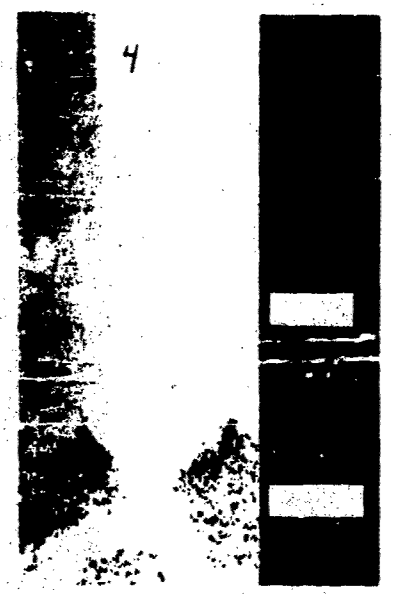

Figure 7. Full View of Front of Metal Strip Showing Corrosion Area.

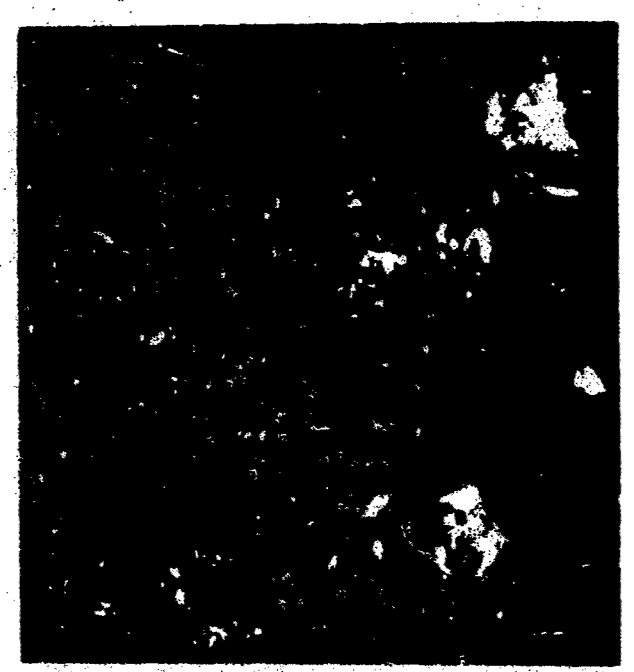

Figure 8. Close Up View of Typical Corrosion with Corrosion Products not Removed.

SAMPLE NO. 940

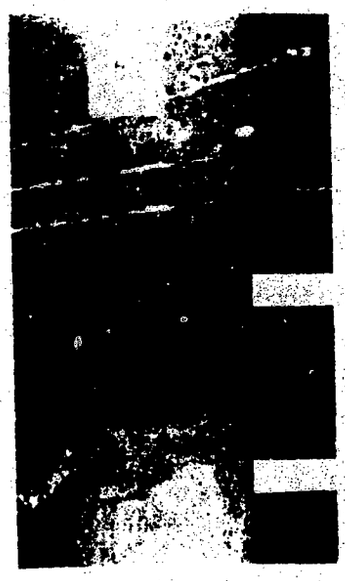

Figure 9. Full View of Back of Metal Strip Showing Corrosion Area.

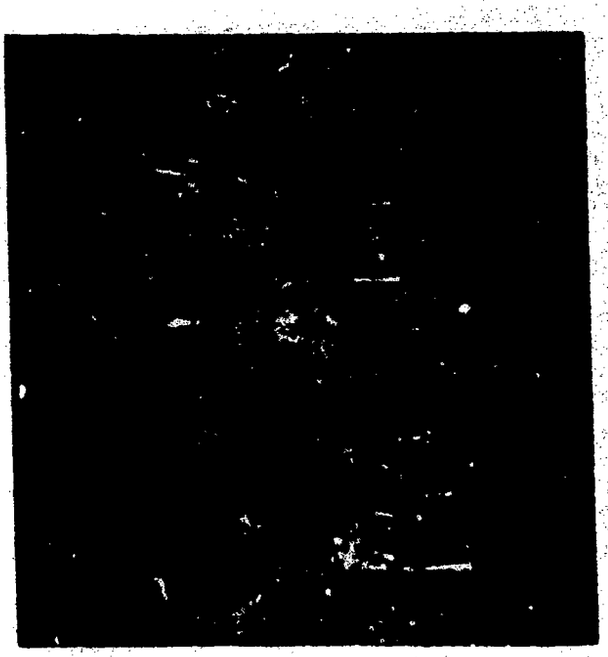

Figure 10. Close Up View of Typical Tubercle Formation.

SAMPLE NO. 2287

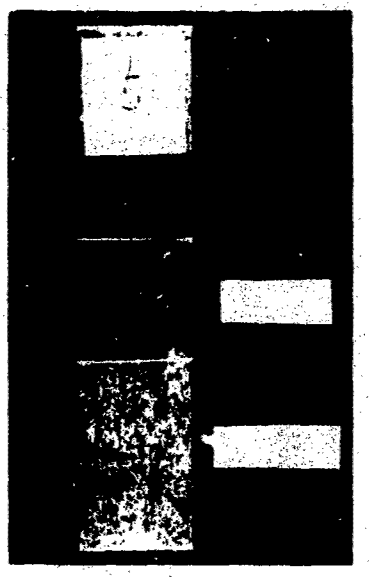

Figure 11. Full View of Metal Strip - Corrosion not Visible.

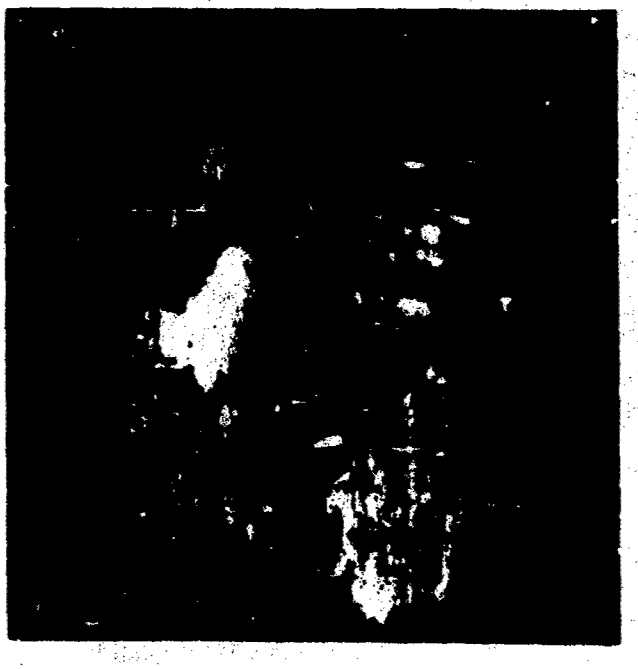

Figure 12. Close Up View of Corrosion Just Beginning.

TABLE 1
RELATIVE NUMBERS OF BACTERIA IN FLUID PHASES
BEFORE AND AFTER TREATMENT WITH 2% DICHROMATE
(ORGANISMS PER ML OF SAMPLE)

Sample No.	Before Dichromate		Water-Fuel Mixture After Dichromate	
	Water	Fuel	48 Hours	7 Days
904	540	0	0	0
924	50	0	0	0
926	0	0	1	0
940	0	0	TNTC	TNTC
2287	0	0	TNTC	0

TABLE 2
RELATIVE NUMBERS OF BACTERIA ON TUBERCLES*
BEFORE AND AFTER TREATMENT WITH 2% DICHROMATE
(ORGANISMS PER ML OF SAMPLE)

Sample No.	White Tubercle				Brown Tubercle			
	Top		Bottom		Top		Bottom	
	before	48 hrs	before	48 hrs	before	48 hrs	before	48 hrs
904	18	0	2	0	1	0	2	0
924	2	8	3	TNTC	6	6	0	TNTC
926	3	2	1	TNTC	2	TNTC	0	TNTC
940	TNTC	2	77	3	1	3	TNTC	4

* All were negative for growth after 7 days of exposure to 2% dichromate

TABLE 3
ORGANISMS USED IN SIMULATED TANK EXPERIMENT

Stock No.	Description	Source
UD-1	gvc, translucent	fuel, water, tubercle
UD-2	gpc, opaque, lemon yellow	tubercle
UD-3	gpr, Bacillus sp., mucoid	water
UD-4	gpr, thin transparent	fuel, tubercle
UD-5	gpr, opaque, dry white	fuel, tubercle
UD-6	gpr, opaque cream	tubercle
UD-7	gpr, opaque gray	tubercle
UD-8	gpr, salmon, dry, wrinkled	tubercle
UD-9	gnr, opaque gray	water, tubercle
UD-10	gnr, transparent	water, tubercle
UD-11	gnr, gray mucoid	tubercle
UD-12	gnr, transparent, brown soluble pigment	water, tubercle
UD-13	gnr, translucent	tubercle
UD-14	gnr, translucent	tubercle
UD-15	gpr, gray, waxy	filter membrane
UD-16	Streptomyces sp.	filter membrane
UD-17	Actinomyces sp.	filter membrane

gpc Gram positive cocci
gvc Gram variable cocci
gpr Gram positive rod
gnr Gram negative rod

TABLE 4
AN ESTIMATE OF CORROSION ON ALUMINUM STRIPS AFTER FOUR WEEKS INCUBATION IN THE PRESENCE OF EACH OF 17 FUEL ISOLATES

Method of Inoculation	Degree of Corrosion		
	+++ (extensive)	++ (moderate)	+ (slight)
1. One colony of each test organism was inoculated directly on separate aluminum strips	UD-1 UD-8 UD-9 UD-11 UD-16 UD-17	UD-3 UD-4 UD-5 UD-6 UD-7 UD-10 UD-12 UD-13 UD-14 UD-15	UD-2
2. One colony of each test organism was inoculated into the broth medium in which the aluminum strips were placed.	UD-1 UD-7 UD-12	UD-2 UD-3 UD-4 UD-6 UD-8 UD-9 UD-10 UD-11 UD-13 UD-14 UD-15 UD-16 UD-17	UD-5

TABLE 5

EVALUATION OF BACTERIOCIDAL ACTION OF MIL-I-27686 ANTI-ICING ADDITIVE

"test" tubes: % anti-icer assayed & incubation time	[1] Growth of subcultures from the various experimental ("test") tubes. (TSA* streak plates, read at 24 hrs. & 48 hrs.)																					
Test Organisms:	Uninoculated control		UD-2		UD-5		UD-7		UD-8		UD-10		B-3		B-12		Y-1		F-3		F-4		F-6		F-9		B-22	
Hours:	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
0% anti-icer: 24 hrs.	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	+	+
48 hrs.	-	-	-	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+	-	+	-	-	-	-	-	+
72 hrs.	+	+	-	-			-	+	-	-	-	+	+	+	+	+			+	+	-	+	-	-	-	+	+	+
7 days	-	-	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
25 days	-	-	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	-	-	+	+
10% anti-icer: 24 hrs.	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	+	-		+	+	-	+	-	-	-	+	-	+
48 hrs.	-	-	-	-	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
72 hrs.	-	+	-	-			-	-	-	-	-	+			-	+			-	+	-	-	-	-	-	+	+	-
7 days	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
25 days	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15% anti-icer: 24 hrs.	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-
48 hrs.	-	-	-	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-
72 hrs.	-	+	-	-			-	-	-	-	-	+	-	-	+	+			-	+	-	-	-	-	-	+	-	-
7 days	-	-	-	-	+	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25 days	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20% anti-icer: 24 hrs.	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	-	-	+	+	-	+	-	+	-	+	-	-
48 hrs.	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-
72 hrs.	-	-	-	-			-	-	-	-	-	+	-	-	-	-			-	+	-	-	-	-	-	+	-	-
7 days	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
25 days	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] + = growth
- = no growth

*TSA = trypticase-soy agar

TABLE 6
DATA ON ADDITIVES TESTED FOR INHIBITION OF MICROBIAL GROWTH

Sample No.	Organisms: Millimeters of Growth Inhibition					Additives Soluble In:[1]	
	UD-1	UD-3	UD-9	UD-11	F-2	H_2O	Fuel
A. Liquid Additives							
OCC- A	10	13	10	12	8	+	-
B	10	11	8	14	7	+	-
C	13	8	10	11	7	+	-
D	9	3	4	4	3	-	+
E	8	5	4	4	-	-	+
F	12	14	10	6	-	-	+
G	20	13	6	10	-	+	-
H	8	4	5	5	5	+	-
J	9	6	12	8	6	+	-
K	13	8	9	12	4	+	+
L	11	8	11	7	4	+	+
M	10	5	14	7	5	+	+
N	13	3	9	6	6	+	+
P	13	7	16	8	5	-	+
Q	15	6	5	4	7	+	-
R	10	5	4	5	7	+	-
S	10	1	1	2	1	+	+
T	13	4	4	4	5	+	-
U	18	1	1	2	0	+	+
V	18	4	3	3	7	+	-
W	15	6	5	4	3	+	+
X	20	6	5	5	-	+	-
CCW-A	?	1	1	7		-	+
CCW-B	20	15	9	3		+	-

1. Soluble = +
   Insoluble = -

TABLE 6 (Cont.)

Sample No.	Organisms					Additives Soluble In:	
	Milimeters of Growth Inhibition						
	UD-1	UD-3	UD-9	UD-11	F-2	H_2O	Fuel
CCW-C	18	9	9	10		+	+
CCW-D	10	2	0	0		-	+
GCC-A	1	1	1	1		-	+
MC-A	?	22	18	24		+	-
CZ-A	?	20	19	25		-	-
S-A	4	11	10	8		+	±
AAC-A	0	1	0	0		+	+
WRGC-A	18	18	17	20		-	+
B. Solid Additives							
BL-A	9	1	1	0		±	±
BL-B	9	2	1	1		±	±
OCC-A	10	8	5	7		-	-
OCC-B	8	7	6	6		-	+
HC-A	8	0	0	0		+	-
HC-B(liquid)	4	2	0	0		-	-
HC-C	13	1	1	0		+	-
HC-D	10	2	2	0		+	+
HC-E	9	3	3	0		+	-
HC-F	0	0	0	0		+	+
UCC-A	20	9	8	2	20	+	-
UCC-B	14	7	5	2	16	±	-
UCC-C	22	8	5	19	23	±	±
UCC-D	28	6	5	20	25	+	-
ECC-A	5	0	1	0	4	-	+
ECC-B	0	0	0	0	0	-	+
HPCC-A	0	0	0	0	2	-	+

TABLE 6 (Cont.)

Sample No.	Organisms					Additives Soluble In:	
	Milimeters of Growth Inhibition						
	UD-1	UD-3	UD-9	UD-11	F-2	H_2O	Fuel
HPCC-B	0	0	0	0	1	-	+
HPCC-C	17	12	13	15	15	±	+
HPCC-D	3	0	2	1	3	-	+

TABLE 7
FTD SAMPLES

*Data Sheet

Date: FTD Sample No.:	Isolates From Sample: (TSA plates) (48 hrs. -37°C)	Growth of Test Organisms Added to the FTD Samples Organisms:					
		R-12	UD-2	UD-5	B-12	F-4	Uninoculated Control
12/11/62							
22-63	-	+	NOT TESTED				
23-63	-	-					
25-63	-	-					
5/18/63							
63B2	-	NOT TESTED	-	-	-	-	-
63B7	+		+	+	+	+	+
63B9	-		-	-	-	-	-
63B11	-		-	+	-	+	-
63B12	-		-	+	-	-	-
63B17	-		-	-	-	-	-
63B18	-		-	-	-	-	-
63B19	-		-	-	-	-	-
63B20	-		-	+	-	-	-
63B21	-		-	-	-	-	-

*Data reported as + (growth) or - (no growth) on TSA plates within 48 hours incubation at 30°C.

Appendix I

Constituents of Microbiological Media

Nutrient Broth

Bacto-Beef Extract	3.0g
Bacto-Peptone	5.0g
Distilled Water	1000 ml

Nutrient Agar (NA)

Bacto-Beef Extract	3.0g
Bacto-Peptone	5.0g
Bacto-Agar	15.0g
Distilled Water	1000 ml

Sabourand Dextrose Agar

Neopeptone, Difco	10.0g
Bacto-Dextrose	40.0g
Bacto-Agar	15.0g
Distilled Water	1000 ml

Mineral Salts Medium (Bushnell-Haas) (B-H)

This medium provided the basis of a nutrient substrate in which jet fuel was the only source of carbon.

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2g
Calcium chloride ($CaCl_2$)	0.02g
Potassium phosphate, monobasic (KH_2PO_4)	1.0g
Amonium nitrate (NH_4NO_3)	1.0g
Potassium phosphate, dibasic (K_2HPO_4	1.0g
Ferric chloride ($FeCl_3$)(15g/25ml aqueous solution)	2 drops
Distilled Water	1000 ml

Adjust to pH 7.0 - 7.2 with dilute Sodium Hydroxide (NaOH).
Solid medium is prepared by the addition of 2.0% agar.

Medium for Iron-Depositing Bacteria

Ammonium sulfate $(NH_4)_2SO_4$	0.5g
Sodium nitrate ($NaNO_3$)	0.5g
Dipotassium phosphate (K_2HPO_4)	0.5g
Magnesium sulfate ($mgSO_4 \cdot 7H_2O$)	0.5g
Calcium chloride ($CaCl_2$)	0.2g
Ferric ammonium citrate	10.0g
Distilled Water	1000 ml

Sterilize ferric ammonium citrate separately and add when cool.
Solid medium is prepared by the addition of 2.0% agar.

Thioglycollate Broth

Trypticase	17.0g
Phytone	3.0g
Dextrose	6.0g
Sodium chloride	2.5g
Sodium thioglycollate	0.5g
Agar	0.7g
ℓ-cystine	0.25g
Sodium sulfite	0.1g
Distilled Water	1000 ml

Trypticase Soy Broth (TSB)

Trypticase	17.0g
Phytone	3.0g
Sodium chloride	5.0g
Dipotassium phosphate	2.5g
Dextrose	2.5g
Distilled Water	1000 ml

Trypticase Soy Agar (TSA)

Trypticase	15.0g
Phytone	5.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled Water	1000 ml

Appendix I (cont.)

Sea Water Agar

Bacto-Agar	15.0g
Aged sea water	1000 ml

Appendix II

Stock Cultures**

Stock Number	Organism or Gram Reaction	Source
UD-1	gvc	University of Dayton
UD-2	gpc	" "
UD-3	Bacillus sp.	" "
UD-4	gpr	" "
UD-5	Bacillus sp.	" "
UD-6	gpr	" "
UD-7	gpr	" "
UD-8	Sphaerotilus sp.	" "
UD-9	gnc	" "
UD-10	gnr	" "
UD-11	gnr	" "
UD-12	gnr	" "
UD-13	gnr	" "
UD-14	gnr	" "
UD-15	Bacillus mycoides	" "
UD-16	Streptomyces marinus	" "
UD-17	Actinomyces sp.	" "
UD-18	Pseudomonas stutzeri	" "
B-1	Aerobacter aerogenes	General Dynamics/Fort Worth
B-2	Escherichia coli	" "
B-3	Pseudomonas aeruginosa	" "
B-5	Staphylococcus aureus	" "
B-8	Bacillus cereus	" "
B-9	Bacillus subtilis	" "

Appendix 2 (cont.)

Stock Number	Organism or Gram Reaction	Source	
B-11	Proteus vulgaris	General Dynamics/Fort Worth	
B-12	Pseudomonas fluorescens	"	"
B-13	Sarcina lutea	"	"
B-14	Serratia marcescens	"	"
B-15	Staphylococcus albus	"	"
B-17	Micrococcus radiodurans	"	"
B-18	Aluminum corrosion No. 308	"	"
B-19	Aluminum corrosion No. 304	"	"
B-20	Flavobacterium arborescens	"	"
B-21	Sphaerotilus natans	"	"
B-22	Clostridium sporogenes	"	"
Y-1	Rhodotorula rubra	"	"
F-1	Spicaria violacea	"	"
F-2	Fusarium roseum	"	"
F-3	Aspergillus niger	"	"
F-4	Cladosporium resinae	"	"
F-5	Aspergillus tamarii	"	"
F-6	Penicillum ochrochloron	"	"
F-7	Alternaria tenuis	"	"
F-8	Fusarium moniliforme	"	"
F-9	Chaetomium globosum	"	"

** Maintained on Trypticase Soy Agar Slants and Bushnell-Haas Broth overlaid with JP-4.