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The Aerobic Pseudomonads: a Taxonomic Study

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Dedicated to L. E. DEN DOOREN DE JONG, who laid the foundations upon which this work was built

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SUMMARY

A collection of 267 strains, representing many of the principal biotypes among aerobic pseudomonads, has been subjected to detailed study, with particular emphasis on biochemical, physiological and nutritional characters. A total of 146 different organic compounds were tested for their ability to serve as sources of carbon and energy. Other characters that were studied included: production of extracellular hydrolases; nitrogen sources and growth factor requirements; H_2-chemolithotrophy; denitrifying ability; pigment production; ability to accumulate poly-β-hydroxybutyrate as a cellular reserve material; biochemical mechanisms of aromatic ring cleavage; and nature of the aerobic electron transport system. The resultant data have revealed many hitherto unrecognized characters of taxonomic significance. As a consequence, it has become possible to recognize among the biotypes examined a limited number of species which can be readily and clearly distinguished from one another by multiple, unrelated phenotypic differences.

INTRODUCTION

The aerobic pseudomonads are an important assemblage of eubacteria which has so far proved resistant to satisfactory taxonomic analysis. We shall describe in this paper a methodology that provides taxonomically significant information about these organisms, as shown by the fact that it permits the division of a large collection of strains into a relatively small number of clusters, distinguishable from one another by multiple, unrelated phenotypic differences. Most of these clusters appear to merit recognition as bacterial species.

We offer the following definition of the aerobic pseudomonads:

Unicellular rods, with the long axis straight or curved, but not helical. Motile by means of one or more polar flagella. Gram-negative. Do not form spores, stalks, or

sheaths. The energy-yielding metabolism is respiratory, never fermentative or photosynthetic. All use molecular oxygen as a terminal oxidant; and some can use denitrification as an alternative, anaerobic respiratory mechanism. All are chemo-organotrophs; some are facultative chemolithotrophs that use H_2 as an energy source.

One additional group property which appears significant, but which cannot yet be fully evaluated, is the base content of the deoxyribonucleic acid. In the species that we have examined, the DNA contains between 58 and 69 moles % of guanine and cytosine (Mandel, 1966). However, it seems unwise to set absolute limits for the base content of the DNA until other biotypes, which possibly deserve to be included, have been subjected to analysis.

This definition excludes many other groups of Gram-negative, polarly flagellated bacteria. The chemoheterotrophic spirilla, the caulobacters, and the *Leptothrix–Sphaerotilus* group are excluded on structural grounds. The specifications with respect to energy-yielding metabolism exclude all pseudomonads and vibrios capable of fermentative metabolism (*Aeromonas*, *Zymomonas*, *Photobacterium*, *Vibrio* in the strict sense); *Desulfovibrio*; and photosynthetic bacteria. The statement concerning chemolithotrophic nutrition excludes chemolithotrophic pseudomonads such as *Nitrosomonas*, *Ferrobacillus* and *Thiobacillus*. The only Gram-negative, polarly flagellated groups which it seems desirable to separate from the aerobic pseudomonads, but which are not rigorously excluded by the proposed definition, are the small-celled parasitic vibrioid bacteria for which the genera *Campylobacter* (Sebald & Véron, 1963) and *Bdellovibrio* (Stolp & Starr, 1963) have been recently proposed. Since the DNA of *Campylobacter* spp. contains about 40 moles % of guanine and cytosine (Sebald & Véron, 1963), a separation may prove feasible on this character alone.

The aerobic pseudomonads constitute a large and fairly diverse array of bacteria. There is general agreement that some of them belong to the genus *Pseudomonas*. However, the circumscription of this genus at present is far from clear, and many of the bacteria that we include among the aerobic pseudomonads are now most commonly classified in other genera: *Xanthomonas*, *Methanomonas*, *Hydrogenomonas*, *Comamonas*, *Vibrio sensu lato*, *Acetomonas* (*Gluconobacter*), and possibly also *Alginomonas*, *Cellulomonas* and *Cellvibrio*. We doubt the usefulness of this generic hypertrophy, and would assign all these organisms provisionally to the single genus *Pseudomonas*, using the group definition of aerobic pseudomonads already presented as a generic definition. Eventually, generic subdivision may prove useful; but, if and when it is undertaken, it should be based on criteria more substantial, and much more thoroughly evaluated, than those which now purportedly separate such genera as *Xanthomonas*, *Hydrogenomonas* and *Comamonas* from *Pseudomonas*.

It is exceedingly difficult to give a satisfactory account of the nature and extent of the aerobic pseudomonads, because the existing descriptions of the constituent subgroups are so fragmentary that the boundaries between them are in many cases by no means clearly defined; some subgroups are recognized primarily on pigmentation, others on pathogenicity, other on physiological attributes that have been specifically selected by the methods used to isolate them from nature. With due recognition of the hazardous nature of the enterprise, it nevertheless seems desirable to attempt an initial survey of the group.

The most extensively studied subgroup of aerobic pseudomonads consists of the

fluorescent pseudomonads, primarily characterized by their ability to produce water-soluble, yellow-green fluorescent pigments of unknown chemical nature. Simple fluorescent pseudomonads are common inhabitants of soil and water, and were first described by Flügge (1886). Flügge recognized two biotypes, distinguishable on the character of gelatin liquefaction, which now bear the names of *Pseudomonas fluorescens* (liquefying) and *P. putida* (non-liquefying). Since then, many other species of simple fluorescent pseudomonads from soil and water have been named on the basis of fragmentary descriptions; their differences from the two classical species, if any, are not clear. As first shown by den Dooren de Jong (1926), simple fluorescent pseudomonads are nutritionally versatile, and can use a very wide range of simple organic compounds as sole sources of carbon and energy.

The bacteria pathogenic for higher plants include many simple fluorescent pseudomonads, and numerous species have been defined largely or solely on the basis of their ability to infect specific plant hosts. This proliferation of specific names for phytopathogenic fluorescent pseudomonads is probably not justifiable (Starr, 1959); and it is still not really clear in what respects, if any, phytopathogenic strains differ from the simple fluorescent pseudomonads commonly found in soil and water.

Several other species have been recognized by virtue of their ability to synthesize, in addition to the group-specific fluorescent pigment, a type-specific phenazine pigment. From the available descriptions, some of these phenazine producers (*Pseudomonas chlororaphis*, *P. aureofaciens*, *P. lemonnieri*) appear to resemble closely in most phenotypic respects the simple fluorescent pseudomonads of the type of *P. fluorescens*. However, *P. aeruginosa*, the type species of the genus *Pseudomonas*, has several properties in addition to the production of a specific phenazine pigment that set it apart from the rest of the group. It is the only fluorescent pseudomonad pathogenic for mammals; it has a temperature maximum higher than that of the other fluorescent pseudomonads (Haynes, 1951); and its flagellation is invariably monotrichous, while nearly all other fluorescent strains are multitrichous (Lautrop & Jessen, 1964).

The ability to produce phenazine pigments also occurs in a group of non-fluorescent pseudomonads, originally isolated from soil in Trinidad (Morris & Roberts, 1959). These bacteria accumulate poly-β-hydroxybutyrate as a cellular reserve material, a property not found in the fluorescent group (Morris & Roberts, 1959). Although this group has been studied by several workers (Forsyth, Hayward & Roberts, 1958; Sneath, 1960), it has not received a formal taxonomic designation. As we shall show, it is a very distinctive, nutritionally versatile biotype which we propose to recognize as a new species, *Pseudomonas multivorans*.

Apart from the fluorescent group and *Pseudomonas multivorans*, the aerobic pseudomonads are either non-pigmented, or produce yellow or red cellular pigments. The character of cellular pigmentation has major diagnostic importance, since the genus *Xanthomonas* was created by Dowson (1939) for certain yellow phytopathogenic pseudomonads. Starr & Stephens (1964) have recently characterized the pigments of the *Xanthomonas* group as new and unusual carotenoids. However, the production of yellow cellular pigments is also characteristic of some hydrogenomonads, and not uncommon in chemoheterotrophic pseudomonads isolated by other enrichment methods or by non-selective methods from water and soil. The relation of these pigmented pseudomonads to the xanthomonads is at present obscure.

The group of hydrogenomonads is now in practice defined by the specific enrichment method used to isolate them from natural sources: selection for ability to grow chemolithotrophically with H_2. Most of the named species are very poorly described. The detailed nutritional study by Doudoroff (1940) on one species, *Pseudomonas saccharophila*, showed that it is a versatile chemo-organotroph, and the less detailed nutritional information about other biotypes suggests that this may be true of all hydrogenomonads. The relations of these organisms to other yellow and non-pigmented aerobic pseudomonads are unknown.

Another group (many of which produce yellow or red cellular pigments) consists of bacteria isolated by specific enrichment at the expense of methane, methanol, formate, oxalate and lower amines. These compounds do not seem to be attacked by other kinds of pseudomonads (or, indeed, by most other aerobic bacteria); hence the capacity to utilize them is probably a character of taxonomic significance. The best-characterized member of this group is *Pseudomonas methanica*, for which methane and methanol are the only organic compounds which support growth (Dworkin & Foster, 1956). Strains isolated by enrichment with methanol, oxalate and lower amines have a somewhat wider nutritional spectrum (den Dooren de Jong, 1926; Peel & Quayle, 1961; Stocks & McCleskey, 1964).

The older literature on bacterial denitrification (e.g. Burri & Stutzer, 1895; Künnemann, 1898; van Iterson, 1902) contains fragmentary descriptions of many species of aerobic pseudomonads, both fluorescent and non-fluorescent, which were enriched from nature by virtue of their denitrifying capacity. The one well-characterized non-fluorescent denitrifier is *Pseudomonas stutzeri* (van Niel & Allen, 1952).

Apart from *P. aeruginosa*, the only *Pseudomonas* species pathogenic for mammals is *P. pseudomallei*, the causative agent of melioidosis, a disease confined to warm, humid tropical regions.

Many bacteria that seem to conform to our definition of aerobic pseudomonads have been isolated from soil, fresh water and sea water by selection for the ability to grow at the expense of complex polysaccharides (cellulose, agar, chitin, alginic acid) and a number of specific, physiologically defined genera (e.g. *Cellulomonas*, *Cellvibrio*, *Alginomonas*) have been proposed for them. We shall not attempt to cite here the voluminous literature on these organisms; but it should be kept in mind that they may well constitute a large and varied group of aerobic pseudomonads, which has so far never been subjected to systematic comparative study.

Aerobic enrichments with a variety of simple organic compounds may yield non-pigmented aerobic pseudomonads in addition to, or in place of, fluorescent pseudomonads (den Dooren de Jong, 1926; Gray & Thornton, 1928; Marcus & Talalay, 1956). Several species have been named among such isolates; but only one, *Pseudomonas acidovorans* (den Dooren de Jong, 1926), has been adequately described.

Many non-pigmented aerobic pseudomonads have been directly isolated on complex media from soil, water, milk, or specimens submitted to the clinical laboratory. Among the better-characterized species in this category are *Pseudomonas maltophilia* (Hugh & Ryschenkow, 1960), *P. alcaligenes* (Hugh & Ikari, 1964) and *P. putrefaciens* (Derby & Hammer, 1931).

Lastly, it should be noted that the acetic acid bacteria with polar flagellation (genus *Acetomonas* or *Gluconobacter*) conform to our definition of aerobic pseudomonads.

Origin and plan of the present study

In attempting to characterize some of our own isolates of aerobic pseudomonads, we reached the conclusion that the diagnostic tests in current use for this group of bacteria do not yield a sufficiently broad picture of the phenotypic traits to permit a satisfactory definition of species. These tests largely originate from the array of diagnostic tests devised for the identification of coliform bacteria, a fact which explains the heavy dependence on acid production in carbohydrate broth cultures incubated under semi-aerobic conditions. Some years ago, Hugh & Leifson (1953) discovered that the basal medium used in making such tests with coliform bacteria is unreliable for detecting acid production from carbohydrates by aerobic pseudomonads; these organisms often produce little acid from carbohydrates, and are almost all powerful ammonifiers of the complex nitrogenous materials in the medium, so that cultures may become strongly alkaline even though the carbohydrate that they contain has been extensively decomposed. Hugh & Leifson accordingly modified the test medium by greatly decreasing the concentration of peptone, and their modified medium has now become the standard one for determining sugar utilization by aerobic pseudomonads. Even this modified medium does not necessarily give readily interpretable results. For example, many fluorescent pseudomonads can oxidize maltose and lactose to the corresponding bionic acids, but cannot grow at the expense of these sugars (Kluyver, de Ley & Rijven, 1951). Furthermore, it is by no means certain that all these strictly aerobic bacteria will in fact produce a detectable amount of acid during growth at the expense of carbohydrates. However, a much more general criticism of this heavy dependence on determinations of carbohydrate utilization can be made. For most nutritionally versatile aerobic pseudomonads, sugars and sugar alcohols represent at best a very small fraction of the potentially utilizable range of carbon sources, so that, if tests are confined to this chemical class, other diagnostically more valuable nutritional characters may be completely ignored. Indeed, as we shall show, several nutritionally versatile species are virtually or completely unable to use sugars and sugar alcohols as carbon sources. In recent taxonomic studies, the ability to utilize malonate and citrate have been commonly determined; these again are tests which obviously derive from work on coliform bacteria, and are not necessarily informative when applied to aerobic pseudomonads. Some workers (Lysenko, 1961; Davis & Park, 1962; Park, 1962; Iizuka & Komagata, 1963*a*, *b*) have tested growth on a limited range of additional compounds (aromatic substances, organic acids, alcohols), but these attempts to extend the nutritional spectrum have been at best half-hearted.

Despite superficial similarities, the aerobic pseudomonads differ very greatly from coliform bacteria in most biochemical and physiological respects. A good methodology for the study of aerobic pseudomonads is likely to be one which probes deeply their most striking and ecologically significant group character, not shared to any degree by coliform bacteria: namely, the ability to use a wide variety of organic compounds as carbon and energy sources for aerobic growth. The foundations for this approach were laid by den Dooren de Jong (1926) in a study of many strains, both fluorescent and non-fluorescent. Unfortunately, the major part of this study was published in Dutch in a thesis of limited distribution, with the result that it has

been completely ignored in all later taxonomic studies dealing with the aerobic pseudomonads. In view of its importance, a brief summary appears warranted.

The problem which den Dooren de Jong set out to study was the nature of the bacterial flora responsible for the aerobic mineralization of simple organic compounds. In order to obtain some idea of the nutritional versatility of known bacterial types, he first examined the ability of 13 different strains of aerobic bacteria to grow at the expense of several hundred different organic compounds, supplied individually as sole sources of carbon and energy in an otherwise mineral medium. The strains included three bacilli, five coliform bacteria, *Mycobacterium phlei*, a *Micrococcus* species, a *Sarcina* species, a *Spirillum* species, and *Pseudomonas fluorescens*. Only *M. phlei* and *P. fluorescens* proved capable of using a varied and extensive range of organic compounds. In the light of present knowledge, this series of experiments cannot be considered satisfactory, since some of the species that were tested (e.g. *Bacillus polymyxa* and *Proteus vulgaris*) are now known to require growth factors, not supplied in the basal medium of den Dooren de Jong. The apparently restricted range of compounds able to support their growth may have reflected the nutritional inadequacy of the basal medium.

Many of the organic compounds included in this initial survey were unable to support growth of any of the bacteria tested. Fifty of these compounds—representing a chemically very heterogeneous assortment—were then used individually as carbon and energy sources in aerobic enrichment cultures, inoculated with soil. After inoculation, the cultures were incubated without agitation at 30°. When surface growth occurred, a loopful of material was transferred to a second flask containing a medium of the same composition, and from this flask a pure culture of the predominant organism was isolated by streaking on the homologous agar medium. The procedure of isolation was rigorously selective; den Dooren de Jong reported that each enrichment medium which gave rise to bacterial growth yielded upon plating a virtually uniform population. The bacterial strains obtained from this set of enrichments were compared on the basis of morphology, colony form and pigmentation. The great majority of successful enrichments yielded pseudomonads of two types, designated as group A (15 enrichments) and group C (15 enrichments). Three enrichments yielded mycobacteria. Five, all furnished with methyl- or ethylamines, yielded representatives of a distinctive group of amine-oxidizing bacteria, which den Dooren de Jong subsequently described elsewhere in considerable detail (den Dooren de Jong, 1927). Six enrichments yielded Gram-negative rods which, despite the absence of flagella, were classified by den Dooren de Jong as *Pseudomonas* strains ('group B'). The organisms of group B were probably nutritionally non-exacting representatives of the genus *Moraxella* (Audureau, 1940; Lwoff & Audureau, 1941), to judge from the one authentic surviving strain that we have been able to examine.

Every strain isolated from this series of enrichments was subjected to very detailed nutritional study, during which ability to grow on over 200 organic compounds, furnished as sole sources of carbon and energy, was determined. This analysis revealed that the *Pseudomonas* strains of groups A and C, initially distinguished on the basis of colony appearance and pigmentation, were likewise different from one another with respect to nutritional spectra. The strains of group A were all typical fluorescent pseudomonads which did not liquefy gelatin, and were therefore

assigned by den Dooren de Jong to the species *P. putida*. The strains of group C were non-pigmented. Their nutritional homogeneity, coupled with their manifold and clear-cut nutritional differences from the group A strains, led den Dooren de Jong to create for them a new species, which he named *P. acidovorans*.

Many other strains of fluorescent pseudomonads were isolated by den Dooren de Jong by less selective methods. Eight were obtained from cultures furnished with complex organic materials (e.g. peptone, yeast autolysate, fibrin) and inoculated with soil, and 34 by the direct plating of tap water or river water on yeast agar. These additional strains were also screened, though less extensively, in order to establish their nutritional spectra.

The analysis of this great body of nutritional information reveals many facts of both ecological and taxonomic interest. In all, some 90 organic compounds could be utilized as carbon and energy sources by one or more of the strains examined. Considering only data for *Pseudomonas acidovorans* and the fluorescent group, these compounds can be divided into three categories: 'universal' substrates, attacked by nearly all strains tested; 'species-specific' substrates; and, lastly, substrates utilized by occasional strains of both groups.

The central core of universal substrates included the acids of the tricarboxylic acid cycle; lactic, pyruvic and gluconic acids; several lower fatty acids; and several amino acids, notably alanine, aspartic and glutamic acids, leucine, isoleucine, phenylalanine and tyrosine. The most unusual universal substrates were saccharic acid, mucic acid, quinic acid, uric acid and *p*-hydroxybenzoic acid.

The utilization of other compounds proved to be species-specific. The higher dicarboxylic acids (adipic to suberic) could not be attacked by any of the fluorescent pseudomonads tested, but were excellent substrates for *Pseudomonas acidovorans*. Conversely, six amines (including putrescine, spermine and benzylamine) could be attacked by nearly all fluorescent pseudomonads, but by no strain of *P. acidovorans*. The two groups also differed markedly with respect to utilization of carbohydrates. Apart from one strain of *P. acidovorans* reported to grow on glucose, strains of this species could not attack any of the five common aldopentoses and aldohexoses tested. Glucose was a universal substrate for the fluorescent pseudomonads, and many of them could attack other aldoses as well. Two amino acids served as excellent differential substrates. Arginine could be attacked by 14 out of 15 fluorescent pseudomonads, and by no strain of *P. acidovorans*; α-aminocaproic acid was used by 12 strains out of 15 of *P. acidovorans*, and by not one strain of fluorescent pseudomonad out of 57 tested. Suggestive, but less clear-cut, differences existed with respect to the utilization of aromatic substrates. Benzoic acid was a universal substrate for fluorescent pseudomonads, but was used by only a small minority of strains of *P. acidovorans*; the converse pattern occurred with respect to utilization of *m*-hydroxybenzoic acid.

This analysis accordingly permitted a distinction on many different nutritional criteria between the simple fluorescent pseudomonads and a second, nutritionally versatile group of non-pigmented pseudomonads, the bacteria for which den Dooren de Jong created *Pseudomonas acidovorans*. The total neglect which befell this work is strikingly shown by the fact that no later paper on the taxonomy of the aerobic pseudomonads so much as mentions *P. acidovorans*, even though it is without doubt one of the best-characterized species in the genus *Pseudomonas*. The frequency

of its occurrence in the enrichment cultures of den Dooren de Jong suggests that it is probably a very common soil bacterium; and, as we shall show, it has been repeatedly isolated by later workers, and described under a variety of other names.

Other interesting facts were revealed by den Dooren de Jong's data on the fluorescent pseudomonads. Of the 57 strains belonging to this group which he studied, 23 were isolated from soil, and 34 from water. The source was almost perfectly correlated with the property of gelatin liquefaction: all 34 strains of aquatic origin were liquefiers, whereas only one of the 23 strains from soil possessed this property. The ability to liquefy gelatin is the single criterion that was classically used to distinguish between *Pseudomonas fluorescens* (liquefying) and *P. putida* (non-liquefying). The data of den Dooren de Jong accordingly indicate that the distinction may be ecologically significant. Some of the compounds tested in the nutritional screening of these strains showed promise as differential substrates. Hippuric acid, creatine and α-amylamine were used by virtually all the strains from soil (*P. putida* types), and by no strain of aquatic origin (*P. fluorescens* types). In the light of this work, the later failure of Rhodes (1959) to find any conventional diagnostic criteria of use for the subdivision of a large collection of simple fluorescent pseudomonads from soil and water is interesting. Rhodes concluded that the simple fluorescent pseudomonads constitute a group in which phenotypic variation is essentially continuous; but den Dooren de Jong's study, which was not cited by Rhodes, provides evidence that her phenotypic scanning of these bacteria was of such a nature that taxonomically significant traits had been overlooked. Accordingly, the taxonomic conclusions that she reached, even though buttressed by computer analysis (Rhodes, 1961) cannot be accepted as valid.

Our primary aim in the present study has been to extend the methodology of den Dooren de Jong, by determining detailed nutritional spectra for a wider range of biotypes among the aerobic pseudomonads than those which he examined. We have coupled this with an examination of other characters which seemed to us in the light of previous work to be of potential taxonomic value.

The choice of biological material

Ideally, the definition of a bacterial species should rest on a comparative study of many strains of different origin. Such comparison is necessary to establish whether a given character is species-specific, or peculiar to one or a minority of the strains that compose the species. The minimal number of strains necessary to define a species is certainly arguable, and depends to a considerable extent on the degree of phenotypic isolation between the species in question and its closest congeners. As a rough working rule, we believe that the number of strains examined in the taxonomic analysis of a bacterial group should be at least 10–20 times as great as the anticipated number of species. In order to keep our study within reasonable bounds, we arbitrarily decided to exclude from the analysis certain subgroups. The excluded groups were: the phytopathogens; the aerobic pseudomonads able to use methane, methanol, oxalate, and lower amines; the polysaccharide decomposers; the acetic acid bacteria; and aerobic pseudomonads of marine origin. A few fluorescent strains isolated from sea water were examined, but they were not indigenous marine organisms by the criteria of MacLeod (1965).

Even with these exclusions, the collection eventually assembled consisted of

about 350 strains. In this paper, we shall discuss a total of 267 strains representing the fluorescent group (175 strains), *Pseudomonas acidovorans* and related types (26 strains), *P. multivorans* sp.nov. (19 strains), *P. stutzeri* (17 strains), *P. maltophilia* (23 strains), and *P. alcaligenes* and related types (7 strains). Characterizations of *P. pseudomallei* (26 strains) and *P. mallei* (15 strains) are being published separately (Redfearn, Palleroni & Stanier, 1966); and *P. lemoignei* sp.nov. (1 strain) has been recently characterized by our methods (Delafield *et al.* 1965). We shall draw on the data of these two publications for purposes of comparative analysis. A study of the hydrogenomonads (about 50 strains) is now in progress, and will be the subject of a later report (Davis, Doudoroff, Palleroni & Stanier, in preparation).

METHODS

Standard media

Most media were prepared from a *standard mineral base*, which contained per litre: 40 ml. of $Na_2HPO_4 + KH_2PO_4$ buffer (M; pH 6·8); 20 ml. of Hutner's vitamin-free mineral base (Cohen-Bazire, Sistrom & Stanier, 1957); and 1·0 g. of $(NH_4)_2SO_4$. This basal medium is easy to prepare, and provides all necessary minerals, including trace elements. It is heavily chelated with nitrilotriacetic acid and EDTA, and forms a copious precipitate upon autoclaving. The precipitate redissolves as the medium cools, to form a water-clear solution. Yeast extract (YE) medium was prepared by adding Difco yeast extract 5 g./l. to the standard mineral base, and yeast agar (YA) medium by the further addition of Difco agar 20 g./l. These complex media were used for the routine cultivation, purification and maintenance of stocks.

Chemically defined media were prepared by adding an appropriate organic carbon and energy source to the standard mineral base; the concentration was generally 1–3 g./l.

Unless otherwise stated, cultures were incubated at 30°. Stock cultures were transferred at monthly intervals, and stored at 5° after growth had occurred.

Screening of organic compounds as sources of carbon and energy for growth

Every strain was tested for ability to grow at the expense of 146 different organic compounds. These tests were performed by replica plating (Lederberg & Lederberg, 1952). The test media were prepared by adding each organic compound at the appropriate concentration to the standard mineral base, solidified by the addition of 1% (w/v) of Ionagar. In order to avoid browning of the agar, it was sterilized separately from the mineral base (both at double strength), and mixed with it after removal from the autoclave. Each test series included a control plate without added organic compound. The use of Ionagar in place of less highly purified agars almost completely eliminated background growth on the control plates, which greatly facilitated the reading of the results.

The choice of substrate concentration in a nutritional survey of this nature is a delicate problem. If the concentration is too high, false results can occur. False negative results may be caused by toxicity; and false positive ones by the presence of utilizable organic contaminants in an intrinsically non-utilizable chemical. For this

reason, it is advisable to keep the substrate concentration as low as possible. As a general rule, we used a concentration of 0·1 % (w/v). For organic acids and bases, this concentration expressed the weight of the organic anion or cation, even though they were added to the medium as salts. The only exceptions were sugars used at a concentration of 0·2 % (w/v), and phenol 0·025 % (w/v). This concentration of phenol, although low, still allows unambiguous growth of positive strains. A concentration of 0·05 % is already toxic to some strains which are capable of using phenol.

The above-mentioned concentrations support good growth by strains that are able to use the compound under test, provided that the plate is not too crowded. In order to avoid early substrate exhaustion, the plates were furnished with a relatively large volume of medium (35 ml.), and the number of patches placed on each plate was limited to a maximum of 23.

Many of the organic compounds used were of unknown thermostability. When thermostability was known, the compounds were sterilized by autoclaving with the mineral base, except for volatile compounds, which were sterilized by filtration. When thermostability was in doubt, the compounds were always sterilized by filtration. Geraniol and naphthalene, which are volatile hydrophobic substances, were not sterilized or added to the medium, but placed in the inverted lid of the Petri dish after its inoculation; their effective concentrations in the medium are accordingly unknown.

For successful replication of the highly motile aerobic pseudomonads, it is essential to use well-dried agar plates. To ensure this, the uninoculated plates were placed in an incubator at 37° for 2–3 days after their preparation. Once dry, they were stored at room temperature in plastic bags, which minimized both contamination and further water loss. They could be kept for 4–5 weeks before use.

When a group of strains was to be screened, a set of master plates was prepared by hand patching on yeast agar. After growth had occurred, each plate could be used to prepare as many as ten submasters by replication on the same medium. When the submaster plates had grown, each one could be used to print nine different test plates, followed by a terminal yeast agar plate to control the successful replication of all patches. Accordingly, a single master plate could be used to test a maximum of 23 different strains on a maximum of 90 different substrates. A few strains produced growth on solid media which could not be successfully replicated, or else did not grow well on yeast agar; they were screened by inoculating the test plates with a small drop of a liquid culture, grown to a carbon limit.

The inoculated test plates were first scored after 48 hr, and examined again after a further 48 hr. Geraniol plates, on which growth was very slow, were scored only after 7 days of incubation. Scoring was visual. The growth was recorded as: 0 (growth no greater than on the control plate without carbon source); + (good growth); ± (scanty growth, but significantly more than on the control plate); and +M (late growth of a few colonies, in the area of the patch, presumed to arise from mutants in the inoculum).

When strains of markedly different nutritional character were tested together on a plate, the results were sometimes difficult to interpret, either because of cross-feeding (particularly marked with sucrose and acetamide) or of starvation of slowly growing patches surrounded by rapidly growing ones. In view of these problems,

the initial random screening was followed by a second series of screenings, in which the strains were re-assorted so that only those of one nutritional type were printed on one set of plates.

Instances of marginal growth on plates (dubious ± values) were further examined by tests of growth in liquid media, where the yield could be determined quantitatively by turbidimetry. This control proved important in some cases, since relatively little total growth on the plates suffices to produce a ± reading. Our experience with sucrose utilization provides a good example, and also illustrates the danger of assuming too lightly that a particular compound is thermostable. We at first sterilized this non-reducing disaccharide either by autoclaving a concentrated aqueous solution (25%, w/v) and mixing with the medium after sterilization, or by autoclaving the medium containing the sucrose in the final concentration. In the plate screenings, almost every fluorescent pseudomonad was scored as either + or ± on sucrose, despite the fact that some members of this group (notably *Pseudomonas aeruginosa*) have been reported by many workers not to attack sucrose, as judged by tests with the medium of Hugh & Leifson (1953). Tested in liquid sucrose media, most of the strains scored as ± showed very low growth yields. The whole collection was then retested on plates prepared with filter-sterilized sucrose; practically all the strains originally scored as ± now failed to grow. Evidently, sucrose does decompose to a slight extent on autoclaving in aqueous solution, and some of the decomposition products are utilizable for growth by nutritionally versatile aerobic pseudomonads. This slight thermal instability does not interfere with tests for sucrose utilization assessed in terms of acid production.

The organic compounds tested as substrates were:

(*a*) Carbohydrates and sugar derivatives: D-ribose, D-xylose, D-arabinose, L-arabinose, D-fucose, L-rhamnose, D-glucose, D-mannose, D-galactose, D-fructose, sucrose, trehalose, maltose, cellobiose, lactose, starch, inulin, gluconate, 2-ketogluconate, saccharate, mucate, salicin.

(*b*) Fatty acids: acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate, heptanoate, caprylate, pelargonate, caprate.

(*c*) Dicarboxylic acids: oxalate, malonate, succinate, maleate, fumarate, glutarate, adipate, pimelate, suberate, azelate, sebacate, eicosanedioate.

(*d*) Hydroxyacids: D-malate, L-malate, D-(−)-tartrate, L-(−)-tartrate, *meso*-tartrate, DL-β-hydroxybutyrate, DL-lactate, glycollate, DL-glycerate, hydroxymethylglutarate, poly-β-hydroxybutyrate.

(*e*) Miscellaneous organic acids: citrate, α-ketoglutarate, pyruvate, aconitate, laevulinate, citraconate, itaconate, mesaconate.

(*f*) Polyalcohols and glycols: erythritol, mannitol, sorbitol, *meso*-inositol, adonitol, glycerol, ethyleneglycol, propyleneglycol, 2,3-butyleneglycol.

(*g*) Alcohols: methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, geraniol.

(*h*) Non-nitrogenous aromatic and other cyclic compounds: D-mandelate, L-mandelate, benzoylformate, benzoate, *o*-hydroxybenzoate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, phthalate, isophthalate, terephthalate, phenylacetate, phenylethanediol, naphthalene, phenol, quinate, testosterone.

(*i*) Aliphatic amino acids: glycine, L-α-alanine, D-α-alanine, β-alanine, L-serine, L-threonine, L-leucine, L-isoleucine, DL-norleucine, L-valine, L-aspartate, L-gluta-

mate, L-lysine, DL-arginine, DL-ornithine, DL-citrulline, DL-α-aminobutyrate, γ-aminobutyrate, DL-α-aminovalerate, δ-aminovalerate.

(*j*) Amino acids and related compounds containing a ring structure: L-histidine, L-proline, L-tyrosine, L-phenylalanine, L-tryptophan, D-tryptophan, L-kynurenine, kynurenate, anthranilate, *m*-aminobenzoate, *p*-aminobenzoate.

(*k*) Amines: methylamine, ethanolamine, benzylamine, putrescine, spermine, histamine, tryptamine, butylamine, α-amylamine.

(*l*) Miscellaneous nitrogenous compounds: betaine, sarcosine, creatine, hippurate, pantothenate, acetamide, nicotinate, trigonelline.

(*m*) Paraffin hydrocarbons: *n*-dodecane, *n*-hexadecane.

In all cases, the purest commercially available preparations were used.

Hydrocarbon utilization. The utilization of hydrocarbons as sole sources of carbon and energy cannot easily be determined on solid media because of the difficulty of obtaining a properly emulsified preparation of these water-insoluble compounds. This property was therefore determined in tubes containing 4–5 ml. liquid mineral medium to which a few drops of the compound had been added under aseptic conditions from a filter-sterilized sample. In our tests we have only used highly purified *n*-dodecane and *n*-hexadecane. The tubes were incubated at 30° on a rotary shaker and the results recorded daily for a period of 7 days.

Tests for chemolithotrophic growth with H_2

Our standard mineral base was not suitable for growth at the expense of H_2; nearly all hydrogenomonads tested grew poorly or not at all chemolithotrophically when tested in it, although they grew excellently at the expense of organic compounds in the same basal medium. Consequently, the simple non-chelated mineral base of Doudoroff (1940) was used in all tests for chemolithotrophy. It contains (per litre): 33 ml. $Na_2HPO_4 + KH_2PO_4$ buffer (M, pH 6·8); 1 g. NH_4Cl; 0·5 g. $MgSO_4.7H_2O$; 0·05 g. $FeCl_3.6H_2O$; 0·005 g. $CaCl_2$. The last two ingredients were autoclaved separately at tenfold strength, to avoid formation of a precipitate. Solid medium was prepared by addition of Ionagar to 10 g./l. (separately sterilized).

Tests were performed by patching strains on plates of this medium and incubating them at 30° in a desiccator containing an atmosphere of (%, v/v) 65, hydrogen; 5, CO_2; 6, oxygen; 24, nitrogen. Control plates were incubated in air. When growth on the experimental plate was no greater than on the control after incubation for 2 weeks, the strain in question was presumed to be incapable of growing chemolithotrophically at the expense of H_2. All strains which appeared positive by the plate test were examined for their ability to grow in the homologous liquid medium under the same conditions of incubation, and only when growth also occurred under these conditions were they deemed to be hydrogen chemolithotrophs.

Tests for denitrification

Tests were conducted in yeast extract (YE) medium, supplemented with 10 g. glycerol, 10 g. KNO_3 and 1 g. Ionagar/l. Each culture was first grown for 24 hr in an unsealed tube containing 5 ml. of this medium incubated without mechanical agitation. A loopful of this semi-aerobic culture was transferred to a tube containing 10 ml. of the same medium, molten but cooled to about 40°. The contents were mixed, and the tube was chilled briefly to allow the medium to become semi-solid. An

overlayer of 2–3 ml. of 1·0 % (w/v) Ionagar in water provided an anaerobic seal and a trap for gas production. Vigorous denitrifiers like *Pseudomonas aeruginosa* produce dense turbidity in this medium after 18–24 hr of incubation at 30°, soon followed by abundant gas production. Other denitrifying pseudomonads develop more slowly, and the tubes should be incubated 5 days before recording a negative result. With some strains, heavy anaerobic growth occurred without visible gas production; such strains were reported as positive. Pre-cultivation in the same medium under semi-aerobic conditions is important, since in some species prolonged aerobic cultivation in a nitrate-free medium seems to result in a loss or weakening of denitrifying ability, presumably as a result of mutation and selection. The pre-cultivation step selects once more for denitrifying ability, and this ensures prompt growth of potential denitrifying strains when placed under rigorously anaerobic conditions.

We have also examined growth under denitrifying conditions in defined media, prepared and used as described above. These media were devoid of yeast extract, and furnished with either glycerol or Na lactate (0·5 %, w/v) as carbon and energy source. The results were closely comparable, but some organisms (notably *P. stutzeri*) grew and produced gas more promptly in the defined media.

Determination of pigment production

Ability to produce specific pigments is a very useful determinative character among aerobic pseudomonads. However, synthesis of the two commonest types of pigments found in these bacteria (water-soluble fluorescent pigments and phenazine pigments) is highly dependent on the composition of the medium, and hence not manifested under all conditions of growth. King, Ward & Raney (1954) developed empirically two complex media, with different mineral additions (media A and B, hereafter termed 'King A' and 'King B') which are very helpful in detecting these two classes of pigments. King A favours the production of phenazine pigments, though some strains will also produce a slight amount of fluorescent pigment on it; King B favours the production of fluorescent pigment, and in most cases completely suppresses production of phenazine pigments. Although these media were devised specifically for the differentiation of *Pseudomonas aeruginosa* from non-phenazine-producing fluorescent pseudomonads, we found them to be satisfactory for the recognition of two other phenazine producers, *P. chlororaphis* and *P. aureofaciens*. They are prepared as slopes, and inoculated by streaking. Cultures were examined for pigmentation after 24, 48, and 72 hr (incubation at 30°). Véron (1961) recommended longer periods of incubation, but we have not found this to be necessary.

The phenazine pigments characteristic of *Pseudomonas aeruginosa* and *P. aureofaciens* (pyocyanine, phenazine α-carboxylic acid, respectively) are water-soluble, and can be recognized by the characteristic colours that they impart to slopes of King A. One phenazine pigment of *P. chlororaphis*, chlororaphin, is very sparingly soluble in water, and accumulates as isolated green crystals in the growth at the butt of a King A slope; the other pigment, oxychlororaphin, is relatively soluble, and imparts a pale yellow colour to the slope.

One other fluorescent pseudomonad, *Pseudomonas lemonnieri*, is characterized by the production of a water-insoluble chloroform-soluble blue pigment which is probably

a phenazine (Starr, Blan & Cosens, 1960). The blue pigment could not be elicited by growing the organisms in the King media. Production of the blue pigment also failed in both the complex and the chemically defined media used throughout our work. In investigating this point further, we found that the pigment could be formed on a solid medium of the following composition (%, w/v): Bacto peptone, 0·5; glucose, 1·0; Bacto agar, 2; in tap water. The pigment was not produced when the medium contained in addition phosphate buffer (M/30, pH 6·8). Two strains of our collection (83, 84), which had the same origin but were received from different laboratories, produced the blue pigment. The pigment appeared after 24 hr of incubation in the colonies of strain 84, while the colonies of strain 83 showed after a few days several blue-pigmented sectors. Strain 143 totally failed to produce the blue colour even after long incubation, although the pigment had been observed in the colonies of the original isolate. The reappearance of the blue variants in strain 83 and the apparent total loss of the character in strain 143 suggest that the production of the blue pigment is a property that is counter-selected under conditions of laboratory maintenance.

Among the non-fluorescent pseudomonads, many strains of the species that we designate as *Pseudomonas multivorans* produce phenazine pigments of undetermined structure. Apparently two such pigments may be synthesized, both of which are water-soluble under neutral or alkaline conditions; one is yellow and the other is purple (Morris & Roberts, 1959). Strains belonging to this species may produce both pigments, yellow pigment, or none. Both pigments are formed abundantly on King A medium, which is accordingly a useful medium for detection. Both the organisms and the slope appeared coloured.

The majority of the strains of *Pseudomonas maltophilia* produce a pale yellow cellular pigment which we have not studied in detail. This pigment is soluble in water and is not extracted by any of the organic solvents used for the carotenoid pigments. We have also noted that the strains of *P. stutzeri* are capable of producing a faintly yellow diffusible pigment on the King B medium. This pigment is not fluorescent and has not been further characterized.

Biochemical methods

Screening for ring fission mechanisms. In recent years it has become evident that aerobic pseudomonads can de-aromatize two of the central intermediates in the metabolism of aromatic compounds, (catechol, protocatechuic acid), by two different mechanisms, *ortho* and *meta* cleavage (Dagley, Evans & Ribbons, 1960). These two modes of cleavage channel the degradation of a given aromatic compound after ring fission into two completely different metabolic pathways. Hence it seemed probable *a priori* that the mode of cleavage would be a taxonomically significant character. Two strains may both utilize *p*-hydroxybenzoate as a growth substrate; on this nutritional character, they appear identical. But, if it can be shown that they use different cleavage mechanisms for attack on the benzenoid nucleus, a whole series of enzymic differences is at once revealed. The problem was to devise a simple way of testing for the difference. This was solved by Dr Keiichi Hosokawa, whose elegant unpublished method we systematically applied to the collection.

Strains are grown as patches on chemically defined media containing an aromatic substrate as sole source of carbon and energy. We have most commonly used

p-hydroxybenzoate as a substrate; this compound is decomposed via protocatechuate. Each patch is scraped off and suspended in 0·02 M-tris buffer (pH 8·0). The suspension is toluenized and 2 ml. samples are furnished with 20 μmoles of either catechol or sodium protocatechuate. When *meta* cleavage occurs, the mixture turns bright yellow within a few minutes. The tube is shaken for 1 hr at 30°, and then tested for the presence of β-ketoadipate (indicative of an *ortho* cleavage) by the Rothera reaction (Kilby, 1948). An unusual deep purple Rothera reaction is given by this β-keto acid. The development of this colour is strong presumptive evidence of *ortho* cleavage. A parallel control experiment, using patches of organism grown on yeast agar plates, was always done to determine whether the observed activity had been specifically induced by growth on the aromatic substrate.

Assay of the arginine dihydrolase system. We assayed this dihydrolase system as follows. The organisms, grown on mineral+lactate medium, were suspended in M/30 phosphate buffer (pH 6·8) to an optical density of 200 Klett units (green filter 54). Four ml. of bacterial suspension in a test tube were gassed with N_2, and 1 ml. of 10^{-3} M-arginine was added. The mixture was gassed again with N_2 and the tube stoppered and incubated at 30° for 2 hr. At the end of the incubation, the tube was immersed in a boiling water bath for 15 min., and arginine determined in a 1 ml. sample. As control, we used an identical suspension which was heated immediately after the addition of arginine, and thereafter subjected to the same treatment. Arginine was determined by the quantitative method of Rosenberg, Ennor & Morrison (1956).

Oxidase test. The oxidase test was performed by soaking a strip of filter paper with 2–3 drops of a 1% (w/v) aqueous solution of *N*,*N*′-dimethyl-*p*-phenylenediamine, and immediately smearing a loopful of bacteria from a slope culture on the moist area. Oxidase-positive strains turn dark red within a few seconds. The test can also be performed with 0·2% (w/v) aqueous 2,6-dichlorophenolindophenol, first reduced with dithionite by careful addition of the reducing agent until the blue colour just disappears. With this reagent, oxidase-positive strains turn blue.

Determination of cytochrome difference spectra. Bacteria were grown overnight in shaken flasks of yeast extract medium, harvested, washed, and resuspended in 0·03 M-phosphate buffer (pH 6·8) to a concentration equiv. 2·0–2·5 mg. dry-wt./ml. Two samples of this suspension were placed in cuvettes (1 cm. light path), and gassed with N_2 (O_2-free) for at least 2 min. The absorbances were compared over the range from 390 to 650 mμ, by using a Cary recording spectrophotometer with a sensitive slide wire (total optical density span: 0·2 unit). In one cuvette, the components of the electron transport system were then oxidized by the addition of 3 drops of 3% H_2O_2, and this suspension was immediately used as a blank to record absorbance over the same spectral range in the reduced suspension. The difference spectrum so obtained (reduced minus oxidized), corrected for minor differences of absorbance (reduced minus reduced) caused by the fact that absorbances of the two cuvettes are never exactly comparable at this degree of sensitivity, shows the peaks of the reduced components of the cytochrome system. To identify precisely the peaks in the Soret (γ) region, the determination must be repeated with a more dilute suspension.

Production of extracellular enzymes. The capacity to produce extracellular proteases was determined with gelatin as substrate, by the plate method described by

Skerman (1959). Patches of growth on plates of Skerman's medium were allowed to grow for 48 hr, and the plates then flooded with an acidic solution of mercuric chloride, which precipitated the unhydrolysed gelatin in the agar. When the clear zone around the patch was not more than 2 mm. in extent, gelatinase production was recorded as weak ($\pm$). Wider zones of clearing were recorded as +. Some strains showed partial clearing under the patch, not extending beyond its edge. These we recorded as −, since the clearing might simply reflect partial autolysis of the bacteria with liberation of proteolytic endoenzymes.

The egg-yolk reaction, useful for internal subdivision of fluorescent pseudomonads, was done on the medium recommended by the *Manual of Microbiological Methods* (1957), patched with the strains to be tested. A positive reaction is shown by the formation of a white precipitate surrounding the patch of growth, which generally appears after 24 hr, although readings should be continued for 6 days.

Starch hydrolysis was tested by patching strains on yeast agar supplemented with 0·2 % (w/v) soluble starch, and flooding the plates with Lugol's solution after incubation for 48 hr.

The production of extracellular lipases was tested by patching strains on the Tween 80 medium recommended by Sierra (1957). Hydrolysis of the detergent is shown by formation in the medium of a precipitate of calcium oleate. The time of appearance of a turbid halo surrounding the patch of growth is very variable; we arbitrarily scored as positive those strains which showed such a halo after not more than 6 days of incubation. A weak reaction occurring after a longer period may simply reflect autolysis of the bacteria and liberation of intracellular esterases.

The production of an extracellular esterase capable of depolymerizing poly-β-hydroxybutyric acid was determined by patching strains on mineral agar plates overlayered with a suspension of poly-β-hydroxybutyric acid (0·25 %, w/v) in mineral agar. Positive strains showed growth of the patch and clearing of the surrounding medium. This is a test of great taxonomic value among aerobic pseudomonads, but unfortunately the substrate, a natural product not commercially available, is tedious to prepare. We used polymer granules extracted from *Bacillus megaterium* and prepared as described by Delafield *et al.* (1965).

Cytological observations

We examined sizes of organisms and were unable to find consistent differences among the strains of different species; therefore, no mention will be made of this character.

Flagellar staining was done by the method of Leifson (1951) on suspensions of bacteria prepared from young slope cultures on yeast agar. In evaluating flagellation as 'monotrichous' or 'multitrichous', we used where necessary the statistical method of analysis recommended by Lautrop & Jessen (1964).

The accumulation of poly-β-hydroxybutyric acid as an intracellular reserve material is an extremely valuable taxonomic character among aerobic pseudomonads. It was detected by microscopic examination of bacteria under phase contrast, and confirmed where necessary by staining smears with Sudan Black or by chemical extraction (Williamson & Wilkinson, 1958). The accumulation of cellular organic reserve materials is favoured under conditions of nitrogen starvation (Doudoroff & Stanier, 1959); and in some purple bacteria, which can form both

glycogen and poly-β-hydroxybutyrate reserves, the formation of poly-β-hydroxybutyrate is dependent on the provision of an organic substrate metabolically closely related to it. These considerations led us to determine poly-β-hydroxybutyrate accumulation on bacteria grown in a special chemically defined medium, consisting of standard mineral base with $(NH_4)_2SO_4$ at a low concentration (0·2 g./l.), furnished with DL-β-hydroxybutyrate (5 g./l.) as carbon and energy source. The medium was used in liquid form (5 ml./tube); cultures were mechanically agitated, and examined after 24–48 hr, when growth had become nitrogen limited. Under these circumstances, polymer-accumulating species regularly contained extensive intracellular deposits of the polymer, readily observable by microscopic examination of wet mounts. Although β-hydroxybutyrate is a carbon and energy source for most aerobic pseudomonads, a few cannot use it. For such organisms, either Na acetate or Na succinate (5 g./l.) was used instead.

Temperature relationships

Ability to grow at 4, 37, 39 and 41° was determined in tubes of yeast extract medium, inoculated with a loopful of culture grown for 12–18 hr in the same medium at 30°. The tubes incubated at 4° were read after 10 days, the others after 24 hr. Growth was recorded as abundant (+) or slight (±). Water baths were used for all determinations; the cultures were not shaken.

Nitrogen sources and growth factor requirements

With the exception of *Pseudomonas maltophilia*, all species described in this paper grow well in standard chemically defined medium, furnished with NH_4^+ as nitrogen source and any utilizable organic carbon and energy source. No strain of *P. maltophilia* will grow under these conditions. It was therefore necessary to determine the minimal nutritional requirements of this species, as a preliminary to examining its carbon and energy requirements. Tests with supplements of amino acids, vitamins, purines and pyrimidines led to the discovery that all strains of this species require methionine, but no other organic growth factor. (In the process of screening amino acids, we observed that leucine was also stimulatory; but this effect is probably attributable to the fact, reported by Demain (1965), that many commercial preparations of leucine are contaminated with methionine.)

All chemically defined media used for the growth and nutritional screening of *P. maltophilia* accordingly had to be supplemented with methionine, which was added to 20 mg./l.

All strains of the collection were tested for their ability to use nitrate as a nitrogen source, and to fix atmospheric nitrogen. Such tests were conducted in tubes of the standard mineral base, in which NH_4^+ was either replaced by KNO_3 (1 g./l.) or omitted. Lactate (1 g./l.) was provided as source of carbon and energy.

Determination of base composition of DNA

The moles % of guanine + cytosine (GC) in the DNA of many of our strains was determined by Dr Manley Mandel, from measurements of buoyant density in CsCl gradients (Mandel, 1966).

Miscellaneous tests

Certain *Pseudomonas* species are reported to produce indole (Haynes, 1957). However, during an extensive survey of the mechanisms of tryptophan dissimilation by pseudomonads (Stanier & Hayaishi, 1951) the one strain found to produce indole (Tsuchida, Hayaishi & Stanier, 1952) was later shown to have been mis-identified, and to be in reality a coliform bacterium (Stanier, 1952). There is, accordingly, no reliable biochemical evidence for the occurrence of tryptophanase among aerobic pseudomonads. To resolve this question, we tested the entire collection for indole production by the customary procedures (Skerman, 1959). The only organisms which gave a pink colour upon addition of the Ehrlich reagent to the culture were certain strains of *P. aeruginosa*, which had formed pyocyanine during growth. Since pyocyanine is red in the acid form, these strains were retested for indole production by the Goré (1921) modification of the usual reaction, in which detection of indole is dependent upon its volatility. The results were then totally negative. We conclude that no aerobic pseudomonad so far studied is able to produce indole, and that this test is diagnostically valueless in the taxonomic analysis of the group. False positives can result from the presence of pyocyanine in the medium.

The oxidation of indole to the insoluble blue compound, indigotin, originally described by Gray (1928) as the salient property of *P. indoloxidans*, was tested on plates of the medium recommended by Gray, except that Na acetate was substituted for glycerol as the carbon source for strains unable to use glycerol. In positive strains, the patch and the adjacent agar turn blue, as a result of the deposition of crystals of indigotin.

Tests for the capacity to grow anaerobically with glucose as a fermentable substrate were conducted in tubes of yeast extract medium supplemented with 1 % (w/v) glucose and 0·1 % (w/v) Ionagar, and overlayered after inoculation with a plain agar seal.

Biological materials

The nucleus of the collection consisted of unidentified *Pseudomonas* strains isolated in our laboratory in Berkeley by enrichment methods over the past 20 years. It was supplemented by many additional strains received from other sources. Before inclusion, each strain was streaked on yeast agar to check its purity and homogeneity. Many of the strains proved to be heterogeneous in colony form. The most common type of colonial heterogeneity was represented by a mixture of relatively translucent, flat colonies and more opaque, raised, compact colonies. This was particularly frequent among fluorescent pseudomonads, and, since our past experience with this group indicated that the translucent flat colony is the wild-type form, we attempted as far as possible to select this form for the establishment of stock cultures. In a few cases where both colony forms were isolated from a single strain and subjected to detailed analysis, no other phenotypic differences between them were observed.

Each strain was also examined for Gram reaction, motility, and ability to grow anaerobically with glucose. Only strains which were Gram-negative, actively motile straight or curved rods and incapable of anaerobic growth with glucose were included.

At the time of its addition to the collection, each strain was given a serial number ($1 \rightarrow n$), and we shall designate strains by these numbers in the discussion of the results. Every strain has now been placed in the American Type Culture Collection, and therefore also carries an ATCC accession number. Specific information on sources, past histories, and ATCC or other collection numbers will be given in the strain descriptions, which are included with the descriptions of the species groups or the individual species.

RESULTS

Primary differentiation of species and species groups

Nearly all the 267 individual strains that we have examined can be assigned to one of 10 species (Table 1). Fortunately, eight of these species have been named by earlier workers. Some can be placed in subgeneric categories which share many common properties, and which we term 'groups'. Taxonomically, the most complex

Table 1. *Specific assignments of 267 individual strains examined in the present study*

Subgeneric group	Species	No. of strains
Fluorescent group	*Pseudomonas aeruginosa*	29
	P. fluorescens	94
	P. putida	41
	Unassigned	11
Acidovorans group	*P. acidovorans*	15
	P. testosteroni	9
	Unassigned	2
Alcaligenes group	*P. alcaligenes*	1
	P. pseudoalcaligenes sp.nov.	6
	P. multivorans sp.nov.	19
	P. stutzeri	17
	P. maltophilia	23
Total		267

of these is the 'fluorescent group', in which we recognize three species: *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida*. *P. aeruginosa* shows a high degree of internal phenotypic uniformity, but both *P. fluorescens* and *P. putida* can in turn be subdivided into a number of distinct 'biotypes', to which we assign identifying letters. Some of these fluorescent biotypes may eventually prove worthy of specific recognition; but, in our opinion, such recognition is not now justified. The 'acidovorans group' contains two species, *P. acidovorans* and *P. testosteroni*. The 'alcaligenes group' contains two species, *P. alcaligenes* and *P. pseudoalcaligenes* sp.nov. The remaining three species that we recognize are all phenotypically somewhat isolated, both from the three groups so far described, and from one another. They are *P. maltophilia*, *P. stutzeri* and *P. multivorans* sp.nov.

Tables 2 and 3 list the phenotypic characters which are most useful for distinguishing these 10 species: in effect, these tables constitute a diagnostic key. In order to make these tables as complete as possible, we have also included data for the 'pseudomallei group' (*Pseudomonas pseudomallei* and *P. mallei* comb.nov.), taken from the paper of Redfearn *et al.* (1966); and for *P. lemoignei*, taken from the paper of Delafield *et al.* (1965). These are the only other members of the genus *Pseudomonas* so far fully characterized by our methods. Other described species which are

probably valid ones (e.g. *P. putrefaciens* and *P. diminuta*) cannot for this reason be included in the tables.

Tables 2 and 3 by no means include all the characters that we have found to be of some diagnostic value; in particular, many other diagnostically significant nutritional properties could have been added. In these tables, we have emphasized the characters which in our experience are of greatest *general* value for presumptive specific identification; the lists are minimal. If need be, the differentiation between closely related species can be strengthened by more extensive tests. For example, the difficult differentiation between *Pseudomonas fluorescens* and *P. putida*, which depends on only three of the characters listed in Tables 2 and 3, can be strengthened by determining the reaction on egg yolk, and the utilization of other carbohydrates and higher amines. Similarly, the differentiation between *P. acidovorans* and *P. testosteroni*, which depends on six of the characters listed in Table 3, can be strengthened by testing utilization of fructose, malonate, L-tartrate and D-tryptophan. These are all nutritional characters that we have omitted from Table 3 because they are of relatively restricted diagnostic value.

In Tables 2 and 3, the notation 'v' indicates that the character in question is variable for a particular species. Such characters may still of course be of diagnostic value for certain strains, if related species are uniformly either + or − for the same character. It should be emphasized that many of the physiological characters to which we have assigned '+' values may be absent from an occasional strain, but are overwhelmingly positive for the group in question. For example, we have listed fluorescent pigment production as '+' for all species of the fluorescent group, although non-fluorescent strains occur. Even such a characteristic trait of the fluorescent pseudomonads as the ability to grow on glucose is absent from one of the 175 strains that we examined. In contrast, the physiological characters to which we have assigned '−' values are truly absent from every strain of the species. Where this is not so, we have used the symbol '−(+)' to show that rare strains may possess the character in question. One example is the utilization of *m*-hydroxybenzoate by *Pseudomonas fluorescens* and *P. putida*, positive for a total of seven out of 135 strains assigned to these two species. Despite these sporadic exceptions, failure to grow on *m*-hydroxybenzoate is a useful nutritional character for distinguishing fluorescent pseudomonads as a group from *P. multivorans* and the acidovorans group, where nearly all strains can use it.

Rare exceptions to the predominant specific phenotype do not seriously interfere with the correct specific assignations, since a strain that is aberrant in one or two characters is in almost every instance still identifiable on the basis of the other characters listed in Table 2, 3. Hence these thirty-nine selected characters should suffice to permit a correct presumptive identification of most strains that belong to one of the listed species.

Taxonomic significance of poly-β-hydroxybutyrate metabolism in aerobic pseudomonads

The suggestion of Forsyth *et al.* (1958) that poly-β-hydroxybutyrate accumulation might be a useful differential character among aerobic pseudomonads has been confirmed by our work. In fact, this is probably the best single character for the primary subdivision of the genus *Pseudomonas*. The polymer producers are

Table 2. *General characters of diagnostic value for the differentiation of species of aerobic pseudomonads*

	Fluorescent group			Pseudomallei group*			Acidovorans group		Alcaligenes group				
	P. aeruginosa	*P. fluorescens*	*P. putida*	*P. multivorans*	*P. pseudomallei*	*P. mallei*	*P. acidovorans*	*P. testosteroni*	*P. alcaligenes*	*P. pseudoalcaligenes*	*P. stutzeri*	*P. maltophilia*	*P. lemoignei*†
No. of flagella	1	> 1	> 1	> 1	> 1	0	> 1	> 1	1	1	1	> 1	1
Poly-β-OH-butyrate as cellular reserve	−	−	−	+	+	+	+	+	−	v	−	−	+
Pigments: Fluorescent	+	+	+	−	−	−	−	−	−	−	−	−	−
Pigments: Phenazine	+	v	−	v	−	−	−	−	−	−	−	−	−
Methionine requirement	−	−	−	−	−	−	−	−	−	−	−	+	−
Denitrification	+	v	−	−	+	v	−	−	−	−	+	−	−
Growth at 4°	−	+	v	−	−	−	−	−	−	−	−	−	−
Growth at 41°	+	−	−	v	+	+	−	−	+	+	v	+	−
Extracellular hydrolases: Gelatin	+	+	−	+	+	+	−	−	+	v	+	−	−
Extracellular hydrolases: Poly-β-OH-butyrate	−	−	−	−	+	+	−	v	−	−	−	+	−
Extracellular hydrolases: Starch	−	−	−	−	+	v	−	−	−	−	+	−	−
Oxidase reaction	+	+	+	+	+	+	+	+	+	+	+	−	+
Arginine dihydrolase	+	+	+	−	+	+	−	−	+	v	−	−	−
Cleavage mechanism for diphenols	*ortho*						*meta*		None		*ortho*	None	

* Data of Redfearn *et al.* (1966). † Data of Delafield *et al.* (1965). v = variable.

Table 3. *Nutritional characters of diagnostic value for the differentiation of species of aerobic pseudomonads*

	Fluorescent group				Pseudomallei group*		Acidovorans group		Alcaligenes group					
Utilization as sole carbon and energy source of:	*P. aeruginosa*	*P. fluorescens*	*P. putida*	*P. multivorans*	*P. pseudomallei*	*P. mallei*	*P. acidovorans*	*P. testosteroni*	*P. alcaligenes*	*P. pseudoalcaligenes*	*P. stutzeri*	*P. maltophilia*	*P. lemoignei*†	
D-Fucose	−	−	−	+	+	+	−	−	−	−	−	−	−	
D-Glucose	+	+	+	+	+	+	−	−	−	−	+	+	−	
Trehalose	−	+	−	+	+	+	−	−	−	−	−	+	−	
Cellobiose	−	−	−	+	+	+	−	−	−	−	−	+	−	
Maltose	−	−	−	−(+)	+	v	−	−	−	−	+	+	−	
Starch	−	−	−	−	+	v	−	−	−	−	+	−	−	
Inositol	−	+	−	+	+	+	v	−	−	−	−	−	−	
Mannitol	+	+	−(+)	+	+	+	+	−	−	−	v	−	−	
Geraniol	+	−	−	−	−	−	−	−(+)	−	−	−	−	−	
2-Ketogluconate	+	+	+	+	+	v	−	−	−	−	−	−	−	
Maleate	−	−(+)	−	−	−	−	+	−	−	−	−	−	−	
Glycollate	−	−(+)	−(+)	v	−	−	+	+	−	−	+	−	−	
DL-Lactate	+	+	+	+	+	+	+	+	+	+	+	+	−	
Pelargonate	+	+	+	+	+	v	−	−	+	+	+	−	−	
Adipate	+	v	−	+	+	v	+	+	−	−	−(+)	−	−	
m-hydroxybenzoate	−	−(+)	−(+)	+	−	−	+	+	−	−	−	−	−	
Testosterone	−	−(+)	−(+)	+	−	−	−	+	−	−	−	−	−	
Acetamide	+	−	v	v	−	−	+	−	−	−	−	−	−	
Arginine	+	+	+	+	+	+	−	−	+	+	−	−	−	
Valine	+	+	+	v	+	v	−	−	−	−	+	−	−	
Norleucine	−	−	−	−	−	−	+	+	−	−	−	−	−	
D-Tryptophan	−	−	−	−	−	−	+	−	−	−	−	−	−	
δ-Aminovalerate	+	v	+	+	+	v	+	−	+	+	−	−	−	
Betaine	+	+	+	+	+	+	−	−	−	+	−(+)	−	−	
Putrescine	+	+	+	+	+	v	−	−	−	+	+	−	−	

* Data of Redfearn *et al.* (1966); † Data of Delaficld *et al.* (1965).

\+ = positive. −(+) = usually negative, occasionally positive. − = always negative.

P. multivorans, *P. pseudomallei*, *P. mallei*, *P. acidovorans*, *P. testosteroni* and *P. lemoignei* sp.nov. (Delafield *et al.* 1965). This character is completely absent from the fluorescent group, *P. stutzeri* and *P. maltophilia*. Only the alcaligenes group is variable, though predominantly positive: two out of six strains of *P. pseudoalcaligenes* fail to produce demonstrable polymer accumulations.

In considering these distributional regularities, it is important to realize that the ability to use poly-β-hydroxybutyrate as a cellular reserve material is, biochemically speaking, a complex character. As Merrick & Doudoroff (1961) showed, the polymer is synthesized from poly-β-hydroxybutyryl-CoA by a specific polymerase, and endogenously hydrolysed to the free hydroxy acid by a system which includes a proteinaceous activator, as well as at least two specific hydrolases. A multienzyme system is accordingly involved in the catalysis of this peripheral cycle on the pathway of fatty acid synthesis.

The ability to attack exogenous poly-β-hydroxybutyrate by means of extracellular enzymes is also a taxonomically valuable character. It is possessed by some, but not all, of the species able to synthesize the polymer as a cellular reserve material, and never occurs in species unable to do so. As Delafield *et al.* (1965) showed, the extracellular depolymerase system of *Pseudomonas lemoignei* is quite different from the enzyme system responsible for endogenous hydrolysis of polymer reserves in *Rhodospirillum rubrum* (Merrick & Doudoroff, 1964). Hence the capacity to hydrolyse and grow at the expense of exogenous poly-β-hydroxybutyrate may be an enzymic character independent of the capacity to use the polymer as an intracellular reserve. Species which regularly produce an extracellular depolymerase are *P. pseudomallei* and *P. lemoignei*; the character is variable in *P. testosteroni* and *P. mallei*.

Significance of denitrifying ability

The ability to use nitrate in place of O_2 as a terminal electron acceptor, and hence to grow well anaerobically in a nitrate-containing medium, is another multi-enzyme character that is of considerable taxonomic significance among the aerobic pseudomonads. It is a universal property of *Pseudomonas aeruginosa* (as already shown by Jessen, 1965), *P. pseudomallei*, *P. mallei* and *P. stutzeri*, but is absent from all other species discussed here except *P. fluorescens*. In *P. fluorescens*, some of the subgroups that we recognize as biotypes (B, C, D, F) are uniformly able to denitrify, while others (A, E, G) lack this property. It should be noted that each of these biotypes is distinguishable from the remaining ones by several additional characters, metabolically unrelated to the property of denitrification. Hence denitrification retains some degree of taxonomic significance even within the one species where it is variable, since it serves, in conjunction with other characters, to define biotypes.

The oxidase reaction and its relation to the cytochrome system

The oxidase reaction is positive for every species that we have examined except *Pseudomonas maltophilia*, which was also reported by Thibault (1961) and by Hugh & Leifson (1963) to be oxidase negative. The biochemical basis of the differences between aerobic bacteria in their response to this commonly used diagnostic test is not known. In an attempt to throw some light on this point, we determined dif-

ference spectra (reduced–oxidized) on heavy suspensions of a few representative strains of each species.

The results (Table 4) indicated that the specific cytochrome complement may be an extremely useful taxonomic character for the differentiation of groups and species among the aerobic pseudomonads, although this cannot be definitely established until more strains of each species have been examined. It is particularly notable that the fluorescent, acidovorans and alcaligenes groups all appear internally homogeneous with respect to cytochrome complement. In species that contain a cytochrome of the *c* type, the α-band of the *b*-type cytochrome lies on the shoulder of the *c* peak, and cannot be precisely located; indeed, its very presence in *Pseudomonas stutzeri*, which has an exceptionally high content of *c*-type cytochrome, is somewhat doubtful. However, every species examined shows a β-band at about 527–530 mμ, reportedly characteristic of *b*-type cytochromes (Smith, 1961).

Table 4. *Wavelengths (mμ) of the main absorption peaks in the cytochrome difference spectra of selected strains of the different species of aerobic pseudomonads*

	α-Bands. Cytochrome type			β-Bands. Cytochrome type		Principal Soret band
Species	*a*	*b*	*c*	*b*	*c*	
P. aeruginosa	.	*c.* 560	552	530	523	428
P. fluorescens	.	*c.* 560	552	530	523	427
P. putida	.	*c.* 560	552	530	523	425
P. acidovorans	600	.	553	530	524	425
P. testosteroni	600	.	553	530	523	423
P. alcaligenes	600	*c.* 560	553	530	523	428
P. pseudoalcaligenes	600	*c.* 560	551	528	522	423
P. multivorans	628, 597	*c.* 560	554	527	523	428
P. stutzeri	.	(*c.* 560)	552	530	523	423
P. maltophilia	628, 597	558	.	530	.	430
P. lemoignei	.	557	552	530	523	422

With regard to the interpretation of the oxidase reaction, it can be seen that *Pseudomonas maltophilia* differs from all other species by virtue of not showing an α-band at 551–554 mμ, or a β-band at 522–524 mμ. These spectral features are both characteristic of cytochromes of the *c* type.

Mr Paul Baumann (unpublished data) has found an analogous situation in the genus *Moraxella*, another group of aerobic bacteria that includes both oxidase-positive and oxidase-negative species: only oxidase-positive strains of *Moraxella* have peaks in their difference spectra referable to a cytochrome of the *c* type. These multiple correlations suggest that a positive oxidase test may indicate the presence of a cytochrome *c* in the terminal respiratory chain, although other possible explanations cannot yet be completely excluded.

The constitutive synthesis of arginine dihydrolase and its relation to the utilization of arginine as a carbon source

The anaerobic conversion of arginine to ornithine, ammonia and CO_2 through action of the so-called 'arginine dihydrolase' system (Hills, 1940) was first described in fluorescent pseudomonads by Slade, Doughty & Slamp (1954) and Sherris, Preston & Shoesmith (1957). This is a reaction of great physiological interest, since

it is accompanied by substrate-level generation of a high-energy phosphate bond (Slade *et al.* 1954), and thus constitutes a mechanism for the anaerobic synthesis of ATP. Indeed, as Sherris *et al.* (1957) have shown by simple but very elegant experiments, it provides the energy necessary for the maintenance of motility under anaerobic conditions by fluorescent pseudomonads, which as a rule rapidly become non-motile when their oxygen supply is depleted, like other strict aerobes. Their data suggested that the arginine dihydrolase system is synthesized constitutively by fluorescent pseudomonads, but is inactive on externally supplied arginine in the presence of O_2, becoming functional only under anaerobic conditions. We have confirmed this surprising fact by more rigorous and detailed unpublished experiments. The method which we recommend for the analysis of arginine dihydrolase activity (see section on Methods) gives positive results only when the enzyme system is constitutively synthesized.

Table 5. *The utilization of arginine by selected strains of Pseudomonas species*

Group	Species	No. of strains examined	No. of positive strains: Constitutive arginine dihydrolase	No. of positive strains: Use of arginine as carbon and energy source
Fluorescent	*P. aeruginosa*	4	4	4
	P. fluorescens	3	3	3
	P. putida	3	3	3
	P. multivorans	8	0	8
Pseudomallei*	*P. pseudomallei*	5	5	5
	P. mallei	10	10	10
Acidovorans	*P. acidovorans*	4	0	0
	P. testosteroni	4	0	0
Alcaligenes	*P. alcaligenes*	1	1	1
	P. pseudoalcaligenes	6	4†	5‡
	P. stutzeri	13	0	0
	P. maltophilia	2	0	0
	P. lemoignei	1	0	0

* Data of Redfearn *et al.* (1966).
† Strains 66 and 417 negative.
‡ Strain 417 negative.

Using less precise and stringent conditions for the study of the reaction, Sherris *et al.* (1959) and Thornley (1960) attempted to determine the taxonomic value of arginine dihydrolase production as a general character to distinguish aerobic pseudomonads from other Gram-negative eubacterial rods (e.g. coliform bacteria). Their data (obtained principally on fluorescent strains) suggested that it might be useful for this purpose. We have not examined every strain in our collection for the character of constitutive arginine dihydrolase synthesis, but we have tested a number of typical strains representative of each species; and the results shown in Table 5 are based on these samplings. In confirmation of the results of Sherris *et al.* (1959) and Thornley (1960), we find that every fluorescent strain examined is positive. However, this character is far from universal among the other species

examined, being present only in the pseudomallei and alcaligenes groups. As also shown in Table 5, the ability to use arginine as a carbon and energy source for aerobic growth is not necessarily correlated with the possession of a constitutive arginine dihydrolase system, since arginine is a good growth substrate for *Pseudomonas multivorans*, which does not contain a constitutive arginine dihydrolase system. Other aerobic pseudomonads (the acidovorans group, *P. stutzeri*, *P. maltophilia*, *P. lemoignei*) neither contain arginine dihydrolase, nor use arginine as an aerobic source of carbon and energy. In summary, the ability to synthesize arginine dihydrolase constitutively appears to be a useful character for the internal differentiation of species in the genus *Pseudomonas*, and not a generic trait.

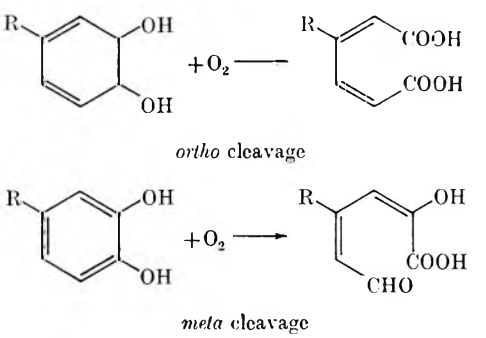

Fig. 1. Mechanisms for the oxygenative cleavage of *ortho*-diphenols among pseudomonads.

Certain aromatic ring cleavage mechanisms

A biochemical character which we have examined systematically for the first time is the mechanism of cleavage of two diphenols, catechol and protocatechuate, the last aromatic intermediates in the oxidative metabolism of many aromatic compounds. Two modes of cleavage of these compounds have been described in various pseudomonads (Dagley *et al.* 1960): so-called '*ortho* cleavage' which results in the formation of a *cis-cis*-muconic acid; and so-called '*meta* cleavage' which results in the formation of an α-hydroxy-*cis-cis*-muconic semialdehyde (Fig. 1). Since the metabolic fates of the two classes of products of aromatic ring cleavage are wholly distinct, the cleavage mechanism in effect determines a long series of subsequent biochemical steps, so that two species which differ in cleavage mechanism differ also in many other specific enzymic respects.

The simple aromatic acid most extensively utilized by aerobic pseudomonads is *p*-hydroxybenzoate, which is degraded via protocatechuate. We have therefore tested the great majority of strains which can grow on *p*-hydroxybenzoate for the mode of ring cleavage of protocatechuate by *p*-hydroxybenzoate-grown organisms. Control tests on organisms of the same strains grown on yeast agar were always done, and usually gave negative results, thus confirming many past reports that the enzymes which mediate this cleavage reaction are strictly and specifically inducible. However, occasional strains of the fluorescent group contained the *ortho*-cleavage enzyme for protocatechuate after growth on yeast extract, suggesting that this enzyme is either partially constitutive, or induced by components of yeast extract in the strains in question.

The results of these tests (Table 6) show that the mechanism of cleavage of protocatechuate is a taxonomically significant character, since all the strains of a given species able to perform this reaction use the same cleavage mechanism. Secondly, it is evident that the *meta* cleavage mechanism is relatively rare, being confined exclusively to the two species of the acidovorans group, *Pseudomonas acidovorans* and *P. testosteroni*. The *ortho* cleavage of protocatechuate is characteristic of the entire fluorescent group, *P. pseudomallei*, *P. multivorans* and *P. stutzeri* (although very few strains of the last-mentioned species are able to grow on *p*-hydroxybenzoate).

Table 6. *Mechanisms for ring cleavage of protocatechuate among aerobic pseudomonads, tested after growth on p-hydroxybenzoate*

Species	No. of strains examined	No. of strains showing *ortho* cleavage	No. of strains showing *meta* cleavage
P. aeruginosa	5	5	0
P. fluorescens	62	62	0
P. putida	34	34	0
Unclassified fluorescent pseudomonads	8	8	0
P. multivorans	8	8	0
P. pseudomallei	6	6	0
P. acidovorans	15	0	15
P. testosteroni	9	0	9
P. stutzeri	3	3	0

Some universally negative nutritional characters of the strains examined

None of the 267 strains examined can grow chemolithotrophically at the expense of H_2. This fact shows that hydrogen chemolithotrophy is neither of random nor of wide occurrence among aerobic pseudomonads, and confers on it a taxonomic significance in the subdivision of this bacterial group that has hitherto been assumed by taxonomists without evidence. We do not consider it a suitable generic character, but at least it can now be used with some confidence as one of the characters of value in the definition of certain *Pseudomonas* species.

None of the 267 strains examined can grow on methanol, methylamine or oxalate. Hence the ability to utilize these compounds can also now be regarded as a significant taxonomic character among aerobic pseudomonads; it is presumably confined to a restricted segment of the genus *Pseudomonas*.

Lastly, it should be noted that none of our strains can grow in an otherwise suitable defined medium that is free from combined nitrogen compounds. Nitrogen fixation is, accordingly, a property absent from the group.

Nutritional versatility of the strains examined

As mentioned in the introduction, the unusually wide range of organic compounds utilizable as carbon and energy sources by *Pseudomonas fluorescens* was established through the comparative nutritional survey of den Dooren de Jong (1926) of a variety of different aerobic bacteria. This particular aspect of his work became well known, as a result of its detailed presentation in Marjory Stephenson's influential book *Bacterial Metabolism* (1939). *P. fluorescens* is, accordingly, often cited as the

epitome of nutritional versatility among bacteria. Since the quantitative aspects of the data on utilization of the 146 organic compounds tested as carbon and energy sources in our study are of some interest in this connexion, and are not easily extractable from the tabulations to be given in later sections, we have summarized them here. In Fig. 2, the range in the number of compounds utilizable by the strains that we assign to each species is expressed in bar form. Where many different strains of a species have been examined, the distribution within the bar is approximately Gaussian, so that the majority of strains cluster around the centre of each bar. In the case of *P. multivorans*, the bar has been prolonged by a dotted line, to

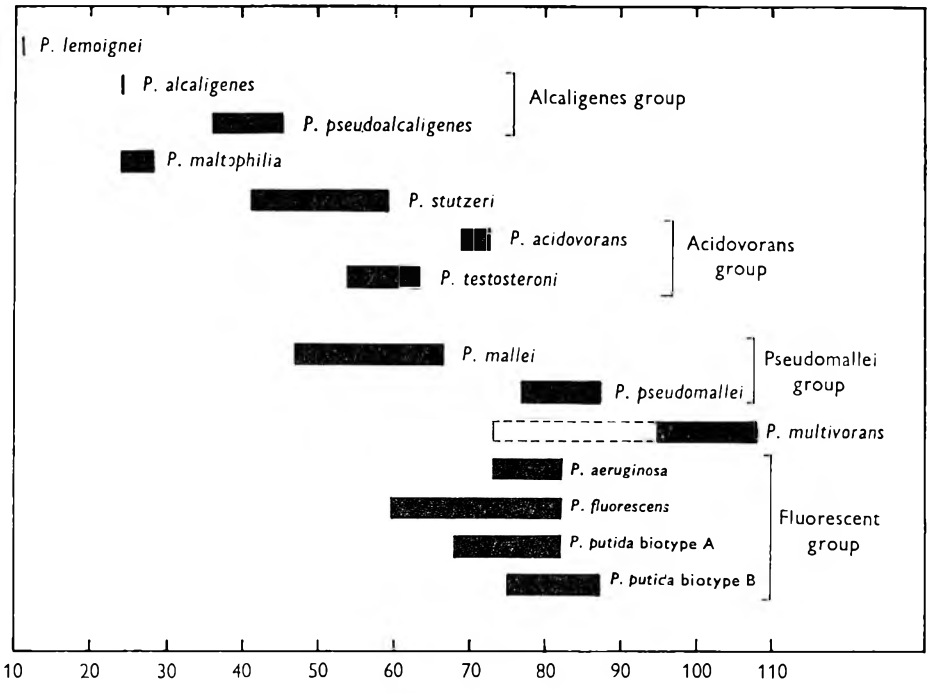

Fig. 2. The numerical range of organic compounds utilizable as sole sources of carbon and energy by certain species of aerobic pseudomonads. The values shown are based on tests with 146 different organic compounds. Data for the pseudomallei group are taken from the paper of Redfearn *et al.* (1966); data for *P. lemoignei* are taken from the paper of Delafield *et al.* (1965) and supplemented by further unpublished information.

indicate a single highly defective strain which falls well outside the normal distribution curve for this species.

In the fluorescent group, we have separated the data for biotypes A and B of *Pseudomonas putida*, since biotype B stands out as the most versatile of fluorescent pseudomonads (75–87 substrates used). The ranges for *P. putida* biotype A (66–82), *P. fluorescens* (59–82) and *P. aeruginosa* (76–82) are slightly lower and quite similar. The very wide span for *P. fluorescens* (all biotypes) is attributable partly to the very large number of strains included, and partly to the fact that the various biotypes differ nutritionally in minor respects from one another. The range for *P. pseudomallei*

(77–87 substrates used) is almost identical with that for *P. putida* biotype B, so that *P. pseudomallei* equals the best record in the fluorescent group. Both are well exceeded by *P. multivorans* (95–108 substrates used, if the single defective strain with a value of 73 is excluded). In the acidovorans group, *P. acidovorans* (68–73) falls only slightly below the fluorescent pseudomonads, while *P. testosteroni* uses markedly fewer compounds (53–63). This group is followed by *P. stutzeri*, whose characteristic nutritional irregularity is shown by a wide numerical span (41–59). At the lower end of the scale are *P. pseudoalcaligenes* (36–45), *P. alcaligenes* (24; one strain), *P. maltophilia* (24–28) and *P. lemoignei* (11; one strain). Fig. 2 confirms the fact, already evident from the nutritional properties of *P. methanica* (Dworkin & Foster, 1956), that the nutritional versatility of different species in the genus *Pseudomonas* differs very widely. It also shows that the numerical extent of the nutritional range is in some cases (e.g. *P. multivorans*) a highly distinctive specific character.

THE FLUORESCENT GROUP

Origins of the strains

The strains belonging to this group will be listed according to their specific assignments.

I. Strains assigned to *Pseudomonas aeruginosa*

45. *P. aeruginosa* strain LA P-6 of Dr S. C. Rittenberg. Isolated from hydrocarbon enrichment. ATCC 17423.
52. *P. aeruginosa* strain B-903 of Dr W. C. Haynes, U.S. Department of Agriculture, Peoria, Ill. ATCC 17429.
54. Same source as 52; strain B-23. ATCC 17431.
55. Same source as 52; strain B-1781. ATCC 17432.
57. Same source as 52; strain B-248. ATCC 17434.
58. Strain of unknown origin obtained from the departmental stock collection, Department of Bacteriology, University of California, Berkeley. ATCC 17435.
131. *P. aeruginosa* strain H-1c of Dr B. Holloway. ATCC 17503.
132. *P. aeruginosa* strain Ph-1, isolated by Dr G. Hegeman from a denitrification enrichment in Berkeley. ATCC 17504.
277–296. Independent strains isolated at several different hospitals from a variety of clinical specimens. Collection received from Dr Vera L. Sutter, School of Dentistry, San Francisco Medical Center. ATCC 17643–17662.
416. Strain received from Dr O. Jessen. ATCC 17831.

II. Strains assigned to *Pseudomonas fluorescens* biotype A

12. *P. fluorescens* strain 216 of den Dooren de Jong (1926). Isolated from tap water. ATCC 17397.
126. Strain mesaconic-12 of Dr M. Shilo. Isolated from soil by mesaconate enrichment. ATCC 17498.
182. Strain PJ 73 of Dr O. Jessen. Included in biotype 63 (group IV) by Jessen (1965). ATCC 17550.
184. Strain W4L-2 isolated from water by enrichment with lactate in Berkeley. ATCC 17552.
185. Strain W2L-1, isolated from water by enrichment with lactate in Berkeley. ATCC 17553.

The following 18 strains (186–216) were studied by Jessen (1965) and are all representative of his biotype 63, group V.

186. Strain PJ 79. Isolated and named *P. fluorescens* in the Inst. Infect. Diseases. Tokyo. ATCC 17554.
187. Strain PJ 139. Originally named *P. angulata*. ATCC 17555.
188. Strain PJ 160. Originally named *P. marginata* (NCPPB 316). ATCC 17556.
189. Strain PJ 227. *Pseudomonas* sp., culture 1208 from the Centre International Collection de Culture, Lausanne. ATCC 17557.
192. Strain PJ 239. This strain has been proposed by Dr Muriel E. Rhodes as neotype for *P. fluorescens*. ATCC 13525.
196. Strain PJ 288. Isolated from sputum in Hadeslev, Jutland. ATCC 17563.
197. Strain PJ 290. Isolated from throat swab in Hjørring, North Jutland. ATCC 17564.
198. Strain PJ 302. Isolated from pleural drainage in Slagalse, Mid-Sealand. ATCC 17565.
199. Strain PJ 311. Isolated from stored blood in Holbaek, North Sealand. ATCC 17566.
200. Strain PJ 367. Isolated from sputum (hospital sample) in Copenhagen. ATCC 17567.
201. Strain PJ 368. Isolated from saliva in the Seruminstitut, Copenhagen. ATCC 17568.
202. Strain PJ 372. From heart blood (autopsy), Copenhagen. ATCC 17569.
203. Strain PJ 376. From pleural drainage, Copenhagen. ATCC 17570.
208. Strain PJ 722. From polluted sea water, Øresund, south of Copenhagen. ATCC 17575.
209. Strain PJ 776. Isolated from polluted sea water, Øresund, north of Copenhagen. ATCC 17576.
211. Strain PJ 826. From ground water in south of Sealand. ATCC 17578.
215. Strain PJ 849. From surface water (natural stream) in south of Sealand. ATCC 17582.
216. Strain PJ 885. From ground water, south of Jutland. ATCC 17583.
392. Strain B-2075 of Dr W. C. Haynes, Peoria. Strain 4 of Lysenko, isolated from a caterpillar and named *P. chlororaphis*. ATCC 17813.

III. Strains assigned to *Pseudomonas fluorescens* biotype B

2. Strain P3T-1, isolated from water by L-tryptophan enrichment. ATCC 17387.
93. Strain TR-10 of Dr S. H. Hutner. Isolated from tryptophan enrichment. ATCC 17467.
108. Strain 52-22 of W. R. Sistrom. From naphthalene enrichment. ATCC 17482.

The following strains (400–415) were studied by Jessen (1965), and all are representatives of his biotype 61, group V, except strain 415, which he placed in biotype 62, group V.

400. Strain PJ 185. Named *P. marginalis* (NCPPB 247). Isolated from lettuce. ATCC 17815.
401. Strain PJ 187. Obtained by Dr H. Lautrop from the Edinburgh and East of Scotland School of Agriculture under the name *P. marginalis*. Isolated from dahlia. ATCC 17816.

402. Strain PJ 188. Obtained from Dr W. C. Haynes. (NRRL strain B-981.) ATCC 17817.
403. Strain PJ 251. Obtained from Dr K. Klinge. (Strain R-2.) ATCC 17818.
404. Strain PJ 283. Isolated from pleural fluid. ATCC 17819.
405. Strain PJ 362. Isolated from fresh water. ATCC 17820.
406. Strain PJ 365. Isolated from fresh water. ATCC 17821.
407. Strain PJ 379. Obtained from Dr J. Ørskov, Statens Serum Institut. ATCC 17822.
408. Strain PJ 380. Isolated from fresh water. ATCC 17823.
409. Strain PJ 381. Isolated from fresh water. ATCC 17824.
410. Strain PJ 384. Isolated from fresh water. ATCC 17825.
411. Strain PJ 672. Isolated from sea water. ATCC 17826.
412. Strain PJ 833. Isolated from pond water. ATCC 17827.
413. Strain PJ 851. Isolated from tap water. ATCC 17828.
414. Strain PJ 883. Isolated from well. ATCC 17829.
415. Strain PJ 383. Isolated from fresh water. ATCC 17830.

IV. Strains assigned to *Pseudomonas fluorescens* biotype C

18. Strain P-7 from the Western Utilization Research and Development Division of the U.S. Department of Agriculture, Albany, California. Psychrophilic organism isolated from hen's egg and named *P. fluorescens*. ATCC 17400.
50. Strain P3T-3, isolated in Berkeley from L-tryptophan enrichment of water sample. ATCC 17427.

The following 13 strains were studied by Jessen (1965); his biotype and group classification is indicated in each case.

181. Strain PJ 70. Isolated by O. Lysenko (strain 295). Biotype 52, group IV. ATCC 948.
191. Strain PJ 236. Received from Dr Muriel E. Rhodes as strain 22.1. Biotype 49, group IV. ATCC 17559.
194. Strain PJ 253. Received from Dr K. Klinge as strain R-60. Biotype 49, group IV. ATCC 17561.
204. Strain PJ 682. Isolated from polluted sea water, Øresund, Denmark. Biotype 49, group IV. ATCC 17571.
205. Strain PJ 686. Isolated from polluted sea water, Øresund, Denmark. Biotype 49, group IV. ATCC 17572.
207. Strain PJ 693. Isolated from polluted sea water, Øresund, Denmark. Biotype 49, group IV. ATCC 17574.
210. Strain PJ 824. Isolated from ground water, from Ribe, Jutland. Biotype 49, group IV. ATCC 17577.
212. Strain PJ 832. Isolated from ground water, Mors, Jutland. Biotype 49, group IV. ATCC 17579.
213. Strain PJ 834. From polluted surface water, canal, South Copenhagen. Biotype 49, group IV. ATCC 17580.
214. Strain PJ 848. From polluted natural stream, South Copenhagen. Biotype 49, group IV. ATCC 17581.
217. Strain PJ 905. From polluted sea water, Greenland (Sukkertoppen). Biotype 49, group IV. ATCC 17584.
218. Strain PJ 929. Same origin as 217. Biotype 52, group IV. ATCC 17585.
219. Strain PJ 969. From garden soil, Arreskov, Funen. Biotype 49, group IV. ATCC 17586.

V. Strains assigned to *Pseudomonas fluorescens* biotype D

All strains of this group were received from Dr W. C. Haynes, U.S. Department of Agriculture, Peoria, Illinois, as *P. chlororaphis.*

30. Strain B-560. ATCC 9446.
31. Strain B-561. ATCC 9447.
32. Strain B-977. ATCC 17411.
35. Strain B-1541. ATCC 17414.
388. Strain B-2266. ATCC 17809.
389. Strain B-1854. ATCC 17810.
390. Strain B-1095. ATCC 17811.
391. Strain B-1869. ATCC 17812.
392. Strain B-2075. ATCC 17813.
393. Strain B-1632. ATCC 17814.
394. Strain B-1632. Colony variant ATCC 17814.

VI. Strains assigned to *Pseudomonas fluorescens* biotype E

All strains of this group were received from Dr W. C. Haynes, Northern Utilization Research and Development Division (U.S. Department of Agriculture, Peoria, Illinois) as *P. aureofaciens.*

36. Strain B-1482R. Isolated at NRRL (Peoria) from a sample of farm soil in 1953. ATCC 17415.
37. Strain B-1543P. Isolated at NRRL from farm soil in 1953. ATCC 17416.
38. Strain B-1576. Received from the late Professor A. J. Kluyver as *Pseudomonas polychromogenes.* Isolated from clay that had been suspended in kerosene for 3 weeks. ATCC 13985.
39. Strain B-1681. Isolated NRRL from forest soil. ATCC 17417.
40. Strain B-1855. Isolated at NRRL from lake water. ATCC 17418.
41. Strain B-2265. Isolated by Dr A. Fuchs in 1959 as *P. aureofaciens* var. *nonliquefaciens.* Air contaminant. ATCC 17419.
86. Strain B-1855. Isolated from a water sample from Illinois. ATCC 17461.

VII. Strains assigned to *Pseudomonas fluorescens* biotype F

83. *P. lemonnieri* strain B-1864 of Dr W. C. Haynes, who received it from Dr P. H. A. Sneath as strain LA. Isolated by Hugo & Turner (1957). ATCC 17458.
143. Strain PL, isolated in Berkeley by Miss Diana Loeb from hydrocarbon enrichment. ATCC 17513.

VIII. Strains assigned to *Pseudomonas fluorescens* biotype G

1. Strain SCT-1, isolated in Berkeley from a tryptophan enrichment of water sample. ATCC 17386.
33. *P. chlororaphis* strain B-1079 received from Dr W. C. Haynes, Peoria. ATCC 17412.
34. Ibid., strain B-1098. ATCC 17413.
99. Strain RYS-3 of R. Y. Stanier. Isolated from L-tryptophan enrichment. ATCC 17473.
124. Strain W4-GLY-1, isolated in Berkeley from glycerol enrichment of water sample. ATCC 17496.

149. Fluorescent pseudomonad strain 2440-40 of Dr M. Starr. Isolated from soil by Dr H. Stolp. ATCC 17518.
164. Ibid., strain 2440-15. ATCC 17533.
166. Ibid., strain 2440-28. ATCC 17535.
169. Ibid., strain 2440-183. ATCC 17538.
171. Ibid., strain 2440-105. ATCC 17540.
172. Ibid., strain 2440-110. ATCC 17541.
195. Strain PJ 274 of Dr O. Jessen. Isolated from sputum, Haderslev, Jutland. Included in biotype 52, group IV, by Jessen (1965). ATCC 17562.
206. Strain PJ 692 of Dr O. Jessen. Isolated from polluted sea water, Øresund, Denmark. Included in biotype 52, group IV, by Jessen (1965). ATCC 17573.
267. *P. schuylkilliensis* strain B9 obtained from Dr M. Mandel. ATCC 17634.
269. *P. geniculata* strain B-1606, obtained from Dr M. Mandel. ATCC 17636.
271. *P. geniculata*, strain B-1612 of Dr M. Mandel. ATCC 17638.
272. Ibid., strain B-1603. ATCC 14150.

IX. Strains assigned to *Pseudomonas putida* biotype A

5. *P. putida* strain 5 of den Dooren de Jong (1926). Isolated from soil by enrichment with amylamine. Received from Laboratorium voor Microbiologie, Delft. ATCC 17390.
6. *P. putida* strain 9 of den Dooren de Jong (1926). Isolated from soil by enrichment with propionate. Same source as 5. ATCC 17391.
7. *P. putida* strain of den Dooren de Jong (1926). Isolated from soil by enrichment with phenylaminoacetate. Same source as 5. ATCC 17392.
8. *P. putida* strain 39 of den Dooren de Jong (1926). Isolated from soil by enrichment with heptanoic acid. Same source as 5. ATCC 17393.
26. Strain KA 14-1 isolated by Dr Keichii Hosokawa in Berkeley from soil by kynurenate enrichment. ATCC 17408.
42. Strain LAF-1 of Dr S. C. Rittenberg, University of California, Los Angeles. Nicotine oxidizer. ATCC 17420.
43. Strain PS-1 of Dr S. C. Rittenberg, University of California, Los Angeles. ATCC 17421.
44. Ibid., strain PS-2. ATCC 17422.
49. Strain MB-15 of Dr K. Hosokawa. Isolated from soil by benzoate enrichment. ATCC 17426.
51. Strain M-6 of Dr K. Hosokawa. Isolated from soil by anthranilate enrichment. ATCC 17428.
56. Strain W-1, isolated in Berkeley by Miss Margaret Farley from arginine enrichment from water. ATCC 17433.
76. Strain C 1-A of Dr I. C. Gunsalus, University of Illinois. Isolated from soil by camphor enrichment. ATCC 17452.
77. Strain C1-B. Same source and origin as 76. ATCC 17453.
81. Strain S-2 of Dr E. E. Snell. Isolated from soil by canavanine enrichment. ATCC 17456.
82. Strain MS of Dr E. E. Snell. Isolated from soil by α-methyl-serine enrichment. ATCC 17457.

87. Strain A.3.1 of R. Y. Stanier. Isolated from soil by alanine enrichment. ATCC 17462.
89. Strain A.3.8 of R. Y. Stanier. Isolated from soil by malate enrichment. ATCC 17464.
90. Strain A.3.12 of R. Y. Stanier. Isolated from soil by lactate enrichment. ATCC 12633.
100. Strain received under the designation *P. ovalis* from Dr Hans Kornberg. ATCC 17474.
111. Strain 53-2 of Dr W. R. Sistrom. Isolated from soil by naphthalene enrichment. ATCC 17485.
115. Strain tyr of Dr M. Shilo. Isolated from soil by tyrosine enrichment. ATCC 17487.
118. Strain meso-1, isolated by Dr M. Shilo from soil by *meso*-tartrate enrichment. ATCC 17490.
119. Strain L-2 of Dr M. Shilo. Isolated from soil by L-tartrate enrichment. ATCC 17491.
122. Strain D-malic-2 of Dr M. Shilo. Isolated from soil by D-malic enrichment. ATCC 17494.
128. Strain MMD of Dr M. Shilo. Isolated from soil by tartrate enrichment. ATCC 17500.
130. Strain N-9 of Dr E. J. Behrman. Isolated from soil by nicotinic acid enrichment. ATCC 17502.
144. Strain B of Dr I. C. Gunsalus, University of Illinois. ATCC 17514.
145. Strain HT, isolated in 1962 by Dr S. Dagley from soil by enrichment on phenylacetate. ATCC 17515.
154. Strain 2440-112 received from Dr M. Starr, University of California, Davis. Isolated by Dr H. Stolp from soil. ATCC 17523.
160. Ibid., strain 2440-133. ATCC 17529.
266. Unidentified strain received from Dr M. J. Coon, Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan. ATCC 17633.
276. Strain isolated by Dr W. B. Jakoby from soil by tartrate enrichment. ATCC 17642.

X. Strains assigned to *Pseudomonas putida* biotype B.

53. Strain B-3 received from Dr W. C. Haynes. Originally classified as *P. aeruginosa*. ATCC 17430.
96. Strain TR-26 isolated by Dr S. H. Hutner from soil by L-tryptophan enrichment. ATCC 17470.
98. Strain RYS-2, isolated in Berkeley from soil by L-tryptophan enrichment. ATCC 17472.
107. Strain 52-19, isolated in Berkeley by Dr W. R. Sistrom from soil by saligenin enrichment. ATCC 17481.
110. Strain 53-18, isolated by Dr N. Walker at Rothamsted by naphthalene enrichment. ATCC 17484.
153. Strain 2440-103 received from Dr M. P. Starr. ATCC 17522.
157. Strain 2440-4. ATCC 17526.
158. Strain 2440-176. ATCC 17527.
167. Strain 2440-87. ATCC 17536.

XI. Unclassified strains of the fluorescent group

Eleven strains of fluorescent pseudomonads could not be assigned to any of the species or biotypes previously listed.

10. Strain 112 of den Docren de Jong (1926), isolated from water. ATCC 17395.
17. Strain P-1 from the Western Utilization Research and Development Division of the U.S. Department of Agriculture, Albany, California. Psychrophilic fluorescent pseudomonad isolated from egg. ATCC 17399.
47. Strain F-67 of Dr E. R. Blakley (National Research Council, Canada). Isolated from a cinnamate enrichment culture. ATCC 17425.
59. Strain W-2 isolated by Miss M. Farley in Berkeley from benzoate enrichment. ATCC 17436.
91. Strain A.3.18; originally received from U.S. Department of Agriculture, Peoria, Illinois, as B-8. ATCC 17465.
92. Strain A.3.19, same source as 91; B-14. ATCC 17466.
95. Strain TR-23, isolated by Dr S. H. Hutner from soil by enrichment with tryptophan. ATCC 17469.
101. Strain NIC-24, isolated in Berkeley from soil by enrichment with nicotinate. ATCC 17475.
109. Strain 53-17 of Dr W. R. Sistrom. Isolated by Dr N. Walker at Rothamsted, by enrichment with naphthalene. ATCC 17483.
127. Strain DMM of Dr M. Shilo. Isolated in Berkeley from enrichment with tartrate. ATCC 17499.
322. Strain 62-FC of Dr M. Véron, Institut Pasteur, Paris. Isolated from mineral oil and designated as *P. denitrificans*. ATCC 17688.

General group characters

The production of water-soluble, yellow-green fluorescent pigments is frequently encountered in the aerobic pseudomonads, and has long been used as a major character in the taxonomic subdivision of the genus *Pseudomonas*. It has never been clearly established, however, whether the production of this type of pigment is correlated with other characters that would justify the recognition of the fluorescent pseudomonads as a special subgroup. This is an important problem from the determinative standpoint, since production of fluorescent pigments is strongly influenced by nutritional factors, and can fail completely on media which support excellent growth. Furthermore, it has been shown that occasional strains otherwise typical of *P. aeruginosa* (the one easily identifiable fluorescent species) may be completely unable to produce fluorescent pigment (Véron, 1961; Jessen, 1965).

Of the 175 strains studied by us, 20 did not produce the group-specific fluorescent pigment on slopes of King B medium, or on any other medium used in their examination. These strains might be capable of fluorescent pigment production under some circumstances, but we did not further pursue this question, because our analysis had by then revealed that the members of this generic subgroup share additional properties which permit their identification even when pigment production fails. We shall therefore preface the description of individual species with a discussion of the common group characters.

Fluorescent pseudomonads do not accumulate poly-β-hydroxybutyrate as a

Table 7. *Fluorescent group. The utilization of organic compounds as carbon and energy sources by fluorescent pseudomonads, based on the analysis of 175 strains*

The compounds listed are ones that are: (*a*) used by a majority of strains, and (*b*) used by a few or no strains. Accordingly, they are not diagnostically useful for the internal differentiation of species in the fluorescent group.

Compound	No. of positive strains	% of positive strains
Carbohydrates and sugar derivatives		
D-Arabinose	0	0
D-Fucose	0	0
L-Rhamnose	10	6
D-Glucose	174	99
D-Fructose	159	91
Maltose	1	1
Cellobiose	0	0
Salicin	0	0
Lactose	0	0
Starch	0	0
Gluconate	174	99
2-Ketogluconate	169	97
Fatty acids		
Acetate	175	100
Caprcate	168	96
Heptanoate	172	98
Caprylate	174	99
Pelargonate	175	100
Caprate	174	99
Dicarboxylic acids		
Malonate	160	91
Succinate	175	100
Maleate	3	2
Fumarate	175	100
Glutarate	170	97
Hydroxyacids		
L-Malate	175	100
D-(−)-Tartrate	16	9
DL-β-OH-Butyrate	173	99
DL-Lactate	175	100
Glycollate	5	3
DL-Glycerate	169	97
Poly-β-OH-butyrate	0	0
Miscellaneous organic acids		
Citrate	174	99
α-Ketoglutarate	173	99
Pyruvate	174	99
Aconitate	162	93
Polyalcohols and glycols		
Glycerol	173	99
Ethyleneglycol	3	2
2,3-Butyleneglycol	112	64

Compound	No. of positive strains	% of positive strains
Non-nitrogenous aromatic and other cyclic compounds		
D-Mandelate	8	5
Benzoate	113	65
o-OH-Benzoate	12	7
m-OH-Benzoate	7	4
p-OH-Benzoate	167	95
Phenylethanediol	6	3
Phthalate	1	1
Isophthalate	0	0
Terephthalate	0	0
Naphthalene	4	2
Phenol	11	6
Quinate	167	95
Testosterone	12	7
Aliphatic amino acids		
L-α-Alanine	174	99
D-α-Alanine	172	98
β-Alanine	174	99
L-Threonine	0	0
L-Leucine	166	95
L-Isoleucine	160	91
L-Norleucine	0	0
L-Valine	164	94
L-Aspartate	175	100
L-Glutamate	175	100
L-Lysine	145	83
DL-Arginine	173	99
DL-Ornithine	157	90
DL-Citrulline	132	75
DL-α-Aminobutyrate	4	2
γ-Aminobutyrate	174	99
δ-Aminovalerate	162	93
Amino acids and related compounds containing a ring structure		
L-Histidine	168	96
L-Proline	173	99
L-Tyrosine	169	97
D-Tryptophan	1	1
Amines		
Ethanolamine	125	71
Putrescine	172	98
Spermine	171	98
Miscellaneous nitrogenous compounds		
Betaine	173	99
Sarcosine	164	94
Pantothenate	14	8

cellular reserve material, and are unable to attack this polymer by means of extracellular enzymes. They grow well with either nitrate or ammonia as sole nitrogen source. All are oxidase-positive. We have examined only a minority of our strains for constitutive arginine dihydrolase production; but every strain examined gave positive results.

The general nutritional spectrum of these bacteria (Table 7) has many characteristic qualitative features, some of which are valuable in distinguishing them from other aerobic pseudomonads. Two hexoses, glucose and fructose, and two sugar acids, gluconate and 2-ketogluconate, are practically universal substrates. One exceptional strain (41) did grow on either glucose or gluconate; and occasional strains did not grow on either fructose or 2-ketogluconate. No strain of this group grew on D-fucose, D-arabinose, cellobiose, lactose or starch; only one strain grew on maltose, and 10 on L-rhamnose.

Among the fatty acids, acetate, propionate, caproate, heptanoate, caprylate, pelargonate and caprate were used by nearly all strains. Utilization of the C_4 and C_5 fatty acids (butyrate, isobutyrate, valerate, isovalerate) was much less common, and reflects the failure of some biotypes of *Pseudomonas fluorescens* to grow on these compounds. In the dicarboxylic acid series, malonate, succinate and glutarate were used by most strains; but the higher homologues (C_6–C_{10}) were much more rarely attacked; the figures for their utilization largely reflect the fact that they are substrates for *P. aeruginosa*. Other aliphatic organic acids used by most or all strains of the group were: fumarate, L-malate, DL-lactate, DL-β-hydroxybutyrate, DL-glycerate, citrate, α-ketoglutarate, aconitate, pyruvate. Glycollate and maleate were used by only four and three strains, respectively.

A number of simple aromatic compounds are substrates for many fluorescent pseudomonads. The most widely utilized was *p*-hydroxybenzoate, which supported growth of 167 strains. Benzoate and anthranilate were used by much smaller total numbers of strains, primarily because benzoate cannot be used by many strains of *Pseudomonas fluorescens* and anthranilate cannot be used by many strains of *P. putida*. The *o*- and *m*-monohydroxybenzoic acids were used only by occasional strains (12 and 7, respectively). The alicyclic acid, quinate, used by 167 strains, was metabolized through an aromatic pathway, since it is converted metabolically to protocatechuate, the first intermediate in the oxidation of *p*-hydroxybenzoate.

Among the amino acids used by most strains as carbon and energy sources were α-alanine, β-alanine, leucine, isoleucine, valine, aspartate, glutamate, arginine, ornithine, histidine, proline, tyrosine, γ-aminobutyrate, δ-aminovalerate. Two amino acids, threonine and norleucine, were not used by any strain, and α-aminobutyrate was used by only one strain. Other very commonly used nitrogenous organic compounds were the polyamines, putrescine and spermine, and the *N*-methyl compounds, betaine and sarcosine.

By selection among the common group characters described above, it is possible to construct a relatively brief list of traits which are diagnostic for fluorescent pseudomonads as a subgroup among the aerobic pseudomonads. These traits, and their frequencies in the 175 strains examined, are listed in Table 8. From the frequencies, one can construct what we term an 'ideal' phenotype, by scoring characters of high frequency as + and characters of low frequency as −. With respect to the sixteen characters which constitute the ideal phenotype, conformity

within the group is excellent: 133 strains show perfect conformity, 38 deviate in one character, and four deviate in two characters (Table 9). These characters were chosen for their value in permitting the differentiation of fluorescent pseudomonads from all the other species and species groups which we have studied. The effectiveness with which they achieve this is also shown in Table 9.

Table 8. *The group characters of greatest differential value in the recognition of the fluorescent pseudomonads, based on the analysis of 175 strains*

Characters	No. of positive strains	Ideal phenotype
1. Production of fluorescent pigment	155	+
2. Poly-β-hydroxybutyrate as cellular reserve material	0	−
3. Utilization of poly-β-hydroxybutyrate as exogenous substrate	0	−
Utilization of		
4. D-Fucose	0	−
5. D-Glucose	174	+
6. Cellobiose	0	−
7. Starch	0	−
8. 2-Ketogluconate	167	+
9. Pelargonate	175	+
10. Benzoate and/or *p*-hydroxybenzoate	172	+
11. *m*-hydroxybenzoate	7	−
12. β-Alanine	174	+
13. Norleucine	0	−
14. Arginine	173	+
15. Putrescine and/or spermine	173	+
16. Betaine and/or sarcosine	175	+

Table 9. *Fluorescent group. Number of strains of different species or groups of species of aerobic pseudomonads conforming to the selected sixteen characters (Table 8) which define the 'ideal' fluorescent pseudomonad*

	No. of characters of 'ideal' phenotype												
	16	15	14	13	12	11	10	9	8	7	6	5	4
Fluorescent group	133	38	4	.	.	.	.	.	.	.	.	.	.
Acidovorans group	.	.	.	.	.	.	.	.	1	.	13	8	4
Pseudomallei group	.	.	.	.	.	.	15	13	9	2	1	1	.
P. multivorans	.	.	.	.	.	19	.	.	.	.	.	.	.
P. stutzeri	.	.	.	.	.	.	4	12	1	.	.	.	.
P. mallophilia	.	.	.	.	.	.	.	.	.	23	.	.	.
Alcaligenes group	.	.	.	.	1	6	.	.	.	.	.	.	.
P. lemoignei	.	.	.	.	.	.	.	.	.	1	.	.	.

The three species that most closely approach the ideal phenotype of fluorescent pseudomonads are *Pseudomonas multivorans*, of which all 19 strains deviated in five of the characters chosen; *P. pseudoalcaligenes*, of which all six strains deviated in five characters; and *P. alcaligenes*, of which the single strain examined deviated in only four characters. Accordingly, the characters chosen as diagnostic for the group may be considered highly satisfactory for determinative purposes, except, possibly, in making the distinction from *P. alcaligenes*.

All major biotypes now recognized among the fluorescent pseudomonads are represented in the collection that we have studied, with the one exception of the phytopathogens. It is therefore not certain that this latter subgroup will conform in its entirety to the proposed ideal phenotype of the fluorescent group. Our fragmentary observations on the phytopathogenic pseudomonads are worth mentioning in this connexion. We have completely characterized four named strains, which were received incidentally from Jessen (1965) among representatives of his group V, biotypes 61 and 63. Two of these (our strains 187 and 188), belonging to Jessen's biotype 61, were labelled *Pseudomonas angulata* and *P. marginata*, respectively. They are typical representatives of *P. fluorescens* biotype A. The other two (our strains 400 and 401), belonging to Jessen's biotype 63, were independent isolates labelled *P. marginalis*, and are typical representatives of *P. fluorescens* biotype B. This experience suggests (and the data of Jessen, 1965, are fully concordant) that some phytopathogenic pseudomonads cannot be distinguished on any cultural, physiological or biochemical criteria from *P. fluorescens*; such organisms will obviously fit the ideal phenotype proposed above.

We also examined cursorily a small collection of phytopathogenic fluorescent strains received from Dr M. P. Starr; these included four strains labelled *Pseudomonas syringae*, two labelled *P. morsprunorum* and one labelled *P. tabaci*. These strains constitute a group of gelatin-liquefying fluorescent pseudomonads clearly distinguishable from any biotype of *P. fluorescens* with which we are familiar. Their most striking characteristic is an extremely low growth rate, not only in chemically defined media, but even in yeast extract medium. The organisms from yeast extract cultures were large, swollen and elongated. These observations suggest that the strains in question may have a partial requirement for one or more growth factors not present in yeast extract. However, they can grow (very slowly) in chemically defined media, which has permitted us to make a preliminary scan of their nutritional spectra. They seem to have a far lower degree of nutritional versatility than any other fluorescent pseudomonads. Accordingly, it is quite possible that one additional fluorescent species, consisting of these types of fluorescent plant pathogens, will eventually deserve recognition; and, in view of their apparent nutritional defects, these strains may deviate significantly from the ideal phenotype of the group that we have proposed on the basis of the study of its free-living members. It should be noted that the results of Jessen (1965) also support this conclusion. He placed most of the phytopathogenic fluorescent strains which he examined in a special group (VI), and commented on the unusually slow growth of many of them. Included in Jessen's group VI were strains labelled *P. syringae* and *P. morsprunorum*.

Internal subdivision of the fluorescent group

The only fluorescent pseudomonad now recognizable with reasonable certainty on the basis of several unrelated characters is the type species of the genus *Pseudomonas*, *P. aeruginosa*. The characters now most commonly used for its recognition are: pyocyanine production; growth at 41–43°; and production of slime in a chemically defined medium containing 2-ketogluconate (Haynes, 1951). By far the most extensive comparison of this species with other fluorescent pseudomonads has been made recently by Jessen (1965), who examined a collection of 859 fluorescent strains, of which 354 were identified as *P. aeruginosa*. Jessen has clearly demon-

strated that *P. aeruginosa* is a distinctive and internally uniform biotype. Apart from the characters already mentioned, Jessen found that the strains of this species shared the following characters: denitrifying ability; gelatin liquefaction; negative egg-yolk reaction; failure to grow at 5°; failure to produce acid from sucrose, adonitol, dulcitol, sorbitol or erythritol; acid production from mannitol; monotrichous flagellation, provided that the assessment of this character is made on a statistical basis, as suggested by Lautrop & Jessen (1964).

The classification of other fluorescent pseudomonads remains highly unsatisfactory. Three species (*Pseudomonas lemonnieri*, *P. chlororaphis*, *P. aureofaciens*) have each been defined on a single property, the ability to produce a specific phenazine pigment. Haynes & Rhodes (1962) suggested that *P. chlororaphis* closely resembles the gelatin-liquefying simple fluorescent pseudomonads. Liu (1960), in a serological study on the extracellular antigens of *Pseudomonas* strains, found that *P. aureofaciens*, *P. chlororaphis* and many strains of haemolytic lecithinase-positive *P. fluorescens* showed extensive cross-reactions, which suggested a close relationship among these species. Another important character which clearly differentiates *P. chlororaphis* and *P. aureofaciens* from *P. aeruginosa*, and at the same time justifies associating the first two species with the simple fluorescent pseudomonads, is the fact that both *P. chlororaphis* and *P. aureofaciens* show a guanine+cytosine (GC) content of 63·5 moles % in their DNA. This GC content is similar to that of some simple fluorescent pseudomonads, and markedly lower than the average of 66·5 % found for our strains of *P. aeruginosa* (Mandel, 1961). All these facts do not fit the conclusion of Lysenko (1961), who considered *P. chlororaphis* and *P. aureofaciens* to be closely allied to *P. aeruginosa*.

Among the simple (non-phenazine-producing) fluorescent pseudomonads, the two classical species, *Pseudomonas fluorescens* and *P. putida*, were originally distinguished by Flügge (1886) under the designations *B. fluorescens liquefaciens* and *B. fluorescens non-liquefaciens* on the basis of gelatin liquefaction, a character possessed by the former but not the latter. At least 20 other species (exclusive of the many species of plant pathogens) have since been named; but the descriptions are fragmentary and the alleged differences between named species are tenuous. To judge from recent studies of this group, the primary taxonomic question seems to be whether even the distinction between *P. fluorescens* and *P. putida* can be successfully maintained. Klinge & Gräf (1959) regarded it as valid, and emphasized the egg-yolk reaction as an additional property characteristic of *P. fluorescens* and not shared by *P. putida*. The data of den Dooren de Jong (1926) suggested that some nutritional characters overlooked by later workers might provide additional means for making the distinction. Rhodes (1959, 1961), on the other hand, found it impossible to make clear-cut subdivisions among 134 strains of the *P. fluorescens–P. putida* group.

The most thorough taxonomic analysis of the simple fluorescent pseudomonads is that of Jessen (1965). On the basis of twenty-nine diagnostic tests, Jessen found that 354 strains of *Pseudomonas aeruginosa* showed sufficient uniformity to justify their grouping in a single biotype; but application of the same tests to 505 strains of simple fluorescent pseudomonads revealed the existence of no less than 81 distinct biotypes. By clustering biotypes that differed from one another in no more than two characters, Jessen sorted the bulk of these strains into five principal groups designated as II–VI; *P. aeruginosa* constituted group I. These five groups included

Table 10. *Diagnostic characters used in the differentiation of six groups of fluorescent pseudomonads (Jessen, 1965)*

Group	Acid formation in Hugh & Leifson (1953) medium from											Egg-yolk reaction	Growth at		
	Sucrose	Maltose	Trehalose	Raffinose	Erythritol	Adonitol	Sorbitol	Mannitol	Inositol	NO_3 reduction	Gelatin liquef.	Levan from sucrose	5°	37°	42°
I (*P. aeruginosa*)	−	−	v	−	−	−	−	+	−	+ + +	+	−	−	+	+
II (192 strains)	v	−	v	−	−	v	−	v	−	− *	v	−	+	v	−
III (26 strains)	−	+	v	−	−	−	−	v	v	−	v	−	+	−	−
IV (185 strains)	−	−	+	−	+	+ †	v	+	+	v	v	−	+	−	−
V (65 strains)	+	−	+ ‡	v	+	v	+	+	+ ‖	v	+	+	+	v	−
VI (22 strains)	+	−	+	v	+ §	−	+	+	+ ‖	−	v	+ ¶	+	−	−

Exceptions to the above patterns: *, 8 positive strains; †, 11 negative strains; ‡, one negative strain; §, three negative strains; ‖, one negative strain; ¶, two negative strains.

48 biotypes, but accounted for a much larger fraction of individual strains (440). The remaining 65 strains, scattered through 33 biotypes, constituted intermediate groups. The characters that distinguish the six groups of Jessen are shown in Table 10.

The largest single biotype in Jessen's collection of simple fluorescent pseudomonads, biotype 11 of his group II, corresponds to *Pseudomonas putida*, while his groups IV and V comprised the bulk of the strains that correspond to *P. fluorescens*. His group VI consisted entirely of phytopathogenic strains, although a few phytopathogens were assigned to groups IV and V. Group III consisted of 26 strains, mostly of marine origin, which do not seem readily equatable with any named species in the fluorescent group. Jessen concluded that the extent of intergrading among the simple fluorescent pseudomonads is too great to allow the recognition of species among them.

We have examined a considerably smaller collection of strains than Jessen, but our phenotypic characterization of these strains has been more extensive than his. We have reached much the same general conclusions. Twenty-nine of the 175 fluorescent pseudomonads in our collection could be readily identified as *Pseudomonas aeruginosa* on the basis of the accepted criteria. The screening of carbon and energy sources revealed several hitherto unrecognized nutritional characters which strengthen the differentiation of this species from other fluorescent pseudomonads. In all respects, these 29 strains showed a high degree of uniformity; we therefore fully concur with Jessen that *P. aeruginosa* constitutes a relatively isolated and uniform biotype. The remaining 146 strains were far less uniform, and we agree with Jessen that their subdivision offers a difficult taxonomic problem. Although we have not fully resolved this problem, we believe that the distinction between the two classical species, *P. fluorescens* and *P. putida*, can be maintained on the basis of several different criteria; and that most of the simple fluorescent pseudomonads can be fitted into one or other of these two species. Of the 146 strains which remained after segregation of strains identified as *P. aeruginosa*, 94 could be assigned to *P. fluorescens* (including all other phenazine producers), and 41 to *P. putida*. The remaining 11 strains could not be satisfactorily assigned to either of these species, and will be separately described but not named. In effect, therefore, we propose, for the present, the recognition of only three species in the fluorescent group: *P. aeruginosa*, *P. fluorescens*, *P. putida*. Of these three species, *P. aeruginosa* is internally relatively uniform; but *P. fluorescens* and *P. putida* are not. *P. fluorescens* includes seven biotypes and *P. putida* two biotypes, which are distinguishable from one another by several different characters. Table 11 lists the characters that we believe to be of greatest value in differentiating among the three species of fluorescent pseudomonads. We shall discuss each character in turn.

Flagellar number. In agreement with Lautrop & Jessen (1964) we have found that all strains of *Pseudomonas aeruginosa* are monotrichous, whereas all our remaining strains of fluorescent pseudomonads are multitrichous.

Extracellular enzyme production. The ability to secrete extracellular hydrolytic enzymes (proteases, lecithinases, lipases) is common to *Pseudomonas aeruginosa* and *P. fluorescens*, but almost completely lacking in *P. putida*. In determining these properties we have depended (like most previous workers) on relatively crude and biochemically imprecise methods of assay, namely, tests for the hydrolysis of gelatin and of Tween 80, and for the occurrence of an egg-yolk reaction.

The production of extracellular proteases, as evidenced by the hydrolysis of gelatin, is a universal property of both *Pseudomonas aeruginosa* and *P. fluorescens*, but never occurs in *P. putida*. The egg-yolk reaction is positive only in *P. fluorescens*, though not universal in this species; 77 of the 94 strains examined gave a positive reaction. The mechanism of this reaction is not understood, but it seems to reflect the ability to produce extracellular lecithinases, since Klinge & Gräf (1959) have shown by direct analysis that egg-yolk-positive strains of *P. fluorescens* can liberate inorganic phosphate from lecithin. However, Klinge & Gräf (1959) also found that

Table 11. *Characters of differential value for the internal subdivision of the fluorescent pseudomonads*

	P. aeruginosa (29 strains)	*P. fluorescens* (94 strains)	*P. putida* (41 strains)
Flagellar no. ...	1	> 1	> 1
	No. of positive strains		
Extracellular enzyme production:			
(*a*) Proteases (liquefaction of gelatin)	29	94	0
(*b*) Lecithinases (egg-yolk reaction)	6	77	0
(*c*) Lipases (hydrolysis of Tween 80)	29	58	3
Temperature relations:			
(*a*) Growth at 4°	0	93	20
(*b*) Growth at 41°	29	0	0
Denitrification	29	46	0
Production of pyocyanine	25	0	0
Nutritional properties			
Carbohydrates and sugar derivatives			
D-Xylose	0	51	11
L-Arabinose	0	64	14
D-Mannose	0	91	13
D-Galactose	0	83	7
Sucrose			
Utilization	0	66	3
Levan formation	0	62	0
Trehalose	0	93	0
Saccharate	0	75	40
Mucate	0	84	41
Dicarboxylie acids			
Adipate	29	13	0
Pimelate	25	9	0
Suberate	19	10	0
Azelate	28	11	0
Sebacate	29	13	0
Hydroxyacids			
L-(+)-Tartrate	0	9	25
hydroxymethylglutarate	0	43	0
Other organic acids			
Citraconate	0	36	7
Polyalcohols and glycols			
Erythritol	0	61	1
Sorbitol	0	53	3
meso-Inositol	0	85	0
Adonitol	0	47	1
Alcohol			
Geraniol	29	0	0

Table 11 (*cont.*)

	P. aeruginosa (29 strains)	*P. fluorescens* (94 strains)	*P. putida* (41 strains)
	No. of positive strains		
Non-nitrogenous aromatic and other cyclic compounds			
L-Mandelate	29	3	3
Phenylacetate	0	15	38
Aliphatic amino acids			
Glycine	22	0	34
DL-α-Aminovalerate	0	2	23
Amines			
Benzylamine	0	4	41
Histamine	29	32	40
Butylamine	0	17	38
α-Amylamine	0	27	38
Miscellaneous nitrogenous compounds			
Creatine	0	2	33
Hippurate	0	2	30
Acetamide	29	0	11
Trigonelline	0	34	41
Paraffin hydrocarbon			
n-Hexadecane	21	0	0

P. aeruginosa was a powerful producer of lecithinase, although it typically does not give an egg-yolk reaction. These facts suggest that a positive egg-yolk reaction may denote the ability to produce a specific type of lecithinase, other lecithinases not producing the turbidity characteristic of the egg-yolk reaction.

Lipase production, which we have detected by the hydrolysis of Tween 80, seems to be a less useful character. It is invariably positive in *Pseudomonas aeruginosa*, and was possessed by 58 out of 95 strains of *P. fluorescens*, and 3 out of 41 strains of *P. putida*.

A careful enzymological and immunological study of the extracellular hydrolases produced by fluorescent pseudomonads is much needed. It might well make the taxonomic differentiation between *Pseudomonas aeruginosa* and *P. fluorescens* on the basis of such characters far more precise and reliable than it now is.

Temperature relations. Every strain of *Pseudomonas aeruginosa* grows abundantly at 41°; no strain of *P. fluorescens* or *P. putida* can do so. No strain of *P. aeruginosa* can grow at 4°, whereas every strain of *P. fluorescens* can do so. The ability to grow at 4° was variable in *P. putida*; 20 strains did so.

Denitrification. The capacity to denitrify is universal in *Pseudomonas aeruginosa*, and always absent from *P. putida*. It is a variable character in *P. fluorescens*, found in certain biotypes, but not in others; somewhat less than half of the strains examined were denitrifiers.

Phenazine pigment production. Pyocyanine was produced on slopes of King A medium by 25 of the 29 strains identified as *Pseudomonas aeruginosa*. Strains capable of producing other phenazine pigments were assigned on the basis of overall phenotype to *P. fluorescens*. Specifically, 10 strains shown to produce chlororaphin, 7 strains shown to produce phenazine-α-carboxylic acid, and 1 strain which produced

the blue pigment characteristic of *P. lemonnieri* all fall within the general confines of *P. fluorescens*. In our opinion, the ability to produce these pigments is not a character of sufficient importance to justify the recognition of *P. chlororaphis*, *P. aureofaciens* and *P. lemonnieri* as species distinct from *P. fluorescens*, which these organisms resemble in other respects. None of the strains of *P. putida* was observed to produce phenazine pigments.

Nutritional patterns. Strains of *Pseudomonas fluorescens* characteristically utilize an extensive range of carbohydrates and polyalcohols, whereas strains of *P. aeruginosa* and most strains of *P. putida* do not. For purposes of differentiation, the two most valuable compounds of this class appear to be trehalose and *meso*-inositol. No strain of *P. aeruginosa* or *P. putida* utilized either compound; trehalose was utilized by 93 out of 94 strains of *P. fluorescens*, and inositol by 85 strains. Sucrose utilization is of considerably less value; although sucrose is never used by *P. aeruginosa*, it was used by only 65 out of 94 strains of *P. fluorescens*, and by four strains out of 41 of *P. putida*. *Pseudomonas fluorescens* characteristically attack this sugar by means of levan sucrase (Fuchs, 1959); only five of these Sucrose-positive strains examined by us did not produce levan from sucrose. The rare sucrose-positive strains of *P. putida*, on the other hand, were not levan-formers.

Two very valuable nutritional characters for the recognition of *Pseudomonas aeruginosa* are the ability to grow on the long-chain alcohol geraniol, possessed by all strains; and the ability to grow on one of two aliphatic hydrocarbons, dodecane or hexadecane, possessed by 25 of the 29 strains tested. None of these three compounds can be used by any strain of *P. fluorescens* or *P. putida*.

Pseudomonas putida and *P. aeruginosa* characteristically grow on the complete series of fatty acids from acetate to caprate (C_2 to C_{10}). However, some biotypes of *P. fluorescens* grew very poorly on the *n* and iso C_4 and C_5 fatty acids; in fact, failure to grow on two or more of this group of four fatty acids is practically diagnostic for *P. fluorescens*. This character is, however, not infallible for the recognition of *P. fluorescens*, since three of the biotypes included (biotypes B, D, E) did not share it.

Good growth on the higher homologues of the dicarboxylic acid series (C_6 to C_{10}) is characteristic for *Pseudomonas aeruginosa*. Only two strains of *P. putida* attacked any of these acids, but their growth was very poor. Most strains of *P. fluorescens* likewise did not grow on them, with the exception of some strains belonging to biotype C.

Among the polyalcohols, erythritol, sorbitol, *meso*-inositol and adonitol are never used by *Pseudomonas aeruginosa* and only by exceptional strains of *P. putida*. These compounds were, on the other hand, attacked by at least half of the *P. fluorescens* strains.

The aliphatic alcohols, ethanol, propanol, butanol and isobutanol, are universal substrates for *Pseudomonas aeruginosa* and almost universal for *P. putida*. However, three of the biotypes of *P. fluorescens* (A, E, F) failed completely to grow on ethanol, *n*-propanol and isobutanol. Consequently, the failure to use the alcohols is a sufficient, but not a necessary, criterion for the recognition of *P. fluorescens*.

All strains of *Pseudomonas aeruginosa* grew on L-mandelate, an aromatic substrate only used rarely by *P. fluorescens* (3 strains) and *P. putida* (3 strains). Phenylacetate is an aromatic substrate that is used by most strains of *P. putida*, but never

attacked by *P. aeruginosa*. Most biotypes of *P. fluorescens* are likewise characteristically phenylacetate negative, with the exceptions of biotypes D and E.

Among the amino acids, glycine is never utilized by *Pseudomonas fluorescens*, but can be used by a large majority of the strains of both *P. aeruginosa* and *P. putida*. α-Aminovalerate was used by 23 strains of *P. putida* but by no strain of *P. aeruginosa* or *P. fluorescens*.

Table 12. *Selected characters for the differentiation of the species of fluorescent pseudomonads*

A. Characters for the differentiation between *Pseudomonas aeruginosa* and *P. fluorescens*

	P. aeruginosa	*P. fluorescens*
1. Monotrichous flagellation	+	−
2. Pyocyanine production	+	−
3. Growth at 4°	−	+
4. Growth at 41°	+	−
5. Egg-yolk reaction	−	+
6. Levan formation from sucrose	−	+
7. Utilization of trehalose	−	+
8. Utilization of inositol	−	+
9. Utilization of geraniol	+	−
10. Utilization of acetamide	+	−

B. Characters for the differentiation between *P. aeruginosa* and *P. putida*

	P. aeruginosa	*P. putida*
1. Monotrichous flagellation	+	−
2. Pyocyanine production	+	−
3. Growth at 41°	+	−
4. Liquefaction of gelatin	+	−
5. Denitrification	+	−
6. Utilization of geraniol	+	−
7. Utilization of phenylacetate	−	+
8. Utilization of benzylamine	−	+
9. Utilization of creatine	−	+
10. Utilization of hippurate	−	+

C. Characters for the differentiation between *P. fluorescens* and *P. putida*

	P. fluorescens	*P. putida*
1. Gelatin liquefaction	+	−
2. Egg-yolk reaction	+	−
3. Utilization of trehalose	+	−
4. Utilization of inositol	+	−
5. Utilization of two or more of the following nitrogenous compounds: benzylamine, creatine, hippurate and glycine	−	+

Pseudomonas putida characteristically grows well on several higher amines (benzylamine, butylamine, α-amylamine) that are not used by *P. aeruginosa*, or by the overwhelming majority of strains of *P. fluorescens*. By far the most useful compound of this class for differential purposes is benzylamine, used by all strains of *P. putida*, and by only four strains of *P. fluorescens*. Creatine and hippurate are two other nitrogenous organic compounds characteristically utilized by *P. putida*, but not by *P. aeruginosa* or *P. fluorescens*. No strain of *P. aeruginosa* attacked either creatine or hippurate; only two strains of *P. fluorescens* attacked each of these

compounds. However, their utilization by *P. putida* is not universal: 33 strains used creatine and 30 hippurate. A nutritional character which has been proposed as specific for *P. aeruginosa* is the ability to grow on acetamide (Bühlmann, Vischer & Bruhin, 1961). All 29 strains tested possessed this property. However, it was also possessed by 11 of the 41 strains of *P. putida*, though by no strain of *P. fluorescens*.

The alkaloid trigonelline (a derivative of nicotinic acid) is a universal substrate for *Pseudomonas putida* and never utilized by *P. aeruginosa*. Two biotypes of *P. fluorescens* (B, G) grow well on it; the remainder are negative, with the exception of four strains of biotype A.

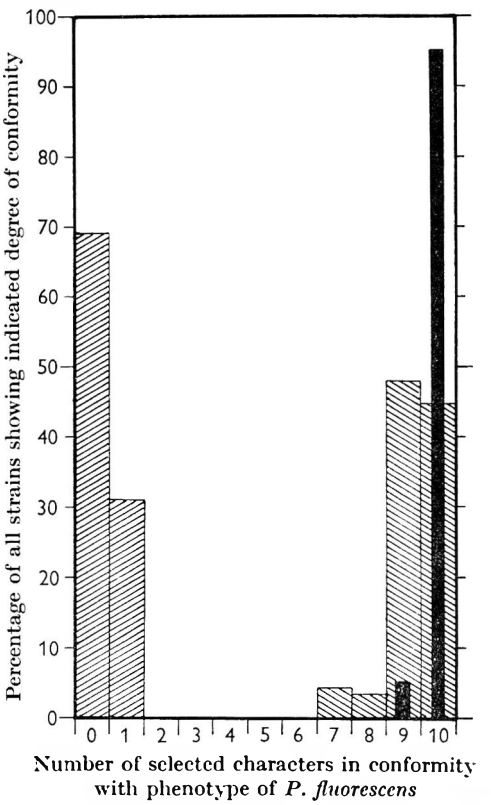

Fig. 3. The differentiation between *Pseudomonas aeruginosa* and *P. fluorescens*, based on a group of ten selected contrasting characters. ▨, *P. aeruginosa*; ▧, *P. fluorescens* (all biotypes); ■, *P. fluorescens* (phenazine-producing biotypes).

From our complete data on the properties of the three fluorescent species, it is possible to select relatively small constellations of characters which permit unambiguous separations between three species (Table 12). A set of ten characters serves to differentiate between *Pseudomonas aeruginosa* and *P. fluorescens*; another set of ten characters differentiates between *P. aeruginosa* and *P. putida*; and a set of five characters differentiates between *P. fluorescens* and *P. putida*. The sharpness with which these selected characters permit interspecific differentiation is shown graphically in Figs. 3–5.

In Fig. 3 all the strains of *Pseudomonas aeruginosa* and of all the biotypes of *P. fluorescens* have been scored on a percentile basis for the extent of their conformity to the ten selected differential characters possessed by *P. fluorescens*. It can be seen that almost 70% of the strains of *P. aeruginosa* possessed none of these characters, while slightly more than 30% possessed one of them. Ninety-three per cent of the strains of *P. fluorescens* possessed either nine or ten of these characters; the remaining 7% possessed either seven or eight. We have calculated separately the degree of conformity of all phenazine-producing strains assigned to

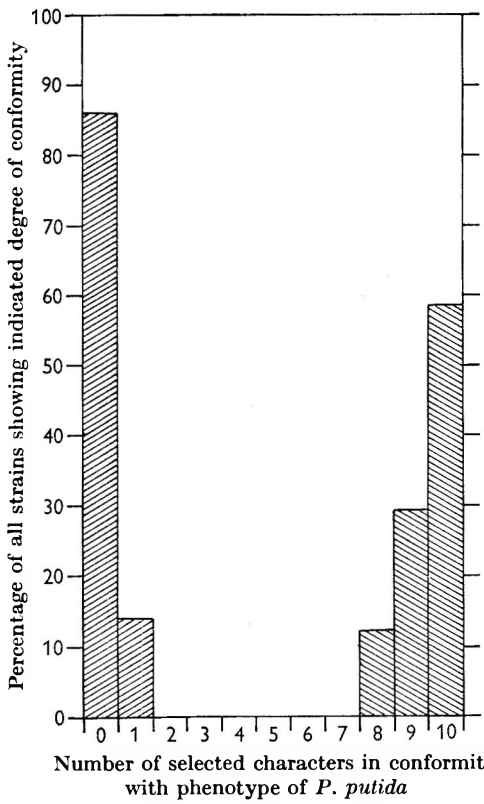

Fig. 4

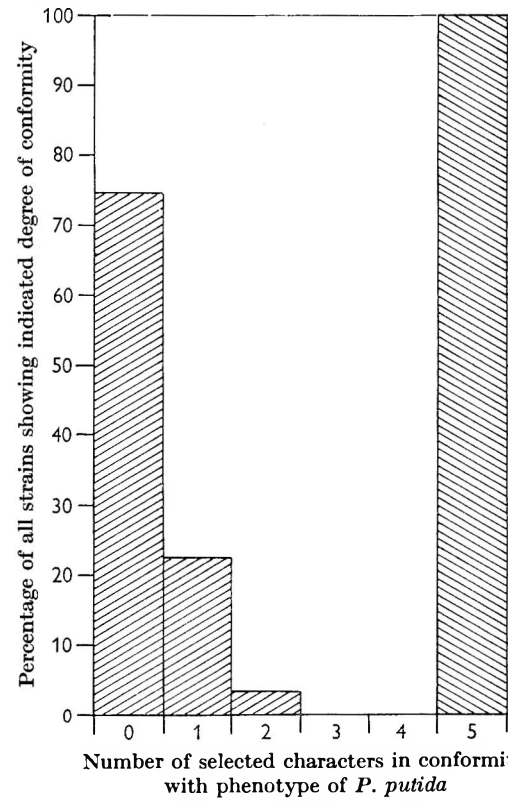

Fig. 5

Fig. 4. The differentiation between *Pseudomonas aeruginosa* and *P. putida*, based on a group of ten selected contrasting characters. ▨, *P. aeruginosa*; ▧, *P. putida* (both biotypes).

Fig. 5. The differentiation between *Pseudomonas fluorescens* and *P. putida*, based on a group of five selected contrasting characters. ▨, *P. fluorescens* (all biotypes); ▧, *P. putida* (both biotypes).

P. fluorescens (biotypes D, E, F); these data are also shown in Fig. 3 as narrow black bars. Ninety-five per cent of the phenazine-producing strains conformed to all ten characters of *P. fluorescens* and 5% to nine of them. It is accordingly easy to distinguish these phenazine-producing fluorescent pseudomonads from *P. aeruginosa* on the basis of the characters here used.

The extent of conformity of all strains of *Pseudomonas aeruginosa* and *P. putida*

(all biotypes) to the ten selected characters distinctive of *P. putida* is shown in Fig. 4. Again, the separation can be considered highly satisfactory. Figure 5 shows the extent of conformity of all strains of *P. fluorescens* and *P. putida* to the five characters distinctive of *P. putida*. Although the separation in this case is adequate for determinative purposes, it is less clear-cut that in the two cases previously considered. All strains of *P. putida* show perfect conformity with respect to these five characters. Almost 75% of the strains of *P. fluorescens* show no conformity; but 22% conform with respect to one character and 3% with respect to two characters.

Pseudomonas aeruginosa

The properties of *Pseudomonas aeruginosa* which are valuable for its differentiation from other fluorescent pseudomonads have already been discussed. Accordingly, in this section we shall describe, without further comment, the detailed phenotypic traits of the 29 strains that we have examined.

Flagellation: monotrichous in all.

Pyocyanine production: all except strains 57, 278, 282, 287.

Fluorescent pigment: all except strains 57, 280, 281, 283, 284, 285, 286, 416.

Strains 281 and 283 to 295, inclusive, also produced on King B medium slopes a brownish red diffusible pigment which became very dark in time, and masked the presence of other pigments.

Denitrification: vigorous in all.

Gelatin liquefaction: positive in all.

Egg-yolk reaction: negative in all.

Hydrolysis of Tween 80: positive in all, but weak and delayed. In Table 13, we have listed those carbon and energy sources used by 90% or more of the strains. Table 14 lists substrates that were not universally used, and indicates specifically the negative strains.

Pseudomonas fluorescens

The characters which we propose for the differentiation of *Pseudomonas fluorescens* from *P. putida* and *P. aeruginosa* have already been discussed, and we shall therefore introduce the detailed description of this species by a general survey of its seven biotypes, and of the properties which serve for their recognition. The principal properties which we have found to be of value for this purpose are: phenazine pigment production: denitrification; levan formation from sucrose; utilization of L-arabinose, D-xylose, saccharate, certain fatty acids, hydroxymethylglutarate, sorbitol, adonitol, certain glycols and alcohols, benzoylformate, phenylacetate, butylamine, trigonelline (Table 15). The possibility of subdividing the gelatin-liquefying fluorescent pseudomonads (excluding *P. aeruginosa*) on the basis of levan formation and denitrification has been previously suggested (e.g. by Fuchs, 1959); it is accordingly gratifying to find that a primary subdivision on these characters is correlated with differences in many other unrelated nutritional properties. The ability to produce specific phenazine pigments, heretofore used as the sole justification for the recognition of three gelatin-liquefying species, *P. chlororaphis* (= our biotype D), *P. aureofaciens* (= our biotype E) and *P. lemonnieri* (= our biotype F) is also a significant internal marker in *P. fluorescens*, since it is correlated with a number of nutritional characters. However, these three biotypes

show such high overall phenotypic resemblances to non-phenazine-producing strains of *P. fluorescens* that in our opinion it is unjustified to accord them more than varietal status.

Table 13. *Pseudomonas aeruginosa. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose
- D-Glucose
- D-Fructose*
- Gluconate
- 2-Ketogluconate

Fatty acids
- Acetate
- Propionate
- Butyrate
- Isobutyrate
- Valerate
- Isovalerate
- Caproate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate
- Adipate
- Azelate*
- Sebacate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate*
- Laevulinate
- Itaconate
- Mesaconate

Polyalcohols and glycols
- Mannitol
- Glycerol
- Propyleneglycol*
- 2,3-Butyleneglycol*

Alcohols
- Ethanol
- *n*-Propanol
- *n*-Butanol
- Isobutanol
- Geraniol

Non-nitrogenous aromatic and other cyclic compounds
- L-Mandelate
- Benzoylformate*
- Benzoate
- *p*-Hydroxybenzoate*
- Quinate*

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Leucine*
- L-Aspartate
- L-Glutamate
- L-Lysine*
- DL-Arginine
- DL-Ornithine
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine*
- L-Kynurenine
- Anthranilate

Amines
- Putrescine
- Spermine
- Histamine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine*
- Acetamide

Paraffin hydrocarbons
- (None)

* Substrate not utilized by all strains. Negative strains are shown in Table 14.

The properties of specific phenazine pigment production, levan formation from sucrose and denitrification enable us to define six biotypes (A–F) which are positive for at least one of these characters, and which are further differentiable with a

considerable degree of sharpness by nutritional characters. These biotypes encompass 77 of the 94 strains that we assign to *Pseudomonas fluorescens*, leaving a residue of 17 strains which have the general characteristics of the species, but are negative for all the primary characters used to distinguish the other six biotypes. These we have assembled in a final biotype, G, which is far more heterogeneous, as shown by the irregularity of the nutritional data. Biotype G is frankly a provisional group, which lacks the uniformity characteristic of the other six biotypes.

Table 14. *Pseudomonas aeruginosa. Substrates utilized by a variable number of the strains*

Substrate	Total positive strains	Negative strains
D-Fruetose	28	290
Pimelate	25	279, 284, 289, 296
Suberate	19	132, 279, 281, 284, 286, 288, 289, 290, 294, 296
Azelate	28	52
D-Malate	3	All except 282, 292, 293
Aconitate	28	416
Propyleneglycol	28	57
2,3-Butyleneglycol	27	45, 416
Benzoylformate	28	279
p-Hydroxybenzoate	28	52
Quinate	26	52, 282, 290
Glycine	22	52, 55, 57, 131, 290, 291, 294
L-Serine	4	All except 281, 282, 293, 416
L-Leucine	28	291
L-Isoleucine	15	132, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290
L-Valine	25	54, 52, 131, 132
L-Lysine	26	52, 54, 55
L-Citrulline	22	52, 54, 55, 58, 131, 132, 416
L-Tyrosine	28	57
L-Phenylalanine	7	All except 45, 54, 55, 57, 132, 279, 293
L-Tryptophan	21	279, 280, 284, 289, 290, 294, 296, 415
Kynurenate	13	54, 58, 132, 277, 278, 279, 280, 281, 283, 287, 288, 289, 290, 292, 295, 296
Ethanolamine	19	277, 279, 280, 281, 285, 286, 287, 291, 292, 293
Sarcosine	26	288, 289, 290
Dodecane	4	All except 45, 58, 131, 132
Hexadecane	21	55, 57, 278, 287, 294, 295, 296

Biotype A. With respect to the general characters of the species, biotype A is typical in almost all respects, being uniformly positive for fluorescent pigment production, gelatin liquefaction, egg-yolk reaction, and growth at 4°. One strain (12) did not hydrolyse Tween 80. Substrates used by 90% or more of the strains are listed in Table 16; and substrates used by only a fraction of the strains in Table 17, which also lists specifically the negative strains.

Biotype B. Not all strains of biotype B produce fluorescent pigment on King B medium; negative for this character were strains 400, 402, 405, 409, 413, 414. All grow at 4°. All liquefy gelatin, but do not hydrolyse Tween 80, and characteristically give a weak or negative egg-yolk reaction; only strains 2, 400, 402, 403, 411, 414 and

Table 15. *Pseudomonas fluorescens. Characters which serve to differentiate seven biotypes among the 94 strains*

	No. of positive strains (percentages in parentheses)						
	Biotype A (24 strains)	Biotype B (19 strains)	Biotype C (15 strains)	Biotype D (10 strains)	Biotype E (7 strains)	Biotype F (2 strains)	Biotype G (17 strains)
Production of							
Chlororaphin	0 (0)	0 (0)	0 (0)	10 (100)	0 (0)	0 (0)	0 (0)
Phenazine-α-carboxylic acid	0 (0)	0 (0)	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)
Insoluble blue phenazine pigment	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)
Levan formation from sucrose	24 (100)	19 (100)	0 (0)	10 (100)	7 (100)	2 (100)	0 (0)
Denitrification	0 (0)	19 (100)	15 (100)	10 (100)	0 (0)	2 (100)	0 (0)
Utilization of							
L-Arabinose	23 (96)	18 (95)	0 (0)	0 (0)	7 (100)	2 (100)	14 (82)
D-Xylose	22 (92)	15 (79)	0 (0)	0 (0)	0 (0)	1 (50)	13 (76)
Saccharate	24 (100)	18 (95)	0 (0)	10 (100)	7 (100)	2 (100)	14 (82)
Propionate	22 (92)	19 (100)	13 (87)	9 (90)	7 (100)	0 (0)	14 (82)
Butyrate	0 (0)	15 (79)	0 (0)	9 (90)	4 (57)	2 (100)	6 (35)
Isobutyrate	0 (0)	17 (89)	0 (0)	0 (0)	1 (14)	0 (0)	1 (6)
Valerate	6 (25)	17 (89)	9 (60)	10 (100)	7 (100)	0 (0)	8 (47)
Hydroxymethylglutarate	21 (87)	17 (89)	0 (0)	0 (0)	0 (0)	0 (0)	5 (29)
Sorbitol	22 (92)	19 (100)	2 (13)	0 (0)	0 (0)	2 (100)	8 (47)
Adonitol	22 (92)	2 (11)	15 (100)	0 (0)	0 (0)	0 (0)	8 (47)
Propylenegiycol	0 (0)	18 (95)	12 (80)	0 (0)	0 (0)	0 (0)	8 (47)
Ethanol	0 (0)	18 (95)	12 (80)	10 (100)	0 (0)	0 (0)	8 (47)
n-Propanol	0 (0)	18 (95)	12 (80)	10 (100)	0 (0)	0 (0)	8 (47)
Benzoylformate	0 (0)	1 (5)	0 (0)	10 (100)	7 (100)	0 (0)	3 (18)
Phenylacetate	0 (0)	0 (0)	0 (0)	6 (60)	7 (100)	0 (0)	2 (12)
Butylamine	0 (0)	1 (5)	0 (0)	8 (80)	5 (71)	0 (0)	3 (18)
Trigonelline	3 (12)	16 (84)	0 (0)	0 (0)	0 (0)	0 (0)	15 (88)

415 are positive for this latter character. They share with the strains of biotype A the property of levan formation from sucrose, but differ in being, without exception, vigorous denitrifiers. As shown in Table 15, most strains of biotypes A and B share a nutritional property otherwise absent from *Pseudomonas fluorescens*,

Table 16. *Pseudomonas fluorescens biotype A. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose
- D-Xylose*
- L-Arabinose*
- D-Glucose
- D-Mannose
- D-Fructose
- Sucrose
- Trehalose
- Gluconate
- 2-Ketogluconate
- Saccharate
- Mucate

Fatty acids
- Acetate
- Proprionate*
- Caproate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate*

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate
- Itaconate*
- Mesaconate*

Polyalcohols and glycols
- Mannitol
- Sorbitol*
- Adonitol*
- Glycerol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- *p*-Hydroxybenzoate*
- Quinate

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Serine*
- L-Leucine
- L-Isoleucine
- L-Valine*
- L-Aspartate
- L-Glutamate
- L-Lysine*
- DL-Arginine
- DL-Ornithine*
- γ-Aminobutyrate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline*
- L-Tyrosine
- L-Tryptophan*

Amines
- Ethanolamine*
- Putrescine*
- Spermine*

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine

Paraffin hydrocarbons
- (None)

* Substrate not utilized by all strains. Negative strains are shown in Table 17.

namely, ability to grow on hydroxymethylglutarate. The most significant differences in nutritional respects between biotypes A and B concern the utilization of certain fatty acids and alcohols. Most strains of biotype B grow well on butyrate and isobutyrate, propyleneglycol, ethanol and *n*-propanol, whereas none of the strains of biotype A can do so. Another notable feature of biotype B is the high

frequency of utilization of trigonelline, otherwise attacked by only three strains of *P. fluorescens* (all in biotype A). Substrates used by 90 % or more of the strains of biotype B are shown in Table 18; and substrates used by only a fraction of the strains are shown in Table 19, which also lists specifically the negative strains.

Table 17. *Pseudomonas fluorescens biotype A. Substrates utilized only by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Xylose	22	184, 185
L-Arabinose	23	12
Propionate	22	182, 186
Valerate	6	All except 184, 185, 201, 202, 209, 216
Isovalerate	20	12, 126, 211, 392
DL-Glycerate	23	203
Hydroxymethylglutarate	21	184, 185, 198
Laevulinate	6	All except 186, 187, 196, 199, 200, 201
Citraconate	13	126, 184, 185, 201, 202, 203, 209, 215, 216, 392
Itaconate	22	184, 185
Mesaconate	22	184, 185
Erythritol	20	12, 184, 185, 216
Sorbitol	22	184, 185
Inositol	21	184, 185, 216
Adonitol	22	184, 185
2,3-Butyleneglycol	6	All except 187, 189, 198, 199, 203, 215
n-Butanol	4	All except 185, 192, 196, 208
Benzoate	8	All except 126, 188, 189, 196, 199, 201, 209, 216
o-Hydroxybenzoate	1	All except 187
p-Hydroxybenzoate	23	189
L-Serine	23	12
L-Valine	23	392
L-Lysine	22	126, 392
DL-Ornithine	22	184, 185
DL-Citrulline	20	184, 185, 192, 211
δ-Aminovalerate	19	126, 208, 209, 216, 392
L-Proline	23	12
L-Phenylalanine	4	All except 12, 184, 185, 192
L-Tryptophan	22	184, 185
L-Kynurenine	20	184, 185, 208, 216
Kynurenate	12	12, 184, 185, 192, 196, 200, 202, 203, 208, 211, 216, 392
Anthranilate	19	12, 184, 185, 192, 201
Ethanolamine	23	392
Putrescine	22	182, 392
Spermine	23	182
Histamine	13	12, 126, 186, 187, 188, 192, 196, 198, 208, 211, 216
Tryptamine	2	All except 184, 185
Creatine	1	All except 208
Trigonelline	3	All except 184, 185, 215

Biotype C. The strains of biotype C all produce fluorescent pigment and grow at 4°. They all liquefy gelatin and give the egg-yolk reaction. Only a minority can hydrolyse Tween 80, the positive strains being 191, 194, 204, 207, 210, 212. All strains of this biotype are denitrifiers. Unlike the strains of biotypes A and B, they

are unable to grow on sucrose, or to synthesize levan from it. Biotype C also differs from most strains of biotypes A and B by its total inability to grow on L-arabinose, D-xylose or saccharate. It shares with biotype A the inability to grow on butyrate and isobutyrate; but most strains do grow well on propyleneglycol, ethanol and

Table 18. *Pseudomonas fluorescens biotype B. Substrates utilized by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose
- L-Arabinose*
- D-Glucose
- D-Mannose
- D-Galactose
- Sucrose
- Trehalose
- Gluconate
- 2-Ketogluconate
- Saccharate*
- Mucate*

Fatty acids
- Acetate
- Proprionate
- Valerate*
- Isovalerate*
- Caproate*
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate*

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate*
- Hydroxymethylglutarate*

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate

Polyalcohols and glycols
- Mannitol
- Sorbitol
- Inositol
- Glycerol
- Propyleneglycol*
- 2,3-Butyleneglycol

Alcohols
- Ethanol*
- *n*-Propanol*
- Isobutanol*

Non-nitrogenous aromatic and other cyclic compounds
- (None)

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- DL-Arginine
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Proline
- L-Tyrosine

Amines
- Putrescine
- Spermine

Miscellaneous nitrogenous compounds
- Betaine

Paraffin hydrocarbons
- (None)

* Substrate not used by all strains. Negative strains shown in Table 19.

n-propanol, characters absent from biotype A. Nine of the 15 strains of biotype C share another set of nutritional characters unusual among fluorescent pseudomonads, with the exception of *Pseudomonas aeruginosa*; they grow well and uniformly on the series of dicarboxylic acids from adipate to sebacate. This is one of the characters which den Dooren de Jong believed to distinguish *P. acidovorans* from fluorescent pseudomonads. It is indeed a very nearly universal property in the acidovorans group; but its presence in *P. aeruginosa* and *P. fluorescens* biotype C destroys its value as a differential character for distinguishing the fluorescent

Table 19. *Pseudomonas fluorescens biotype B. Substrates utilized by only a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Xylose	15	108, 400, 402, 409
L-Arabinose	18	409
L-Rhamnose	4	All except 401, 403, 414, 415
D-Fructose	13	404, 405, 406, 408. 410, 412
Saccharate	18	409
Mucate	18	410
Butyrate	15	2, 93, 400, 402
Isobutyrate	1	2, 93
Valerate	17	2, 93
Isovalerate	17	2, 93
Caproate	17	2, 93
Glutarate	17	400, 402
D-Malate	6	All except 403, 407, 409, 413, 414, 415
D-(−)-Tartrate	4	All except 401, 403, 411, 414
meso-Tartrate	1	All except 413
Glycollate	1	All except 93
DL-Glycerate	17	400, 402
Hydroxymethylglutarate	17	93, 413
Laevulinate	1	All except 402
Citraconate	6	All except 93, 401, 403, 411, 414, 415
Itaconate	14	2, 407, 412, 413, 415
Mesaconate	13	2, 93, 407, 412, 413, 415
Erythritol	14	2, 93, 405, 407, 413
Adonitol	2	All except 2, 415
Ethyleneglycol	1	All except 93
Propyleneglycol	18	2
Ethanol	18	93
n-Propanol	18	93
n-Butanol	14	2, 93, 400, 402, 411
Isobutanol	17	2, 93
L-Mandelate	1	All except 93
Benzoylformate	1	All except 93
Benzoate	3	All except 93, 407, 413
L-Serine	14	2, 93, 108, 405, 412
L-Leucine	14	400, 402, 405, 406, 408
L-Valine	17	400, 402
L-Lysine	11	2, 108, 400, 402, 404, 405, 407, 412
L-Arginine	17	400, 402
DL-Ornithine	14	2, 400, 402, 407, 413
DL-Citrulline	12	2, 401, 403, 407, 413, 414, 415
L-Histidine	15	405, 406, 409, 412
L-Phenylalanine	7	All except 2, 93, 400, 401, 402, 407, 413
L-Tryptophan	14	400, 401, 403, 407, 413
L-Kynurenine	15	401, 403, 407, 413
Anthranilate	9	All except 2, 93, 406, 408, 410, 411, 412, 414 415
Ethanolamine	10	2, 400, 403, 404, 405, 406, 408, 409, 410
Histamine	1	All except 413
Tryptamine	2	All except 108, 415
Butylamine	1	All except 413
α-Amylamine	4	All except 2, 93, 108, 413
Sarcosine	14	405, 406, 408, 410, 412
Trigonelline	16	408, 409, 413

group from the acidovorans group. The fluorescent strain described recently by Janota-Bassalik & Wright (1964), which was capable of growing on higher dicarboxylic acids and of denitrifying, is obviously a representative of *P. fluorescens* biotype C.

Substrates used by 90 % or more of the strains of biotype C are shown in Table 20;

Table 20. *Pseudomonas fluorescens biotype C. Substrates used by 90 % or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose
- D-Glucose
- D-Mannose
- D-Galactose*
- D-Fructose*
- Trehalose
- Gluconate
- 2-Ketogluconate

Fatty acids
- Acetate
- Proprionate*
- Caproate*
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate

Polyalcohols and glycols
- Erythritol
- Mannitol
- *meso*-Inositol
- Adonitol
- Glycerol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- *p*-Hydroxybenzoate*
- Quinate*

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Serine
- L-Leucine
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- L-Lysine
- DL-Arginine
- DL-Ornithine
- DL-Citrulline
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine
- L-Tryptophan
- L-Kynurenine
- Kynurenate
- Anthranilate

Amines
- Ethanolamine
- Putrescine
- Spermine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine
- Pantothenate

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are shown in Table 21.

and substrates used by only a fraction of the strains in Table 21, which also lists specifically the negative strains.

Biotype D. Biotype D comprises all save three of the strains which we received with the specific designation *Pseudomonas chlororaphis*; the exceptions are strain 392, which is a typical representative of biotype A; and 33 and 34, which we have

assigned to biotype G. In none of these three strains could we observe production of chlororaphin.

All the strains which we assign to biotype D produced chlororaphin on King A medium, and all save one (strain 393) produced fluorescent pigment on King B medium. All grow at 4°. All liquefy gelatin, give the egg-yolk reaction and hydrolyse

Table 21. *Pseudomonas fluorescens biotype C. Substrates used by only a fraction of the strains*

Substrate	Total of positive strains	Negative strains
L-Rhamnose	4	All except 191, 194, 205, 207
D-Galactose	14	204
D-Fructose	14	204
Mucate	10	18, 50, 204, 210, 212
Propionate	13	181, 194
Valerate	9	18, 181, 191, 194, 204, 207
Isovalerate	9	18, 50, 191, 194, 204, 212
Caproate	18	18, 50
Maleate	8	All except 204, 210, 212
Adipate	9	181, 213, 214, 217, 217, 218, 219
Pimelate	9	181, 213, 214, 218, 219
Suberate	9	181, 213, 214, 217, 218, 219
Azelate	9	181, 213, 214, 217, 218, 219
Sebacate	9	181, 213, 214, 217, 218, 219
Aconitate	12	204, 210, 212
Laevulinate	3	All except 181, 191, 194
Citraconate	6	All except 18, 181, 191, 194, 205, 207
Itaconate	6	All except 181, 191, 194, 204, 210, 212
Mesaconate	6	All except 181, 191, 204, 210, 212
Sorbitol	2	All except 181, 218
Propyleneglycol	12	204, 210, 212
2,3-Butyleneglycol	9	181, 191, 194, 204, 210, 212
Ethanol	12	204, 210, 212
n-Propanol	12	204, 210, 212
n-Butanol	10	50, 204, 207, 210, 212
Isobutanol	12	204, 210, 212
Benzoate	6	All except 18, 204, 213, 214, 217, 219
m-Hydroxybenzoate	1	All except 210
p-Hydroxybenzoate	14	212
Quinate	14	50
L-Lysine	14	50
L-Tyrosine	14	212
L-Phenylalanine	3	All except 181, 194, 214
Kynurenate	13	50, 210
Anthranilate	13	18, 212
Benzylamine	1	All except 210
Tryptamine	5	All except 191, 194, 204, 210, 212
α-Amylamine	5	All except 191, 194, 204, 210, 212
Sarcosine	14	212
Creatine	1	All except 210
Hippurate	2	All except 18, 181
Pantothenate	13	18, 50

Tween 80. All produce levan from sucrose and denitrify. The properties of levan formation and denitrification are shared with biotype B, from which biotype D is, however, differentiable by several nutritional characters as well as by chlororaphin

production. No strain of biotype D can use L-arabinose, D-xylose, isobutyrate, hydroxymethylglutarate, sorbitol, propyleneglycol or trigonelline; most strains of biotype D use benzoylformate, phenylacetate and butylamine, which are rarely or never used by biotype B (see Table 15).

Table 22. *Pseudomonas fluorescens biotype D. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose
- D-Glucose
- D-Mannose
- Sucrose
- Trehalose
- Gluconate
- 2-Ketogluconate
- Saccharate
- Mucate

Fatty acids
- Acetate
- Propionate*
- Butyrate*
- Valerate
- Isovalerate
- Caproate*
- Heptanoate
- Pelargonate
- Caprylate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate*

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Itaconate
- Mesaconate

Polyalcohols and glycols
- Mannitol
- *meso*-Inositol
- Glycerol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- Benzoylformate
- Benzoate
- *p*-Hydroxybenzoate
- Quinate

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Leucine
- L-Isoleucine
- L-Valine*
- L-Aspartate
- L-Glutamate
- DL-Arginine
- γ-Aminobutyrate
- δ-Aminovalerate*

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine
- L-Tryptophan
- L-Kynurenine
- Anthranilate

Amines
- Putrescine
- Spermine
- Histamine*

Miscellaneous nitrogenous compounds
- Betaine

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are given in Table 23.

Substrates used by 90% or more of the strains of biotype D are shown in Table 22; and substrates used by only a fraction of the strains in Table 23, which also lists specifically the negative strains.

Biotype E. Biotype E contains the seven strains which were received as *Pseudomonas aureofaciens*. All produced fluorescent pigment on King B medium, and an

orange pigment (presumably phenazine-α-carboxylic acid) on King A medium. All grow at 4°. All hydrolyse gelatin, strain 41 very weakly; and, with the exception of this strain, all give a positive egg-yolk reaction and hydrolyse Tween 80. All produce levan from sucrose, but none can denitrify.

Table 23. *Pseudomonas fluorescens biotype D. Substrates used by only a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Galactose	3	30, 32, 35, 388, 391, 393, 394
D-Fructose	7	388, 393, 394
Propionate	9	388
Butyrate	9	391
Caproate	9	394
Adipate	1	All except 389
D-Malate	6	32, 35, 388, 391
L-(+)-Tartrate	6	32, 35, 388, 391
DL-Glycerate	9	391
Aconitate	6	32, 35, 388, 391
Laevulinate	5	32, 388, 389, 390, 391
2,3-Butylene glycol	4	32, 35, 388, 389, 390, 391
Erythritol	4	30, 388, 389, 390, 393, 394
Ethanol	1	All except 394
n-Propanol	1	All except 394
n-Butanol	4	388, 389, 390, 391, 393, 394
o-Hydroxybenzoate	1	All except 389
m-Hydroxybenzoate	2	All except 32, 388
Phenylacetate	6	30, 31, 393, 394
Testosterone	1	All except 391
L-Serine	6	388, 389, 390, 391
L-Valine	9	388
L-Lysine	6	388, 389, 390, 391
DL-Ornithine	5	32, 388, 391, 393, 394
L-Citrulline	5	32, 388, 391, 393, 394
δ-Aminovalerate	9	388
L-Phenylalanine	4	30, 388, 389, 391, 393, 394
Kynurenate	1	All except 391
Ethanolamine	8	390, 391
Histamine	9	391
Butylamine	8	32, 388
α-Amylamine	7	32, 388, 391
Sarcosine	8	388, 390

Strain 41 is atypical of the entire fluorescent group with respect to the utilization of glucose and related compounds. It is the only fluorescent pseudomonad which cannot grow on glucose or gluconate; one of two strains which cannot use glycerol; and one of five which cannot use 2-ketogluconate. Indeed, the only two sugars able to support its growth are the sterically related compounds galactose and L-arabinose. Strain 41 is none the less a vigorous levan former; hence it can cleave sucrose, even though it cannot use this disaccharide as a carbon source, owing to inability to use glucose, the monosaccharide liberated by the action of levan sucrase.

In the general character of its nutritional spectrum biotype E most closely resembles biotype D (= *Pseudomonas chlororaphis*). The principal respects in which

it differs from biotype D are ability to use L-arabinose, and inability to use ethanol and propanol, coupled of course with failure to denitrify and the production of a slightly different kind of phenazine pigment. (The orange pigment of *P. aureofaciens* is the free acid of phenazine-α-carboxamide, oxychlororaphin; chlororaphin

Table 24. *Pseudomonas fluorescens biotype E. Substrates used by 85 % or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose*
- L-Arabinose
- D-Glucose*
- D-Mannose*
- D-Galactose
- D-Fructose*
- Sucrose*
- Trehalose*
- Gluconate*
- 2-Ketogluconate*
- Saccharate
- Mucate

Fatty acids
- Acetate
- Propionate
- Valerate
- Isovalerate
- Caproate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate
- Itaconate
- Mesaconate

Polyalcohols and glycols
- Mannitol
- *meso*-Inositol
- Glycerol*

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- Benzoylformate
- Benzoate*
- *p*-Hydroxybenzoate
- Phenylacetate
- Quinate

Aliphatic amino acids
- L-α-Alanine
- β-Alanine
- L-Serine
- L-Leucine
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- DL-Arginine
- DL-Ornithine
- γ-Aminobutyrate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine
- L-Phenylalanine
- L-Tryptophan
- L-Kynurenine
- Anthranilate

Amines
- Ethanolamine
- Putrescine
- Spermine
- Histamine*
- α-Amylamine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are given in Table 25.

is a reduced dimer of phenazine-α-carboxamide; Sneath, 1960). The two biotypes D and E are accordingly easily differentiable from one another. Biotype E shares with biotype A the ability to form levan from sucrose, and the inability to denitrify; but they differ from one another in many nutritional characters.

Characters which are absent from biotype E but overwhelmingly positive in biotype A include: utilization of xylose, hydroxymethylglutarate, sorbitol and adonitol; conversely, all strains of biotype E can use benzoylformate and phenylacetate, while none of biotype A can do so. Substrates used by six out of seven or by all strains of biotype D are shown in Table 24; substrates used by only a fraction of the strains in Table 25, which also lists the negative strains.

Table 25. *Pseudomonas fluorescens biotype E. Substrates utilized by only a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Ribose	6	41
D-Glucose	6	41
D-Mannose	6	41
D-Fructose	6	41
Sucrose	6	41
Trehalose	6	41
Gluconate	6	41
2-Ketogluconate	6	41
Butyrate	4	37, 39, 40
Isobutyrate	1	All except 41
Laevulinate	5	36, 37
Glycerol	6	41
2,3-Butyleneglycol	5	38, 41
Benzoate	6	37
DL-Citrulline	2	All except 36, 86
DL-α-Aminovalerate	1	All except 36
δ-Aminovalerate	4	37, 38, 41
Kynurenate	1	All except 36
Histamine	6	41
Butylamine	5	36, 37

Biotype F. This biotype, which corresponds to the named species *Pseudomonas lemonnieri*, is unfortunately represented by only two strains, so that the weight which should be placed on the characters that appear to differentiate it from other biotypes of *P. fluorescens* is uncertain. Strain 83 is an authentic strain studied by Hugo & Turner (1957), whereas strain 143 was isolated in Berkeley. The recognition of *P. lemonnieri* by means of its most distinctive character, the production of a blue insoluble phenazine pigment (Starr *et al.* 1960), is not easy in our experience. In the first place, King A medium, which is otherwise a very reliable medium for demonstrating the production of phenazine pigments, fails completely with strains of this biotype; and it is necessary to use another special medium (see Methods). Even on this special medium, the results were erratic; strain 83 produced only blue sectors, indicating that the population was genetically heterogeneous with respect to the character of specific phenazine production; and strain 143 gave negative results, even though it had been tentatively identified as *P. lemonnieri* by virtue of its characteristic colour upon primary isolation several years ago. However, in other respects these two independent isolates are very similar, and share a number of properties which seem to set them apart from the biotypes so far described. Both produce fluorescent pigment on King B medium. Both liquefy gelatin and give an egg-yolk reaction; one strain (83) hydrolyses Tween 80. Both grow at 4°. Both are

levan formers and denitrifiers. Hugo & Turner (1957) reported monotrichous flagellation for the strains of *P. lemonnieri* studied by them. However, we found both the strains studied by us to be multitrichous; this has been confirmed by Dr H. Lautrop (personal communication).

Table 26. *Pseudomonas fluorescens biotype F. Substrates utilized by the two strains*

Carbohydrates and sugar derivatives
- D-Ribose
- L-Arabinose
- D-Glucose
- D-Mannose
- D-Galactose
- D-Fructose
- Sucrose
- Trehalose
- Gluconate
- Saccharate
- Mucate

Fatty acids
- Acetate
- Butyrate
- Caproate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- D-Malate
- L-Malate
- L-(+)-Tartrate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate
- Citraconate

Polyalcohols and glycols
- Mannitol
- Sorbitol

Polyalcohols and glycols (*cont.*)
- *meso*-Inositol
- Glycerol
- 2,3-Butyleneglycol

Alcohols
- *n*-Butanol

Non-nitrogenous aromatic and other cyclic compounds
- Benzoate
- *o*-Hydroxybenzoate
- Quinate

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Serine
- L-Leucine
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- L-Lysine
- DL-Arginine
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine
- L-Phenylalanine

Amines
- Ethanolamine
- Putrescine
- Spermine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine

Paraffin hydrocarbons
- (None)

On the properties of denitrification and levan formation, the strains resemble biotypes B and D, from which however they are distinguishable by failure to grow on propionate, valerate, isovalerate, ethanol and *n*-propanol. Furthermore, biotype F can be distinguished from biotype B by failure to grow on hydroxymethylglutarate, propyleneglycol and trigonelline; and from biotype D by failure to grow on benzoylformate, phenylacetate and butylamine, and by growth on L-arabinose and sorbitol.

The compounds which can be utilized as sources of carbon and energy are shown in Table 26. The strains differ in only three nutritional respects: utilization of D-xylose and 2-ketogluconate (positive for strain 83) and of ornithine (positive for strain 143).

Table 27. *Pseudomonas fluorescens biotype G. Substrates utilized by 90 % or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose*
- D-Glucose
- D-Mannose*
- D-Fructose
- Trehalose
- Gluconate
- 2-Ketogluconate

Fatty acids
- Acetate
- Caproate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate*

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate*
- Pyruvate
- Aconitate

Polyalcohols and glycols
- Mannitol*
- Glycerol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- *p*-Hydroxybenzoate
- Quinate

Aliphatic amino acids
- L-α-Alanine
- β-Alanine
- L-Serine*
- L-Leucine*
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- DL-Arginine
- DL-Ornithine*
- γ-Aminobutyrate

Amino acids and related compounds containing a ring structure
- L-Histidine*
- L-Proline
- L-Tyrosine

Amines
- Putrescine*
- Spermine*

Miscellaneous nitrogenous compounds
- Betaine*
- Sarcosine
- Trigonelline*

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are given in Table 28.

Biotype G. This is much the least satisfactory biotype of *Pseudomonas fluorescens* and consists essentially of strains with the general character of the species which remained unaccounted for after the recognition of biotypes A–F. All except strains 164 and 172 produce fluorescent pigment. All are gelatin liquefiers; most give an egg-yolk reaction (negative: strains 169, 172, 206, 267) and hydrolyse Tween 80 (negative: strains 149, 206, 267, 272). One strain (267) is unable to grow at 4°, an otherwise invariable property of *P. fluorescens*. None can denitrify. None can form levan from sucrose, though strains 99, 164, 172 and 269 can grow at the expense of this sugar, thus apparently constituting exceptions to the rule that *P. fluorescens* attacks sucrose by means of a levan sucrase. Substrates used by 90 % or more of the strains are shown in Table 27; and substrates used by only a fraction of the

strains, in Table 28. The characteristic nutritional irregularity of biotype G, shown in the data of Table 15, has already been mentioned. However, this irregularity is at least in part a reflexion of the fact that biotype G assembles representatives of what might well have emerged (had more strains been examined) as additional biotypes of *P. fluorescens*. Some indication of this can be obtained from the perusal of Table 28, which shows certain repeated clusterings of strains. This is particularly true for strains 1, 99 and 124, which differ from the rest of biotype G in many nutritional characters, both positive and negative.

Table 28. *Pseudomonas fluorescens biotype G. Substrates utilized by only a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Ribose	16	124
D-Xylose	13	1, 99, 124, 271
L-Arabinose	14	1, 99, 124
L-Rhamnose	2	All except 267, 272
D-Mannose	15	124, 164
D-Galactose	14	1, 99, 124
Sucrose	4	All except 99, 164, 172, 269
Saccharate	14	1, 99, 124
Mucate	13	1, 99, 124, 272
Propionate	14	267, 269, 271
Butyrate	3	All except 1, 99, 124
Isobutyrate	1	All except 1
Valerate	5	All except 1, 99, 124, 206, 269
Isovalerate	8	33, 34, 149, 164, 166, 172, 267, 271, 272
Glutarate	16	164
Adipate	3	All except 1, 99, 124
Suberate	1	All except 1
Azelate	2	All except 1, 99
Sebacate	4	All except 1, 99, 124, 272
D-Malate	4	All except 195, 267, 269, 271
L-Serine	15	99, 124
L-Leucine	15	267, 272
L-Lysine	13	33, 34, 267, 271
DL-Ornithine	15	169, 271
DL-Citrulline	10	169, 171, 172, 206, 269, 271, 272
DL-α-Aminobutyrate	1	All except 166
DL-α-Aminovalerate	1	All except 1
δ-Aminovalerate	14	33, 34, 267
L-Histidine	16	272
L-Phenylalanine	10	1, 124, 166, 171, 195, 271, 272
L-Tryptophan	11	164, 169, 171, 172, 206, 269
L-Kynurenine	11	164, 169, 171, 172, 206, 269
Kynurenate	2	All except 99, 124
Anthranilate	11	164, 169, 171, 172, 206, 269
Ethanolamine	13	164, 169, 172, 269
Benzylamine	3	All except 1, 124, 171
Putrescine	16	169
Spermine	16	169
Histamine	3	All except 1, 99, 124
Tryptamine	1	All except 272
Butylamine	3	All except 1, 99, 124
α-Amylamine	4	All except 1, 99, 124, 149
Betaine	15	124, 267

Table 28 (*cont.*)

Substrate	Total of positive strains	Negative strains
Nicotinate	9	1, 99, 164, 169, 171, 172, 206, 269
Trigonelline	15	1, 99
D-(−)-Tartrate	1	All except 169
L-(+)-Tartrate	1	All except 267
meso-Tartrate	11	1, 99, 124, 169, 206, 272
Hydroxymethylglutarate	5	All except 33, 34, 149, 166, 195
α-Ketoglutarate	16	267
Laevulinate	1	All except 99
Citraconate	9	1, 33, 34, 99, 124, 169, 172, 206
Itaconate	9	99, 164, 169, 171, 172, 206, 269, 272
Mesaconate	7	All except 1, 33, 34, 149, 166, 195, 271
Erythritol	8	1, 99, 164, 169, 171, 172, 206, 269, 271
Mannitol	16	124
Sorbitol	8	1, 99, 124, 164, 169, 171, 172, 206, 269
meso-Inositol	11	164, 169, 171, 172, 206, 269
Adonitol	8	1, 99, 124, 164, 169, 171, 172, 206, 269
Propyleneglycol	8	All except 1, 99, 124, 149, 166, 195, 267, 272
2,3-Butyleneglycol	8	All except 1, 33, 34, 99, 149, 166, 195, 272
Ethanol	8	All except 1, 99, 124, 149, 166, 195, 267, 272
n-Propanol	10	33, 34, 171, 172, 206, 269, 271
n-Butanol	12	33, 34, 206, 267, 271
Isobutanol	11	33, 34, 172, 206, 269, 271
L-Mandelate	2	All except 99, 124
Benzoylformate	3	All except 1, 99, 124
Benzoate	5	All except 1, 99, 124, 164, 171
Phenylacetate	2	All except 99, 124
Testosterone	1	All except 124

Pseudomonas putida

Of the 175 strains belonging to the fluorescent group that we studied, 52 are unable to hydrolyse gelatin or to give an egg-yolk reaction, do not denitrify, do not grow at 41°, and do not produce phenazine pigment. By virtue of this constellation of characters, they are separable both from *Pseudomonas aeruginosa* and from the seven biotypes of *P. fluorescens*. The failure to hydrolyse gelatin is, of course, the one character which has classically defined *P. putida* (= *P. fluorescens non-liquefaciens* Flügge), and we were therefore initially inclined to regard all these strains as being representative of *P. putida*. In nutritional respects, however, they are not an internally uniform group. A total of 32 strains constitute a fairly homogeneous major subgroup, and we have decided arbitrarily to consider them the central biotype of *P. putida*; we shall term this subgroup biotype A. Another nine strains, internally homogeneous, resemble biotype A in most respects, but are more versatile nutritionally, and will be described as *P. putida* biotype B. The remaining 11 strains present unsolved taxonomic problems, and we shall simply describe them as 'unclassified strains of the fluorescent group'.

Biotype A. All 32 strains produce fluorescent pigment on King B medium. Only two (42, 144) can hydrolyse Tween 80. The ability to grow at 4° is relatively rare,

being possessed only by strains 6, 26, 49, 56, 90, 100, 111, 115, 119, 122, 145 (eleven in all). Substrates used by 90% or more of the strains are shown in Table 29; substrates used by a fraction of the strains are listed in Table 30, with negative strains included.

Table 29. *Pseudomonas putida biotype A. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Glucose
- D-Fruetose*
- Gluconate
- 2-Ketogluconate
- Saccharate
- Mucate

Fatty acids
- Acetate
- Propionate
- Butyrate*
- Valerate
- Isovalerate
- Caproate
- Heptanoate*
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Succinate
- Fumarate
- Glutarate*

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate*
- Pyruvate
- Aconitate

Polyalcohols and glycols
- Glycerol*

Alcohols
- Ethanol*
- *n*-Propanol*
- *n*-Butanol*
- Isobutanol

Non-nitrogenous aromatic and other cyclic compounds
- Benzoate
- *p*-Hydroxybenzoate
- Phenylacetate*
- Quinate*

Aliphatic amino acids
- L-α-Alanine*
- D-α-Alanine*
- β-Alanine
- L-Leucine
- L-Isoleucine
- L-Valine*
- L-Aspartate
- L-Glutamate
- L-Lysine
- DL-Arginine
- DL-Ornithine
- DL-Citrulline
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine
- L-Phenylalanine*

Amines
- Benzylamine
- Putrescine
- Spermine*
- Histamine
- Butylamine*
- α-Amylamine*

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine
- Trigonelline

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are listed in Table 30.

Biotype B. The nine strains of biotype B, with the exception of 98, produce fluorescent pigment. One strain (153) hydrolyses Tween 80. This group of strains can be most readily distinguished from biotype A by their ability to grow on L-tryptophan and L-kynurenine (used by no strain of biotype A) and on anthranilate (used by only two strains of biotype A, 51 and 276). In some respects, biotype B is more fluorescens-like than biotype A: all strains can grow at 4°, and the utilization of sugars is more extensive than in biotype A. The most useful sugar for the dif-

ferentiation between biotypes A and B is galactose, used by no strain of biotype A, and by seven out of nine strains of biotype B. Biotype B has a significantly wider general nutritional spectrum than biotype A, as shown by the much higher frequencies with which such substrates as testosterone, phenol, salicylate, naphthalene and tryptamine are attacked. Substrates used by 90% or more of the strains are shown in Table 31. Table 32 gives the list of substrates attacked by a fraction of the strains, with negative strains indicated. The only two substrates utilized by a large fraction of the strains of biotype A and by few or none of the strains of biotype B are α-aminovalerate (used only by strain 110 of biotype B) and nicotinate (Table 33). The nutritional characters of tryptophan, kynurenine, galactose and nicotinate utilization appear to be the only absolute criteria for differentiating the two biotypes.

Unclassified strains

Of the 11 strains in this category, all save one (109) produce fluorescent pigment. Eight of them (10, 17, 47, 59, 91, 92, 127, 322) could be regarded as strains of *Pseudomonas putida*, which they resemble in failing to denitrify, to hydrolyse gelatin or to give an egg-yolk reaction, and in rarely hydrolysing Tween 80 (only strain 322 positive). A possible exception is strain 10, which was described by den Dooren de Jong (1926) as liquefying gelatin. In our hands, however, strain 10 has given a faint reaction under the patch by the test of Skerman (1959) after several days of incubation, probably attributable to lysis and release of intracellular proteases. What distinguishes these eight strains from biotypes A and B of *P. putida* is essentially their much more limited nutritional versatility, as shown by the data in Tables 34 and 35. The many typical nutritional characters of *P. putida* which they lack would have conferred an undesirable heterogeneity on this species had they been included.

The remaining three unclassified strains (95, 101, 109) present a different problem. They are the only strains which appear to be intermediate between *Pseudomonas fluorescens* and *P. putida* with respect to the key nutritional characters that otherwise serve well to distinguish these species. They are putida-like in the sense that none can denitrify, hydrolyse gelatin or give an egg-yolk reaction; and only one (strain 101) can hydrolyse Tween 80. All grow at 4°. Two (95, 101) can use trehalose, a character universally positive in *P. fluorescens* and universally negative in *P. putida*; and they share with several biotypes of *P. fluorescens* the inability to grow on butyrate, isobutyrate, ethanol or *n*-propanol. Strain 109 cannot grow on trehalose, but is fluorescens-like in its ability to use D-xylose, L-arabinose, D-galactose, hydroxymethylglutarate, sorbitol or adonitol; and in its failure to use butyrate. The nutritional characters of these three exceptional strains are shown in Tables 36 and 37.

Neotype strains in the fluorescent group

According to the catalogue of the American Type Culture Collection (1964) no less than three different neotype strains have been proposed for *Pseudomonas aeruginosa*: the cultures carrying ATCC numbers 10145, 14209 and 14216. We were unfortunately not aware of this fact until recently, and none of these strains was included in our study. However, in view of the phenotypic uniformity and

Table 30. *Pseudomonas putida biotype A. Substrates used by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Ribose	17	8, 26, 49, 51, 56, 77, 87, 100, 111, 115, 118, 119, 130, 144, 266
D-Xylose	5	All except 5, 8, 87, 100, 119
L-Arabinose	6	All except 6, 100, 118, 119, 128, 130
D-Mannose	6	All except 5, 87, 119, 145, 266, 276
D-Fructose	30	44, 100
Sucrose	3	All except 145, 154, 160
Butyrate	31	100
Isobutyrate	28	8, 100, 111, 115
Heptanoate	31	118
Malonate	21	5, 42, 43, 44, 49, 51, 56. 87
Glutarate	31	89
D-Malate	18	5, 8, 42, 43, 44, 56, 87, 100, 118, 119, 128, 130, 154, 160
D-(−)-Tartrate	5	All except 6, 82, 100, 119, 128
D-(+)-Tartrate	23	5, 6, 8, 118, 122, 130, 145, 154, 160
meso-Tartrate	16	5, 6, 8, 42, 43, 44, 49, 51, 56, 87, 100 119, 130, 145, 154, 160
Glycollate	3	All except 51, 82, 145
α-Ketoglutarate	31	89
Laevulinate	8	All except 7, 49, 81, 118, 130, 266, 276
Citraconate	2	All except 118, 130
Itaconate	6	All except 8, 100, 118, 119, 130, 276
Mesaconate	5	All except 8, 100, 118, 119, 130
Erythritol	1	All except 118
Mannitol	3	All except 8, 100, 144
Sorbitol	1	All except 118
Adonitol	1	All except 8
Glycerol	31	122
Ethyleneglycol	1	All except 145
Propyleneglycol	26	5, 7, 8, 115, 118, 130
2,3-Butyleneglycol	23	6, 7, 8, 81, 89, 100, 113, 119, 130
Ethanol	31	8
n-Propanol	31	8
n-Butanol	30	8, 100
D-Mandelate	7	All except 6, 7, 49, 81, 89, 90, 111
L-Mandelate	2	All except 89, 90
Benzoylformate	15	All except 6, 7, 26, 42, 43, 44, 49, 81, 89, 90, 111, 115, 122, 145, 276
o-Hydroxybenzoate	3	All except 51, 111, 115
m-Hydroxybenzoate	3	All except 43, 44, 56
p-Hydroxybenzoate	30	76, 77
Phenylacetate	29	8, 100 and 111
Phenylethanediol	5	All except 81, 89, 90, 111, 160
Naphthalene	1	All except 111
Phenol	5	All except 51, 76, 77, 89, 144
Quinate	30	76, 77
Testosterone	1	All except 130
Glycine	26	8, 81, 100, 118, 119, 130
L-α-Alanine	31	160
D-α-Alanine	31	8
L-Serine	20	5, 6, 7, 51, 76, 77, 82, 89, 119, 122, 128, 144
L-Valine	31	5
DL-α-Aminobutyrate	2	All except 76, 266
DL-α-Aminovalerate	22	8, 26, 56, 81, 100, 115, 118, 119, 130, 276

Table 30 (*cont.*)

Substrate	Total of positive strains	Negative strains
L-Phenylalanine	31	145
Kynurenate	4	All except 8, 26, 100, 115
Anthranilate	2	All except 51, 276
Ethanolamine	17	6, 7, 26, 49, 51, 76, 77, 81, 82, 87, 89, 90, 118, 130, 266
Spermine	31	81
Tryptamine	7	All except 6, 7, 89, 115, 118, 122, 130
Butylamine	29	8, 49, 100
α-Amylamine	29	8, 49, 100
Creatine	28	82, 128, 144, 266
Hippurate	23	81, 89, 100, 118, 122, 128, 130, 144, 266
Pantothenate	1	All except 145
Acetamide	10	All except 43, 44, 51, 56, 76, 77, 87, 145, 154, 160
Nicotinate	21	5, 6, 7, 49, 82, 115, 122, 128, 144, 266

distinctiveness of *P. aeruginosa*, it seems probable that almost any strain would be a satisfactory neotype.

A neotype strain has been proposed for *Pseudomonas fluorescens* (NCTC 10038 = ATCC 13525) by Rhodes (1959). Since she did not accept as valid the distinction between *P. fluorescens* and *P. putida*, it was chosen by her to be representative of this whole assemblage. We have examined the strain in question (our designation: 192), which proves to be a typical representative of *P. fluorescens* biotype A. We therefore concur (on different grounds) with the proposal of Rhodes. Acceptance of this neotype will have the consequence of fixing the designation *P. fluorescens* on our biotype A, if subsequent work shows that some or all of the biotypes of *P. fluorescens* merit species rank.

Apparently no neotype strain has been proposed for *Pseudomonas putida*. Accordingly, we propose our strain 90 (former designation A.3.12 = ATCC 12633), which is a typical member of *P. putida* biotype A. This particular strain has been used over the past 20 years in many different biochemical and physiological studies, and there is consequently a large body of information about its metabolism and physiology.

Nomenclatural problems in the fluorescent group

The account of the properties of *Pseudomonas fluorescens* has presented the evidence which leads us to classify the three phenazine producers *P. chlororaphis*, *P. aureofaciens* and *P. lemonnieri* as biotypes of *P. fluorescens*. These three names could perhaps be preserved as varietal designations for our biotypes D, E and F.

A much more difficult nomenclatural problem is presented by the many named gelatin-liquefying fluorescent species which do not produce phenazine pigments. Most of these are plant pathogens; and the question of the possible synonymy of these species with *Pseudomonas fluorescens* must await a detailed comparative study of the phytopathogenic fluorescent pseudomonads by the methods which we have used to characterize *P. fluorescens*. However, Haynes (1957) also recognized in the latest (7th) edition of *Bergey's Manual of Determinative Bacteriology* a total of nine

Table 31. *Pseudomonas putida biotype B. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- L-Arabinose*
- D-Glucose
- D-Fructose
- Gluconate
- 2-Ketogluconate
- Saecharate*
- Mucate

Fatty acids
- Acetate
- Propionate
- Butyrate
- Valerate
- Isovalerate
- Caproate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate

Polyalcohols and glycols
- Glycerol

Alcohols
- Ethanol*
- *n*-Propanol*
- *n*-Butanol
- Isobutanol*

Non-nitrogenous aromatic and other cyclic compounds
- Benzoate
- *p*-Hydroxybenzoate
- Phenylacetate
- Quinate
- Testosterone*

Aliphatic amino acids
- Glycine
- L-α-Alanine
- D-α-Alanine
- β-Alanine
- L-Serine*
- L-Leucine
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- L-Lysine*
- DL-Arginine
- DL-Ornithine
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine*
- L-Proline
- L-Tyrosine
- Phenylalanine*
- L-Tryptophan
- L-Kynurenine
- Anthranilate

Amines
- Benzylamine
- Putrescine
- Spermine
- Histamine*
- Tryptamine
- Butylamine
- α-Amylamine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine
- Trigonelline

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are listed in Table 32.

non-phytopathogenic, gelatin-liquefying species in addition to *P. fluorescens*. They are: *P. reptilivora*, *P. caviae*, *P. boreopolis*, *P. effusa*, *P. fairmontensis*, *P. myxogenes*, *P. schuylkilliensis*, *P. geniculata*, *P. septica*. We have examined one strain with the label *P. schuylkilliensis* (strain 267) and three with the label *P. geniculata* (strains 269, 271, 272). All were assigned to *P. fluorescens* biotype G. Since there is nothing in the published descriptions of any of these nine species which would permit a

distinction from *P. fluorescens*, we believe that they should all be reduced to synonomy with it, unless good evidence to the contrary can be obtained through a new examination of the type strains, if any of these is still in existence.

An entirely comparable nomenclatural problem exists for the fluorescent species which do not liquefy gelatin. There is a number of named plant pathogens in this

Table 32. *Pseudomonas putida biotype B. Substrates used by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Ribose	4	53, 96, 98, 107, 110
D-Xylose	6	96, 153, 157
L-Arabinose	8	98
D-Mannose	7	110, 153
D-Galactose	7	98, 153
Sucrose	1	All except 158
Saccharate	8	98
Isobutyrate	7	98, 107
Adipate	1	All except 107
Sebacate	1	All except 107
D-Malate	2	All except 96, 107
D-(−)-Tartrate	2	All except 98, 158
L-(+)-Tartrate	2	All except 96, 107
Laevulinate	3	53, 96, 98, 110, 153, 157
Citraconate	5	96, 98, 110, 157
Mannitol	5	98, 110, 157, 158
Sorbitol	2	All except 53, 107
2,3-Butyleneglycol	3	53, 107, 110, 153, 157, 158
Ethanol	8	107
n-Propanol	8	107
Isobutanol	8	107
D-Mandelate	1	All except 53
L-Mandelate	1	All except 53
Benzoylformate	1	All except 53
o-Hydroxybenzoate	6	96, 98, 158
Phenylethanediol	1	All except 53
Naphthalene	2	All except 107, 110
Phenol	6	53, 158, 167
Testosterone	8	110
L-Serine	8	98
L-Lysine	8	107
DL-Citrulline	5	96, 153, 157, 158
DL-α-Aminovalerate	1	All except 110
L-Histidine	8	53
L-Phenylalanine	8	107
D-Tryptophan	1	All except 110
Kynurenate	6	96, 98 and 110
Ethanolamine	3	All except 98, 158, 167
Histamine	8	98
Creatine	5	98, 107, 153, 157
Hippurate	7	98, 110
Acetamide	1	All except 110

category, the status of which *vis-à-vis Pseudomonas putida* cannot be decided at present. Haynes (1957) also listed several non-phytopathogenic species, in addition to *P. putida*, namely: *P. striata*, *P. ovalis*, *P. incognita*, *P. rugosa*, *P. convexa*,

Table 33. *Pseudomonas putida biotypes A and B. Selected characters diagnostically important for the differentiation of biotypes A and B*

Character	Biotype A		Biotype B	
	No. of positive strains	% of positve strains	No. of positive strains	% of positive strains
Growth at 4°	11	34	9	100
Utilization of				
D-Xylose	5	16	6	67
L-Arabinose	6	19	8	89
D-Mannose	6	19	7	78
D-Galactose	0	0	7	78
Mannitol	3	9	5	56
o-Hydroxybenzoate	3	9	6	67
Phenol	5	16	6	67
Testosterone	1	3	8	89
α-Aminovalerate	22	69	1	11
L-Tryptophan	0	0	9	100
L-Kynurenine	0	0	9	100
Anthranilate	2	6	9	100
Tryptamine	7	22	9	100
Nicotinate	21	66	0	0

Table 34. *Unclassified fluorescent pseudomonads. Substrates utilized by seven or all eight strains (10, 17, 47, 59, 91, 92, 127, 322)*

Carbohydrates and sugar derivatives
- D-Glucose
- Gluconate

Fatty acids
- Acetate
- Propionate
- Caproate*
- Caprylate*
- Pelargonate
- Caprate*

Dicarboxylic acids
- Succinate
- Fumarate
- Glutarate*

Hydroxyacids
- L-Malate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate*
- α-Ketoglutarate
- Pyruvate*

Polyalcohols and glycols
- Glycerol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- Quinate*

Aliphatic amino acids
- L-α-Alanine
- D-Alanine*
- β-Alanine*
- L-Serine*
- L-Leucine*
- L-Isoleucine*
- L-Valine*
- L-Aspartate
- L-Glutamate
- DL-Arginine
- γ-Aminobutyrate*
- δ-Aminovalerate*

Amino acids and related compounds containing a ring structure
- L-Histidine*
- L-Proline*

Amines
- Putrescine
- Spermine*

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine

Paraffin hydrocarbons
- (None)

* Substrates utilized by seven out of eight strains. Negative strains are indicated in Table 35.

Table 35. *Unclassified fluorescent pseudomonads. Substrates utilized by a fraction of the eight strains*

Substrate	Total of positive strains	Negative strains
D-Ribose	3	10, 47, 59, 127, 322
D-Xylose	1	All except 92
L-Arabinose	2	All except 10, 92
D-Mannose	1	All except 92
D-Galactose	2	All except 10, 92
D-Fructose	6	10, 91
Maltose	1	All except 322
2-Ketogluconate	4	17, 47, 127, 322
Saccharate	5	17, 47, 91
Mucate	5	17, 47, 91
Butyrate	5	10, 59, 127
Isobutyrate	4	10, 59, 91, 322
Valerate	5	10, 59, 92
Isovalerate	3	10, 59, 92, 127, 322
Caproate	7	10
Heptanoate	6	10, 92
Caprylate	7	10
Caprate	7	322
Malonate	4	17, 91, 92, 322
Glutarate	7	127
D-Malate	4	10, 59, 92, 127
D-α-Alanine	7	10
β-Alanine	7	10
L-Serine	7	10
L-Leucine	7	322
L-Isoleucine	7	322
L-Valine	7	10
L-Lysine	5	10, 127, 322
DL-Ornithine	6	10, 59
DL-Citrulline	6	10, 127
γ-Aminobutyrate	7	322
δ-Aminovalerate	7	322
L-Histidine	7	322
L-Proline	7	92
L-Tyrosine	6	10, 92
L-Phenylalanine	5	10, 127, 322
Ethanolamine	5	10, 59, 92
Benzylamine	1	All except 17
Spermine	7	92
Histamine	2	All except 59, 92
Butylamine	4	10, 59, 92, 127
α-Amylamine	3	10, 17, 92, 127, 322
Creatine	1	All except 92
Hippurate	1	All except 92
Nicotinate	1	All except 92
Trigonelline	3	10, 17, 47, 91, 322
D-(−)-Tartrate	3	17, 47, 91, 127, 322
L-(+)-Tartrate	1	All except 127
meso-Tartrate	4	10, 47, 59, 322
DL-β-Hydroxybutyrate	6	92, 127
Hydroxymethylglutarate	1	All except 127
Citrate	7	322
Pyruvate	7	10
Aconitate	3	10, 17, 47, 91, 322
Laevulinate	1	All except 322

Table 35 (*cont.*)

Substrate	Total of positive strains	Negative strains
Citraconate	1	All except 322
Itaconate	2	All except 92, 322
Mesaconate	2	All except 92, 322
Mannitol	1	All except 92
meso-Inositol	1	All except 92
Propyleneglycol	6	10 and 322
2,3-Butyleneglycol	5	10, 92, 322
Ethanol	5	47, 92, 127
n-Propanol	5	47, 92, 127
n-Butanol	3	10, 47, 59, 92, 127, 322
Isobutanol	6	10, 322
Benzoylformate	1	All except 59
Benzoate	2	All except 92, 322
p-Hydroxybenzoate	6	92, 322
Phenylacetate	3	10, 59, 92, 127, 322
Quinate	7	322
Glycine	2	All except 127, 322

Table 36. *Unclassified fluorescent pseudomonads. Substrates utilized by strains 95, 101 and 109*

Carbohydrates and sugar derivatives
- D-Ribose
- D-Glucose
- D-Mannose
- D-Fructose
- Gluconate
- 2-Ketogluconate
- Mucate

Fatty acids
- Acetate
- Propionate
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate

Polyalcohols and glycols
- Mannitol
- Glycerol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- (None)

Aliphatic amino acids
- L-α-Alanine
- β-Alanine
- L-Leucine
- L-Isoleucine
- L-Aspartate
- L-Glutamate
- DL-Arginine
- DL-Citrulline
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline

Amines
- Ethanolamine
- Putrescine
- Spermine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine
- Trigonelline

Paraffin hydrocarbons
- (None)

Table 37. *Unclassified fluorescent pseudomonads. Substrates used by one or two of the strains 95, 101 and 109*

Substrate	Strain 95	101	109	Substrate	Strain 95	101	109
D-Xylose	−	−	+	*n*-Propanol	−	−	+
L-Arabinose	−	−	+	*n*-Butanol	−	±	−
D-Galactose	−	−	+	Benzoate	+	−	−
Sucrose	+	+	−	*o*-Hydroxybenzoate	−	−	+
Trehalose	+	+	−	*m*-Hydroxybenzoate	+	−	−
Saccharate	−	+	+	*p*-Hydroxybenzoate	+	+	−
Caproate	+	−	+	Naphthalene	−	−	+
D-Malate	−	−	+	Quinate	+	+	−
D-(−)-Tartrate	−	−	+	Testosterone	+	+	−
L-(+)-Tartrate	−	+	−	Glycine	±	+	−
meso-Tartrate	−	−	+	D-α-Alanine	+	+	−
Glycollate	−	−	+	L-Serine	−	+	−
Hydroxymethylglutarate	−	−	+	L-Valine	+	+	−
Laevulinate	−	±	−	L-Lysine	+	±	−
Citraconate	−	−	+	DL-Ornithine	+	+	−
Itaconate	±	−	+	L-Tyrosine	+	−	−
Mesaconate	−	−	+	L-Phenylalanine	+	±	−
Sorbitol	−	−	+	L-Tryptophan	+	−	−
meso-Inositol	+	±	−	L-Kynurenine	+	+	−
Adonitol	−	−	+	Anthranilate	±	±	−
Ethyleneglycol	−	−	+	Histamine	−	+	+
Propyleneglycol	−	−	+	Butylamine	+	−	−
2,3-Butyleneglycol	−	−	±	α-Amylamine	+	−	−
Ethanol	−	−	+	Nicotinate	−	+	+

P. eisenbergii. There is nothing in the published descriptions of these species which would permit their differentiation from *P. putida*, so again we recommend reduction to synonomy, unless type strains still exist which can be shown to differ significantly from *P. putida* as we have described it.

Many workers (e.g. Lautrop & Jessen, 1964) apply the name *Pseudomonas ovalis* to strains which we consider to be typical of *P. putida*. This name, first used by Chester (1901), is derived from the characteristic shape of the organisms, which are small, plump and oval. In our experience, this shape occurs, either exclusively or predominantly, in many strains of *P. putida*. However, other typical strains of *P. putida* are longer and are indistinguishable on a morphological basis from *P. fluorescens*. Jessen (1965) has made similar comments about the variability of shape in his biotype 11, group II, which encompasses strains that we would assign to *P. putida*. It therefore seems evident that the shape of the organisms cannot be considered a reliable character for the definition of a species among the fluorescent pseudomonads which do not liquefy gelatin; and there is nothing else in Chester's description of *P. ovalis* which justifies a separation from *P. putida*. Since *P. putida* is clearly the older specific name, first applied in 1889 by Trevisan, and derived from Flügge's trinomial *Bacillus fluorescens putidus*, we cannot see any justification for the continued use of the name *P. ovalis*.

THE ACIDOVORANS GROUP

Twenty-six strains were assigned to this group, within which we recognize two species: *Pseudomonas acidovorans* den Dooren de Jong (15 strains), and *P. testosteroni* Marcus & Talalay (nine strains). Two strains could not be given a definite specific assignment, but appear on their general characters to be members of this group.

Origins of the strains

(*a*) Strains assigned to *Pseudomonas acidovorans* den Dooren de Jong (1926)

14. Strain 7 of den Dooren de Jong (1926). Isolated from soil by aerobic enrichment with acetamide. Received from Laboratorium voor Microbiologie, Delft. ATCC 15668.
24. *P. desmolytica* strain 4B of Dr M. Yano (no published description). Isolated from soil by aerobic enrichment with *p*-hydroxybenzoate. Received from Institute of Applied Microbiology, Tokyo. ATCC 17406.
29. *P. indoloxidans* Gray (1928). Isolated from soil through aerobic enrichment with indole. Authentic strain; ATCC 9355.
61. Strain 60.79 of Dr P. Thibault, Institut Pasteur. Strain 452-3 of Dr R. Hugh. Isolated from a pharyngeal swab. ATCC 17438.
62. Strain 60.78 of Dr P. Thibault. Strain 553-3 of Dr R. Hugh. Isolated from pharyngeal swab. ATCC 17439.
80. Strain A of Dr U. Bachrach (1957). Received from Professor S. Dagley, Leeds University. ATCC 17455.
102. Strain B_2 aba of Professor H. Kornberg, Leicester University; originally isolated at Oxford by Dr June Lascelles. ATCC 17476.
103. Strain Nic SH_2 of Dr S. H. Hutner, Haskins Laboratories, N.Y. Isolated from soil by aerobic enrichment with nicotinate about 1947. ATCC 17477.
105. Strain RYS-4. Isolated from soil by aerobic enrichment with tryptophan by R. Y. Stanier at Berkeley in 1950. ATCC 17479.
106. Strain Tr-7 of Dr S. H. Hutner, Haskins Laboratories, N.Y. Isolated from soil by aerobic enrichment with tryptophan about 1947. ATCC 17480.
114. Strain 5 of Dr M. Shilo. Isolated from soil by aerobic enrichment with maleate at Berkeley in 1956. ATCC 17486.
125. Strain 11 of Dr M. Shilo. Isolated from soil by aerobic enrichment with mesaconate at Berkeley in 1956. ATCC 17497.
129. Strain 59 of Dr M. Shilo. Isolated from soil by aerobic enrichment with maleate at Berkeley in 1956. ATCC 17501.
146. *P. desmolytica* Gray & Thornton (1928). Authentic strain; ATCC 15005.
148. Strain C-I-O of Professor I. C. Gunsalus, University of Illinois. Origin uncertain. ATCC 17517.

(*b*) Strains assigned to *Pseudomonas testosteroni* Marcus & Talalay (1956)

15. Strain 21 of den Dooren de Jong (1926). Isolated from soil by aerobic enrichment with fumarate. Received from Laboratorium voor Microbiologie, Delft; ATCC 15667.

16. Strain 31 of den Dooren de Jong (1926). Isolated from soil by aerobic enrichment with bromosuccinate. Same source as strain 15. ATCC 15668.
25. *P. desmolytica* strain 1123 from Institute of Applied Microbiology, Tokyo. Isolated from soil by aerobic enrichment with anthranilate. ATCC 17407.
27. Strain KA-11 of Dr K. Hosokawa. Isolated from soil by aerobic enrichment with kynurenate in 1963 at Berkeley. ATCC 17409.
28. Strain KA-14-2. Same history as strain 27. ATCC 17410.
78. *P. testosteroni*, type strain of Marcus & Talalay (1956). Isolated at Berkeley from soil by Dr Paul Talalay, using aerobic enrichment with testosterone, about 1953. Authentic strain received via Professor I. C. Gunsalus in 1964. ATCC 11996.
79. NCTC 8893. Received from Professor S. Dagley, Leeds University, in 1964. ATCC 17454.
138. Strain FD-30 of Dr F. Delafield. Isolated from soil by aerobic enrichment with poly-β-hydroxybutyrate at Berkeley in 1961. ATCC 17510.
139. Strain FD-32. Same history as 138. ATCC 17511.

(*c*) Unclassified strains

60. Strain A-35 of Dr P. Thibault, Institut Pasteur. Isolated from human pleural fluid. ATCC 17437.
298. Strain 6084 of Dr P. Thibault, Institut Pasteur. ATCC 17664.

General group characters

The acidovorans group consists of non-pigmented, nutritionally versatile aerobic pseudomonads which share a distinctive nutritional spectrum and certain unique metabolic properties. All strains are multitrichous. When grown at 30° on yeast agar slopes, the strains of *Pseudomonas testosteroni* show a predominance of the monotrichous type of flagellation. The flagellar index, as defined by Lautrop & Jessen (1964), is low; but it increases considerably when the organisms are grown in liquid medium at 10–12°. According to these authors, therefore, the flagellation should be considered as multitrichous. In two strains (78, 79) it was also possible to show the formation of lateral flagella of shorter wavelength when the organisms were grown at low temperatures. Strain 78 has a higher percentage of this type of flagella.

All strains of the group can accumulate poly-β-hydroxybutyrate as a cellular reserve material. Four strains of *Pseudomonas testosteroni* (15, 16, 138, 139) attack poly-β-hydroxybutyrate by means of extracellular enzymes and use it as an exogenous carbon source; this property is absent from the rest of the group. Thus the ability to produce extracellular enzymes for the hydrolysis of poly-β-hydroxybutyrate is not a universal property in this species, as it is in *P. pseudomallei* (Redfearn *et al.* 1966) and in certain hydrogenomonads (unpublished observations). It should be noted that this property was selected in the isolation of strains 138 and 139, since they were enriched with poly-β-hydroxybutyrate as carbon and energy source.

None of the members of this group hydrolyses gelatin or gives an egg-yolk reaction; all can hydrolyse Tween 80. Growth factors are not required, and ammonia or nitrate can be used as nitrogen source. No strain can denitrify. The temperature range is relatively narrow; growth does not occur at either 4° or 41°, except for

the aberrant strain 298, which grows at 41°. Several strains grow at 37°, although poorly. The oxidase reaction is positive.

Table 38 lists the organic compounds that can be used as carbon and energy sources by 90% or more of the strains of *Pseudomonas acidovorans* and *P. testosteroni*; this list accordingly represents the common nutritional core characteristic

Table 38. *Acidovorans group. Substrates utilized by 90% or more of the strains*

Carbohydrates and sugar derivatives
- Gluconate
- Saccharate
- Mucate

Fatty acids
- Acetate
- Propionate
- Butyrate
- Valerate
- Isovalerate
- Caproate

Dicarboxylic acids
- Succinate
- Fumarate
- Glutarate
- Adipate
- Pimelate
- Suberate
- Azelate
- Sebacate

Hydroxyacids
- D-Malate
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- Glycollate
- DL-Glycerate
- Hydroxymethylglutarate*

Miscellaneous organic acids
- Citrate
- Citraconate
- Itaconate
- α-Ketoglutarate
- Pyruvate
- Aconitate
- Laevulinate

Polyalcohols and glycols
- (None)

Alcohols
- *n*-Butanol

Non-nitrogenous aromatic and other cyclic compounds
- *m*-Hydroxybenzoate
- *p*-Hydroxybenzoate
- L-Tyrosine
- L-Phenylalanine
- L-Kynurenine*
- Kynurenate*

Aliphatic amino acids
- Glycine*
- L-α-Alanine*
- D-α-Alanine*
- L-Leucine
- L-Isoleucine
- L-Aspartate
- L-Glutamate
- L-Norleucine
- L-Glutamate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline

Amines
- (None)

Miscellaneous nitrogenous compounds
- Hippurate*

Paraffin hydrocarbons
- (None)

* Not utilized by all strains. Negative strains are given in Tables 39, 40.

for the group. Tables 39 and 40 supplement the nutritional picture for *P. acidovorans* and *P. testosteroni*, respectively; they list all compounds used by a fraction of the strains, together with specific indications of the negative strains.

Although *Pseudomonas acidovorans* and *P. testosteroni* are nutritionally versatile, they cannot attack glucose or most other carbohydrates. As a consequence, they can be easily confused with the much less nutritionally versatile alcaligenes group when examined by customary methods. The pattern of fatty acid utilization is highly characteristic. With some exceptions for isobutyrate, the fatty acids from

acetate to caproate are excellent substrates. One strain of *P. acidovorans* grows on heptanoate, and caprate supports the growth of eight strains of the same species; caprylate and pelargonate are completely non-utilizable by both species. The dicarboxylic acids, from succinate to sebacate, support uniformly excellent growth.

Table 39. *Pseudomonas acidovorans. Compounds used by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
Mannitol	14	114
meso-Inositol	7	14, 29, 61, 80, 105, 114, 125, 129
Glycerol	5	All except 24, 29, 114, 125, 146
Heptanoate	1	All except 103
Caprate	8	14, 102, 103, 105, 106, 114, 146
Isobutanol	4	All except 24, 29, 103, 148
2,3-Butyleneglycol	7	29, 61, 62, 80, 114, 125, 129, 146
D-Tryptophan	14	62
L-Kynurenine	14	62
Kynurenate	14	62
Phenol	1	All except 114
Glycine	14	114
D-α-Alanine	14	61
β-Alanine	12	105, 106, 114
DL-α-Aminobutyrate	14	61
γ-Aminobutyrate	13	24, 114
DL-α-Aminovalerate	13	61, 103

Table 40. *Pseudomonas testosteroni. Compounds used by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
Isobutyrate	6	25, 28, 79
D-(−)-Tartrate	2	All except 138, 139
meso-Tartrate	4	25, 27, 28, 78, 79
Poly-β-hydroxybutyrate	4	All except 15, 16, 138, 139
Mesaconate	4	All except 16, 25, 27, 138
Hydroxymethylglutarate	8	25
n-Propanol	7	15, 16
Isopropanol	1	All except 25
Geraniol	2	79, 138
Benzoylformate	8	15
Benzoate	7	15, 25
Phthalate	2	All except 138, 139
Terephthalate	2	All except 138, 139
Nicotinate	6	25, 27, 78
Trigonelline	6	25, 27, 78
L-α-Alanine	7	15, 16
L-Valine	7	All except 25
DL-α-Aminovalerate	2	All except 25, 27
Hippurate	8	15

Most strains grow on five organic acids (glycollate, citraconate, itaconate, mesaconate, hydroxymethylglutarate) which are not widely utilized by other aerobic pseudomonads, and never all attacked by one strain of other species. The pattern

with respect to aromatic acids is likewise unusual. Two aromatic acids, *m*- and *p*-hydroxybenzoate, are universal substrates. However, no strain can grow on anthranilate or salicylate, and benzoate is used only by some strains of *P. testosteroni*. The pattern of amino acid utilization is also distinctive. Norleucine, which is wholly unutilizable by the fluorescent pseudomonads, *P. multivorans* and *P. pseudomallei* (all nutritionally versatile organisms) supports the growth of all acidovorans strains. Fourteen out of 15 strains of *P. acidovorans* grow on DL-α-aminobutyrate,

Table 41. *Acidovorans group. The group characters of greatest differential value in the recognition of the acidovorans group, based on the analysis of 26 strains*

Characters	Number of positive strains	Ideal phenotype
1. Poly-β-hydroxybutyrate as cellular reserve material	26	+
Utilization of		
2. L-arabinose	0	−
8. D-Glucose	0	−
4. D-Galactose	0	−
5. 2-Ketogluconate	0	−
6. Saccharate	25	+
7. Pelargonate	0	−
8. Adipate, pimelate, suberate, azelate, sebacate	26	+
9. Glycollate	24	+
10. Laevulinate	26	+
11. Itaconate	26	+
12. *m*-Hydroxybenzoate	25	+
13. Norleucine	25	+
14. Putrescine	0	−

Table 42. *Acidovorans group. Number of strains of different species or groups of species of aerobic pseudomonads conforming to the selected fourteen characters (Table 41) which define the ideal phenotype of the acidovorans group*

	No. of characters of 'ideal' phenotype														
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
Acidovorans group	24	.	1	1	.	.	.	.	.	.	.	.	.	.	.
Fluorescent group	.	.	.	.	.	.	.	.	1	24	31	44	48	27	.
Pseudomallei group	.	.	.	.	.	.	.	.	.	1	23	10	7	.	.
P. multivorans	.	.	.	.	.	.	.	.	13	6	.	.	.	.	.
P. stutzeri	.	.	.	.	.	.	.	1	8	8	.	.	.	.	.
P. maltophilia	.	.	.	.	.	.	.	.	.	23	.	.	.	.	.
Alcaligenes group	.	.	.	.	.	.	.	.	4	2	1	.	.	.	.
P. lemoignei	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.

a property shared with only three other strains in our collection. In contrast, no strain can grow on serine, lysine, arginine or ornithine, and only one strain on valine. These are all amino acids commonly used by other nutritionally versatile *Pseudomonas* species. Lastly, it should be noted that the acidovorans group is completely unable to attack higher amines and *N*-methyl compounds which are generally used by other nutritionally versatile species. Although the nutritional spectrum of the acidovorans group shows some degree of overlap with the nutritional spectrum of

the fluorescent pseudomonads, *P. multivorans* and *P. pseudomallei*, in many particulars it is a complementary one.

We selected a total of fourteen characters to constitute an ideal phenotype for the acidovorans group (Table 41). Every strain of *Pseudomonas acidovorans* and *P. testosteroni* conforms perfectly to this ideal phenotype, while the two unclassified strains that we have assigned to the group deviate from it in two and three characters, respectively. This is shown in Table 42, together with the degree of conformity of the strains of all other species to the ideal phenotype of the acidovorans group. The degree of separation is very satisfactory.

Table 43. *The differences between Pseudomonas acidovorans (15 strains) and P. testosteroni (9 strains)*

GC (moles %) of DNA, average value	*P. acidovorans* 66·8	*P. testosteroni* 61·8
Growth on	(Numbers of positive strains)	
D-Fructose	15	0
Mannitol	14	0
Malonate	15	0
Maleate	15	0
L-(+)-Tartrate	15	0
Quinate	15	0
Ethanol	15	0
Phenylacetate	15	0
D-Tryptophan	14	0
L-Tryptophan	15	0
Benzoylformate	0	8
Benzoate	0	7
β-Alanine	12	0
DL-α-Aminobutyrate	14	0
γ-Aminobutyrate	13	0
δ-Aminovalerate	15	0
Acetamide	15	0
Testosterone	0	9

Differentiation of two species: Pseudomonas acidovorans and P. testosteroni

As a result of our early screening of the acidovorans group, we were inclined to consider that it contained only one nutritionally somewhat irregular species. However, the analyses of Mandel (1966) have shown that strains of this group can be sharply subdivided on the guanine+cytosine (GC) content of their DNA, some showing a value of about 67 moles % GC, and others of about 62. A closer scrutiny of the nutritional data then revealed that many of the 'irregularities' were systematically correlated with differences in GC content. The correlated differences in GC content and in nutritional properties (Table 43) evidently justify the recognition of two species. For reasons that will be discussed shortly, we assign the strains of high GC content to *P. acidovorans* and those of low GC content to *P. testosteroni*.

Nomenclatural problems and designations of type strains

The recognition of two species in the acidovorans group posed a nice nomenclatural problem. One of the original strains to which den Dooren de Jong (1926) gave the name *Pseudomonas acidovorans* (strain 14) is in the high GC group, and the other

two (strains 15, 16) in the low GC group. Since there is no objective way of determining which most clearly typifies *P. acidovorans*, we have arbitrarily decided to retain this name for strain 14; accordingly, the high GC group becomes *P. acidovorans* den Dooren de Jong, with strain 14 as the type. Our choice automatically reduces to synonomy the later specific names for strain 29 (*P. indoloxidans* Gray, 1928) and for strain 146 (*P. desmolytica* Gray & Thornton, 1928). One additional nomenclatural point requires discussion. Hugh (*ATCC Catalogue*, 7th edition, 1964) has named strain 61 (ATCC 12816) *Comamonas terrigena*, a new combination for *Vibrio terrigenus* Günther (1894). Günther's strain is long since lost, and his description of it had the inadequacy typical of that era. In our opinion, it could never be certainly recognized upon re-isolation from nature, and the specific name might well have been consigned to oblivion. However, Hugh (1962) chose to revive it as an earlier synonym for *Vibrio percolans* Mudd and Warren (1923), a strain of which (ATCC 8461) still exists, and has been designated by Hugh as the neotype. We have briefly examined this strain, and find that it cannot grow on a chemically defined medium. Whatever it may be, it is clearly not identical with our strain 61 (ATCC 12816), which Hugh also considered to be '*Comamonas terrigena*'. The epithet 'terrigena' therefore cannot be used as an earlier synonym for 'acidovorans'.

In the low GC group, the oldest isolates are strains 15 and 16, which den Dooren de Jong (1926) assigned to *Pseudomonas acidovorans*, a name we propose to reserve for the high GC group. Strain 25, received under the label *P. desmolytica*, does not resemble Gray & Thornton's type strain of this species (our strain 146), which falls in the high GC group, and is therefore synonymous with *P. acidovorans*. The only other name previously applied to a low GC strain, *P. testosteroni* Marcus & Talalay (1956), becomes the correct designation for this group; and the strain of Marcus & Talalay (our strain 78) is the holotype. *P. testosteroni* was named on the basis of a single striking character, its ability to grow on testosterone and related steroids. Fortunately, this nutritional character proves to be taxonomically significant, since it is possessed by all low GC strains and by no high GC strains (Table 43).

The danger of naming a species on the basis of one striking character possessed by a single isolate is well illustrated by *Pseudomonas indoloxidans* of Gray (1928), now a synonym for *P. acidovorans*. Gray's strain could oxidize indole to the water-insoluble blue compound, indigotin, which accumulated as crystals in and around the bacterial growth on an indole-containing medium. The test for indigotin formation must be conducted on a medium furnished with a utilizable carbon and energy source, since indole itself cannot so serve. Tested under these conditions, Gray's original strain (our strain 29) still forms indigotin abundantly, *but is the only strain in the acidovorans group to do so.* In all other respects, strain 29 is typical of *P. acidovorans*. We have screened all other strains in our collection for the ability to form indigotin from indole and have found only three (strains 109, 110, 187) which possess this property. All three are fluorescent pseudomonads. It is accordingly evident that this striking biochemical property has no taxonomic significance.

Ecological considerations and enrichment

The frequency with which strains of the acidovorans group have been isolated from soil by aerobic enrichment with a variety of organic compounds (see strain histories) shows that this group is widespread in soil. The nutritional data suggest

enrichment methods which should be relatively or absolutely specific for the bacteria of this group. Our own experience shows that they often predominate in aerobic enrichments with higher dicarboxylic acids and kynurenate incubated at 30°. Enrichments at the same temperature with either maleate or D-tryptophan should be highly specific for *Pseudomonas acidovorans*, since these compounds are very rarely used by strains belonging to other species of aerobic pseudomonads.

Table 44. *Properties of strains 60 and 298. Only positive nutritional characters included*

Character	Strain no. 60	Strain no. 298	Character	Strain no. 60	Strain no. 298
GC in DNA (moles%)	59	64	Citraconate	−	+
Growth on			Itaconate	+	+
Gluconate	+	+	Mesaconate	−	+
Acetate	+	+	α-Ketoglutarate	+	+
Propionate	+	+	Pyruvate	+	+
Butyrate	+	+	Aconitate	+	+
Valerate	+	+	Laevulinate	+	+
Isovalerate	+	+	*m*-Hydroxybenzoate	+	−
Caproate	+	+	*p*-Hydroxybenzoate	+	+
Succinate	+	+	L-Tyrosine	+	−
Fumarate	+	+	L-Phenylalanine	+	−
Glutarate	+	+	D-α-Alanine	+	−
Adipate	+	+	L-Leucine	+	+
Pimelate	+	+	L-Isoleucine	+	+
Suberate	+	+	Norleucine	+	−
Azelate	+	+	L-Aspartate	+	+
Sebacate	+	+	L-Glutamate	+	+
D-Malate	+	+	L-Proline	+	+
L-Malate	+	+			
DL-β-Hydroxybutyrate	+	+			
DL-Lactate	+	+			

Properties of unclassified strains

The two remaining strains that we assign somewhat tentatively to the acidovorans group are 60 and 298. As shown in Table 44 their DNA has a GC content of 59 (strain 60) and of 64 (strain 298) %. Both strains accumulate poly-β-hydroxybutyrate as a cellular reserve material. These two strains are unable to grow on more than 20 compounds which are used by *Pseudomonas testosteroni*, so that they give the appearance of being physiological mutilates which have lost a large number of enzyme systems basic to the acidovorans group, but still retain a few of its distinctive properties. The most serious objection to regarding them as defective strains of *P. testosteroni* is that the GC content of their DNA is not identical with that of typical *P. testosteroni* strains.

THE ALCALIGENES GROUP

Seven strains of our collection have been assigned to this group, which we divide into two species: *Pseudomonas alcaligenes* (one strain), and *P. pseudoalcaligenes* (six strains).

Origins of the strains

63. Received from Dr P. Thibault, Institut Pasteur, Paris, as strain X-59. Isolated 1956 from sinus discharge. ATCC 17440.
65. Received from Dr P. Thibault, Institut Pasteur, Paris, as strain R-160. Isolated 1960 as contaminant of culture medium. ATCC 17442.
66. Received from Dr P. Thibault, Institut Pasteur, Paris, as strain 6076. ATCC 17443.
142. Strain 1577 of Dr R. Hugh. Isolated from swimming pool water. Suggested as neotype (Hugh & Ikari, 1964). ATCC 14909.
297. Received from Dr P. Thibault as strain 55.111. Isolated from rabbit blood culture. ATCC 17663.
299. Received from Dr P. Thibault, NCTC 8769. ATCC 17665.
417. *P. alcaligenes* ATCC 12815.

General group characters

Monias (1928) briefly described a group of non-pigmented pseudomonads, unable to liquefy gelatin or produce acid from sugars, to which he gave the name *Pseudomonas alcaligenes*. Similar strains were encountered by Thibault (1961) in a systematic study of bacteria which presented the physiological characters of the genus *Alcaligenes*. Thibault showed that the category of motile Gram-negative rods which are oxidase-positive and do not hydrolyse gelatin or produce acid from sugars includes both peritrichous and polar forms. In effect, they are distinguishable in terms of conventional diagnostic criteria only by the mode of insertion of the flagella. The 16 polarly flagellated strains studied by Thibault could, in turn, be subdivided on the basis of the number of flagella: some were monotrichous, others multitrichous. Through the kindness of Dr P. Thibault, we have been able to examine many of his polarly flagellate strains.

Ikari & Hugh (1963) examined by conventional taxonomic tests 12 strains which they considered to be representative of *Pseudomonas alcaligenes* Monias; they proposed a neotype strain for the species (Hugh & Ikari, 1964). Even in terms of an extended series of tests, this organism was defined almost entirely by negative physiological criteria: the only positive physiological criteria for its recognition given by Hugh & Ikari (1964) were the presence of catalase, ability to attack citrate, to reduce nitrate to nitrite and to give a positive oxidase reaction. These authors also showed that the flagellation was monotrichous.

The characteristics of the acidovorans group as revealed by conventional diagnostic tests are very similar to those of the alcaligenes group, and the two cannot in fact be distinguished by such cultural and physiological properties as attack on gelatin, oxidase reaction, and reactions in the carbohydrate medium of Hugh & Leifson (1953). This was revealed when we examined the collection of strains received from Dr P. Thibault under the tentative designation of *Pseudomonas alcaligenes*. Two were typical strains of *P. acidovorans*, and two were atypical strains of the acidovorans group. There remained a residue of strains which did not possess the characters of the acidovorans group. We have compared these strains with the neotype strain of *P. alcaligenes* proposed by Hugh & Ikari (strain 142), and with one other strain which had been studied by Hugh & Ikari (strain 417).

Table 45. *Alcaligenes group. Substrates utilized by all strains*

Carbohydrates and sugar derivatives
(None)

Fatty acids
Acetate
Propionate
Butyrate
Caprylate
Pelargonate
Caprate

Dicarboxylic acids
Succinate
Fumarate

Hydroxyacids
L-Malate
DL-Lactate

Miscellaneous organic acids
α-Ketoglutarate
Pyruvate

Polyalcohols and glycols
(None)

Alcohols
(None)

Non-nitrogenous aromatic and other cyclic compounds
(None)

Aliphatic amino acids
L-α-Alanine
β-Alanine
L-Leucine
L-Glutamate
γ-Aminobutyrate
δ-Aminovalerate

Amino acids and related compounds containing a ring structure
(None)

Amines
Spermine

Miscellaneous nitrogenous compounds
(None)

Paraffin hydrocarbons
(None)

Table 46. *Alcaligenes group. Substrates utilized by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Fructose	5	142
Valerate	5	142, 417
Caproate	3	66, 142, 297, 417
Glutarate	6	142
D-Malate	6	142
DL-β-Hydroxybutyrate	6	142
DL-Glycerate	5	66, 142
Citrate	3	66, 297, 299, 417
Itaconate	6	142
Mesaconate	6	142
Glycerol	2	All except 297, 299
Propyleneglycol	5	63, 142
Ethanol	6	142
n-Propanol	6	142
n-Butanol	6	142
Glycine	3	63, 66, 142, 417
D-α-Alanine	5	142, 417
L-Serine	6	142
DL-Arginine	6	417
DL-Ornithine	1	All except 66
L-Histidine	5	66, 417
L-Proline	4	63, 66, 417
L-Tyrosine	6	142
L-Phenylalanine	6	142
Ethanolamine	6	142
Putrescine	6	142
Betaine	6	142
Sarcosine	3	63, 142, 297, 299

All these strains show monotrichous flagellation. Four of them (63, 5, 297, 299) accumulate poly-β-hydroxybutyrate as a cellular reserve material, but none of them can attack the polymer by means of extracellular enzymes. None is a denitrifier. Growth factors are not required. Both ammonia and nitrate can serve as sole nitrogen sources. All are oxidase-positive. None can grow at 4°, but all grow at 41°. although the growth of strains 3, 65 and 417 at this temperature is relatively poor, Using Skerman's method, we found a feeble attack on gelatin by strains 142, 299 and 417; Hugh & Ikari (1964) reported strain 142 to be gelatinase negative.

Table 45 gives the list of the substrates attacked by all the strains of the alcaligenes group, and Table 46 lists those substrates used by a fraction of the strains. Only 19 out of the total of 146 compounds tested are universal substrates. Beyond this, the proposed neotype strain of Hugh & Ikari (strain 142) diverges in nutritional respects from the remaining six strains, which constitute an internally homogeneous group. Strain 142 fails to grow on fructose, glutarate, D-malate, DL-β-hydroxybutyrate, itaconate, mesaconate, ethanol, *n*-propanol, *n*-butanol, L-serine, L-tyrosine, L-phenylalanine, ethanolamine, putrescine and betaine, a total of fifteen compounds used by all other strains. Citrate, used by strain 142 as reported by Hugh & Ikari (1964), is used by two of the six other strains. All strains except 417 can grow on DL-arginine, though growth of some is feeble.

Table 47. *Alcaligenes group. The group characters of greatest differential value in the recognition of the alcaligenes group, based on the analysis of seven strains*

Characters	No. of positive strains	Ideal phenotype
1. Monotrichous flagellation	7	+
2. Growth at 41°	7	+
3. Denitrification	0	−
Utilization of		
4. Glucose	0	−
5. Gluconate	0	−
6. Caprylate	7	+
7. *m*-Hydroxybenzoate	0	−
8. *p*-Hydroxybenzoate	0	−
9. β-Alanine	7	−
10. L-Serine	6	+
11. L-Aspartate	0	−
12. Spermine	7	+

The nutritional spectrum of the group recalls in many respects that of the fluorescent pseudomonads; but it is much narrower, notably in the areas of sugars and sugar-acids, and of aromatic compounds. The ability of some of the members of the group to accumulate poly-β-hydroxybutyrate, and the inability to use sugars (with the exception of fructose) are characters of the alcaligenes group also found in the acidovorans group. However, an ideal phenotype which separates the alcaligenes group from all other aerobic pseudomonads can be easily constructed (Table 47). Table 48 shows the degree of conformity of all species and groups to this ideal phenotype.

Differentiation of two species: Pseudomonas alcaligenes and P. pseudoalcaligenes, sp. nov.

In the light of the nutritional data, it seems evident that there are two distinct species in the alcaligenes group: *Pseudomonas alcaligenes*, uniquely represented in our collection by strain 142, the proposed neotype strain of Hugh & Ikari (1964); and a second species, represented by the other six strains, which is nutritionally more versatile. The data reported by Mandel (1966) for the GC content of the DNA support this conclusion: strain 142 has 66 % GC, and all other strains, 63. We propose to recognize strains 3, 65, 66, 297, 299 and 417 as a new species, *P. pseudoalcaligenes*, with strain 63 (Thibault's strain x-59) as the type strain.

Table 48. *Alcaligenes group. Number of strains of different species and groups of species of aerobic pseudomonads conforming to the selected twelve characters (Table 47) which define the ideal phenotype of the alcaligenes group*

	No. of characters of 'ideal' phenotype										
	12	11	10	9	8	7	6	5	4	3	2
Alcaligenes group	6	1	.	.	.	.	.	.	.	.	.
Fluorescent group	.	.	.	.	.	6	92	64	10	3	.
Acidovorans group	.	.	.	.	.	.	.	1	.	13	12
Pseudomallei group	.	.	.	.	.	2	15	11	9	4	.
P. multivorans	.	.	.	.	.	.	8	8	3	.	.
P. stutzeri	.	.	.	.	.	2	2	9	3	1	.
P. maltophilia	.	.	.	.	.	.	4	19	.	.	.
P. lemoignei	.	.	.	.	.	1	.	.	.	.	.

The ecology of the alcaligenes group is at present wholly obscure. Strains have been isolated only by direct plating on complex media, either from clinical specimens or from water samples.

PSEUDOMONAS MULTIVORANS SP. NOV.

We propose this name for a homogeneous and distinctive group of aerobic pseudomonads, particularly remarkable for the range and number of organic compounds that they can use as carbon and energy sources. This group was first described, but not named, by Morris & Roberts (1959). They studied 16 strains from soil and two strains from river water, isolated in Trinidad by direct plating on glucose yeast-extract peptone agar. These pseudomonads accumulate large amounts of poly-β-hydroxybutyrate, a property explored by Forsyth *et al.* (1958) and Hayward, Forsyth & Roberts (1959), who were the first to emphasize the absence of this property from fluorescent pseudomonads and its probable value in the taxonomy of the genus *Pseudomonas*. Another feature of many strains in this group is conspicuous and unusual pigmentation: according to Morris & Roberts, they form 'characteristic opaque yellow colonies which in some strains change after 2–3 days to an intense reddish-purple colour'.

Under the label of *Chromobacterium ianthinum*, one of the Trinidad strains (strain 426 of our collection) was examined by Sneath (1956, 1960) in the course of his taxonomic analysis of the genus *Chromobacterium*. He concluded that it did not belong to this genus, and did not fit any of the classical descriptions of *C. ianthinum* (Sneath, 1960). The brief characterization of the group by Morris & Roberts and the

fuller description of one strain by Sneath seem to be the only published accounts of the properties of this species. Our description is based on the study of 19 strains. Two were isolated in Berkeley; the remaining 17 included some of the original Trinidad isolates, together with other strains later isolated in Great Britain.

Origins of the strains

85. Received from Dr W. C. Haynes, Northern Regional Research Laboratory Peoria, Illinois, under the designation B 2320. Isolated by Miss M. B. Morris (at the time, curator of the Hankey Culture Collection, Trinidad) from the Maraval River, Trinidad. ATCC 17460.
104. Isolated at Berkeley from soil by Dr G. Hegeman, using D-mandelate enrichment at 30°. ATCC 17478.
249. Strain S-4-AN, isolated at Berkeley from soil using anthranilate enrichment at 41°. ATCC 17616.
382. Isolated by Dr A. C. Hayward, Commonwealth Mycological Institute, Kew, Surrey, England, in 1958 from forest soil, Trinidad (isolate 8). Other designations: Sneath D 335, and NCIB 9085. ATCC 17759.
383. Isolated by Dr A. C. Hayward in 1958 from forest soil, Trinidad (isolate 61). Other designations: NCIB 9086, and Sneath D 336. ATCC 17760.
384. Isolated by Dr A. C. Hayward in 1958 from rotting tree trunk, Trinidad (isolate 7c). Other designations: NCIB 9087 and Sneath D 337. ATCC 17761.
385. Received from Dr A. C. Hayward under the designation IMI B 2056. Isolated by Dr R. G. Mitchell from urine in urinary tract infection at Bristol Royal Infirmary, 1964. ATCC 17763.
386. Received from Dr A. C. Hayward, isolated by Dr R. G. Mitchell from baby's thermometer in Bristol Royal Infirmary. ATCC 17763.
387. Received from Dr A. C. Hayward under the designation IMI B 2572, NCIB 8507. Studied by Sneath (1960) under the designation D 338. ATCC 17764.
396. Received from Dr A. C. Hayward as strain B 2394. Isolated from urine in urinary tract infection of child, at Bristol Royal Infirmary, 1964. ATCC 17765.
397, 398, and 399. Received from Dr A. C. Hayward under the designations B 2443, B 2253 and B 2384. These three strains have the same origin as 396. ATCC 17766, 17767 and 17768, respectively.
420. Received from Mr W. Hodgkiss, Torry Research Station, Aberdeen, Scotland (NCIB 9088; HCC 79). From soil, Trinidad. ATCC 17769.
421. Received from Mr W. Hodgkiss (NCIB 9089 and HCC B 175). From soil, Trinidad. ATCC 17770.
422. Received from Mr W. Hodgkiss (NCIB 9091; HCC B 338). From soil, Trinidad. ATCC 17771.
423. Received from Mr W. Hodgkiss (NCIB 9092; HCC B 339). From soil, Trinidad. ATCC 17772.
424. Received from Mr W. Hodgkiss (NCIB 9093; HCC B 340). ATCC 17773.
425. Received from Mr W. Hodgkiss (Sneath TI = Sneath B 269; NCIB 9135). Isolated from forest soil in Trinidad. ATCC 17774.

General properties

All strains are multitrichous. All accumulate poly-β-hydroxybutyrate as a cellular reserve material, but are unable to attack exogenous poly-β-hydroxybutyrate. Sixteen of the 19 strains hydrolyse gelatin (negative strains are 249, 385, 398); and, with the exception of one strain (398), they give an egg-yolk reaction. All are vigorous lipase producers, as judged by the hydrolysis of Tween 80. None can denitrify. They do not require growth factors, and can use both ammonia and nitrate as nitrogen sources. They are oxidase positive. Half of the strains grow well at 41°, and all can grow at 37°. None can grow at 4°.

Morris & Roberts (1959) divided their strains into two subgroups on the basis of pigmentation: some were yellow on glucose yeast-extract peptone agar, others various shades of brown, red, violet and purple. From a strain of the latter type, they isolated a purple pigment, and identified it unequivocally as a phenazine derivative. In our experience, pigment production is a very variable character in any given strain. Purple-pigmented strains show a bewildering variety of colours when grown on solid chemically defined media with a series of different carbon sources, the pigments being sometimes exclusively associated with the organisms, sometimes also diffusible. In accord with Morris & Roberts, we find that some strains never show red or purple pigmentation, producing only yellow pigments which are partly cellular and partly diffusible. Two strains in our collection (249, 385) are completely non-pigmented. Strains 85, 383, 387, 420, 421, 422 and 425 produce the purple diffusible pigment, and strains 104, 382, 386, 396, 397, 398, 399, 423 and 424 give a yellow diffusible pigment. These two pigments are readily produced in the King A medium. Differences in pigmentation are not correlated with any of the other phenotypic characters that we have studied, and therefore seem to be devoid of taxonomic significance.

Compounds which are used by 90 % or more of the strains of *Pseudomonas multivorans* are shown in Table 49. Details for substrates utilized by a fraction of the strains are given in Table 50.

Five strains (249, 396, 397, 398, 399) lack a series of nutritional characters otherwise practically constant for the group; they cannot grow on glycollate, histamine, testosterone, acetamide or (with the exception of 249) *o*-hydroxybenzoate. Strain 385 appears to be the most defective in the whole collection; it is unable to grow at the expense of valerate, isovalerate, caproate, caprylate, caprate, D-malate, hydroxymethylglutarate, citraconate, benzoate, DL-citrulline, kynurenate, anthranilate, ethanolamine, benzylamine, spermine, butylamine, α-amylamine, sarcosine or hippurate, which are practically universal substrates for the rest of the strains.

Apart from this, the group shows a striking nutritional uniformity. In a qualitative sense, the nutritional spectrum overlaps that of the fluorescent pseudomonads, with which *Pseudomonas multivorans* also shares the *ortho* type of ring cleavage in the metabolism of benzoate and *p*-hydroxybenzoate. The only compounds utilized by a substantial number of fluorescent pseudomonads that are not utilized by *P. multivorans* are D-arabinose, erythritol, D-(−)-tartrate, itaconate, mesaconate, geraniol, propyleneglycol, glycine, L-leucine, creatine and pantothenate; but none of these compounds is a universal substrate in the fluorescent group. There is, however, a remarkable *quantitative* difference between the nutritional

Table 49. *Pseudomonas multivorans. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Ribose
- D-Arabinose
- L-Arabinose
- D-Fucose
- D-Glucose
- D-Mannose
- D-Galactose
- D-Fructose
- Sucrose
- Trehalose*
- Cellobiose
- Salicin
- Gluconate
- 2-Ketogluconate
- Saccharate
- Mucate

Fatty acids
- Acetate
- Butyrate
- Isobutyrate*
- Valerate*
- Isovalerate*
- Caproate*
- Heptanoate
- Caprylate*
- Pelargonate
- Caprate*

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate
- Adipate
- Pimelate
- Suberate
- Azelate
- Sebacate

Hydroxyacids
- D-Malate*
- L-Malate
- *meso*-Tartrate
- DL-β-Hydroxybutyrate
- DL-Lactate
- DL-Glycerate
- Hydroxymethylglutarate*

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate
- Aconitate*
- Laevulinate
- Citraconate*

Polyalcohols and glycols
- Mannitol
- Sorbitol
- *meso*-Inositol
- Adonitol
- Glycerol
- 2,3-Butyleneglycol

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- Benzoate*
- *m*-Hydroxybenzoate
- *p*-Hydroxybenzoate
- Phenylacetate
- Quinate

Aliphatic amino acids
- L-α-Alanine
- β-Alanine
- L-Threonine
- L-Aspartate
- L-Glutamate
- L-Lysine
- DL-Arginine
- DL-Citrulline*
- γ-Aminobutyrate
- δ-Aminovalerate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine
- L-Phenylalanine
- L-Tryptophan
- L-Kynurenine*
- Kynurenate*
- Anthranilate*

Amines
- Benzylamine*
- Putrescine
- Spermine*
- Tryptamine
- Butylamine*
- α-Amylamine*

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine*

Paraffin hydrocarbons
- (None)

* Not used by all strains. Negative strains are listed in Table 50.

Table 50. *Pseudomonas multivorans. Substrates utilized by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Xylose	9	85, 383, 384, 385, 387, 420, 421, 422, 423, 425
L-Rhamnose	15	421, 423, 424, 425
Trehalose	18	398
Maltose	3	All except 384, 423, 425
Propionate	16	249, 385, 387
Isobutyrate	18	249
Valerate	18	385
Isovalerate	18	385
Caproate	18	385
Caprylate	18	385
Caprate	18	385
D-Malate	17	383, 385
L-(+)-Tartrate	12	85, 383, 384, 385, 387, 420, 425
Glycollate	13	249, 385, 396, 397, 398, 399
Hydroxymethylglutarate	17	249, 385
Aconitate	18	425
Citraconate	17	249, 385
Ethanol	10	249, 383, 384, 385, 420, 421, 422, 423, 425
n-Propanol	8	249, 383, 384, 385, 387, 420, 421, 422, 423, 424, 425
n-Butanol	12	249, 420, 421, 422, 423, 424, 425
Isobutanol	7	All except 85, 104, 386, 396, 397, 398, 399
L-Mandelate	4	All except 104, 382, 386, 424
Benzoylformate	15	249, 385, 387, 421
Benzoate	17	385, 387
o-Hydroxybenzoate	14	385, 396, 397, 398, 399
Phthalate	1	All except 249
Phenol	9	249, 382, 384, 385, 386, 396, 397, 398, 399, 423
Testosterone	13	249, 385, 396, 397, 398, 399
L-Serine	16	423, 424, 425
L-Isoleucine	7	All except 85, 104, 382, 420, 422, 423, 424
L-Valine	8	85, 249, 382, 385, 387, 396, 397, 398, 399, 424, 425
DL-Ornithine	18	399
DL-Citrulline	17	249, 385
D-Tryptophan	2	All except 383, 384
L-Kynurenine	18	422
Kynurenate	18	385
Anthranilate	18	385
Ethanolamine	16	383, 384, 385
Benzylamine	17	385, 387
Spermine	18	385
Histamine	11	249, 385, 387, 396, 397, 398, 399, 421
Butylamine	18	385
α-Amylamine	18	385
Sarcosine	17	385, 422
Hippurate	16	385, 387, 421
Acetamide	13	249, 385, 396, 397, 398, 399
Nicotinate	11	104, 382, 383, 384, 385, 386, 421, 424
Trigonelline	10	104, 382, 383, 384, 385, 386, 421, 424, 425
n-Dodecane	7	All except 85, 383, 387, 420, 422, 423, 425
n-Hexadecane	7	All except 249, 383, 387, 420, 421, 422, 423

spectra of the two groups. No *single* strain of fluorescent pseudomonad that we have examined can grow on the total number of substrates that are used by the least nutritionally versatile strains of *P. multivorans* (excluding the defective strain 385). Particularly characteristic of *P. multivorans* is the very wide range of carbohydrates utilized, coupled with utilization of all the higher dicarboxylic acids and nearly all simple aromatic acids: this is a nutritional constellation which does not occur in the fluorescent group. In addition, all strains of *P. multivorans* can use a few substrates which are completely non-utilizable by the fluorescent pseudomonads that we have examined: D-fucose, cellobiose, salicin and L-threonine.

Table 51. *Pseudomonas multivorans. The characters of greatest differential value in the recognition of P. multivorans, based on the analysis of 19 strains*

Characters	No. of positive strains	Ideal phenotype
1. Poly-β-hydroxybutyrate as cellular reserve material	19	+
Utilization of:		
2. D-Arabinose	19	+
3. D-Fucose	19	+
4. Cellobiose	19	+
5. Saccharate and mucate	19	+
6. 2,3-Butyleneglycol	19	+
7. Sebacate	19	+
8. *meso*-Tartrate	19	+
9. Citraconate	17	+
10. *o*-Hydroxybenzoate	14	+
11. *m*-Hydroxybenzoate	19	+
12. L-Threonine	19	+
13. DL-Ornithine	18	+
14. Tryptamine	19	+

Table 52. *Pseudomonas multivorans. Number of strains of different species or groups of species of aerobic pseudomonads conforming to the selected 14 characters (Table 51) which define the ideal phenotype of Pseudomonas multivorans*

	No. of characters of 'ideal' phenotype														
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
P. multivorans	13	4	2	.	.	.	.	.	.	.	.	.	.	.	.
Fluorescent group	.	.	.	.	.	.	.	.	4	12	43	74	39	3	.
Acidovorans group	.	.	.	.	.	.	.	7	12	5	.	2	.	.	.
Pseudomallei group	.	.	.	.	.	.	.	1	25	15	.	.	.	.	.
P. stutzeri	.	.	.	.	.	.	.	.	.	.	5	8	2	2	.
P. maltophilia	.	.	.	.	.	.	.	.	.	.	.	.	.	23	.
Alcaligenes group	.	.	.	.	.	.	.	.	.	.	.	.	.	5	2
P. lemoignei	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.

Phenotypic comparison between Pseudomonas multivorans and other species of aerobic pseudomonads

In Table 51 we give a list of selected characters used for the definition of the ideal phenotype of *P. multivorans*; 13 of the strains in our collection fit this ideal phenotype in all fourteen characters, four strains coincide in thirteen of the characters, and

two strains in twelve (Table 52). All other groups and species of aerobic pseudomonads examined by us deviate from the *P. multivorans* phenotype in at least seven of the fourteen selected characters.

Proposal of type strain

As type strain for the species *Pseudomonas multivorans* we propose strain 382, one of the Trinidad isolates. This strain produces yellow diffusible pigment, liquefies gelatin, gives the egg-yolk reaction and grows at 41°. It can grow on all the substrates attacked by some *P. multivorans* strains (see Tables 49 and 50) with the exception of maltose, isobutanol, phthalate, phenol, L-valine, nicotinate, trigonelline, *n*-dodecane and *n*-hexadecane.

Ecological considerations and enrichment

It is a remarkable fact that *Pseudomonas multivorans*, by far the most nutritionally versatile aerobic pseudomonad so far examined, should have been first isolated so recently. One possible explanation might be that it is, like *P. pseudomallei* (Redfearn *et al.* 1966), a geographically restricted species, abundant only in tropical regions; this would fit with its high temperature maximum. Unfortunately, the conditions of isolation of the strains from Trinidad and Great Britain throw no light on its ecology. However, the two strains isolated in Berkeley were both obtained by enrichments, albeit unusual ones. Strain 104 emerged from a deliberate search by Dr G. Hegeman for a strain that lacked mandelic racemase, and hence could grow on L-, but not on D-mandelate. An aerobic liquid enrichment containing L-mandelate as carbon and energy source was inoculated with pond water and incubated at 30°. Colonies were isolated by plating on L-mandelate agar medium. This plate was replicated on a plate of D-mandelate agar, and a colony which failed to grow on the replica plate was isolated.

Strain 249 was isolated by one of us from an aerobic liquid enrichment with anthranilate as carbon and energy source, inoculated with soil and incubated at 41°, in the hope of discovering a specific enrichment method for *Pseudomonas aeruginosa*. This hope was unfulfilled, since from several soil samples so enriched the organism which we subsequently recognized as *P. multivorans* was isolated. The same enrichment, conducted at 30°, typically yields fluorescent pseudomonads other than *P. aeruginosa* as the predominant microflora. These facts suggest that, at 30°, *P. multivorans* is likely to be outgrown in aerobic enrichments by fluorescent pseudomonads, other nutritionally versatile aerobic pseudomonads (e.g. *P. acidovorans*, *P. testosteroni*) or members of the *Moraxella* group, even though the medium contains a carbon and energy source that is readily utilizable by *P. multivorans*. Consequently, unless some form of post-selection is applied (as in the isolation of strain 104), *P. multivorans* will probably be overlooked on plates, being a minority member of the enriched population. In enrichments at 41°, on the other hand, competition from other nutritionally versatile aerobic pseudomonads (with the exceptions of *P. aeruginosa*, *P. pseudomallei* and possibly *P. stutzeri*) is automatically eliminated. Further work on this ecological problem is obviously needed.

PSEUDOMONAS STUTZERI

A good general description of this species was published some years ago by van Niel & Allen (1952), who also retraced its chequered taxonomic history. We shall present data, based on the study of 17 strains, which supplement their description.

Origins of the strains

Strains 220 to 229 were received from Dr H. Lautrop, Statens Seruminstitut, Copenhagen. They were isolated from different clinical specimens submitted for bacteriological diagnosis.

220. Strain AB 180. ATCC 17587.
221. Strain AB 201. ATCC 17588.
222. Strain AB 205. ATCC 17589.
223. Strain AB 207. ATCC 17590.
224. Strain AB 212. ATCC 17591.
225. Strain AB 217. ATCC 17592.
226. Strain AB 316. ATCC 17593.
227. Strain AB 317. ATCC 17594.
228. Strain AB 320. ATCC 17595.
229. Strain AB 322. ATCC 17596.
275. Strain 41.4 of van Niel & Allen (1952). Received from Dr P. V. Liu, University of Louisville, Louisville, Ky. ATCC 17641.

Strains 316 to 321 were received from Dr M. Véron, Institut Pasteur, Paris; like the strains of Dr Lautrop, they were isolated from clinical specimens.

316. Strain 62C. ATCC 17682.
318. Strain 62DA. ATCC 17684.
319. Strain 62GN. ATCC 17685.
320. Strain 62HA. ATCC 17686.
321. Strain 62HB. ATCC 17687.
419. Isolated in Berkeley from soil by L-(−)-tartrate enrichment. ATCC 17832.

General properties

All strains that we have examined are monotrichous. Poly-β-hydroxybutyrate is not accumulated as a cellular reserve material; nor can it be attacked by extracellular enzymes when furnished as an exogenous substrate. Gelatin is not hydrolysed, and the egg-yolk reaction is negative. Most strains can hydrolyse Tween 80. Growth factors are not required, and ammonia or nitrate can serve as the nitrogen source. The temperature maximum is high; well over half the strains in our collection can grow at 41°. The oxidase test is positive.

Two outstanding properties have traditionally served to identify this species: its denitrifying ability, and its unusual colony structure. The colonies of freshly isolated strains are light brown in colour and of the rough form, wrinkled, dry and coherent. However, as van Niel & Allen (1952) noted, smooth colony variants frequently predominate after laboratory cultivation for some time. Most of the strains received by us from other laboratories proved to have a smooth colony form.

Furthermore, many of these strains grew anaerobically with nitrate only after a lag of several days; however, once vigorous growth and denitrification had occurred, subcultures into the same medium grew and denitrified promptly. Interestingly enough, several of the strains which had possessed a smooth colony form upon their receipt from other laboratories regained the rough colony form characteristic of fresh isolates from soil after one or two passages through denitrification media. These observations suggest that aerobic cultivation on complex media free of

Table 53. *Pseudomonas stutzeri. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Glucose
- Sucrose
- Maltose*
- Starch*

Fatty acids
- Acetate
- Butyrate*
- Caproate*
- Heptanoate*
- Caprylate
- Pelargonate*
- Caprate

Dicarboxylic acids
- Succinate
- Fumarate
- Glutarate*
- Sebacate*

Hydroxyacids
- L-Malate
- DL-β-Hydroxybutyrate
- DL-Lactate
- Glycollate

Miscellaneous organic acids
- Citrate*
- α-Ketoglutarate*
- Pyruvate
- Citraconate*
- Itaconate*
- Mesaconate*

Polyalcohols and glycols
- Glycerol*
- Propyleneglycol*

Alcohols
- Ethanol
- *n*-Propanol*
- *n*-Butanol*

Non-nitrogenous aromatic and other cyclic compounds
- (None)

Aliphatic amino acids
- Glycine*
- L-α-Alanine
- D-α-Alanine
- L-Glutamate

Amino acids and related compounds containing a ring structure
- L-Proline*

Amines
- (None)

Miscellaneous nitrogenous compounds
- (None)

Paraffin hydrocarbons
- (None)

* Not used by all strains. Negative strains are given in Table 54.

nitrate counterselects both the denitrifying ability and the rough colony form characteristic of *Pseudomonas stutzeri*, a point which should be kept in mind by those who are attempting to identify the species. In our experience, every strain can regain the capacity for active denitrification as a result of prolonged growth under conditions selective for this ability. The 'training' process can often be accelerated by initial cultivation in a nitrate-containing medium under semi-anaerobic conditions followed by transfer to a fresh tube of the same medium incubated under strictly anaerobic conditions. Some but not all strains will also regain the typical colony form as a result of this treatment.

As already mentioned in the section on the oxidase reaction and its relation to the cytochrome system, suspensions of *Pseudomonas stutzeri* show very strong

Table 54. *Pseudomonas stutzeri. Substrates used by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Mannose	4	All except 226, 227, 275, 318
D-Fructose	11	222, 225, 275, 316, 319, 419
Maltose	16	316
Starch	15	229 (strain 316 hydrolyses starch, but does not grow on it)
Gluconate	13	228, 319, 320, 419
Saccharate	7	All except 222, 223, 228, 229, 318, 320, 321
Mucate	7	All except 222, 223, 228, 229, 318, 320, 321
Propionate	7	All except 221, 222, 226, 227, 229, 316, 318
Butyrate	16	275
Valerate	11	220, 229, 275, 318, 321, 419
Isovalerate	14	275, 320, 321
Caproate	15	275, 320
Heptanoate	16	223
Pelargonate	16	223
Malonate	13	228, 229, 320, 419
Glutarate	16	228
Adipate	1	All except 223
Suberate	1	All except 223
Azelate	13	228, 229, 275, 419
Sebacate	15	275, 419
D-Malate	14	223, 227, 419
L-(+)-Tartrate	1	All except 419
DL-Glycerate	5	All except 223, 275, 319, 320, 419
Hydroxymethylglutarate	3	All except 222, 226, 227
Citrate	16	318
α-Ketoglutarate	16	228
Aconitate	11	222, 226, 227
Citraconate	15	223, 419
Itaconate	16	223
Mesaconate	16	223
Mannitol	9	226, 227, 316, 318, 319, 320, 321, 419
Glycerol	16	275
Ethyleneglycol	1	All except 419
Propyleneglycol	16	221
2,3-Butyleneglycol	13	226, 227, 229, 275
n-Propanol	16	221
n-Butanol	16	273
Isobutanol	12	226, 227, 229, 275, 320
Benzoate	2	All except 316, 319
Testosterone	1	All except 226, 227, 318, 419
L-Phenylalanine	3	All except 316, 319
Glycine	16	318
L-Isoleucine	13	275, 319, 321, 419
L-Valine	13	228, 229, 275, 321
L-Aspartate	13	221, 229, 275, 419
γ-Aminobutyrate	6	All except 223, 226, 227, 229, 319, 419
δ-Aminovalerate	4	All except 225, 226, 319, 419
L-Proline	16	419
L-Tyrosine	14	223, 229, 318
Kynurenate	2	All except 223, 275, 319
Ethanolamine	6	All except 220, 224, 225, 316, 319, 320
p-Hydroxybenzoate	4	All except 319
Putrescine	13	229, 316, 321, 419
Spermine	2	All except 319, 320
Betaine	1	All except 223

absorption bands of a cytochrome of the *c* type. This probably explains the characteristic colour of growth on solid media.

The substrates used by all or most strains of *Pseudomonas stutzeri* are shown in Table 53. Detailed data for substrates used by a fraction of the strains are given in Table 54. A total of 72 compounds can be used as carbon and energy sources by some strains of *P. stutzeri*; but only seventeen compounds (24% of the total) are substrates for all strains. These figures clearly indicate the marked nutritional heterogeneity of this species as compared, for instance, with a relatively homogeneous species like *P. multivorans*, for which the number of universally utilized substrates represents about 60% of the total number of compounds used by one or more of the strains of the species. The nutritional spectrum of *P. stutzeri* is very irregular; furthermore, the results of successive screenings have not always been completely reproducible. In these as in other respects, *P. stutzeri* seems to be a species prone to undergo considerable variation.

The nutritional data presented in Table 53 show, however, that *Pseudomonas stutzeri* does possess some characteristic features, although almost every nutritional character useful for the identification of the species is lacking in an occasional

Table 55. *Pseudomonas stutzeri. The group characters of greatest differential value in the recognition of P. stutzeri, based on the analysis of 17 strains*

Characters	No. of positive strains	Ideal phenotype
1. Monotrichous flagellation	17	+
2. Denitrification	17	+
Utilization of:		
3. L-Arabinose	0	−
4. D-Galactose	0	−
5. Cellobiose	0	−
6. Maltose	16	+
7. Starch	16	+
8. 2-Ketogluconate	0	−
9. Propyleneglycol	16	+
10. Glycollate	17	+
11. *p*-Hydroxybenzoate	3	−
12. *β*-Alanine	0	−
13. L-Histidine	0	−

exceptional strain. One of the diagnostically most valuable properties is the ability to grow on maltose and starch, the latter substrate being attacked by extracellular amylases. Other unusual nutritional features are the good growth on glycollate, itaconate, mesaconate and propyleneglycol. With respect to amino acids, useful features are the good growth on glycine, and the failure to grow on histidine. Higher amines and *N*-methyl compounds are rarely and sporadically used. Aromatic compounds are likewise rarely utilized; the typical irregularity of this species is shown by the fact that two strains can grow on benzoate, and three others on *p*-hydroxybenzoate.

Thirteen characters have been selected to define the ideal phenotype of *Pseudomonas stutzeri* (Table 55). As showni n Table 56, twelve strains fit the ideal phenotype in all characters, four strains deviate in one character, and 1 strain in two characters.

These thirteen characters permit a clear differentiation of *P. stutzeri* from other groups and species of aerobic pseudomonads.

In their discussion of this species, van Niel & Allen (1952) noted that denitrifying enrichments furnished with tartrate as a carbon and energy source, first used by van Iterson (1902), are particularly useful for the isolation of *Pseudomonas stutzeri*. We were therefore surprised to discover that none of the 16 strains first examined, including 275, the strain of van Niel & Allen (1952), reportedly isolated by denitrifying enrichment with tartrate, could grow on any isomer of tartrate. We therefore set up a denitrifying enrichment with L-tartrate, using a soil inoculum, which did in fact yield a strain of *P. stutzeri* (strain 419) that could grow on L-tartrate, though not on the D- and *meso*-isomers.

Table 56. *Pseudomonas stutzeri. Number of strains of different species or groups of aerobic pseudomonads conforming to the selected 13 characters (Table 55) which define the ideal phenotype*

	No. of characters of 'ideal' phenotype												
	13	12	11	10	9	8	7	6	5	4	3	2	1
P. stutzeri	12	4	1	.	.	.	.	.	.	.	.	.	.
Fluorescent group	.	.	.	.	.	1	1	27	10	48	35	16	37
Acidovorans group	.	.	.	.	.	.	11	3	12	.	.	.	.
Pseudomallei group	.	.	.	.	.	.	.	.	.	26	6	4	5
P. multivorans	.	.	.	.	.	.	.	.	.	.	.	3	10
P. maltophilia	.	.	.	.	.	.	.	23	.	.	.	.	.
Alcaligenes group	.	.	.	.	.	1	3	2	1	.	.	.	.
P. lemoignei	.	.	.	.	.	1	.	.	.	.	.	.	.

As Palleroni & Doudoroff (1965) pointed out, some investigators have confused *Pseudomonas stutzeri* with a hydrogenomonad, *P. saccharophila*. This confusion is probably caused by similarities in carbohydrate utilization, specifically the ability to utilize starch. These two species are, however, readily distinguishable by other characters, notably denitrifying ability (not possessed by *P. saccharophila*) and H_2 chemolithotrophy (not possessed by *P. stutzeri*). *P. stutzeri* also resembles *P. pseudomallei* in some respects (colony structure, denitrifying ability, high temperature maximum). However, it can be readily distinguished from *P. pseudomallei* by its lesser nutritional versatility and particularly by its failure to accumulate poly-β-hydroxybutyrate, or to decompose this polymer by means of extracellular enzymes.

The strain of van Niel & Allen (strain 275) is atypical of the species (or has become atypical) in several respects. It is therefore not a desirable neotype strain, and we propose instead strain 221.

DNA base composition and internal subdivision of Pseudomonas stutzeri

The GC content of the DNA of our strains of *Pseudomonas stutzeri* has been analysed by Mandel (1966). The analyses revealed the existence of two clearly differentiated subgroups within the species. Strains 220, 223, 224, 225, 228 and 320 have GC contents that average 62 moles %, while strains 221, 222, 226, 227, 229, 275, 316, 318, 319, 321 and 419 show values which cluster around 65 %. These differences in GC content suggested that two species might be combined in *P. stutzeri*. Unfortunately, the difference in GC content is not correlated with a large number of pheno-

typic differences, as in the acidovorans group. The one nutritional character which shows a high degree of correlation with GC content is the ability to grow on propionate, possessed only by strains with 65 moles % GC; but it is absent from strains 275, 319, 321 and 419, all of which do have 65 moles % GC. The ability to grow at high temperatures also appeared to show some correlation with GC content, and we investigated this aspect of the problem further. In attempting to determine the maximum temperature of growth, we found that none of the strains of *P. stutzeri* grow at 45°, but that five strains (221, 222, 229, 316, 318) grew at 44°. These five strains have 65 moles %GC in their DNA. The temperature that seems to define most clearly the two groups of strains is 43°; all high GC strains, with the exception of 275 and 419, can grow at this temperature; none of the strains of low GC content can do so. Strain 419 can grow at 42°; but so does strain 228, which has 62 moles % GC. As can be seen from the nutritional data presented in Tables 53 and 54, strain 419 is highly atypical in nutritional respects. It cannot grow on fructose, gluconate, valerate, malonate, azelate, sebacate, D-malate, citraconate, aconitate, isoleucine, aspartate, proline or putrescine, which are all attacked by the majority of other strains. On the other hand, it is the only strain capable of utilizing ethyleneglycol and L-(+)-tartrate for growth.

Possibly other correlations between phenotypic characters and DNA composition in the strains assigned to *Pseudomonas stutzeri* may be revealed by further investigation. However, it would certainly be premature to split *P. stutzeri* into two species on the basis of the minor and unreliable phenotypic differences just discussed; and we are likewise reluctant to do so purely on the basis of differences in the GC content of the DNAs.

PSEUDOMONAS MALTOPHILIA

This species was named by Hugh & Ryschenkow (1960), who subsequently (1961) published a detailed description of it, based on conventional taxonomic criteria. We have examined 23 strains, consisting of the type strain (strain 67) and a large collection of strains which had been isolated in France or French dependencies, and identified as *Pseudomonas maltophilia* by Thibault (1961).

Origins of the strains

All these strains were received from Dr P. Thibault, Institut Pasteur, Paris, under the designation *Pseudomonas maltophilia.*

67. Strain 6077. Isolated by Hugh & Ryschenkow (1961) from the oropharyngeal region of patient with mouth cancer and described as the type strain. Strain 810-2 of Hugh. ATCC 13637.
68. Strain R-72. Isolated as contaminant of a streptomycin solution. ATCC 17444.
69. Strain X-53. Isolated from pleural pus. ATCC 17445.
70. Strain CB. Obtained from cerebrospinal fluid. ATCC 17446.
71. Strain R-173. Isolated from contaminated tissue culture. ATCC 17447.
72. Strain Hamze. From blood culture. ATCC 17448.
73. Strain X-55. Obtained from a case of arthritis. ATCC 17449.
74. Strain X-80. Isolated from human lung. ATCC 17450.
75. Strain R-123. Isolated from blood culture. ATCC 17451.
300. Strain X-56. Isolated from contaminated tissue culture. ATCC 17666.
301. Strain X-57. Isolated from contaminated tissue culture. ATCC 17667.

302. Strain X-61. Isolated from sinusitis pus. ATCC 17668.
303. Strain R-41. Origin unknown. Received from French Equatorial Africa. ATCC 17669.
304. Strain R-147. Obtained from contaminated tissue culture. ATCC 17670.
305. Strain X-51. Isolated from blood culture. ATCC 17671.
306. Strain X-54. Isolated from sputum. ATCC 17672.
307. Strain X-60. Isolated from blood culture. ATCC 17673.
308. Strain X-62. Obtained from cerebrospinal fluid. ATCC 17674.
309. Strain X-77. Isolated from tissue culture. ATCC 17675.
310. Strain X-83. Isolated from sinusitis pus. ATCC 17676.
311. Strain X-57. Isolated from otitis pus. ATCC 17677.
312. Strain R-122. Isolated from blood culture. ATCC 17678.
313. Strain R-146. Isolated from tissue culture. ATCC 17679.

General properties

Pseudomonas maltophilia is multitrichous. It does not accumulate poly-β-hydroxybutyrate as a cellular reserve material, and cannot attack exogenous polymer. Nutritionally, *P. maltophilia* has proved to be a remarkably uniform biotype, which possesses several very distinctive characters not revealed by previous conventional taxonomic studies (Hugh & Ryschenkow, 1960, 1961). Every strain requires methionine, and only methionine, as growth factor; this requirement has been independently established by Iizuka & Komagata (1964) for five strains of Japanese origin. It is, furthermore, the only species of aerobic pseudomonad examined that cannot use nitrate as a principal nitrogen source. It is also the only oxidase-negative aerobic pseudomonad that we have encountered. All strains hydrolyse gelatin and are strongly lipolytic, but do not give an egg-yolk reaction. They do not denitrify.

Some of the strains produce a yellow intracellular pigment which does not diffuse into the medium. This pigment cannot be extracted from the organisms by treatment with organic solvents; it is, however, water soluble. Although we have not further investigated this point, it seems likely that the yellow colour is due to flavins.

The range of utilizable carbon and energy sources is restricted; only 23 of the 145 compounds tested support the growth of all or most strains (Table 57). Substrates used by a fraction of the strains are listed in Table 58. Notable features of the nutritional spectrum are the ability to use the disaccharides cellobiose, maltose and lactose. In fact, *Pseudomonas maltophilia* is the only species of our collection characterized by the ability to use lactose. The strains grow on lactose rather slowly, and the growth yield does not seem to be very high when lactose is used at the same concentration as the other carbohydrates in the nutritional screening. This poor growth presumably reflects the fact that galactose cannot be attacked by *P. maltophilia*. Other nutritional properties of interest include the very limited range of fatty acids utilized (acetate, propionate, valerate); the failure to grow on glutarate, β-hydroxybutyrate or aspartate, otherwise well-nigh universal substrates for aerobic pseudomonads; and the failure to grow on polyalcohols, aromatic compounds or amines. Strain 72 is aberrant: it can grow at the expense of D-xylose, isobutyrate, ethanol, *n*-propanol and L-aspartate, but not on malonate. The type strain of the

species, which was proposed by Hugh & Leifson (1963), is 67 (ATCC 13637). It can grow on: D-glucose, D-mannose, D-fructose, sucrose, trehalose, maltose, cellobiose, salicin, lactose, acetate, propionate, valerate, malonate, succinate, fumarate, L-malate, DL-lactate, citrate, α-ketoglutarate, pyruvate, aconitate, L-α-alanine, D-α-alanine, L-glutamate, L-histidine, L-proline.

Table 57. *Pseudomonas maltophilia. Substrates used by 90 % or more of the strains*

Carbohydrates and sugar derivatives
- D-Glucose
- D-Mannose
- Sucrose
- Trehalose
- Maltose
- Cellobiose
- Salicin
- Lactose

Fatty acids
- Acetate
- Propionate
- Valerate

Dicarboxylic acids
- Malonate*
- Succinate
- Fumarate

Hydroxyacids
- L-Malate
- DL-Lactate

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Pyruvate

Polyalcohols and glycols
- (None)

Alcohols
- (None)

Non-nitrogenous aromatic and other cyclic compounds
- (None)

Aliphatic amino acids
- L-α-Alanine
- D-α-Alanine
- L-Glutamate

Amino acids and related compounds containing a ring structure
- L-Histidine
- L-Proline

Amines
- (None)

Miscellaneous nitrogenous compounds
- (None)

Paraffin hydrocarbons
- (None)

* Not used by all strains. Negative strains are listed in Table 58.

Table 58. *Pseudomonas maltophilia. Substrates utilized by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Xylose	1	All except 303
D-Fructose	15	69, 74, 300, 301, 303, 307, 310, 312
Isobutyrate	2	All except 300, 302
Malonate	22	72
Aconitate	12	68, 69, 71, 300, 301, 304, 307, 309, 310, 312, 313
Ethanol	1	All except 72
n-Propanol	2	All except 72, 310
L-Aspartate	1	All except 72

Phenotypic comparison between Pseudomonas maltophilia and other species of aerobic pseudomonads. The ideal phenotype of *P. maltophilia*, defined on the basis of a total of ten selected characters, is shown in Table 59. This selected group of characters permits a very satisfactory separation from other species of aerobic

pseudomonads (Table 60). In fact, *P. maltophilia* appears to be by far the most isolated species, in terms of overall phenotype, among the aerobic pseudomonads that we have examined.

Ecological considerations

The ecology of *Pseudomonas maltophilia* is obscure. The restricted nutritional spectrum and requirement for methionine should be serious handicaps in competition with more nutritionally versatile aerobic pseudomonads. Yet the records of its repeated isolation from clinical specimens (Hugh & Ryschenkow, 1961; Dr P. Thibault, personal communication) as well as from water and raw milk (Hugh & Ryschenkow, 1961) suggest that it is widespread in nature. Iizuka & Komagata (1964) isolated it from enrichments with kerosene and crude oil inoculated with soil from petroleum zones in Japan, and reported that fresh isolates utilized hydrocarbons, but rapidly lost this ability when grown on nutrient agar slopes. None of the strains in our collection grew on dodecane or hexadecane.

Table 59. *Pseudomonas maltophilia. The group characters of greatest differential value in the recognition of P. maltophilia based on the analysis of 23 strains*

Characters	No. of positive strains	Ideal phenotype
1. Methionine requirement	23	+
2. Oxidase reaction	0	−
3. Utilization of nitrate as nitrogen source	0	−
Utilization of:		
4. Lactose	22	+
5. Cellobiose	23	+
6. Aspartate	1	−
7. β-Hydroxybutyrate	0	−
8. Glutarate	0	−
9. Glycerol	0	−
10. Maltose	23	+

Table 60. *Pseudomonas maltophilia. Number of strains of different species or groups of species of aerobic pseudomonads conforming to the selected 10 characters (Table 59) which define the ideal phenotype of P. maltophilia*

	No. of characters of 'ideal' phenotype										
	10	9	8	7	6	5	4	3	2	1	0
P. maltophilia	21	2	.	.	.	.	.	.	.	.	.
Fluorescent group	.	.	.	.	.	.	.	.	1	8	166
Acidovorans group	.	.	.	.	.	.	.	.	.	21	5
Pseudomallei group	.	.	.	.	.	.	.	7	32	2	.
P. multivorans	.	.	.	.	.	.	.	.	3	16	.
P. stutzeri	.	.	.	.	.	.	.	1	3	13	.
Alcaligenes group	.	.	.	.	.	.	1	.	4	2	.
P. lemoignei	.	.	.	.	.	.	.	1	.	.	.

DISCUSSION

Nutritional spectra: their taxonomic utility, physiological interpretation, and limitations

The long-ignored discovery of den Dooren de Jong (1926) that the nutritional spectrum of aerobic pseudomonads includes phenotypic traits of deep taxonomic significance has been overwhelmingly substantiated by our work. Every species and group of species can use a characteristic range of organic compounds as carbon and energy sources. The fact that a strain can use a given organic compound as sole source of carbon and energy demonstrates its possession of a special complement of enzymes, necessary to convert this primary substrate into one or more members of the general intermediary metabolic pool of the cell. When the biochemistry of the pathway is known, the nutritional datum immediately provides information about the enzymic constitution of the strain in question. Present biochemical knowledge tells us, for example, that a fluorescent pseudomonad able to grow on L-tryptophan can synthesize the twelve specific enzymes which mediate the conversion of this primary substrate within the cell to L-alanine, succinate and acetyl-CoA (Behrman, 1962). The ability of a fluorescent pseudomonad to grow on L-tartrate, on the other hand, is dependent upon the synthesis of only two specific proteins: a stereospecific permease, and L-tartrate dehydrase which converts the primary substrate immediately to oxalacetate (Shilo, 1957). In other words, nutritional characters, which numerical taxonomists would treat as characters of equivalent weight, are in fact far from equivalent to one another when we examine them in terms of their underlying enzymic mechanisms. In enzymic terms (and hence also in terms of structural genes) growth on L-tryptophan is a 12-unit character, growth on L-tartrate only a 2-unit character. To make really refined phenotypic comparisons among aerobic pseudomonads by the methods of numerical taxonomy (Sokal & Sneath, 1963) we require much more biochemical information about these bacteria than we now possess; and it is largely for this reason that we have resisted the temptation to feed our data to a computer. A purely nutritional analysis can also completely conceal deep biochemical differences. Nearly all strains of the acidovorans and fluorescent groups grow well on *p*-hydroxybenzoate, and with closely similar growth yields. *Nutritionally* speaking, the two groups are phenotypically identical in this particular respect. However, the metabolic pathways which they use for the dissimilation of this substrate diverge after the first step-reaction, and knowledge of this fact, obtainable only by biochemical analysis, leads to the recognition that the members of each group can synthesize at least four specific enzymes not synthesized by the other.

The inability of a strain to use a given compound as sole source of carbon and energy for growth is subject to several possible interpretations. The strain in question may simply be sensitive to the toxic effect of the compound at the concentration used, and may in fact possess the complete array of enzymes required for its utilization. The failure of many strains of *Pseudomonas fluorescens* to grow on butyrate and isobutyrate at a concentration of 0·1 % (w/v), while growing well on the same concentration of higher and lower homologues of the fatty acid series, might well prove to be interpretable in this way.

Failure to grow on a compound may occur despite the presence in the cell of the

complete array of enzymes necessary for its metabolism, if penetration is mediated by a specific permease not synthesized by the strain in question. The failure of some nutritionally versatile species (e.g. *Pseudomonas testosteroni*, *P. acidovorans*) to grow on glucose might be attributable to impermeability.

Lastly, failure to grow on a compound may be caused by the inability to synthesize one or more of the enzymes necessary for its metabolism. If the dissimilation of a compound necessitates synthesis of a considerable number of specific enzymes, the significance that should be attached to absence of growth can be determined only by a biochemical analysis. For example, in the case of L-tryptophan, lack of growth could be explicable as the result of a failure to synthesize only the first enzyme of the specific sequence, tryptophan pyrrolase. This is probably true of *Pseudomonas aeruginosa*, since strains of this species typically grow well on L-kynurenine, the second intermediate of the tryptophan pathway, but not on L-tryptophan. Many of these strains give rise to tryptophan-positive mutants when patches are replicated on tryptophan agar. From the purely determinative standpoint, this is a nuisance, since it means that tryptophan utilization is an irregular character in *P. aeruginosa*, difficult to score in plate tests. However, for a profounder understanding of the specific phenotype it is a deeply significant fact, since it shows that the ability to use tryptophan as a carbon and energy source is immanent in the genome of the species, and requires only the appearance and selection of a mutation in a gene governing synthesis of an early enzyme to achieve full phenotypic expression. If, on the other hand, it could be shown that a species unable to grow on L-tryptophan was unable to synthesize any of the specific enzymes of the tryptophan pathway, this would suggest that the entire set of genes governing the tryptophan pathway might be absent from the genome. With respect to tryptophan metabolism, such an organism would differ radically from *P. aeruginosa*, even though both might be scored nutritionally as 'tryptophan negative'.

Accordingly, the determination of nutritional spectra, useful as these may be for practical taxonomic purposes, represents only a first approximation (and a very crude one) towards characterizing the phenotypes of the aerobic pseudomonads. In effect, our present nutritional findings point the way to a much more refined analysis of the different species at the biochemical level. At least such analyses can now be conducted, as they could not be in the past, on strains which have a meaningful taxonomic identity.

Prospective developments: towards a biochemical taxonomy of the aerobic pseudomonads

As already pointed out, different species may carry out the attack on a common primary substrate in biochemically different ways. This important fact, illustrated particularly clearly by the fluorescent and acidovorans groups, deserves further discussion. The most extensive evidence for the existence of metabolic divergences between the fluorescent and acidovorans groups has been accumulated through many different biochemical studies on the oxidation of aromatic substrates. This body of work is summarized in the form of two comparative metabolic maps (Figs. 6, 7). These figures show how two commonly attacked aromatic compounds, tryptophan and *p*-hydroxybenzoic acid, are metabolized by the two groups. The main features of the metabolic map for the fluorescent group have been known for

many years. However, the metabolic map for the acidovorans group could not be previously constructed, because the biochemical data on which it is based had been obtained piecemeal with various strains, for the most part designated by those who

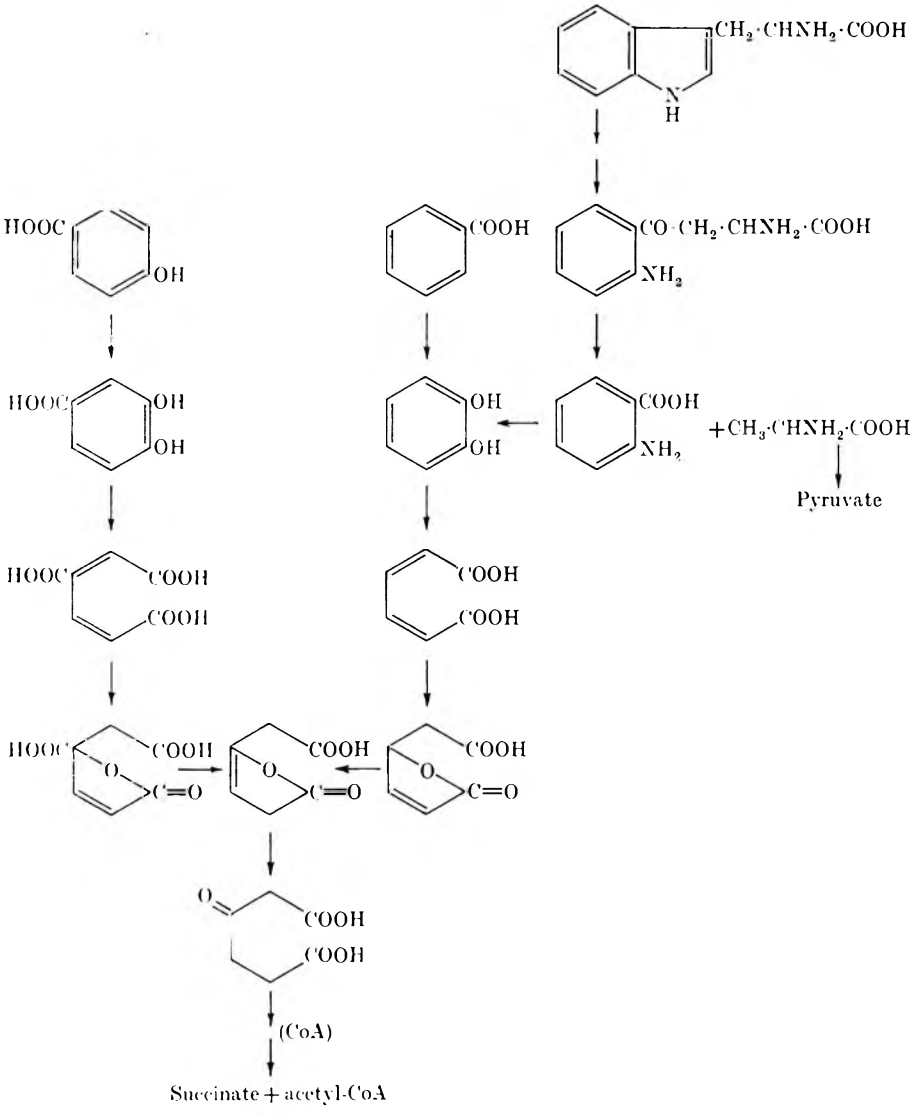

Fig. 6. Convergent pathways for metabolism of L-tryptophan and *p*-hydroxybenzoic acid in the fluorescent group of pseudomonads. The metabolic relationship of benzoate to these two pathways is also shown.

worked with them as '*Pseudomonas* sp.', or 'an unidentified Pseudomonas isolated from soil'. It is the present taxonomic analysis of many of these strains which has enabled us to give coherence to the biochemical information. For example, the existence of two divergent pathways for tryptophan metabolism among aerobic pseudomonads was definitely established through comparative studies as long ago

as 1951 by Stanier, Hayaishi & Tsuchida. These authors noted that fluorescent pseudomonads all used the pathway leading through anthranilate to β-ketoadipate. Of the strains then shown to use the pathway leading through kynurenate, three which survived have now been identified by us as *P. acidovorans* (strains 103, 105,

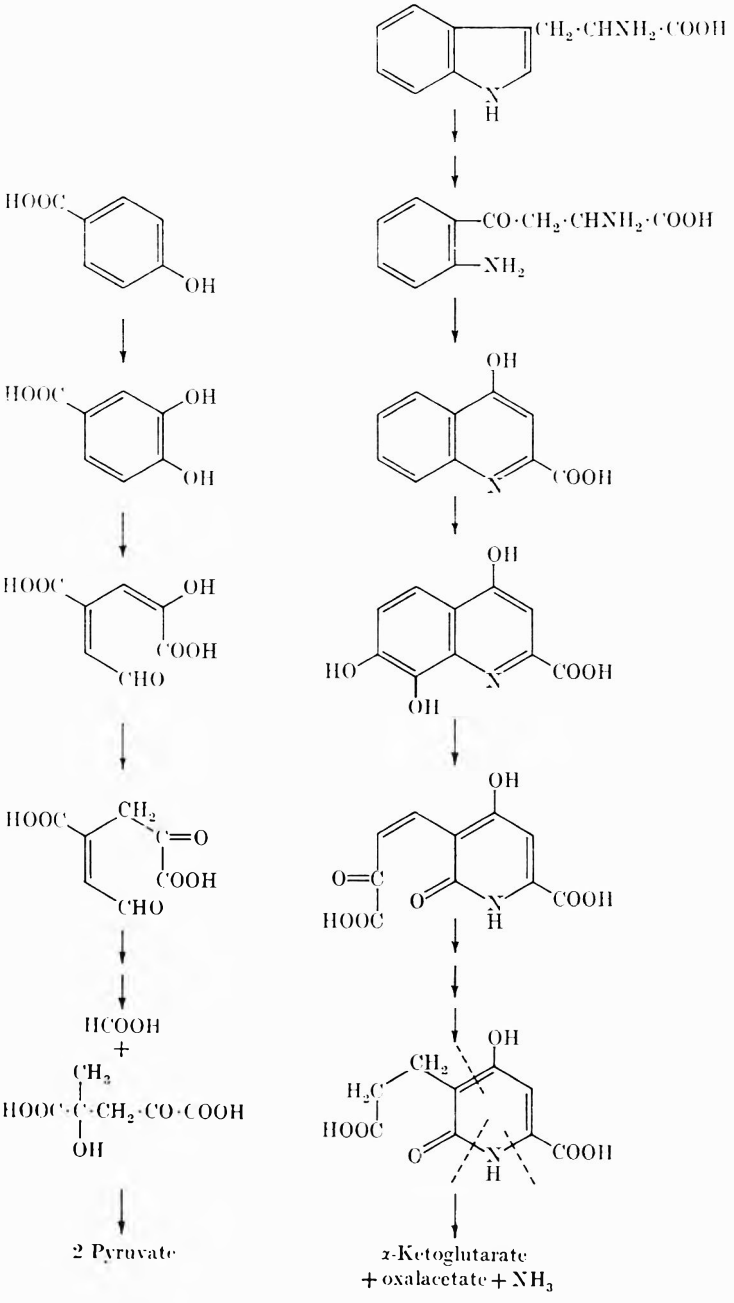

Fig. 7. Divergent pathways for metabolism of L-tryptophan and *p*-hydroxybenzoic acid in the acidovorans group of pseudomonads.

106). Stanier *et al.* (1951) also noted that the ability to oxidize D-tryptophan at a high rate is possessed only by strains that use the quinoline pathway. As we have now shown, the nutritional character of growth on D-tryptophan is a distinctive specific trait of *P. acidovorans*. Consequently, although we have not yet examined the mode of tryptophan metabolism by other strains of this species, it is reasonable to assume that they all use the quinoline pathway. The second species of the acidovorans group, *P. testosteroni*, cannot grow on either isomer of tryptophan; but all strains grow well on kynurenate, and somewhat less well on L-kynurenine. These facts suggest that *P. testosteroni* probably also synthesizes the specific enzymes of the quinoline pathway, but cannot synthesize the two enzymes (tryptophan pyrrolase, formylkynurenine formamidase) used by all aerobic pseudomonads to convert tryptophan to kynurenine.

Under the designation 'Ps. B_2 aba', strain 102 of *P. acidovorans* has been the subject of numerous biochemical investigations by H. L. Kornberg and his associates. Francis, Hughes, Kornberg & Phizackerley (1963) have found that the oxidation of L-malate by this strain is catalysed by a soluble NAD-dependent dehydrogenase, whereas in a strain of *P. putida* the same reaction is catalysed by a particle-bound oxidase. This observation suggests that some of the enzymic differences between the fluorescent and acidovorans groups may be very profound indeed, and extend deep into the central intermediary metabolism of the cell.

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Deoxyribonucleic Acid Base Composition in the Genus *Pseudomonas*

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SUMMARY

The base compositions of deoxyribonucleic acids extracted from aerobic pseudomonads were determined by examination of the buoyant density of each sample in caesium chloride (CsCl) gradients. A collection of 165 strains, representing many of the principal biotypes among aerobic pseudomonads, was subjected to this examination. The similarities and differences in the base compositions of the genetic material have been compared with the taxonomic arrangements suggested by the comparison of phenotypic (largely nutritional and enzymic) features. The data on base composition of the deoxyribonucleic acids of the various cultures support, for the greater part, the conclusions of Stanier, Palleroni & Doudoroff (1966) and of Redfearn, Palleroni & Stanier (1966). Data are also presented on pseudomonads not included in the latter studies.

INTRODUCTION

Taxonomic groupings, if presented as representing natural groupings of organisms by virtue of common descent or heritage, should demonstrate some uniformity of genetic capacity. Evidence of this sort has proved quite difficult to obtain within the microbial world. A recent review of this evidence (Marmur, Falkow & Mandel, 1963*a*) indicates the limits which the paucity of known genetic mechanisms have placed upon the application of biological hybridization as a tool in bacterial taxonomy. Lee, Wahl & Barbu (1956) recognized that different bacteria have quite different proportions of the purine and pyrimidine bases present in their deoxyribonucleic acids (DNA). They also recognized that bacterial species and genera which were classified together were more often similar in base composition than were those of greater separation in current schemes of classification. Lanni (1960) proposed that a minimum requirement for genetic exchange was a similarity of base compositions of the DNA of sexual partners. Within the genus *Bacillus* it was then demonstrated that a necessary, but not sufficient, condition for transformation to occur was identity of base composition (Marmur, Seaman & Levine, 1963*b*).

Colwell & Mandel (1964*a*) showed that groups of Gram-negative bacteria of differing DNA base composition were well separated by the computer groupings of Adansonian analysis. A similar correlation for Gram-positive cocci has been provided by Silvestri & Hill (1965).

At present, there appears to be no reasonable evidence that the mean DNA base composition of an organism is subject to either induced or spontaneous change within the limits of our gross analytical techniques. Claims to the contrary can be

attributed to the failure to recognize contamination as such, or to failures in analytical techniques (De Ley, 1964*a*). We have, therefore, determined the DNA base composition of a large and representative sampling of the strains of pseudomonads reported in the companion papers by Stanier, Palleroni & Doudoroff (1966) and of representatives of *Pseudomonas pseudomallei* and *P. mallei* examined by Redfearn, Palleroni & Stanier (1966). These cultures have been subjected to intensive biochemical characterization, as has been the case for *P. lemoignei* (Delafield *et al.* 1965).

The DNA base compositions reported in the present communication have been deduced from the buoyant densities determined at equilibrium in a caesium chloride (CsCl) gradient. The choice of the method was dictated by several considerations, of which the large number of samples to be examined was the most demanding. Schildkraut, Marmur & Doty (1962) pointed out that purified DNA samples are not required for use in this method: consequently, the preparation time of samples is considerably shortened and the nucleic acids are of high molecular weight because of the lessened risk of shear incidental to increased manipulation during purification. A further advantage of the technique is that only small amounts of material are required for analysis. The disadvantages are that the determinations lack some of the precision of comparisons obtainable by means of optical absorbance changes as a function of temperature. Offsetting this additional precision is the danger of recording spurious melting temperatures as a consequence of inequalities in ionic strength, presence of heavy metals or polyamines as contaminants of the preparations (Schildkraut & Lifson, 1965; Mandel, 1962). Although the presence of an unusual base replacing either thymine or cytosine and glycosylation of the constituent bases may lead to density determinations higher than those predicted by knowledge of the base compositions of the DNA (Schildkraut *et al.* 1962), thus far no such anomaly has been found in any DNA sample isolated from a bacterial source. Reports of discrepancies between base composition determined by melting temperature and buoyant density (Frontali, Hill & Silvestri, 1965) are probably attributable to the uncertainty of the linearity of the relationships of melting temperature to base composition at extreme ratios of guanine+cytosine to adenine+thymine (Marmur & Doty, 1962; Colwell & Mandel, 1964*a*). The determination of buoyant density of DNA samples can consequently be used with confidence to assay reproducibly a large number of samples for comparative estimates of base composition. Additionally, the technique has the bonus advantage of revealing non-Gaussian distributions of molecules in the gradient if this departure is evident (as will be shown for the DNA of two strains of *Pseudomonas stutzeri*). Forty-four replicate determinations of the buoyant density in CsCl of one sample of DNA extracted from a strain of *Caulobacter crescentus* averaged 1·7255 g./cm.^{3} with a standard deviation of 0·0005 g./cm.^{3} and a standard error of less than 0·0001 g./cm.^{3} permitting a convenient comparison with the determinations reported here.

METHODS

Organisms. The strains examined were received on nutrient agar slopes via airmail from the donors. Usually three slopes were shipped to Texas and a fourth retained at the University of California laboratories for diagnostic examination when

necessary. Cultures were often identified only as to number, thus eliminating operator bias.

Preparation of DNA samples. Lysates of bacteria were prepared by washing the growth from the surface of the agar slopes with a small amount of saline EDTA (0·15 M-NaCl + 0·1 M-ethylenediaminetetra-acetic acid, EDTA; pH 8·0) followed by the addition of sodium lauryl sulphate to 2% (w/v). The lysate was deproteinized by shaking with neutralized phenol saturated with saline EDTA, then with chloroform + isoamyl alcohol (24 + 1, by vol.). The nucleic acids were precipitated with 2 vol. of ethanol, collected by spooling on a glass rod, washed twice with 70% (v/v) ethanol in water and then dissolved in 0·15 M-NaCl containing 0·015 M-Na_3 citrate (pH 7·0).

Cultures received as lyophilized samples were cultivated in nutrient broth (Difco) at 30° on a rotary shaker. The cultures were streaked on nutrient agar (Difco) and examined microscopically with phase-contrast optics to assure cultural purity. DNA was isolated and purified by the method of Marmur (1961), following lysis and one deproteinization with phenol as described above. Lysates of *Pseudomonas pseudomallei* and *P. mallei* were prepared within plastic isolation hoods and removed to the room through a phenol solution only after the addition of phenol to the lysate.

Buoyant density. The buoyant density in CsCl solutions and the guanine + cytosine (GC) content were determined as described by Schildkraut *et al.* (1962). Centrifugation was performed in a Spinco Model E analytical ultracentrifuge at 42,040 rev./min. and 25·0° for 22–23 hr. Each sample was also analysed by centrifugation in another instrument at 44,770 rev./min. and 25·0° for 19–21 hr. Photographs of the DNA bands at equilibrium in the gradient were obtained on Kodak Ortho Contrast Film by using monochromatic light at 265 mμ. Each centrifuge cell received approximately 1–2 μg. of the DNA of unknown density and 0·5 μg. of a reference DNA sample from SP 8 bacteriophage (*Bacillus subtilis* host) of a density of 1·742 g./cm.^{3} as calculated by comparison with DNA of *Escherichia coli* taken to be the primary reference of 1·710 g./cm.^{3}. Microdensitometer tracings of the ultraviolet (u.v.) photographs were made with a Joyce–Loebl double-beam recording microdensitometer. The GC content was calculated from the peak of each u.v.-absorbing band by using the equation of Schildkraut *et al.* (1962).

Values of standard deviations and statistical significance were determined with the aid of an IBM 1620 computer and standard program.

RESULTS

The fluorescent pseudomonads

Of the 175 strains studied by Stanier *et al.* (1966), 66 were analysed for DNA base composition. The mean buoyant density calculated from a minimum of duplicate determinations of the density of the DNA of each strain is listed in Table 1. Each species or biotype, in the terminology of Stanier *et al.* (1966), appears to be composed of individuals of homogeneous mean DNA base composition. The organisms comprising the species *Pseudomonas putida* can be divided into two types on the basis of their GC content; biotype A consists of organisms whose mean DNA base composition is 2% higher in GC content than organisms of biotype B and this difference is significant at the 99% confidence level (Table 2). Stanier *et al.* (1966)

Table 1. *Buoyant density and GC content of DNA of representatives of the fluorescent pseudomonads*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Species	Biotype	Strain	Mean density (g./cm.^{3})	Mean value and standard deviation calculated for each group: Density (g./cm.^{3})	GC (moles %)
Pseudomonas aeruginosa	—	45	1·726	1·7259 ± 0·0011	67·2 ± 1·1
		52	1·726		
		55	1·7255		
		58	1·726		
		131	1·727		
		132	1·726		
		278	1·725		
		280	1·725		
		282	1·726		
		286	1·7265		
		291	1·7255		
P. fluorescens	A	12	1·719	1·7193 ± 0·0011	60·5 ± 1·1
		126	1·719		
		182	1·7195		
		392	1·7195		
	B	2	1·719	1·7201 ± 0·0011	61·3 ± 1·1
		400	1·721		
		401	1·7195		
		403	1·720		
		404	1·721		
		411	1·720		
	C	18	1·7195	1·7194 ± 0·0008	60·6 ± 0·8
		50	1·719		
		181	1·720		
		191	1·719		
		213	1·7195		
		217	1·7195		
	D	30	1·7215	1·7222 ± 0·0009	63·5 ± 0·9
		31	1·722		
		32	1·722		
		35	1·7225		
		388	1·7215		
		389	1·7225		
		390	1·722		
		391	1·7225		
		393	1·7225		
		394	1·722		
	E	36	1·722	1·7223 ± 0·0008	63·6 ± 0·8
		37	1·7215		
		38	1·7225		
		39	1·723		
		41	1·722		
		86	1·7225		
	F	83	1·718	1·7182 ± 0·0008	59·4 ± 0·8
		143	1·7185		
	G	33	1·720	1·7193 ± 0·0010	60·5 ± 1·0
		34	1·7195		
		267	1·7195		
		269	1·7185		
		271	1·719		
		272	1·719		

Table 1 (*cont.*)

Species	Biotype	Strain	Mean density (g./cm.³)	Mean value and standard deviation calculated for each group: Density (g./cm.³)	GC (moles %)
P. putida	A	5	1·7205	1·7213 ± 0·0009	62·5 ± 0·9
		6	1·722		
		7	1·721		
		26	1·7215		
		42	1·721		
		90	1·722		
	B	53	1·719	1·7195 ± 0·0011	60·7 ± 1·1
		96	1·7185		
		98	1·720		
		110	1·7195		
		153	1·7205		
		157	1·7195		
		158	1·719		
		167	1·719		
Unclassified		95	1·7185	1·7185	59·7

Table 2. *Significant differences of mean GC content of DNAs of fluorescent pseudomonads*

P. fluorescens biotypes	A	14·537 (28)*								
	B	14·406 (32)*	1·651 (18)‡							
	C	17·690 (32)*	0·460 (18)	1·672 (22)‡						
	D	11·593 (40)*	7·070 (26)*	5·568 (30)*	8·369 (30)*					
	E	9·916 (32)*	7·200 (18)*	5·432 (22)*	8·700 (22)*	0·306 (30)				
	F	15·824 (26)*	2·095 (12)‡	3·719 (16)*	3·162 (16)†	9·375 (24)*	10·330 (16)*			
	G	16·912 (32)*	0·000 (18)	1·878 (22)†	0·439 (22)	8·078 (30)*	7·907 (22)*	2·238 (16)†		
P. putida biotypes	A	12·247 (32)*	4·841 (18)*	3·062 (22)*	5·686 (22)*	2·450 (30)*	2·719 (22)†	7·638 (16)*	5·352 (22)*	
	B	17·519 (36)*	0·531 (22)	1·377 (26)‡	0·223 (26)*	7·787 (34)*	7·344 (26)*	2·718 (20)†	0·609 (26)	4·801 (26)*
			A	B	C	D	E	F	G	A
		P. aeruginosa	*P. fluorescens* biotypes							*P. putida* biotype

Number of degrees of freedom are given in parentheses for values of t calculated by the method of Fisher (1925). Pairs significantly different at the 99 % confidence level are marked *, at the 95 % level †, at the 80 % confidence level ‡. Pairs not marked are not significantly different.

have pointed out that biotype B is more *fluorescens*-like than is biotype A. Indeed this resemblance extends to the GC contents and biotype B of *P. putida* is indistinguishable from *P. fluorescens* biotypes A, B and G on this basis. Biotype A of *P. putida*, on the other hand, has DNA more nearly like *P. fluorescens* biotypes E and

D, but significantly different from both at the 95% confidence level. *P. putida* biotype B thus probably warrants designation as a species. In concert with my Californian colleagues, I also would refrain from such designation until comparisons can be made with the various named plant pathogens or authentic surviving holotypes of the named species which have been reduced to synonomy with *P. putida*.

Table 2 is a display of the statistical tests for significant differences in the mean GC content representative of each set of fluorescent pseudomonads. The data concerning the GC content of the representatives of *Pseudomonas fluorescens* biotypes A, B, C and G clearly demonstrate no highly significant differences in the overall composition of the genetic material. While biotype F appears to be the lowest in GC content of all the fluorescent pseudomonads, too few determinations have been made to separate this biotype from biotype A with any high degree of confidence. Biotypes D and E, on the other hand, are distinctly different in GC content from the remainder of the *P. fluorescens* group. On the basis of this difference and the phenotypic differences set forth by Stanier *et al.* (1966) a specific epithet for *P. chlororaphis* and *P. aureofaciens* appears to be justifiable and one must reject the proposal that these species be reduced to synonymy with *P. fluorescens*. The difference in GC content of as much as 3% between *P. fluorescens* and these phenazine-

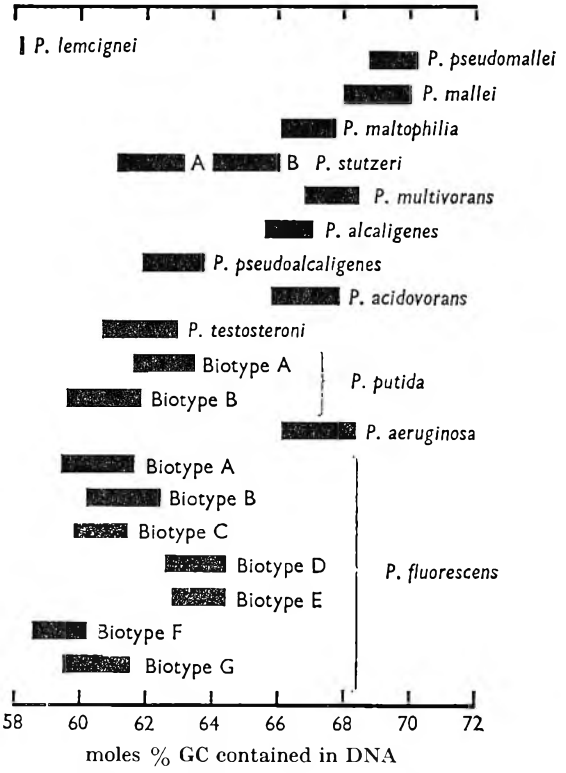

Fig. 1. The GC content of DNAs of the aerobic pseudomonads. Values plotted are means ± one standard deviation. Species and biotypes represent those studied by Stanier *et al.* (1966), the pseudomallei group of Redfearn *et al.* (1966) and the monotypic *Pseudomonas lemoignei* of Delafield *et al.* (1965).

producing organisms sets them apart, but does not preclude the existence of substantial genetic information shared in common. Again, the high degree of phenotypic similarity and of GC content of *P. chlororaphis* and *P. aureofaciens* indicates the advisability of reducing the latter to synonymy with the former. Quite striking is the difference observed when all these fluorescent pseudomonads are contrasted with *P. aeruginosa*. The DNA of the latter species has GC 67·2 %, distinctly separate from the GC content of all other fluorescent pseudomonads. Obviously, the conclusion of Lysenko (1961) that *P. chlororaphis* and *P. aureofaciens* are closely allied to *P. aeruginosa* is not substantiated. This solitary position of *P. aeruginosa* is indeed unfortunate, for this is the sole pseudomonad in which a system for genetic recombination is known (Holloway, 1955; Holloway & Monk, 1959), and if experience with the Enterobacteriaceae, Bacillaceae and *Neisseria* are any guide, interspecific hybridizations of *P. aeruginosa* with other fluorescent pseudomonads are of slight likelihood (see Marmur *et al.* 1963*a*).

Fig. 1 provides a summary of the mean GC contents of the representatives of the fluorescent pseudomonads in comparison with the aerobic pseudomonads described below.

The acidovorans group

The 26 strains placed in the acidovorans group by Stanier *et al.* (1966) have all been examined for GC content of their DNA with results as listed in Table 3. Fifteen of the strains are readily separable into a set possessing 66·8 moles % GC and another 9 strains can be assigned to a set possessing 61·8 % GC. This division of the cultures into the two species *Pseudomonas acidovorans* and *P. testosteroni* is further correlated with the nutritional differences established by Stanier *et al.* (1966). Two strains (60, 298) which bear phenotypic resemblances to the acidovorans group differ sufficiently from each other and from each of the named species in GC content and in nutritional properties to preclude their identification with either of the two species of the acidovorans group. It is possible that these represent additional species which have not otherwise been encountered in the study of Stanier *et al.* (1966).

Stanier *et al.* (1966) point out the discrepancy between the growth of strains 61 and 62, which they received as *Comamonas terrigena* but have identified as typical representatives of *Pseudomonas acidovorans*, and of ATCC 8461, the type culture of *C. terrigena*. The DNA of the latter culture and of its peritrichate mutant have been examined by Leifson & Mandel (1966). By using identical techniques and reference standards, they find the type culture to contain DNA of 64 % GC, or 2–3 % poorer in GC than the DNA of strains 61 and 62. Regardless of what taxonomic lodging *Comamonas terrigena* may eventually find, the epithet *terrigena* cannot be used as an earlier synonym for *P. acidovorans*.

The alcaligenes group

The DNAs of all 7 strains included in this group by Stanier *et al.* (1966) were analysed; Table 4 gives these data. There was no significant difference between the density determinations of the DNAs of 6 strains assigned to *Pseudomonas pseudoalcaligenes*, but the representative of *P. alcaligenes* was significantly richer in GC content by 3·5 %; this difference is significant at the 99·9 % probability level.

When the GC contents of the representatives of the alcaligenes group are contrasted with those of the acidovorans group, *Pseudomonas alcaligenes* DNA does not differ significantly from that of *P. acidovorans*. The 1% difference in GC content

Table 3. *Buoyant density and GC content of DNAs of representatives of the acidovorans group*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Species	Strain	Mean density (g./cm.³)	Mean value and standard deviation calculated for each group: Density (g./cm.³)	GC (moles %)
P. acidovorans	14	1·7255		
	24	1·7255		
	29	1·7255		
	61	1·726		
	62	1·725		
	80	1·725		
	102	1·726		
	103	1·726	1·7255 ± 0·0010	66·8 ± 1·0
	105	1·725		
	106	1·726		
	114	1·727		
	125	1·725		
	129	1·7255		
	146	1·7245		
	148	1·7255		
P. testosteroni	15	1·7205		
	16	1·721		
	25	1·720		
	27	1·7205		
	28	1·721	1·7206 ± 0·0011	61·8 ± 1·1
	78	1·720		
	79	1·720		
	138	1·721		
	139	1·7215		
Unclassified	60	1·7175	1·7175	58·7
	298	1·723	1·723	64·3

Table 4. *Buoyant density and GC content of DNAs of representatives of the alcaligenes group*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Species	Strain	Mean density (g./cm.³)	Mean value and standard deviation calculated for each group: Density (g./cm.³)	GC (moles %)
P. alcaligenes	142	1·725	1·7250 ± 0·0007	66·3 ± 0·7
P. pseudoalcaligenes	63	1·721		
	65	1·721		
	66	1·722		
	297	1·7225	1·7216 ± 0·0009	62·8 ± 0·9
	299	1·721		
	417	1·722		

between *P. pseudoalcaligenes* and *P. testosteroni* is significant at the 95 % confidence level. Hence the GC content can aid in distinguishing these species, even when used only in conjunction with conventional bacteriological diagnostic tests. Neither unclassified member of the acidovorans group has GC contents which match either species of the alcaligenes group.

Pseudomonas multivorans

Of the 19 strains studied by Stanier *et al.* (1966), 12 were analysed for GC content. The data in Table 5 show that the groups of strains examined are quite alike in GC content and the defective strains (385, 396, 397, 399) did not display any difference in this regard.

Table 5. *Buoyant density and GC content of DNAs of representative strains of Pseudomonas multivorans*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Strain	Mean density (g./cm.^{3})	Mean value and standard deviation calculated for each group	
		Density (g./cm.^{3})	GC (moles %)
85	1·7265		
104	1·727		
382	1·7255		
383	1·7255		
384	1·726		
385	1·726		
386	1·7265	1·7263 ± 0·0008	67·6 ± 0·8
387	1·7265		
396	1·727		
397	1·727		
398	1·726		
399	1·7265		

There seems little chance that *Pseudomonas multivorans* could be mistaken for *P. aeruginosa*, *P. maltophilia*, *P. acidovorans*, *P. mallei* or *P. pseudomallei* on cultural and physiological comparison. These high GC representatives all differ in base composition from *P. multivorans* by relatively small amounts, the smallest difference being found with *P. aeruginosa*. The *t*-test indicates a significant difference at 85 % probability; the other species cited above have differences calculated to be of significance at the 95 % probability level or better.

Pseudomonas stutzeri

The 17 strains studied by Stanier *et al.* (1966) were analysed for GC content, with the results shown in Table 6. Two clearly differentiated groups are revealed by this analysis. Group B contains the neotypes proposed by van Niel & Allen (1952) and by Stanier *et al.* (1966); hence, *P. stutzeri* is characterized by DNA of 65 % GC content. Group A differs in having DNA of 62·1 % GC and the disparity is significant at the 99·9 % confidence level. If these bacteria have genomes containing as few as 10^7 nucleotide pairs in their DNA, then this difference represents approxi-

mately $2{\cdot}9 \times 10^4$ GC substitutions for adenine + thymine (AT) pairs in the DNA of organisms of group B as contrasted with group A organisms. If these base-pair substitutions are spread at random, we should predict little or no genetic material in common. Stanier *et al.* (1966) were reluctant to propose the designation of a new

Table 6. *Buoyant density and GC content of DNAs of Pseudomonas stutzeri and P. stanieri sp.nov.*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Group	Strain	Mean density (g./cm.³)	Mean value and standard deviation calculated for each group: Density (g./cm.³)	GC (moles %)
A (*P. stanieri* sp.nov.)	220	1·721		
	223	1·7195		
	224	1·721	1·7209 ± 0·0010	62·1 ± 1·0
	225	1·7215		
	228	1·7215		
	320	1·721		
B (*P. stutzeri*)	221	1·724		
	222	1·7235		
	226	1·7235		
	227	1·7243		
	229	1·724		
	275	1·723	1·7237 ± 0·0010	65·0 ± 1·0
	316	1·7245		
	318	1·724		
	319	1·724		
	321	1·724		
	419	1·724		

species for organisms of group A. Such a separate designation is felt to be necessary by the present author, despite the lack of as clear a set of phenotypic traits correlated with this different base composition of the DNA as was observed in the acidovorans group. The variety of phenotypic traits displayed in the group of cultures studied as *P. stutzeri* would seem to make it quite difficult to demonstrate such a correlation using the data at hand. Sebald & Véron (1963) have already established a precedent for altering nomenclatural designations based upon determinations of DNA base composition, and Campbell & Postgate (1965) have provided descriptions of the species of *Desulfotomacculum* in which the GC content of the described species are definitive, along with one or two other distinguishing phenotypic traits. For these reasons I propose that the organisms of group A be separated from the species *P. stutzeri* and given a specific designation.

A review of the nomenclatural status of *Bacillus denitrificans* II Burri and Stutzer by van Niel & Allan (1952) elucidated the unwarranted confusion attending the classification of this organism and the proper recognition of *Pseudomonas stutzeri* (Lehmann and Newmann) Kluyver as the combined epithets. Further confusion would result if one of the specific synonyms for *P. stutzeri* were now used to describe the species of low GC content, particularly when neither holotypes nor neotypes of the cultures which have borne the synonyms appear to exist for which the base composition can be determined. It is therefore proposed that the new species be

named after Professor R. Y. Stanier in recognition of his contributions to the knowledge of the physiology and taxonomy of the genus *Pseudomonas*. A description follows, based in the main on the data of Stanier *et al.* (1966).

Pseudomonas stanieri sp.nov., description as for *P. stutzeri* conforming in all respects to the ideal phenotype of Stanier *et al.* (1966). DNA contains 62·1 moles % GC. Differentiated from most strains of *P. stutzeri* by the ability to utilize ethanolamine and mannitol and the inability to utilize propionate. No growth at 43°. Strains 224 and 225 conform strictly to this description and strain 224 (strain AB 212 of Dr H. Lautrop, Statens Seruminstitut, Copenhagen; ATCC 17591) is proposed as the holotype.

Strains 223, 320 and 228 are atypical in many nutritional characters. It is possible that these strains may not be *P. stanieri*, but it would be premature to consider the creation of still another species.

Two strains (226, 227) of *Pseudomonas stutzeri* have yielded DNA specimens which display a departure from the Gaussian distribution of DNA molecules in the CsCl gradient found with all other specimens reported in this study (as well as for over 1200 other samples of DNAs of bacterial origin examined in the author's laboratory in the past years). Figure 2 shows the appearance of a microdensitometer tracing of the DNA of strain 227. A DNA satellite is evident on the less dense side of the main DNA band. There was no difference in the position and amount of the satellite band in DNA extracted from 226. To ensure that the satellite was indeed composed of DNA, samples were diluted with water to 1% the salt concentration, boiled for 10 min. and chilled in ice. This denaturation was accomplished at low concentrations of DNA (5–10 μg./ml.) to guard against aggregation phenomena (Marmur, Rownd & Schildkraut, 1963). Upon examination of the CsCl gradient, with DNA of *Escherichia coli* as a reference standard, both satellite and main DNA bands were found to be increased in density by 0·014–0·015 g./cm.^{3} and the bands were increased in distribution width as would be expected if both represented double-stranded DNA. Subcultures of both strains were re-examined by Dr N. J. Palleroni and not found to be contaminated nor to be different from the previous description. The cultures were streaked on nutrient agar and single colonies again streaked. Six clones of each strain were then cultivated in nutrient broth, and the DNAs isolated and purified by the method of Marmur (1961). Again each specimen contained the same satellite and in the same proportion as in the original observations, irrespective of the fact that one sample of each strain was cultivated at 37° rather than 30° as had been the remainder. The satellite DNA represents 10–15% of the total DNA, as estimated from the area under the curve of the ultraviolet-absorbing material. If the satellite DNA corresponded to the presence of a bacterial contaminant in each culture, the contaminant would have to make up approximately that proportion of the population. Since microscopic examination of the suspended cultures did not reveal any tendency for the organisms to aggregate in groups, it is safe to dismiss contamination as the source of the satellite DNA. The satellite DNA is remarkably like that reported to be present in species of *Halobacterium* (Joshi, Guild & Handler, 1963). The latter authors concluded that an episomal element was probably present. Preliminary experiments by Mandel & Goldschmidt (unpublished) would indicate that it is likely that some form of cytoplasmic DNA (plasmid or episome) is present. Growth of strains 226 and 227 in nutrient broth containing neutral acriflavine 25 μg./ml.

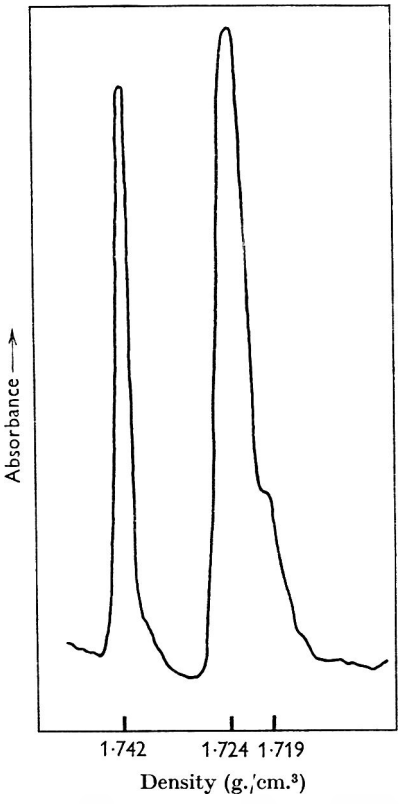

Fig. 2. Microdensitometer tracing of the ultraviolet absorbing bands of purified DNA of *Pseudomonas stutzeri* strain 227. Film exposed after 22 hr at 44,770 rev./min. in 5·7 M-CsCl at 25·0°. The band at the left is that of the reference DNA (0·5 μg.) of SP 8 bacteriophage.

(Hirota & Iijima, 1957) has yielded populations where the satellite DNA is either absent or so decreased in amount as not to be discernible on the ultraviolet photographs of the extracted DNA at equilibrium in the CsCl gradient. Single colonies of strain 227 lacking the satellite are now being studied in the laboratory of Professor Stanier for alteration of phenotypic traits and in this laboratory for the ability to re-acquire the trait by transmission from the wild-type strains. It is of interest that in the data of Stanier *et al.* (1966) strains 226 and 227 deviate from the remainder of their *P. stutzeri* strains in eight common features, five of which are positive characters.

Pseudomonas maltophilia

This species is represented by 4 strains analysed of the 23 studied by Stanier *et al.* (1966). The indicated GC content was 66·9 moles % and all 4 strains were quite uniform in this respect (Table 7). The sample is too small to guarantee that there is no other GC type present, but the uniformity of phenotypic characters present in the 23 strains does not raise any such suspicion.

Table 7. *Buoyant density and GC content of DNA of representative strains of Pseudomonas maltophilia*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Stanier *et al.* (1966).

Strain	Mean density (g./cm.^{3})	Mean value and standard deviation calculated for the group: Density (g./cm.^{3})	GC (moles %)
67	1·726		
72	1·7255	1·7256 ± 0·0008	66·9 ± 0·8
301	1·7255		
303	1·7255		

The pseudomallei-mallei group

Table 8 lists the buoyant densities of the DNA of 6 of the 26 strains of *Pseudomonas pseudomallei* and 7 of the 15 strains of *P. mallei* studied by Redfearn *et al.* (1966). These bacteria had DNA of the highest GC content found in the pseudomonads. The difference in GC content for the two groups is not of high significance (80 % probability).

Table 8. *Buoyant density and GC content of DNAs of representatives of pseudomallei group*

Values given for mean density are rounded averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Strain numbers correspond to designations of Redfearn *et al.* (1966).

Species	Strain	Mean density (g./cm.^{3})	Mean value and standard deviation calculated for each group: Density (g./cm.^{3})	GC (moles %)
P. pseudomallei	NBL 111	1·728		
	NBL 113	1·728		
	NBL 114	1·728		
	NBL 117	1·728	1·7281 ± 0·0007	69·5 ± 0·7
	NBL 121	1·7285		
	NBL 123	1·728		
P. mallei	NBL 1	1·728		
	NBL 2	1·728		
	NBL 4	1·727		
	NBL 7	1·7265	1·7276 ± 0·0010	69·0 ± 1·0
	NBL 16	1·7275		
	NBL 17	1·7278		
	NBL 19	1·7275		

Pseudomonas lemoignei sp.nov.

Delafield *et al.* (1965) have described a new species of aerobic pseudomonad which has been distinguished from the bulk of the pseudomonads by the criteria used by R. Y. Stanier and his collaborators. The holotype and sole strain of this poly-β-hydroxybutrate-digesting strain has DNA of 58·2 % GC (Table 9). It therefore represents the lowest GC content found among the aerobic pseudomonads reported above.

Table 9. *Buoyant density and mean GC content of the DNA of additional cultures received as species of Pseudomonas*

Organism	Strain designation and source	Density (g./cm.^{3})	GC (moles %)
P. lemoignei sp.nov.	Strain 443 of Delafield *et al.* (1965); received as harvest of organisms from Dr N. J. Palleroni, University of California, Berkeley	1·717	58·2
P. pseudoalcaligenes	Strain 63 AD of Dr M. Véron, Institut Pasteur; received from Prof. R. Y. Stanier as 440	1·721	62·2
	Strain 63 AS of Dr M. Véron; received from Prof. R. Y. Stanier as 441	1·722	63·2
P. aeruginosa	ATCC 8707	1·726	67·3
	ATCC 8689	1·726	67·3
	ICPPB 2020; received from Dr M. P. Starr, University of California, Davis	1·7257	67·0
	NRRL B 23; studied as strain 54 by Stanier *et al.* (1966); received from Dr J. Marmur, Albert Einstein Medical College, New York	1·727	68·4
P. polycolor	PP 2; received from Dr M. P. Starr, University of California, Davis	1·726	67·3
P. aminovorans	Strain 26 of den Dooren de Jong (1926); received from Dr E. R. Leadbetter, Amherst College, Amherst	1·722	63·2
P. schuylkilliensis	NRRL B 1104; from Dr W. C. Haynes, U.S. Department of Agriculture, Peoria, Ill.	1·7195	60·7
	NRRL B 1105; from Dr W. C. Haynes	1·7197	60·9
P. geniculata	NRRL B 1603; from Dr W. C. Haynes	1·719	60·2
	NRRL B 2080; from Dr W. C. Haynes	1·725	66·3
	NRRL B 2337; from Dr W. C. Haynes	1·726	67·3
P. atlantica	NCMB 301 Received from Dr J. M. Shewan, Torry Research Station, Aberdeen	1·7025	43·5
P. mallei	NCTC 3709; received from Mr Paul Baumann, University of California, Berkeley	1·728	69·4
P. tabaci	PT 1; received from Dr M. P. Starr, pathogenic for tobacco	1·717	58·2
P. phaseolicola	PM 142; received from Dr M. P. Starr, pathogenic for beans	1·7177	58·9
P. methanica	Harvested organisms received from Dr J. W. Foster, The University of Texas, Austin	1·711	52·1
P. xanthe	F 3·0; received from Dr O. B. Weeks, New Mexico State University, University Park	1·7285	69·9

Other aerobic pseudomonads

Table 9 lists a number of additional cultures which were analysed for the GC content of their DNAs. Two other strains of *Pseudomonas pseudoalcaligenes* have been identified as such by Drs Stanier, Palleroni and Doudoroff; their GC contents fall within the range established for the cultures previously described.

Four additional cultures of *Pseudomonas aeruginosa* were analysed. The first two listed under this name in Table 9 had originally been erroneously described as *P. fluorescens*, as noted in the catalogue of the American Type Culture Collection (1964). All four strains have DNA of GC content characteristic for *P. aeruginosa*. The representative of *P. polycolor* had DNA of a GC content like that of *P. aeruginosa*, adding weight to the proposal of Elrod & Braun (1942) that this plant pathogen is identical with *P. aeruginosa*, despite some differences in cultural characteristics. Dr T. Feary (unpublished results) has examined the sensitivity of this culture to aeruginosa phages and found it to be attacked by more virulent and temperate phages than any other strain of *P. aeruginosa*.

The holotype of *Pseudomonas aminovorans* (den Dooren de Jong, 1926) has 63·2 moles % GC. It cannot be placed with any certainty into any of the species delineated by Stanier *et al.* (1966).

Two additional strains of *Pseudomonas schuylkilliensis* have GC contents not significantly different from the *P. fluorescens* biotype G, in which group Stanier *et al.* (1966) placed strain 267 (NRRL B9). The latter culture was also identified as *P. fluorescens* by Dr W. C. Haynes, who provided these cultures. The epithet should be reduced to synonymy with *P. fluorescens*.

Strains of *Pseudomonas geniculata* pose another problem. The description of this species is that of a fluorescent pseudomonad isolated from the Schuylkill River in Pennsylvania. Strains 269 (NRRL B1606) and 271 (NRRL B1612) were studied by Stanier *et al.* (1966) and placed in *P. fluorescens* biotype B; each has DNA of 60 % GC content. There are an additional 3 strains received as *P. geniculata* in Table 9. One of these (NRRL B1603) has DNA of 60 % GC composition while the other two have 66 or 67 % GC. De Ley & Van Muylem (1963) reported the GC content of *P. geniculata* 338 to be 67·7 %. Their culture was obtained from Lysenko, who proposed it as the neotype for the species (Lysenko, 1961). His description of the organism is that of a non-fluorescent pseudomonad of little enzymic capacity.

Strains NRRL B 2080 and B 2337, with 66–68 % GC have recently been identified by Dr Palleroni as *P. multophilia*. The same is probably true for the organisms studied by De Ley & van Muylem (1963).

The strain of *Pseudomonas atlantica* analysed is shown in Table 9 to have DNA of 43·5 moles % GC. The source of this culture is ostensibly the same as that organism analysed by De Ley & Friedman (1965) and found by them to have DNA of 66·4 % GC. The culture examined in this laboratory was obtained directly from Dr J. M. Shewan (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland), while De Ley & Friedman received their specimen from Dr O. Lysenko, who reported having received it from Dr Shewan. The report of Lysenko (1961) noted that his culture differed in some characters from the original description. Another specimen of an agar-utilizing and alginate-utilizing Gram-negative rod labelled *P. atlantica* was found to have DNA of 55 % GC content

(Colwell & Mandel, 1964*b*). In view of this confusion it is impossible to state what the GC content of *P. atlantica* is and whether it shows any affinity with the genus *Pseudomonas* as defined by Stanier *et al.* (1966).

Table 9 also lists one further specimen of *Pseudomonas mallei*. The GC content of the DNA matches the composition found for the strains provided by Redfearn *et al.* (1966; Table 8).

Data for two phytopathogenic fluorescent pseudomonads, *Pseudomonas tabaci* and *P. phaseolicola*, are given in Table 9. Both have DNA of 58 to 59 moles % GC content, at the low GC extreme of the fluorescent group. Whether these, and other similar fluorescent phytopathogenic pseudomonads, can be accommodated within one of the established biotypes of *P. fluorescens* must await full examination of the nutritional and enzymic properties of this economically important group of bacteria.

Dworkin & Foster (1956) recommended that *Methanomonas methanica* be transferred to the genus *Pseudomonas*. The GC content of the authentic culture provided by Dr J. W. Foster is 52 moles % (Table 9) and would thereby be remote in base composition from those organisms which Stanier *et al.* (1966) and the present author would include in the genus *Pseudomonas*. The representatives of *Hydrogenomonas* (which are to be reported on elsewhere by Doudoroff and collaborators and by myself) have DNA base compositions in the range reported for *Pseudomonas*. The proper generic classification of the methane-oxidizing pseudomonad is obscure.

The last culture listed in Table 9, *Pseudomonas xanthe*, exemplifies another problem in the classification of bacteria. The culture is apparently Gram-negative on routine examination but does not lyse when treated with high concentrations of sodium lauryl sulphate (up to 10 %, w/v). In the author's experience, all Gram-negative bacteria (and a few Gram-positive organisms) lyse promptly upon addition of this detergent. Dr O. B. Weeks has advised me that this strain, as well as others bearing the same name, gives an equivocal Gram reaction by the Hucker method but is definitely Gram positive when stained by Kopeloff's procedure. Zettnow's original culture apparently is not available for comparison.

DISCUSSION

Stanier *et al.* (1966) have proposed a rigorous definition for the genus *Pseudomonas* based upon morphological and physiological properties. The inclusion of a range of DNA base compositions was deferred. The arguments for setting such a range as part of the definition of *Pseudomonas* follow. If *Pseudomonas* was described as containing organisms having DNA with not less than 57 nor more than 70 moles % guanine+cytosine (GC), all the aerobic pseudomonads encountered within this study would be included. Aerobic pseudomonads which are at present classified in other genera, but which might be assigned to the genus *Pseudomonas*, have DNA base compositions which fall within these suggested limits. Analyses have been published for representatives of *Xanthomonas* (Colwell & Mandel, 1964*a*; De Ley & Van Muylem, 1963), for the polarly flagellate *Acetomonas* (*Gluconobacter*), by De Ley & Schell (1963), for *Comamonas* (Leifson & Mandel, 1966; Sebald & Véron, 1963), for *Vibrio sensu lato* (Sebald & Véron, 1963; Colwell & Mandel, 1964*a*), and all have GC contents well within the suggested range. The hydrogenomonads likewise have DNA base compositions within this range (data to be published). No information

is available for representatives of *Alginomonas*, *Cellulomonas* and *Cellvibrio*. The use of the above suggested range of GC contents as diagnostic for *Pseudomonas* would exclude *Methanomonas* if the value for the single culture examined in this study should prove to be representative of the genus.

The morphological and physiological definition does not exclude the newly proposed genera *Campylobacter* (Sebald & Véron, 1963) and *Bdellovibrio* (Stolp & Starr, 1963). The 30–34 moles % GC content of the DNA of *Campylobacter* was one of the chief criteria used by Sebald & Véron (1963) in assigning *Vibrio fetus* and *V. bubulus* to the new genus. Drs Mandel, Starr & Baigent will report elsewhere that representatives of *Bdellovibrio* have DNAs of low GC content and thus may be excluded from *Pseudomonas*.

The marine bacterium *Flavobacterium piscicida* Bein has been found to be polarly flagellate (Hansen, Ingebritsen & Weeks, 1963) and hence reassigned to the genus *Pseudomonas* by Buck, Meyers & Leifson (1963). Mandel, Weeks & Colwell (1965) have shown that 20 strains of this species have DNA of 44·5 moles % GC. The above proposed limit of GC contents would exclude this species from the genus *Pseudomonas*. The computer taxonomic study of Hansen, Weeks & Colwell (1965) showed this phenon to bear a 67–70 % similarity to certain *Pseudomonas* species. The species of *Pseudomonas* bearing the greatest similarity to *P. piscicida* were the strains of Lysenko (1961) labelled *P. geniculata* and *P. atlantica*; my doubts about the identity of these cultures are expressed above. No disservice to the taxonomy of *Pseudomonas* will be done by further chequering the career of the chromogenic ichthyotoxigenic marine bacterium by its exclusion from the genus. This bacterium, as well as the low-GC organisms *P. cruciviae* and *P. atlantica*, should be compared with the marine vibrios of similar base compositions (Colwell & Mandel, 1964*b*).

If the genus *Pseudomonas* is then defined, in part, as having species whose GC contents range from 57 to 70 moles %, how does this compare with other taxonomic units? The compilation of DNA base compositions of Marmur *et al.* (1963*a*) lists members of the genus *Bacillus* as having a still broader range (35–51 % GC), and members of *Corynebacterium* range from 53 to 59 % GC. Mandel, Bergendahl & Pfennig (1965) established that the green sulphur bacteria of the genus *Chlorobium* have at least 51–58 % GC contents of their DNAs. *Caulobacter* species range from 62 to 67 % GC, and the related *Asticcacaulis* has DNA of 55 % GC (Poindexter, 1964). *Lactobacillus* species are reported as having DNAs of 35–53 % GC, by Suzuki & Kitahara (1964).

The family Enterobacteriaceae has representatives whose DNA GC contents are as low as 39 moles % (*Proteus*) and as high as 59 % (*Serratia*). The actinomycetes appear to be more uniform and, though usually accorded higher taxonomic rank, GC contents apparently range from 65 to 75 % GC. In summary, the suggested range of GC contents for definition of the genus *Pseudomonas* cannot *a priori* be accounted as either too narrow or too broad. The decision on the propriety of using DNA base composition as part of a generic definition is then based upon the uniformity provided to the assemblage contained within the taxon.

One major test of the genetic and perhaps phylogenetic relatedness of the aerobic pseudomonads has been initiated by De Ley & Friedman (1965). They found substantial hybrid formation of DNA molecules extracted from various species of

Xanthomonas, *Pseudomonas* and acetic acid bacteria, with the DNA of *X. pelargoni*. Indeed, the degree of relatedness expressed by these estimates of hybrid formation are surprisingly high.

Marmur *et al.* (1963*a*) suggested that the limits of episomal transfer, based upon experiences with the Enterobacteriaceae, could define a family. The apparent episome found in *Pseudomonas stutzeri* may provide a similar test for the pseudomonads. Mandel & Rownd (1964) showed that *Aeromonas liquefaciens* and *Serratia marcescens* contain satellite DNA matching *Escherichia coli* DNA in base composition but present in only one-tenth the proportion of satellite to main DNA found in *P. stutzeri*. The physiological definition excludes *Aeromonas* and *Serratia* from *Pseudomonas* but the notion persists that *Aeromonas* is related to *Pseudomonas* and may serve as an evolutionary link between the enteric bacteria and pseudomonads (De Ley, 1964*b*; Lysenko, 1961). Needless to say, neither molecular hybridizations nor genetic tests have been performed which would lend support to this view; the task is a formidable one.

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A Comparative Study of *Pseudomonas pseudomallei* and *Bacillus mallei*

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SUMMARY

A comparative study of many strains of *Pseudomonas pseudomallei* and *Bacillus mallei* has shown that these two species are very similar with respect to their nutritional and biochemical properties, thus confirming earlier claims of a relationship between them, based on such criteria as pathological and serological properties. *P. pseudomallei* is in all respects a typical and nutritionally highly versatile member of the genus *Pseudomonas*. In view of this fact we propose that *B. mallei* should also be placed in the genus *Pseudomonas*, even though it is a permanently non-motile bacterium. The ecology and possible evolutionary relationships between the two species are discussed in the light of the present findings.

INTRODUCTION

The etiological agent of glanders, which is primarily a disease of horses, was discovered in 1882 by Loeffler & Schütz, and subsequently named *Bacillus mallei* by Zopf (1885). Like certain other non-motile Gram-negative rod-shaped bacteria which are pathogenic for animals, *B. mallei* has spent much of its scientific career in search of a satisfactory generic location. It has been placed at various times in *Pfeifferella* (Buchanan, 1918), *Malleomyces* (Pribram, 1933), *Actinobacillus* (Brumpt, 1910), *Loefflerella* (Holden, 1935) and *Acinetobacter* (Steel & Cowan, 1964). All these genera are poorly defined pigeon holes for non-motile Gram-negative rod-shaped bacteria of uncertain affinities and mostly of pathogenic propensities.

One other Gram-negative true bacterium has long been recognized to resemble *Bacillus mallei*. This is *B. pseudomallei*, the etiological agent of melioidosis, a glanders-like disease of man and other mammals which occurs in some regions of the tropics. *B. pseudomallei* was discovered and described by Whitmore (1913), and his choice of a specific name was determined by its resemblances in pathological and cultural respects to the agent of glanders. Subsequently, Stanton & Fletcher (1925) and Cravitz & Miller (1950) showed that there are also serological relationships between these two bacterial species. For several decades the systematic position of *B. pseudomallei* also remained obscure, and it followed *B. mallei* through *Malleomyces*, *Loefflerella* and *Pfeifferella*. However, *B. pseudomallei* is a motile organism, and several workers (Brindle & Cowan, 1951; Lajudie, Fournier & Chambon, 1953; Wetmore & Gochenour, 1956) have shown that it possesses polar multitrichous flagella. Several studies, of which the most thorough is that of Wetmore & Gochenour (1956), have indicated that *B. pseudomallei* also resembles

aerobic pseudomonads (*Pseudomonas aeruginosa*, *P. stutzeri*) in some cultural and physiological respects. In the 6th edition of *Bergey's Manual*, Haynes (1957) formally included this organism in the genus *Pseudomonas* as *P. pseudomallei*. This generic assignation accords with the fragmentary existing information about its nutritional and biochemical properties. Levine, Dowling, Evenson & Lien (1954) showed that it requires no growth factors, and can grow well in a simple chemically defined medium with several amino acids and organic acids as sources of carbon and energy. Bokman, Levine & Lusby (1957) found that it metabolizes glucose, probably through the pathway of Entner & Doudoroff (1952); this is the characteristic mode of glucose dissimilation by several species of aerobic pseudomonads. Finally, Levine & Wolochow (1960) showed that this organism accumulates poly-β-hydroxybutyrate as a cellular reserve material, a trait that is also not uncommon in non-fluorescent species among the aerobic pseudomonads (Forsyth, Hayward & Roberts, 1958).

Bacillus mallei was placed in the genus *Actinobacillus* by Haupt (1957) in the 7th edition of *Bergey's Manual*. Since then, an additional significant biological resemblance between *Pseudomonas pseudomallei* and *B. mallei* has been discovered. Smith & Cherry (1957) reported that several temperate phages isolated from *P. pseudomallei* could lyse strains of *B. mallei* but not strains of other *Pseudomonas* species.

As an adjunct to the taxonomic analysis of the aerobic pseudomonads undertaken by Stanier, Palleroni & Doudoroff (1966), it appeared desirable to characterize *Pseudomonas pseudomallei* by the same methods; and in view of the indications that *Bacillus mallei* might be a closely related bacterium, it also was examined.

METHODS

The methods used have been fully described by Stanier *et al.* (1966). The routine temperature of incubation was 32°. The results of cultural and physiological tests with *Bacillus mallei* were recorded only after incubation for 10 days, made necessary by the very slow growth of this organism.

The strains examined were received at the Naval Biological Laboratory of the University of California between 1949 and 1963 and subsequently maintained as lyophil preparations (Drs R. J. Heckley & M. M. Johnston, personal communication). Their designations and histories follow.

Pseudomonas pseudomallei

NBL 101. Received 1949 from Army Medical Service Graduate School, Washington, D.C., U.S.A., as strain 1096.

NBL 102. Received 1951 from same source as 101. Strain 295 of Calcutta School of Tropical Medicine (CSTM); strain MP-F of Wetmore & Gochenour (1956).

NBL 103. Received 1951 from same source as 101. Strain 294 of CSTM; strain MP-G of Wetmore & Gochenour (1956).

NBL 104. Received 1951 from the same source as 101. Strain China 3; strain MP-H of Wetmore & Gochenour (1956).

NBL 111. Received 1953 from Institute of Medical Research (IMR), Kuala Lumpur, as strain Wong Fook Mook. Isolated from human infection in Malaya.

NBL 112. Same source as 111; IMR strain P.W., isolated from human infection in Malaya.

NBL 113. Same source as 111; IMR strain Horse, isolated 1947–49 from equine infection in Malaya. Possibly identical with strain MP-E of Wetmore & Gochenour (1956) and that isolated by Davie & Wells (1952).

NBL 114. Same source as 111. IMR strain Ipoh, isolated from human infection in Malaya.

NBL 115. Received 1953 from Microbiology Division, Fort Detrick, Md., U.S.A., as strain AHS-I. Identical with NCTC 8016; referred to by Brindle & Cowan (1951). Isolated 1949 from infected sheep in Australia and probably from the outbreak described by Cottew (1950)

NBL 116. Received 1954 from Microbiology Division, Fort Detrick, Md., U.S.A., as AMGS 1454. All strains with AMGS prefix originated from Army Medical Service Graduate School, Washington, D.C., U.S.A., and are probably human isolates. Isolated 1953 in Thailand.

NBL 117. Same source as 116. Strain AMGS 1455, isolated 1954 in Thailand.

NBL 118. Same source as 116. Strain AMGS 1456, isolated 1954 in Thailand.

NBL 119. Same source as 116. Strain AMGS 1457, isolated 1953 in Thailand.

NBL 120. Same source as 116. Strain AMGS 1458, isolated 1953 in Thailand.

NBL 121. Received 1956 as strain 286 from Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D.C., U.S.A. Isolated 1953 from human infection in U.S.A. (Ziskind, Pizzolato, & Buff, 1954). Strain MP-S of Wetmore & Gochenour (1956).

NBL 122. Received as strain 56-B-I from same source as 121. Isolated 1946 from human infection in U.S.A. (Gutner & Fisher, 1948). Strain MP-R of Wetmore & Gochenour (1956).

NBL 124. Received 1957 from Institüt voor Bacteriologie, Rijksuniversiteit, Utrecht, Netherlands, as Aruba Strain II. Isolated 1955 from infected sheep in Netherlands Antilles. (Sutmöller, Kraneveld & Schaff, 1957).

NBL 125. Same source as 124. Isolated 1954 from human infection in Indonesia.

NBL 126. Same source as 124. Aruba strain 2, with same isolation history as 124.

NBL 128. Received 1958 from Division of Veterinary Medicine, Walter Reed Army Institute of Research, as strain M 1379. Isolated 1957 from human infection U.S.A.

NBL 129. Same origin as 128. Strain M 1444, isolated 1957 from human infection in Panama.

NBL 130. Received from Microbiology Division, Fort Detrick, as strain P.W. 124/61. Isolated 1961 from infected pig in Malaya.

NBL 133. Same source as 130. Strain P.W. 140/60, isolated 1960 from infected horse in Malaya.

NBL 135. Same source as 130. Strain P.W. 62/61, isolated 1961 from infected goat in Malaya.

NBL 137. Received 1963 from Communicable Diseases Center, Atlanta, Ga., U.S.A., as strain 720. Isolated 1962 from human infection in Ecuador (Biegeleisen, Mosquera & Cherry, 1964).

NBL 138. Same origin as 137. Strain 1960.

Of the strains originally isolated in the Americas, three (NBL 121, 122, 128), which were isolated from human infections in the U.S.A., almost certainly represent instances of primary infections contracted in the Far East. Strains isolated in the Caribbean region (NBL 124, 126, 129) and in Equador (NBL 137, 138) originated from primary infections contracted in those geographical areas.

Bacillus mallei

NBL 1. Received 1951 from Professor G. B. Reed, Queens University, Canada, as strain 280 A.

NBL 2. Received 1951 from Army Medical Services Graduate School, Washington, D.C., U.S.A., as strain China (Kweiyang) 3. Originally from the China National Epidemic Prevention Bureau. Isolated 1942 from infected horse.

NBL. 4 Same source as NBL 2. Strain China (Kweiyang) 5, isolated 1942 from infected horse 29.

NBL 5. Same source as NBL 2. Strain 2023. Probably isolated from horse.

NBL 6. Same source and isolation as NBL 5. Strain 2024.

NBL 7. Same source as NBL 2. Strain 3873; strain China 7. Isolated 1944 from human infection.

NBL 8. Received 1951 from Division of Microbiology, Fort Detrick, Md., U.S.A., as strain K (China 6).

NBL 10. Same source as NBL 1. Strain 3873-18, identical in origin with NBL 7.

NBL 11. Same source as NBL 1. Strain 3873-18-3-AP, identical in origin with NBL 10. but subjected to three passages through the hamster.

NBL 12. Received 1952 from Division of Microbiology, Fort Detrick, Md., U.S.A., as strain 120 A.

NBL 16. Received 1952 from Captain Turgut Tulgo, Army Veterinary Laboratory, Ankara, Turkey, as strain V. Isolated 1949 from human infection in Turkey.

NBL 17. Same source as NBL 16. Strain VII. Isolated 1951 from human infection in Turkey.

NBL 18. Same source as NBL 16. Strain VIII. Isolated 1951 from human infection in Turkey.

NBL 19. Same source as NBL 16. Strain IX. Isolated 1951 from human infection in Turkey.

NBL 20. Received from National Collection of Type Cultures, England (strain 3709) under the designation *Loefflerella mallei* (*Acinetobacter mallei*).

RESULTS

The characterization of Pseudomonas pseudomallei

The form and colour of the growth on solid media are high variable in *Pseudomonas pseudomallei*. Colonies can range in structure from extreme rough to mucoid, and in colour from cream to bright orange (Nigg, Ruch, Scott & Noble, 1956). Many different combinations of these two characters were represented among the 26 strains examined. In other respects, however, the strains were remarkably uniform; and the few minor differences in nutritional characters that were observed showed no correlations with differences in pigmentation or colony form.

Except as specifically noted, the following characters are shared by all strains.

Poly-β-hydroxybutyric acid is accumulated as a cellular reserve material. There is no growth under strictly anaerobic conditions in a complex medium containing glucose. There is abundant growth under strictly anaerobic conditions in a complex medium containing nitrate; and with the exception of strains NBL 104 and 125, growth is accompanied by gas production. Growth occurs at 42°, but not at 5°. Growth is excellent in chemically defined media containing ammonia as sole nitrogen source. Gelatin, starch, Tween 80 and poly-β-hydroxybutyric acid are hydrolysed by extracellular enzymes.

Five strains (NBL 111, 113, 114, 117, 121) were subjected to the qualitative tests of Hosokawa (Stanier *et al.* 1966) to determine the pathways which they use for the dissimilation of benzoate and *p*-hydroxybenzoate. In every case, extracts prepared from organisms grown on these two compounds convert the respective diphenolic intermediates, catechol and protocatechuate, to β-ketoadipate. Accordingly, ring cleavage is of the *ortho* type, as in fluorescent pseudomonads and *Pseudomonas multivorans* (Stanier *et al.* 1966).

The same five strains were examined for constitutive synthesis of the arginine dihydrolase system, with positive results in every case. This is a biochemical property that is shared with the fluorescent pseudomonads, but not with *Pseudomonas multivorans* (Stanier *et al.* 1966).

As reported in the paper by Mandel (1966), the DNA samples isolated from 5 strains of *Pseudomonas pseudomallei* are of relatively high guanine + cytosine (GC) content, averaging 69 moles % GC, with very little variation between samples. This value is at the upper limit of the range recorded for aerobic pseudomonads.

As shown in Tables 1–3, the range of organic compounds utilizable as sole sources of carbon and energy is exceptionally wide, and the nutritional patterns of the 26 strains examined are on the whole very homogeneous. No less than 80 of the compounds tested can be used by at least 24 strains, the great majority of them by all strains (Table 1). *Pseudomonas pseudomallei* accordingly ranks among the most nutritionally versatile members of the genus *Pseudomonas*. The list of utilizable compounds includes a large number of carbohydrates, notably fucose, D-arabinose, cellobiose, maltose and starch, all of which are rarely utilized by aerobic pseudomonads. Fatty acids and most dicarboxylic acids, as well as a variety of other organic acids, can support growth. Several aromatic compounds (benzoate, *p*-hydroxybenzoate, anthranilate, phenylacetate) are universally used. Eighteen amino acids, several higher amines and two *N*-methyl compounds are used. The utilizable amino acids include L-threonine, very rarely attacked by aerobic pseudomonads. However, the alcohols, which are used as substrates by most aerobic pseudomonads, are in general not substrates for *P. pseudomallei*. Only three strains (NBL 115, 118, 125) can grow on ethanol. Strain 115 can also grow on *n*-propanol, isopropanol, *n*-butanol isobutanol and ethyleneglycol; and strain 125 on the latter three compounds.

In the general character of its nutritional spectrum *Pseudomonas pseudomallei* resembles most closely the fluorescent pseudomonads and *P. multivorans*, which are also nutritionally versatile groups. However, many characters serve to distinguish *P. pseudomallei* from these aerobic pseudomonads. Distinguishing characters *vis-à-vis* the whole group of fluorescent pseudomonads include: accumulation of poly-β-hydroxybutyric acid as a cellular reserve material; ability to use poly-β-hydroxybutyric acid as an exogenous carbon source; lack of fluorescent pigment

production; utilization of D-arabinose, fucose, maltose, cellobiose and starch. Distinguishing characters *vis-à-vis* *P. multivorans* include: ability to use poly-β-hydroxybutyric acid as an exogenous carbon source; capacity for denitrification; constitutive production of arginine dihydrolase; growth on starch; failure to grow

Table 1. *Pseudomonas pseudomallei. Compounds used as substrates by 23 or more strains*

Carbohydrates and sugar derivatives
D-Ribose
D-Arabinose
D-Fucose
D-Glucose
D-Mannose
D-Galactose
D-Fructose
Sucrose
Trehalose
Maltose
Cellobiose
Salicin
Starch
Gluconate
2-Ketogluconate

Fatty acids
Acetate
Propionate
Butyrate
Isobutyrate
Valerate
Isovalerate
Caproate
Heptanoate
Caprylate
Pelargonate
Caprate

Dicarboxylic Acids
Succinate
Fumarate
Adipate
Suberate
Azelate*
Sebacate

Hydroxyacids
L-Malate
DL-β-Hydroxybutyrate
DL-Lactate
DL-Glycerate
Poly-β-Hydroxybutyrate

Miscellaneous organic acids
Citrate
α-Ketoglutarate
Pyruvate
Aconitate
Levulinate*

Polyalcohols and glycols
Erythritol
Mannitol
Sorbitol
Inositol
Glycerol

Alcohols
(None)

Non-nitrogenous aromatic and other cyclic compounds
Benzoylformate
Benzoate
p-Hydroxybenzoate
Phenylacetate
Quinate

Aliphatic amino acids
L-α-Alanine
D-α-Alanine
β-Alanine
L-Serine*
L-Threonine
L-Isoleucine*
L-Valine
L-Aspartate
L-Glutamate
L-Lysine*
DL-Arginine
γ-Aminobutyrate
δ-Aminovalerate

Amino acids and related compounds containing a ring structure
L-Histidine
L-Proline
L-Tyrosine
L-Phenylalanine
L-Tryptophan
L-Kynurenine
Kynurenate
Anthranilate*

Amines
Ethanolamine
Putrescine
Butylamine*
α-Amylamine

Miscellaneous nitrogenous compounds
Betaine
Sarcosine*
Hippurate

Paraffinic hydrocarbons
(None)

* Strains which fail to use this compound are shown in Table 3; all other compounds are universal substrates.

on *o*- and *m*-hydroxybenzoate, uracil and testosterone. The only other *Pseudomonas* species with which *P. pseudomallei* shares some conspicuous traits is *P. stutzeri*. Both these species can produce peculiar rough wrinkled colonies, quite unlike those of any other well-characterized member of the aerobic pseudomonads. Two other characters shared with *P. stutzeri* are the ability to hydrolyse starch and to deni-

trify. However, the two species can be very easily distinguished by morphological and physiological characters. The flagellation of *P. stutzeri* is monotrichous, that of *P. pseudomallei* multitrichous (Leifson, 1960). *P. stutzeri* can neither accumulate poly-β-hydroxybutyric acid as a cellular reserve material nor use it as an exogenous carbon source, and it is much less versatile nutritionally than *P. pseudomallei*.

Table 2. *Pseudomonas pseudomallei. Compounds not used by any strain*

Carbohydrates and sugar derivatives	Polyalcohols and glycols	Amino acids and related compounds containing a ring structure
L-Arabinose	Propyleneglycol	D-Tryptophan
D-Xylose	2,3-Butyleneglycol	*m*-Aminobenzoate
L-Rhamnose		*p*-Aminobenzoate
Lactose	Alcohols	
Inulin	Methanol	Amines
Saccharate	Geraniol	Methylamine
Mucate		Benzylamine
	Non-nitrogenous aromatic and other cyclic compounds	Tryptamine
Dicarboxylic acids		Miscellaneous nitrogenous compounds
Oxalate	D-Mandelate	Creatine
Malonate	*o*-Hydroxybenzoate	Pantothenate
Maleate	*m*-Hydroxybenzoate	Nicotinate
	Phthalate	
Hydroxy acids	Isophthalate	Paraffinic hydrocarbons
D-(−)-Tartrate	Terephthalate	*n*-Dodecane
L-(+)-Tartrate	Phenylethanediol	
meso-Tartrate	Phenol	
	Testosterone	
Miscellaneous organic acids		
	Aliphatic amino acids	
Citraconate	Glycine	
Itaconate	L-Norleucine	
Mesaconate	DL-Citrulline	
	DL-α-Aminobutyrate	
	DL-α-Aminovalerate	

Characterization of Bacillus mallei

The 15 strains of *Bacillus mallei* examined constitute a reasonably uniform group in terms of the characters examined, although there was more internal variation than in *Pseudomonas pseudomallei*. The form and colour of growth of *B. mallei* on solid media did not show the variations found with *P. pseudomallei*. Colonies ranged from smooth cream to smooth white. An occasional rough white was seen with one of the strains.

Except as specifically noted, the following characters were shared by all strains. Poly-β-hydroxybutyric acid was accumulated as a cellular reserve material. There was no growth under strictly anaerobic conditions in a complex medium containing glucose. There was abundant growth under strictly anaerobic conditions in a complex medium containing nitrate; but only five strains (NBL 1, 2, 6, 7, 10) produced visible gas. Growth occurred at 42° but not at 5°. We were unable to find any statements in the literature about the minimal growth requirements of *Bacillus mallei*. However, every strain grew in chemically defined media containing ammonia as the

sole nitrogen source. The growth in such media was very slow; but even in complex media *B. mallei* grew relatively slowly, as compared with *Pseudomonas pseudomallei*. Gelatin and Tween 80 were hydrolysed by means of extracellular enzymes. Eight strains (NBL 1, 4, 5, 6, 7, 10, 16, 18) hydrolysed starch; and all but two strains (NBL 5, 6) hydrolysed poly-β-hydroxybutyric acid by extracellular enzymes.

Table 3. *Pseudomonas pseudomallei. Substrates utilized by a fraction of the strains*

Substrate	No. of positive strains	Negative strains
Glutarate	18	111, 119, 125, 128, 130, 133, 135, 138
Pimelate	18	101, 103, 111, 119, 121, 122, 125, 128
Azelate	23	111, 117, 119
D-Malate	16	102, 103, 111, 117, 121, 128, 130, 133, 135, 138
Levulinate	24	119, 121
Adonitol	14	102, 103, 111, 112, 113, 114, 115, 118, 121, 130, 133, 135
Ethyleneglycol	2	All except 115, 125
Ethanol	3	All except 115, 118, 125
n-Propanol	1	All except 115
Isopropanol	1	All except 115
n-Butanol	2	All except 115, 125
Isobutanol	2	All except 115, 125
L-Mandelate	18	111, 115, 117, 119, 120, 125, 137, 138
p-Hydroxy-benzoate	24	124, 126
L-Serine	25	118
L-Leucine	2	All except 114, 121
L-Isoleucine	25	111
L-Lysine	25	104
DL-Ornithine	1	All except 104
Anthranilate	25	128
Spermine	18	103, 111, 117, 128, 130, 133, 135, 138
Butylamine	24	103, 121
α-Amylamine	24	103, 121
Sarcosine	23	103, 111, 121
Trigonelline	10	All except 101, 111, 115, 117, 119, 122, 130, 135, 137, 138
n-Hexadecane	4	All except 117, 118, 135, 137

All strains, except NBL 1, produce a constitutive arginine dihydrolase. All strains except NBL 4 and 12, cleave protocatechuate and catechol by *ortho* oxidation.

As shown by Mandel (1966) the DNA of *Bacillus mallei* contains 69% GC, a value identical with that for *Pseudomonas pseudomallei*.

The spectrum of organic compounds utilizable by *Bacillus mallei* as sole sources of carbon and energy resembles that of *P. pseudomallei*, but is considerably less extensive, and also more variable from strain to strain (Tables 4–6). A total of 46 different organic compounds was used by at least 13 of the 15 strains examined; most of them were used by all 15 strains (Table 4). The list includes a constellation of compounds which were also attacked by *P. pseudomallei*, but rarely by other aerobic pseudomonads: fucose, D-arabinose, cellobiose, L-threonine, poly-β-hydroxybutyric acid. Maltose and starch, universally used by *P. pseudomallei*, were used by 8 out of 15 strains of *Bacillus mallei*.

In Table 7 we have summarized the principal nutritional differences between *Bacillus mallei* and *Pseudomonas pseudomallei*. Only four compounds which were

wholly non-utilizable by *P. pseudomallei* were attacked by *B. mallei*: D-xylose and glycine (used by 14 strains); DL-α-aminobutyrate (used by 13 strains); L-arabinose (used by 8 strains). Growth on D-xylose, on glycine and on α-aminobutyrate are, accordingly, the only positive nutritional characters that clearly distinguish *B. mallei* from *P. pseudomallei*. On the other hand, no less than 18 compounds which

Table 4. *Bacillus mallei. Compounds used as substrates by 13 or more strains*

Carbohydrates and sugar derivatives

D-Xylose*
D-Arabinose*
D-Fucose
D-Glucose
D-Mannose
D-Galactose
Sucrose
Trehalose
Cellobiose
Gluconate*

Fatty acids

Acetate
Propionate*

Dicarboxylic acids

Malonate*
Succinate*
Fumarate
Adipate*

Hydroxy-acids

L-Malate
DL-β-Hydroxybutyrate
DL-Lactate
DL-Glycerate*
Poly-β-Hydroxybutyrate

Miscellaneous organic acids

α-Ketoglutarate
Pyruvate*

Polyalcohols and glycols

Mannitol
Sorbitol*
Inositol*
Glycerol

Alcohols

(None)

Non-nitrogenous aromatic and other cyclic compounds

Benzoate
p-Hydroxybenzoate
Phenylacetate
Quinate*

Aliphatic amino acids

Glycine*
L-α-Alanine
D-α-Alanine
β-Alanine*
L-Threonine
L-Aspartate*
L-Glutamate
DL-Arginine
DL-α-Aminobutyrate*
γ-Aminobutyrate

Amino acids and related compounds containing a ring structure

L-Histidine
L-Tyrosine
L-Tryptophan

Amines

(None)

Miscellaneous nitrogenous compounds

Betaine*
Hippurate

Paraffinic hydrocarbons

(None)

* Strains which fail to use this compound are shown in Table 6; all others are universal substrates.

did not support growth of any strain of *B. mallei* were used by all or the great majority of strains of *P. pseudomallei*. As shown in Table 7, the compounds in question are chemically diverse, and include carbohydrates, fatty acids, aromatic compounds, amino acids and amines. Accordingly, it is evident that the specific nutritional differences between *B. mallei* and *P. pseudomallei* are overwhelmingly represented by characters for which *B. mallei* strains are negative; *B. mallei* clearly lacks many enzymic potentialities possessed by *P. pseudomallei*.

Nine compounds were more or less consistently utilized by some strains of *Bacillus mallei* but not by others (Table 8). With the exception of L-arabinose, they are all substrates for *Pseudomonas pseudomallei*, so that strains of *B. mallei* which utilized them resemble *P. pseudomallei* more closely in nutritional spectrum than those which do not. These nutritional differences might appear to justify the recognition of two distinct varieties of *B. mallei*, were it not for an interesting feature

of the strain histories. Strains 7, 10 and 11 are all derived from a single source, a human infection in China. Strains 7 and 10 have been maintained in culture or in lyophil preparation since their isolation in 1944 and are nutritionally identical; they can use all nine compounds listed in Table 8. Strain 10 was subjected, some years after its primary isolation, to three serial passages in the hamster; the strain re-

Table 5. *Bacillus mallei. Compounds not utilized by any of the strains*

Carbohydrates and sugar derivatives	Polyalcohols and glycols	Amino acids and related compounds containing a ring structure
D-Ribose	Erythritol	D-Tryptophan
L-Rhamnose	Ethyleneglycol	L-Kynurenine
Lactose	Propyleneglycol	Kynurenate
Inulin	2,3-Butyleneglycol	*m*-Aminobenzoate
Saccharate		*p*-Aminobenzoate
Mucate	Alcohols	
	Methanol	Amines
Fatty acids	Ethanol	Methylamine
Isobutyrate	*n*-Propanol	Ethanolamine
Valerate	Isopropanol	Benzylamine
Isovalerate	*n*-Butanol	Spermine
Caproate	Isobutanol	Histamine
Heptanoate	Geraniol	Tryptamine
Pelargonate		Butylamine
Caprate	Non-nitrogenous aromatic and other cyclic compounds	α-Amylamine
Dicarboxylic acids	D-Mandelate	Miscellaneous nitrogenous compounds
Oxalate	L-Mandelate	Creatine
Maleate	Benzoylformate	Pantothenate
Eicosanedioate	*o*-Hydroxybenzoate	Acetamide
	m-Hydroxybenzoate	Nicotinate
Hydroxy-acids	Phthalate	
D-(−)-Tartrate	Isophthalate	Paraffinic hydrocarbons
L-(+)-Tartrate	Terephthalate	*n*-Dodecane
meso-Tartrate	Phenylethanediol	*n*-Hexadecane
Glycollate	Phenol	
Hydroxymethylglutarate	Testosterone	
Miscellaneous organic acids	Aliphatic amino acids	
Levulinate	L-Leucine	
Citraconate	L-Isoleucine	
Itaconate	L-Norleucine	
Mesaconate	L-Lysine	
	DL-Ornithine	
	DL-Citrulline	
	DL-α-Aminovalerate	

isolated after these passages is strain 11. Strain 11 can use only two of the compounds listed in Table 8; furthermore, it cannot grow on either sorbitol or aspartate, both of which support the growth of strains 7 and 10. The reliability of strain histories cannot, of course, be taken for granted; but these facts do suggest that the nutritional characters of *B. mallei* may be modifiable by animal passage. Until this possibility has been further studied, the taxonomic significance of the data in Table 8 cannot be assessed.

Proposed neotype strains

The original isolates of *Bacillus mallei* and *Pseudomonas pseudomallei* have long since been lost. Lysenko (1961) proposed a neotype strain for *P. pseudomallei*; but the properties of this strain differ in many respects from those of the strains which

Table 6. *Bacillus mallei. Substrates utilized by a fraction of the strains*

Substrate	No. of positive strains	Negative strains
D-Xylose	14	4
D-Arabinose	14	4
L-Arabinose	8	2, 8, 11, 12, 17, 19, 20
D-Fructose	9	2, 8, 12, 17, 19, 20
Maltose	8	2, 8, 11, 12, 17, 19, 20
Salicin	6	1, 2, 4, 6, 8, 12, 17, 19, 20
Starch	8	2, 8, 11, 12, 17, 19, 20
Gluconate	13	4, 20
2-Ketogluconate	11	1, 2, 4, 20
Propionate	14	1
Butyrate	11	1, 8, 17, 19
Caprylate	8	1, 8, 11, 12, 17, 19, 20
Malonate	13	2, 4
Succinate	13	8, 12
Glutarate	3	All except 8, 18, 19
Adipate	13	4, 19
Pimelate	4	All except 1, 5, 6, 8
Suberate	3	All except 1, 5, 6
Azelate	2	All except 1, 5
Sebacate	1	All except 4
D-Malate	10	4, 8, 12, 19, 20
DL-Glycerate	13	1, 17
Poly-β-hydroxybutyrate	13	5, 6
Citrate	10	8, 11, 12, 17, 19
Pyruvate	13	8, 19
Aconitate	1	All except 1
Sorbitol	14	11
Inositol	14	8
Adonitol	1	All except 7
Quinate	14	8
Glycine	14	2
β-Alanine	14	4
L-Serine	3	All except 16, 18, 20
L-Valine	9	1, 4, 12, 17, 19, 20
L-Aspartate	14	11
DL-α-Aminobutyrate	13	1, 12
δ-Aminovalerate	9	5, 11, 12, 17, 19, 20
L-Proline	8	1, 8, 11, 12, 17, 19, 20
L-Phenylalanine	9	4, 8, 12, 17, 19, 20
Anthranilate	11	2, 4, 6, 10
Putrescine	6	1, 2, 4, 6, 8, 12, 17, 19, 20
Betaine	14	4
Sarcosine	8	1, 2, 4, 8, 12, 19, 20
Trigonelline	8	1, 8, 11, 12, 17, 19, 20

we have examined. Furthermore, the description of Lysenko's strain in the catalogue of the American Type Culture Collection (1964) carries the following comment: '*Pseudomonas aeruginosa* (W. C. Haynes, personal communication).' Hence it seems

necessary to designate another neotype. We propose NBL 121 as the neotype strain of *P. pseudomallei*. Apparently no neotype strain of *B. mallei* has been designated; we propose NBL 7.

Table 7. *Principal nutritional differences between Pseudomonas pseudomallei and Bacillus mallei*

Compound	Fraction of positive strains	
	P. pseudomallei	*P. mallei*
D-Ribose	26/26	0/15
D-Xylose	0/26	14/15
L-Arabinose	0/26	8/15
Isobutyrate	26/26	0/15
Valerate	26/26	0/15
Isovalerate	26/26	0/15
Caproate	26/26	0/15
Heptanoate	26/26	0/15
Pelargonate	26/26	0/15
Caprate	26/26	0/15
Levulinate	24/26	0/15
Erythritol	26/26	0/15
Glycine	0/26	14/15
L-Isoleucine	25/26	0/15
L-Lysine	25/26	0/15
DL-α-Aminobutyrate	0/26	13/15
L-Kynurenine	26/26	0/15
Kynurenate	26/26	0/15
Ethanolamine	26/26	0/15
Butylamine	24/26	0/15
α-Amylamine	24/26	0/15

Table 8. *Differences in nutritional spectrum between two groups of strains of Bacillus mallei, together with the corresponding nutritional properties of Pseudomonas pseudomallei*

Compound	Strains of *B. mallei*														*P. pseudomallei*: fraction of positive strains
	8	11	12	17	19	20	1	2	4	5	6	7	10	16	18	
L-Arabinose	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	0/26
Maltose	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	26/26
Starch	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	26/26
Caprylate	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	26/26
Citrate	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	26/26
L-Phenylalanine	−	+	−	−	−	−	+	+	−	+	+	+	+	+	+	26/26
Trigonelline	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	26/26
L-Proline	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	26/26
Sarcosine	−	+	−	−	−	−	−	−	−	+	+	+	+	+	+	23/26

DISCUSSION

The affinities and taxonomic position of Pseudomonas pseudomallei and Bacillus mallei

Our characterization of *Pseudomonas pseudomallei* and *Bacillus mallei* in terms of physiological and nutritional properties amply confirms the close resemblance between these two species, which has been suggested by others on the basis of simi-

larities in pathogenicity (Whitmore, 1913), antigenic constitution (Stanton & Fletcher, 1925; Cravitz & Miller, 1950) and phage susceptibility (Smith & Cherry, 1957). The phenotypic resemblances assume even deeper significance in view of the virtual identity in the GC content of the respective DNAs (Mandel, 1966). The natural affinity between these two bacterial species seems now established beyond reasonable doubt; consequently, the maintenance of a generic separation between them seems unnecessary and undesirable.

At the same time, the present characterization of *Pseudomonas pseudomallei*—the first which can be considered reasonably complete—fully supports its current assignment to the genus *Pseudomonas*. *P. pseudomallei* conforms in every respect to the generic definition proposed by Stanier *et al.* (1966). This being so, we cannot avoid the conclusion that *Bacillus mallei* should also be placed in the genus *Pseudomonas*, despite its permanent non-motility. We therefore proposed to designate it as *Pseudomonas mallei*, comb.nov.

Polar flagellation has heretofore been considered a cardinal criterion for membership in the genus *Pseudomonas*. It is true that Jessen (1965) has described rare strains of the type species, *Pseudomonas aeruginosa*, which appear to be permanently non-motile; but polar flagellation is characteristic of the overwhelming majority of strains which belong to this species. No strain of *Pseudomonas mallei* has ever been reported to show motility, and the presumption is therefore strong that this species is completely non-motile.

In other groups of Gram-negative polarly-flagellate bacteria, non-motile species have long been associated with motile ones, though generally not within the confines of a single genus. Among photosynthetic bacteria, many species of purple sulphur bacteria are non-motile; and green bacteria are characteristically non-motile, only one species with polar flagella having been described to date. The taxonomic association of polarly flagellate and non-motile species among photosynthetic bacteria has never been questioned by bacterial taxonomists, who have more or less tacitly assumed in this case that the shared physiological and biochemical properties afford an overwhelming presumption of evolutionary affinity. The same line of reasoning justifies the taxonomic association that we now propose between *Pseudomonas pseudomallei* and *P. mallei*.

However, the abandonment of polar flagellation as an essential attribute of the genus *Pseudomonas* does entail some practical disadvantages. It opens the generic portals to a large number of species of non-motile Gram-negative rod-shaped eubacteria now allotted to such ill-defined genera as *Flavobacterium* and *Achromobacter*. The characterization of most of these species is grossly inadequate. Hence, as a matter of practical policy, we believe that they should be declared 'passportless cosmopolitans' until they have been shown to conform to all the criteria proposed by Stanier *et al.* (1966) for the genus *Pseudomonas*, except for the possession of flagella. If any species can pass this test, we see no valid grounds for denying it admission to *Pseudomonas*. It should be recognized that the taxonomic problem raised here has always been immanent in schemes of eubacterial classification which use the mode of flagellation as a major differential character, since such schemes by their very nature leave the many permanently non-motile species in an indeterminate state.

The phenotypic resemblances between *Pseudomonas pseudomallei* and *P. mallei*

are so great that these two species can be regarded as constituting a generic subgroup, analogous to the fluorescent, acidovorans and alcaligenes groups described by Stanier *et al.* (1966). For purposes of differentiating the 'pseudomallei group' as we shall term it, from other aerobic pseudomonads, we have constructed an ideal group phenotype, comprising a total of 16 unrelated characters which are shared by most strains of *P. pseudomallei* and *P. mallei* (Table 9). In Table 10 we have calculated the degree of conformity of all species of the genus *Pseudomonas* which have

Table 9. *The group of characters of the greatest value in the differentiation of Pseudomonas pseudomallei and P. mallei from other aerobic pseudomonads*

Characters	No. of positive strains		Ideal phenotype for the group
	P. pseudomallei	*P. mallei*	
Poly-β-hydroxybutyrate as reserve material	26	15	+
Poly-β-hydroxybutyrate degradation by extracellular enzymes	26	13	+
Denitrification	26	15	+
Fluorescent pigment	0	0	−
Utilization of:			
Starch	26	8	+
Cellobiose	26	15	+
D-Fucose	26	15	+
D-Arabinose	26	14	+
Adipate	26	13	+
Mucate	0	0	−
m-Hydroxybenzoate	0	0	−
p-Hydroxybenzoate	24	15	+
DL-Arginine	26	15	+
L-Threonine	26	15	+
Betaine	26	14	+
Testosterone	0	0	−

been characterized on the basis of the methods here used to the ideal phenotype of the pseudomallei group. Of the 41 strains of the pseudomallei group that we have examined, 39 either conform perfectly to the ideal phenotype, or deviate by a single character; one strain of *P. mallei* deviates by two characters; and one, by three. Among other *Pseudomonas* species, *P. multivorans* most closely approaches the ideal phenotype of the pseudomallei group, all 19 strains of *P. multivorans* deviating from it by 6 characters. In all remaining *Pseudomonas* species, no strain deviates by less than 8 characters.

The existence of a close relationship between *Pseudomonas pseudomallei* and *P. aeruginosa* has been repeatedly suggested by earlier workers. This suggestion stemmed in part from epidemiological and pathological resemblances; and it is true that in these respects there are some interesting parallels to be discussed. On the basis of a comparative study Wetmore & Gochenour (1956) concluded that the resemblances extend to cultural and physiological characters, and alleged that it is difficult to distinguish achromogenic strains of *P. aeruginosa* from *P. pseudomallei*. In reality, as Table 10 shows, the differences between the two species are numerous.

Of the 16 characters selected to define the ideal phenotype of the pseudomallei group, no strain of *P. pseudomallei* possesses less than 15; and no strain of *P. aeruginosa* more than 8. Hence even on this restricted set of traits, the two species are readily differentiable. (The total number of phenotypic traits by which they differ is, of course, much greater. We have not precisely determined it; but this can be readily done by the numerically inclined taxonomist, by using the complete data for *Pseudomonas aeruginosa* (Stanier *et al.* 1966) and the complete data given here for *P. pseudomallei*).

The conclusion reached by Wetmore & Gochenour is evidently incorrect but instructive. It shows how far astray the bacterial taxonomist can be led if he uses a set of 'standard methods' which are either inadequate or inappropriate for the particular bacterial group to which he applies them.

Table 10. *Conformity of strains of different species of aerobic pseudomonads to the selected 16 characters (Table 9) which define the ideal phenotype of the pseudomallei group*

		Total no. of characters shared with ideal phenotype													
		16	15	14	13	12	11	10	9	8	7	6	5	4	3
		No. of strains													
Group	Species														
Pseudomallei	*P. pseudomallei*	24	2	.	.	.	.	.	.	.	.	.	.	.	.
	P. mallei	5	8	1	1	.	.	.	.	.	.	.	.	.	.
	P. multivorans	.	.	.	.	.	.	19	.	.	.	.	.	.	.
Fluorescent	*P. aeruginosa*	.	.	.	.	.	.	.	.	9	20	.	.	.	.
	P. fluorescens	.	.	.	.	.	.	.	.	3	14	31	45	1	.
	P. putida	.	.	.	.	.	.	.	.	.	.	.	29	12	.
Acidovorans	*P. acidovorans*	.	.	.	.	.	.	.	.	.	.	.	.	15	.
	P. testosteroni	.	.	.	.	.	.	.	.	.	.	.	.	9	.
Alcaligenes	*P. alcaligenes*	.	.	.	.	.	.	.	.	.	.	.	1	.	.
	P. pseudoalcaligenes	.	.	.	.	.	.	.	.	.	4	1	1	.	.
	P. stutzeri	.	.	.	.	.	.	.	.	.	.	3	11	2	1
	P. maltophilia	.	.	.	.	.	.	.	.	.	.	.	23	.	.
	P. lemoignei	.	.	.	.	.	.	.	.	.	.	1	.	.	.

Ecological and evolutionary considerations

As a pathogen *Pseudomonas pseudomallei* presents many ecological puzzles. Nigg (1963) summed up half a century of work on this subject in one sentence: 'almost nothing is known about the epidemiology of melioidosis, but textbooks state that rodents constitute the natural reservoir of infection'. We shall now attempt to construct a coherent ecological hypothesis, which takes into account the existing information about the pathogenic behaviour and epidemiology of *P. pseudomallei* and our present findings about its physiological properties. *P. pseudomallei* shows an exceptionally low degree of host specificity. Natural infections have been reported in man, horse, cow, pig, sheep, goat, cat, dog and several different rodents. This wide host spectrum has been confirmed and extended by the experimental studies of Miller *et al.* (1948). The clinical and pathological manifestations of melioidosis are extremely varied. There are no distinctive symptoms, and correct diagnosis is essentially dependent on the isolation of the causative organism.

Once melioidosis has become clinically recognizable, it typically follows a progressive course, and the mortality is very high. However, there are many indications that *Pseudomonas pseudomallei* cannot easily establish itself in the mammalian host. Human melioidosis is a relatively rare disease, even in south-east Asia where its incidence is highest. No case of contact infection has been reported in man, although patients with the disease have often not been isolated. Stanton & Fletcher (1925) stated: 'man is not readily susceptible; otherwise cases of melioidosis would surely have occurred in laboratories where living cultures have been freely handled or among the Asiatic attendants who have looked after hundreds of infected animals' (*sic*).

Clinical findings are relevant in this connexion. Roques & Dauphin (1953) and Alain, Saint-Etienne & Reynes (1949) observed that melioidosis may develop secondarily in patients hospitalized for other reasons, including surgical treatment and non-infectious disease. The detailed histories of the first six human cases recorded in northern Australia (Rimington, 1962) reveal the interesting fact that five of the affected individuals also suffered from chronic debilitating diseases. Melioidosis is evidently apt to develop in individuals with an unusually low degree of resistance. Nigg (1963) showed by serological tests that about 10% of a group of over 500 healthy adult Thai males had been exposed to *Pseudomonas pseudomallei*. This finding also indicates that exposure is rarely followed by a clinically recognizable infection. Taken together, these pathological and epidemiological facts all lead to one conclusion: *P. pseudomallei* does not have the biological profile of a successful parasite. It has little invasive power, and its host and tissue specificity alike are unusually low. To put the matter another way, there is no evidence that *P. pseudomallei* has undergone an evolutionary adaptation to parasitic existence through long participation in a host-parasite relationship.

There has been much speculation about the natural reservoir of *Pseudomonas pseudomallei* in south-east Asia. A reservoir in rats, suggested by Whitmore (1913) without evidence, has been overwhelmingly disproved by subsequent work. At the Institut Pasteur in Saigon (an area of high incidence of melioidosis), over 20,000 autopsies on rats, several thousand of which were accompanied by blood cultures, resulted in only one isolation of *P. pseudomallei* (Alain *et al.* 1949). There is, on the other hand, evidence for the occurrence of *P. pseudomallei* in soil and water in south-east Asia. Chambon (1955) subjected 150 samples of mud and water to bacteriological analysis, and was able to isolate *P. pseudomallei* directly from five of them. Nigg & Johnston (1961) emphasized in this connexion the relevance of an epidemiological observation. Three cases of acute melioidosis have occurred as a sequel to car accidents, in which the victim suffered lacerations that were heavily contaminated with muddy water.

The biological character of *Pseudomonas pseudomallei* as it has emerged from our work is completely in accord with the assumption that this bacterium is a normal inhabitant of soil and water. Its physiological properties, like those of other nutritionally versatile members of the genus (the fluorescent pseudomonads, the acidovorans group, *Pseudomonas multivorans*), potentially equip it to play many roles in the aerobic mineralization of organic matter, and in denitrification. The crucial evidence still lacking is the demonstration that *P. pseudomallei* can be isolated from soil or water by specific enrichment methods. Nevertheless, we consider the pre-

sumptive evidence that *P. pseudomallei* exists in nature as a free-living organism to be strong. In this event, it must be regarded as an accidental pathogen that can on rare occasions find a niche in the animal body, either as a result of abnormally low host resistance, or because exceptionally favourable portals of entry have been created by physical trauma.

The ecology of the other typical *Pseudomonas* species pathogenic for warm-blooded animals, *P. aeruginosa*, presents striking analogies. *Pseudomonas aeruginosa* is a common inhabitant of soil, and has been shown by specific enrichment experiments

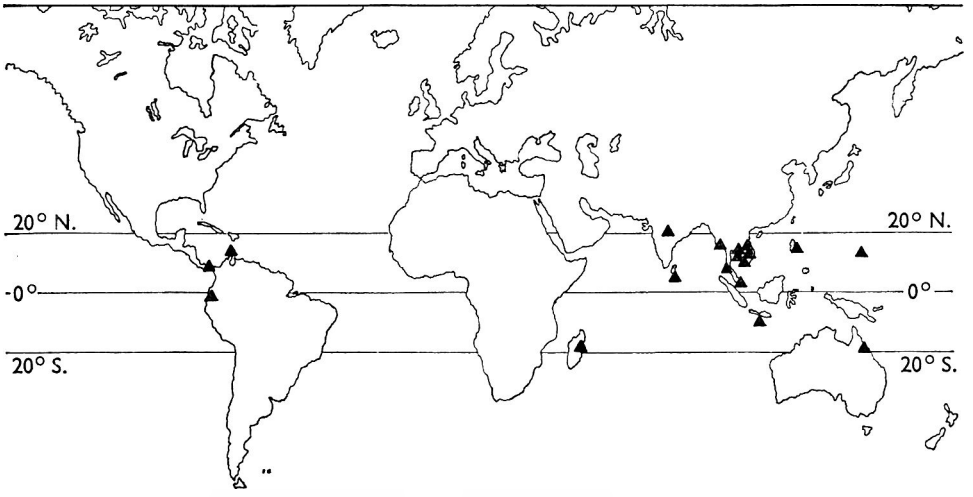

Fig. 1. Known geographical distribution of melioidosis. Each triangle indicates an area from which at least one bacteriologically diagnosed case of the disease has been reported.

to play a role in denitrification (Beijerinck & Minkman, 1910) and in the oxidation of normal alkanes (Konovaltschikoff-Mazoyer & Senez, 1956). Although it has assumed increasing clinical importance in recent decades as a result of its resistance to antibiotic therapy, it also does not have the profile of a successful parasite (Forkner, 1960). Laboratory infections have not been reported; the organism never gives rise to contact infections; and it is typically a hospital pathogen which establishes systemic infections in individuals who, for any one of a variety of medical reasons, have severely lowered degrees of resistance. Successful lodgement classically occurs when extensive artificial portals of entry have been created as a result of burns. Like *P. pseudomallei*, *P. aeruginosa* can be interpreted as a free-living organism which can occasionally develop in the animal body, but has not undergone any evolutionary selection for this rarely used capacity. It is no doubt significant that *P. pseudomallei* and *P. aeruginosa* invariably grow well at 41–42°. Growth at this temperature is irregular in *P. multivorans* and *P. stutzeri*, and fails wholly in all other *Pseudomonas* species except the alcaligenes group, of which too few strains have been examined to draw final conclusions on the temperature range (Stanier *et al.* 1966). The capacity to grow well around 40° is obviously an essential pre-condition for occasional development in mammalian hosts.

However, *Pseudomonas pseudomallei* and *P. aeruginosa* do differ in one major ecological respect. Whereas *P. aeruginosa*, like most free-living bacteria, has a world-

wide distribution, *P. pseudomallei* apparently has not. It must be realized that the geographical distribution of the latter species is now exclusively inferred from its pathological manifestations, which almost certainly give a much too limited picture; but even taking this limitation into account, the facts are striking enough. The overwhelming majority of recorded cases of melioidosis both in man and in other animals have occurred in south-east Asia (Burma, Thailand, Malaya, Indochina, Indonesia). However, sporadic cases have been reported in other tropical areas, some far distant from the major focus. These include: Ceylon, Madagascar, northern Australia, the Caribbean region and Ecuador.* All these sites lie between 20° N. and 20° S. (Fig. 1). Hence it seems fair to assume that *P. pseudomallei* is widely distributed through the tropical regions of the earth; and there is no epidemiological evidence for its occurrence outside them. Believing as we do that *P. pseudomallei* is a free-living organism, we are none the less forced to conclude that it has a limited geographical range, and occurs rarely, if at all, in temperate regions. A systematic study of its geographical ecology will be possible only when a method for its selective isolation from soil and water is devised. Our data on its physiological properties offer many indications of how this might be achieved.

In ecological terms, *Pseudomonas mallei* provides a striking contrast to *P. pseudomallei* and to *P. aeruginosa*. The disease which it causes is almost entirely confined to horses, donkeys and mules, although infection is transmissible from equine hosts to man. Other animal species are susceptible, but do not seem to contract the disease in nature. In the infected animal, the infection can assume two sharply defined clinical forms: glanders, in which the primary focus of infection is the lungs; and farcy, in which the infection spreads (generally from a primary skin lesion) through the lymphatic system (*Topley & Wilson's Principles*, 1955). These authors state: 'several cases of glanders have been reported in laboratory workers; indeed, probably no organism, with the possible exception of *Brucella tularensis*, is so dangerous to work with as the glanders bacillus'.

Glanders is a disease that has been effectively eradicated from many European countries, Canada and the United States by the systematic destruction of infected animals and the application of quarantine measures. The remarkable success of these control programmes indicates that soil and water do not serve as reservoirs for *Pseudomonas mallei*, which can apparently exist in nature only through passage in susceptible animals. Its geographical range is therefore co-terminous with that of its natural hosts. In summary, *P. mallei* is a successful parasite which shows evidence of long participation in a specific host-parasite relationship, to which it has become highly adapted. We consider it to be the only parasite of animals in the genus *Pseudomonas*.

Viewed in this context, the specific differences between *Pseudomonas pseudomallei* and *P. mallei*, so remarkably similar in their general phenotypes, become highly

* Melioidosis is probably far more prevalent in many of these areas than the published reports suggest. The history of the disease in northern Australia (Rimington, 1962) throws an interesting light on this question. The first cases were discovered in 1949 in sheep. The first human case was diagnosed in 1950; and the report on it notes that identification of *Pseudomonas pseudomallei*, isolated from blood samples, was made at the local Animal Health Station. Subsequent outbreaks in a variety of domestic animals made it evident that melioidosis was endemic in the area; and in 1959–60, no less than five additional human cases were diagnosed. Rimington (1962), in discussing these facts, suggests that melioidosis may account for 'some of the undiagnosed fevers that occur in North Queensland'.

significant. With minor exceptions, the characters by which they differ are negative in *P. mallei*. *P. mallei* lacks flagella; it has a much narrower range of carbon and energy sources; and it grows more slowly on artificial media, both chemically defined and complex. If we accept a common evolutionary origin for the two species, an assumption which is difficult to avoid, nearly all the specific structural and physiological differences between them can be interpreted as losses or impairments of function which took place in the *P. mallei* line during the course of its transition from existence in soil and water to obligate dependence on a mammalian host. Equivalent functional regression has not taken place in the *P. pseudomallei* line, because this bacterium (despite its pathogenic potentialities) has never succeeded in establishing a durable host-parasite relationship, and is consequently subject to evolutionary selection exclusively in the context of its activities as a free-living organism. Indeed, these two bacteria may provide an exquisite specific illustration of the evolutionary postulate of Lwoff (1944): adaptation to a parasitic mode of existence is typically accompanied by functional as well as structural regression.

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Note added to proofs

Antisera from rabbits hyperimmunized with formalin-killed cells of both *Pseudomonas pseudomallei* NBL 114 and *Bacillus mallei* NBL 7 were used to perform slide-agglutination tests with all strains of both species. Each antiserum agglutinated to at least the 1/50 dilution all strains of both *P. pseudomallei* and *B. mallei*. Serum from a rabbit hyperimmunized with formalin-killed cells of *Pseudomonas aeruginosa* NCTC 9229 did not agglutinate any strain of *P. pseudomallei* and *B. mallei* at the 1/10 dilution and neither did antisera to *P. pseudomallei* NBL 114 and *B. mallei* NBI 7 agglutinate *P. aeruginosa* NCTC 9229 at the same dilution. No strain of any of the three species was agglutinated by normal rabbit antisera diluted 1/10.