Genetic Transformation in *Pseudomonas*

By N. C. KHAN AND S. P. SEN

*Microbiology Laboratory, Department of Botany, Kalyani
University, Kalyani, West Bengal, India*

(Accepted for publication 5 May 1967)

SUMMARY

Intraspecific and interspecific transformation in *Pseudomonas* has been investigated. Two isoleucine + valine requiring mutants of *Pseudomonas aeruginosa* have been transformed to prototrophy. By using gelatin liquefaction as a marker character it was observed that two strains of *P. fluorescens*, a phytopathogen *P. mangiferaeindicae* and a strain of *Pseudomonas* originally isolated from fish were transformable. When *P. solanacearum* was used as a donor, the transformation frequency of *P. mangiferaeindicae* was very high. DNA from fish *Pseudomonas* 1 was capable of transforming two strains of *P. fluorescens* and *P. mangiferaeindicae* in addition to fish *Pseudomonas* 21. When *P. fluorescens* was treated with the DNA from *P. aeruginosa* transformation frequency was lower; the capacity of pigment production, however, could not be transferred to any of the strains. The highest transformation frequency was obtained towards the end of the log phase of growth of the receptor bacteria, with a DNA concentration of about 10 µg./ml. Optimum temperature for transformation was 25–30° and agitation and starvation in diluted media increased the number of transformants. The significance of the observations has been discussed from the point of view of relationships among the strains and species studied.

INTRODUCTION

Taxonomy of the genus *Pseudomonas* is a matter of considerable controversy. The recognized manuals of bacterial classification describe more than one hundred species under this genus. The wealth of informations available has been critically examined by De Ley (1964) and Stanier, Palleroni & Doudoroff (1966) in two recent reviews. Adansonian analysis has decreased the number of species considerably. Marmur, Falkow & Mandel (1963) emphasized the importance of DNA base composition, DNA hybridization and genetic transformation as tools in bacterial taxonomy. Colwell & Mandel (1964), De Ley & Friedman (1965), Colwell, Citarella & Ryman (1965), Mandel, Weeks & Colwell (1965), De Ley, Park, Tijtgate & Ermengem (1966) and Mandel (1966) have reported the guanine + cytosine (GC) content of the DNA of 44 species belonging to this genus. Though most of the species reveal a GC content of 60–67% the range extends from 37 to 70%. The present communication records our observations about the transformability of twelve strains of *Pseudomonas*, with a view to understanding the inter-relationships of the strains studied.

METHODS

Organisms. The following cultures were used: five strains of *Pseudomonas aeruginosa* including two auxotrophic (isoleucine + valine) mutants, two phytopathogenic strains (*P. solanacearum*, *P. mangiferaeindicae*), one strain of *P. putrefaciens*, two strains of *P. fluorescens* and two other pseudomonads (fish *Pseudomonas* 1, 21)—which were originally isolated from freshwater fish and closely resembled *P. fluorescens*. These latter two strains differed from each other in the production of gelatinase, lipase and lecithinase, in the reduction of nitrate and in the formation of levan (Bullock, Snieszko & Dunbar, 1965). The two strains of *P. fluorescens* (ATCC 12633, and a strain obtained from Dr J. H. Darrell, henceforth be referred to as the Darrell strain) were incapable of liquefying gelatin (a property characteristic of *P. fluorescens*) thus confirming the observations of Bullock *et al.* (1965) and Dr J. H. Darrell (personal communication) concerning these two strains. In this respect these cultures resemble *P. putida* which Flüge (1886) considered to be a non-liquefying biotype of *P. fluorescens*. In absence of other details we shall refer to these strains as belonging to *P. fluorescens*. The strains of *P. mangiferaeindicae* and *P. putrefaciens* also did not liquefy gelatin, contrary to the description of these species in *Bergey's Manual of Determinative Bacteriology* (1957). All the five strains of *P. aeruginosa*, including the auxotrophic mutants, however, liquefied gelatin. This is in agreement with the observations of Stanier *et al.* (1966) that all strains of this species liquefy gelatin. The auxotrophic (isoleucium + valine-requiring) mutants *ilva* A9 and *ilva* C1 were originally obtained from the wild strain *P. aeruginosa* 78. *Pseudomonas solanacearum* had pronounced gelatinase activity. The source of the cultures is given in Table 1. All the cultures were purified twice before use.

Table 1. *Strains of Pseudomonas used and their sources*

Designation	Source
<i>Pseudomonas aeruginosa</i> 78 <i>P. aeruginosa ilva</i> A9 <i>P. aeruginosa ilva</i> C1	Dr J. S. Loutit, Dept. of Microbiology, Medical School, University of Otago, Dunedin, New Zealand
<i>P. aeruginosa</i> LC-1 <i>P. aeruginosa</i> 215 <i>P. fluorescens</i>	Laboratory culture originally obtained from a Calcutta hospital. Dr J. H. Darrell, Dept. of Bacteriology, Post graduate Medical School of London, U.K.
<i>P. fluorescens</i> ATCC 12633 Fish <i>Pseudomonas</i> 1 Fish <i>Pseudomonas</i> 21	Dr G. L. Bullock, Eastern Fish Disease Laboratory, Leetown, W. Va., U.S.A.
<i>P. solanacearum</i> P 25 <i>P. mangiferaeindicae</i> P 24	Indian Type Culture Collection, New Delhi, India
<i>P. putrefaciens</i> NRRL B. 950	Dr W. C. Haynes, Agricultural Research Service, Northern Regional Research Laboratory, Peoria, Ill., U.S.A.

In addition to the *Pseudomonas* cultures the following organisms were also used in the transformation experiments: (i) *Bacillus subtilis* MB 294 (kindly supplied by Dr H. B. Woodruff of Merck, Sharp and Dohme, Rahway, N.J., U.S.A.), (ii) *Sarcina lutea* obtained from the Microbiology Laboratory of Dey's Medical Stores (Manufacturing) Private Ltd., Calcutta, (iii) *Azotobacter chroococcum* AZCF isolated from local soil samples in this laboratory (Sen & Sen, 1965).

Media. The cultures were usually grown in nutrient agar medium of the following composition: Lab Lemco, 10 g.; peptone, 10 g.; sodium chloride, 5 g.; glucose, 1 g.; agar, 20 g.; water to 1000 ml.; pH 7.2. Transformants obtained were maintained in nutrient gelatin medium containing nutrient broth with 15% (w/v) of gelatin (Difco) and without glucose. For maintenance of the auxotrophic mutants Morihara's (1964) medium having the following composition was used: Glucose, 1 g.; NH_4Cl , 1 g.; Na_2HPO_4 , $12\text{H}_2\text{O}$ 1 g.; KH_2PO_4 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g.; CaCO_3 , 0.05 g.; isoleucine, 2 mg.; valine, 2 mg.; distilled water to 1000 ml. For scoring organisms transformed to prototrophy dilutions of the incubation mixture were plated on the minimal medium without valine and isoleucine.

Transformation. Competent bacteria were prepared according to the method of Stuy (1962) with slight modifications. Except in the experiments in which conditions were varied, 0.1 ml. of an 18-hr culture of a receptor strain was suspended in 0.8 ml. of normal saline and 0.1 ml. nutrient broth added, followed by shaking for 30 min. at 30° . The concentration of the recipient bacteria varied between 2 and 3.5×10^7 bacteria/ml. After 'starvation' in this diluted medium for 30 min., 1.0 ml. of DNA solution obtained from the donor strain was added and the mixture incubated for another 30 min. at 30° , followed by treatment with DNase (Worthington Biochemicals Corporation, U.S.A.) 10 $\mu\text{g.}/\text{ml.}$ solution containing 5 $\mu\text{moles MgCl}_2/\text{ml.}$ The number of transformants/ml. was calculated by dilution plating in an appropriate media. In another set of experiments the DNA isolated from donor strains was first incubated with DNase for 5 min. at 37° and 1 ml. of this DNase-treated preparation was included in the transformation medium before the receptor bacteria were introduced. After incubation for 30 min. dilutions of the transformants, if any, were plated as described. Experiments were also done in which attempts were made to transform the receptor bacteria with its own DNA as also with the DNA isolated from such unrelated species as *Sarcina lutea*, *Bacillus subtilis* and *Azotobacter chroococcum*. In the experiments in which the optimum conditions were worked out, only the factor being studied was varied, all other conditions remaining unchanged.

Isolation of transforming DNA. The donor strains were grown in shake cultures for 18–20 hr and the bacteria were collected by centrifugation. Sometimes the donor strains were seeded on nutrient agar medium in Petri dishes, incubated for 18–20 hr and the bacteria harvested by gently scraping them from the surface of the agar. DNA was isolated and purified following the method of Marmur (1961) with slight modifications. Concentration of DNA was measured at 600 $\text{m}\mu$ in a Bausch and Lomb Spectronic-20 colorimeter by the diphenylamine method.

Genetic markers. In most of the experiments described here gelatin liquefying capacity was the marker used. In some of the experiments in which *Pseudomonas aeruginosa* was used as donor, formation of the green diffusible pigment was used as a marker character. For transformation of the auxotrophic (isoleucine + valine) mutants of *P. aeruginosa* to prototrophy the capacity to synthesize isoleucine + valine was used as a marker character.

Transformation assay procedure. Nutrient agar containing 0.4% (w/v) gelatin was used for scoring the transformants following Smith's modification of Frazier's method as described in the *Manual of Microbiological Methods* (1957). Dilutions of the reaction mixture were plated on this medium. The plates were incubated at $28\text{--}30^\circ$ for 24–48 hr. The colonies were transferred to nutrient gelatin tubes and allowed to grow for 24–48 hr

at 28–30°. After incubation, each tube was kept in the refrigerator for several hours and solidification, if any, noted. Transformants capable of liquefying gelatin exhibited no solidification. When liquefaction was poor the incubation period was prolonged to 72 or 96 hr. The presence of gelatinase was also tested according to the following procedure. Ten ml. of a reagent containing 15 g. HgCl_2 dissolved in 100 ml. distilled water + 20 ml. conc. HCl when poured on the medium precipitated any unhydrolysed gelatin, giving a white opacity; absence of opacity surrounding a colony indicated its ability to hydrolyse gelatin. Similar results were also obtained by flooding the plates with a saturated solution of ammonium sulphate.

RESULTS

It was possible to transform six out of the eight strains used as receptor, using gelatin liquefaction or isoleucine + valine requirement as marker character. Neither of these characters, however, could be introduced in the receptor strains when they were treated with the DNA isolated from the same strain. When *Pseudomonas* receptor bacteria were treated with the DNA of such unrelated bacterial species as *Sarcina lutea*, *Bacillus subtilis* or *Azotobacter chroococcum*, the reported GC contents of which are 68–72, 39–45 and 57·5 %, respectively (see Hill, 1966), there was no transformation with respect to the marker characters used, including gelatin liquefaction and pigment production. Incubation of the DNA of donor bacteria with DNase 10 $\mu\text{g./ml.}$ at 37° for 5 min. before the treated DNA was applied to receptor bacteria resulted in a complete loss of its transforming ability in all the cases investigated.

Conditions for transformation

In preliminary experiments it was observed that transformability of the strains could be tested conveniently by using gelatin liquefying capacity as a marker character. Using a gelatin-liquefying strain fish *Pseudomonas* 1 as the donor, and a strain of *Pseudomonas fluorescens* ATCC 12633 as receptor the conditions for transformation were worked out. The age of the receptor bacteria having highest competency and the optimum concentration of donor DNA required for transformation were determined. The effects of the duration of incubation with transforming DNA, agitation of the incubation mixture, temperature and composition of the medium on transformation frequency were also studied. Under optimum conditions the frequency of transformation with these two strains was as high as 4·8 %.

The formation of transformants depended considerably on the physiological state of the receptor strains. Accordingly, transformation experiments were made at different stages of growth. The highest number of transformants was obtained towards the end of the log phase; there was no further increase during the stationary phase. The occurrence of transformants increased with time and the transformation curve resembled considerably the growth curve, the maximum being attained at about 18 hr (Fig. 1).

When the DNA concentration was varied it was observed that transformation frequency increased with increasing concentration of DNA up to a certain limit, beyond which no further increase in the frequency was detected. Fig. 2 shows the response of the receptor strain *Pseudomonas fluorescens* ATCC 12633 to DNA preparations from fish *Pseudomonas* 1. The percentage of transformants increased linearly with concentration, the saturating concentration of transforming DNA being reached at about 10 $\mu\text{g. DNA/ml.}$

Transformation frequency increased rapidly with the time of exposure to DNA (Fig. 3). Within 5 min. 2.5% of the bacteria were transformed; the highest frequency was reached at 20 min.

Fig. 1. Formation of transformants at different phases of growth. Δ — Δ , Growth curve of the receptor strain *Pseudomonas fluorescens* ATCC 12633; $\circ$ — $\circ$, transformation of *P. fluorescens* ATCC 12633 with fish *Pseudomonas* 1 DNA; $\bullet$ — $\bullet$, transformation of *P. fluorescens* ATCC 12633 with *P. aeruginosa* 215 DNA.

Fig. 2

Fig. 3

Fig. 2. Effect of DNA concentration on transformation frequency. $\circ$ — $\circ$, Transformation of *Pseudomonas fluorescens* ATCC 12633; $\bullet$ — $\bullet$, transformation of fish *Pseudomonas* 21. Donor fish *Pseudomonas* 1.

Fig. 3. Effect of the time of incubation with DNA on transformation frequency of *Pseudomonas fluorescens* ATCC 12633 with the DNA of fish *Pseudomonas* 1.

The temperature range 25–30° was found to be the optimum. At temperatures lower than 20° the frequency was nil and at 37° it was negligibly small. Transformation frequency was enhanced when the amount of nutrient broth in the incubation medium was decreased. Highest frequency was observed when the broth was diluted about

10-fold. Agitation of the incubation mixture gave a higher frequency than in the stationary condition, when other conditions were unchanged.

Intraspecific and interspecific transformation

The possibility of intraspecific and interspecific transformation was examined by using the conditions found to be optimum for transformation. Recipient bacteria harvested before the termination of the logarithmic phase of growth were incubated with transforming DNA 10 µg./ml. for 30 min. at 30° in a 10-fold diluted nutrient broth with continuous stirring. Samples of the incubation mixture were then diluted in steps of 10 and plated on appropriate media.

Table 2. *Intraspecific transformation in Pseudomonas aeruginosa*

The marker characterizes (isoleucine + valine) requirement

Donor	Receptor	Transformation frequency (%)
<i>P. aeruginosa</i> 78	<i>P. aeruginosa</i> <i>ilva</i> <i>Cr</i>	4.7
<i>P. aeruginosa</i> 78	<i>P. aeruginosa</i> <i>ilva</i> <i>A9</i>	1.4

Table 3. *Interspecific transformation in Pseudomonas*

Donor	Receptor	Marker	Transformation frequency (%)
<i>P. aeruginosa</i> LC-1	<i>P. fluorescens</i> (Darrell)	Gelatin liquefaction	0.73
<i>P. aeruginosa</i> LC-1	<i>P. fluorescens</i> ATCC 12633		0.13
<i>P. aeruginosa</i> 215	<i>P. fluorescens</i> ATCC 12633		2.25
Fish <i>Pseudomonas</i> 1	<i>P. fluorescens</i> (Darrell)		0.16
Fish <i>Pseudomonas</i> 1	<i>P. fluorescens</i> ATCC 12633		4.8
Fish <i>Pseudomonas</i> 1	Fish <i>Pseudomonas</i> 21		4.0
Fish <i>Pseudomonas</i> 1	<i>P. mangiferaeindicae</i> P 24	Pigment production	3.7
<i>P. solanacearum</i> P 25	<i>P. mangiferaeindicae</i> P 24		11.1
<i>P. aeruginosa</i> LC-1	<i>P. fluorescens</i> (DARRELL)		No transformation
<i>P. aeruginosa</i> LC-1	<i>P. solanacearum</i> P 25		
<i>P. aeruginosa</i> LC-1	<i>P. mangiferaeindicae</i> P 24		
<i>P. aeruginosa</i> LC-1	Fish <i>Pseudomonas</i> 21		
<i>P. aeruginosa</i> LC-1	Fish <i>Pseudomonas</i> 1		
<i>P. aeruginosa</i> 215	<i>P. mangiferaeindicae</i> P 24		
<i>P. aeruginosa</i> 215	<i>P. solanacearum</i> P 25		
<i>P. aeruginosa</i> 215	Fish <i>Pseudomonas</i> 1		

Intraspecific transformation. The possibility of intraspecific transformation in *Pseudomonas aeruginosa* was investigated by using a wild-type strain, *P. aeruginosa* 78, as donor and two isoleucine + valine requiring mutants *ilva Cr* and *ilva A9* as receptors. Both the strains could be transformed to prototrophy (Table 2) and the highest frequency was observed when recipient bacteria 16–18 hr old were used for transformation. The frequency recorded with the *ilva Cr* strain was however much higher than that for the *ilva A9* strain.

Interspecific transformation. Choice of a suitable marker for the demonstration of interspecific transformation among the pseudomonads used posed a problem. Finally,

gelatin-liquefying capacity and pigment production were selected as markers. The results of the transformation experiments are summarized in Table 3.

Fish *Pseudomonas* 1 was a very effective donor, being capable of transforming both the strains of *Pseudomonas fluorescens*, *P. mangiferaeindicae* and fish *Pseudomonas* 21. Of the two strains of *P. fluorescens* used, *P. fluorescens* ATCC 12633 yielded a higher transformation frequency than the Darrell strain.

DNA from *Pseudomonas aeruginosa* was capable of transforming both the strains of *P. fluorescens*, but the gelatin-liquefying capacity only was incorporated; the property of pigment production could not be transferred to any other non-pigment producing strain. *P. mangiferaeindicae* was easily transformed by DNA preparations from *P. solanacearum*, the frequency of transformation being over 10%. *P. mangiferaeindicae* was also transformed by DNA preparations from fish *Pseudomonas* 1; the frequency in this case was 3.7%.

A curious observation was the finding that though both the strains of *Pseudomonas fluorescens*, ATCC 12633 and the Darrell strain, were incapable of liquefying gelatin, when the former was transformed with the latter 2.0% of the transformants liquefied gelatin. Fish *Pseudomonas* 21 also developed gelatinase activity when treated with the DNA of another inactive strain, namely, *P. putrefaciens*. The latter, however, did not transform any of the strains of *P. fluorescens* (Table 4).

Table 4. Development of gelatin-liquefying capacity in *Pseudomonas* strains by genetic transformation

The donor and receptor strains were both incapable of gelatin liquefaction.

Donor	Receptor	Transformation frequency (%)
<i>P. fluorescens</i> (DARRELL)	<i>P. fluorescens</i> ATCC 12633	2.0
<i>P. putrefaciens</i> NRRL B-950	Fish <i>Pseudomonas</i> 21	0.1
<i>P. putrefaciens</i> NRRL B-950	<i>P. fluorescens</i> (DARRELL)	nil
<i>P. putrefaciens</i> NRRL B-950	<i>P. fluorescens</i> ATCC 12633	nil

DISCUSSION

The frequency of transformation depends in a large measure on the similarity of DNA base composition, pairing compatibility of donor and receptor DNAs, the degree of homology within the marker locus and in the rest of the DNA molecule, host-induced modifications, the permeability of the recipient cell membrane to donor DNA and the quality of the DNA preparation used for transformation, in addition to the conditions used for the transformation experiments. The significance of the transformation frequency values for determination of interrelationships must be assessed with these limitations in view. The transformation of the auxotrophic mutants of *Pseudomonas aeruginosa* to prototrophy and the relatively high frequency observed in the transformation of gelatinase - to gelatinase + strains belonging to different species indicate that both intraspecific and interspecific transformation is possible at least among the species of *Pseudomonas* studied here. All the four gelatin-liquefying strains used as donor in the transformation experiments (*P. aeruginosa* LC-1, *P. aeruginosa* 215, fish *Pseudomonas* 1, *P. solanacearum*) have transferred this property to non-liquefying strains belonging to *P. fluorescens*, *P. mangiferaeindicae* and fish *Pseudomonas* 21. The frequency varied from 0.13% to 11.1%; in more than half of

the cases it was above 2%. The pigment-producing capacity of *P. aeruginosa*, however, was not transferred to any of the strains used.

The recipient strains apparently vary considerably in their capacity to integrate the piece of DNA responsible for the marker character. Thus while the wild strain *Pseudomonas aeruginosa* 78 transformed the two isoleucine+valine requiring mutants (*ilva* A9, *ilva* C1) to prototrophy, the efficiency of transformation of the former was only one-third that of the latter. The same observation also holds true for the two strains of *P. fluorescens* used, the Darrell strain and ATCC 12633.

If transformability of a strain with the DNA of another is an indication of the interrelationship of the two, then *Pseudomonas aeruginosa* would appear to be related to *P. fluorescens* since both the strains of *P. aeruginosa* (LC-1, 215) were capable of transforming the Darrell and ATCC 12633 strains of *P. fluorescens*. These two strains of *P. fluorescens* were also transformable with the DNA of fish *Pseudomonas* 1 which also transformed fish *Pseudomonas* 21 with a high frequency. Fish *Pseudomonas* 1 and 21 thus seem to be related with each other and with *P. fluorescens*. On the basis of biochemical and growth characteristics Bullock *et al.* (1965) concluded that fish *Pseudomonas* 1 and 21 were 'closely related to or identical with *P. fluorescens*'.

Fish *Pseudomonas* 1 also transformed the phytopathogen *Pseudomonas mangiferae-indicae* with a frequency of 3.7%. When another phytopathogen *P. solanacearum* was used as donor the frequency rose to 11.1%, the highest recorded for any of the *Pseudomonas* strains. The two phytopathogens thus are probably very closely related. If fish *Pseudomonas* 1 is a biotype of *P. fluorescens* then *P. mangiferae-indicae* and *P. solanacearum* would appear to be near relations of such a biotype. Many phytopathogenic pseudomonads are fluorescent, differing from each other in their capacity to attack specific hosts; they have many characters in common and Starr (1959) and Stanier *et al.* (1966) have questioned the validity of many phytopathogenic species. There are several reports of the failure of phage preparations to distinguish between many phytopathogenic and saprophytic pseudomonads, implying a close interrelationship among them. Many phytopathogenic pseudomonads have also been reported to be antigenically related.

Stanier *et al.* (1966) published extensive observations on the physiological, biochemical, nutritional and growth characteristics of 267 strains of aerobic pseudomonads and have clearly brought out the similarities and dissimilarities of the different species and biotypes. From an Adansonian analysis of several *Pseudomonas* strains Colwell & Mandel (1964) found that the strains of *Pseudomonas* used by them revealed a high degree of mutual similarity suggesting a species level relationship. Some of the *Pseudomonas fluorescens* strains 'clustered' within the *P. aeruginosa* group. Based on maximal intergroup S-values the relationship between the *P. fluorescens* group and the *P. solanacearum* group was found to be about 67%.

The DNA base composition of the strains used here is not known. It may be mentioned that the GC content of the DNA of seven biotypes of *Pseudomonas fluorescens* A, B, C, D, E, F and G has been reported to lie between 58.2 and 64.2% and that of *P. putida* biotypes between 59 and 63% (Mandel, 1966). The similarity of base composition among the *P. fluorescens* biotypes particularly A, B, C, F and G and between these biotypes and *P. putida* is thus considerable, suggestive of the possibilities of successful transformation among them. *P. aeruginosa* DNA is slightly richer in guanine and cytosine, the GC content being 67.3–68.4%. There are reports in the literature

that transformation is possible among strains which differ significantly in their DNA base composition, if the nucleotide sequences are common to the strains and can be recognized and incorporated in the recipient genome (Marmur *et al.* 1963). From DNA hybridization experiments De Ley *et al.* (1966) determined the degree of homology between different species of *Pseudomonas* and have numerically fixed their relatedness. The DNA homology of *P. putida* and *P. aeruginosa* relative to *P. fluorescens* DNA was 70.5 and 63 %, respectively. The corresponding taxonomic similarity between these species as calculated by Lysenko (1961) was 78 and 72 %. The present results obtained from the transformation experiments are thus in good agreement with the tentative conclusions drawn earlier about interrelationship among the pseudomonads from comparative biochemical and morphological studies of the strains, Adansonian analyses and DNA base composition and hybridization data.

We express our deep gratitude to Drs J. H. Darrell, G. L. Bullock, W. C. Haynes, J. S. Loutit and H. B. Woodruff for the gift of the cultures.

REFERENCES

- Bergey's Manual of Determinative Bacteriology* (1957). 7th ed. Ed. by R. S. Breed, E. G. D. Murray and N. R. Smith. Baltimore U.S.A.: Williams and Wilkins Co.
- BULLOCK, G. L., SNIESZKO, S. F. & DUNBAR, C. E. (1965). Characteristics and identification of oxidative pseudomonads isolated from diseased fish. *J. gen. Microbiol.* **38**, 1.
- COLWELL, R. R. & MANDEL, M. (1964). Adansonian analysis and deoxyribonucleic acid base composition of some Gram-negative bacteria. *J. Bact.* **87**, 1412.
- COLWELL, R. R., CITARELLA, R. V. & RYMAN, I. (1965). Deoxyribonucleic acid base composition and Adansonian analysis of heterotrophic, aerobic pseudomonads. *J. Bact.* **90**, 1148.
- DE LEY, J. (1964). *Pseudomonas* and related genera. *A. Rev. Microbiol.* **18**, 17.
- DE LEY, J. & FRIEDMAN, S. (1965). Similarity of *Xanthomonas* and *Pseudomonas* deoxyribonucleic acid. *J. Bact.* **89**, 1306.
- DE LEY, J., PARK, I. W., TIJTGAT, R. & VAN ERMENGEM, J. (1966). DNA homology and taxonomy of *Pseudomonas* and *Xanthomonas*. *J. gen. Microbiol.* **42**, 43.
- FLÜGGE, C. (1886). *Die Mikroorganismen*, 2. Aufl. Leipzig: F. C. W. Vogel.
- HILL, L. R. (1966). An index to deoxyribonucleic acid base composition of bacterial species. *J. gen. Microbiol.* **44**, 419.
- LYSENKO, O. (1961). *Pseudomonas*—an attempt at a general classification. *J. gen. Microbiol.* **25**, 379.
- MANDEL, M. (1966). Deoxyribonucleic acid base composition in the genus *Pseudomonas*. *J. gen. Microbiol.* **43**, 273.
- MANDEL, M., WEEKS, O. B. & COLWELL, R. R. (1965). Deoxyribonucleic acid base composition of *Pseudomonas piscicida*. *J. Bact.* **90**, 1492.
- Manual of Microbiological Methods* (1957). Ed. by Committee on Bacteriological Technic, Society of American Bacteriologists. New York: McGraw-Hill.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. molec. Biol.* **3**, 208.
- MARMUR, J., FALKOW, S. & MANDEL, M. (1963). New approaches to bacterial taxonomy. *A. Rev. Microbiol.* **17**, 329.
- MORIYAMA, K. (1964). Production of elastase and proteinase by *Pseudomonas aeruginosa*. *J. Bact.* **88**, 745.
- SEN, M. & SEN, S. P. (1965). Interspecific transformation in *Azotobacter*. *J. gen. Microbiol.* **41**, 1.
- STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic *Pseudomonas*: a taxonomic study. *J. gen. Microbiol.* **43**, 159.
- STARR, M. P. (1959). Bacteria as plant pathogens. *A. Rev. Microbiol.* **13**, 211.
- STUY, J. H. (1962). Transformability of *Haemophilus influenzae*. *J. gen. Microbiol.* **29**, 537.