Further Observations on Genetic Transformation in *Pseudomonas*

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SUMMARY

Intraspecific transformation in three strains of *Pseudomonas fluorescens* was observed with respect to gelatin liquefaction and chlortetracycline resistance. In interspecific transformation experiments involving 23 pairs of strains and four markers, two yielded positive results: a strain of *P. fluorescens* acquired the property of gelatin liquefaction when transformed with *P. aeruginosa* DNA, and a strain of fish *Pseudomonas*, incapable of growing at 37 °C, did so when transformed with DNA from another strain of *P. aeruginosa*. The guanine–cytosine content of the DNA of the strains investigated ranged between 61.0 and 64.4 mol %. The transforming ability of DNA was destroyed by DNase. The divalent ions Mg^{2+} , Ca^{2+} and Ba^{2+} improved transformation frequency several fold. Actinomycin D inhibited transformation.

INTRODUCTION

In a previous paper (Khan & Sen, 1967) we have described our observations concerning intra- and interspecific transformation in *Pseudomonas*. Intraspecific transformation was reported for *Pseudomonas aeruginosa* with respect to isoleucine and valine requirement. Interspecific transformation of gelatin liquefaction was demonstrated in eight species of *Pseudomonas*. Pigment production, however, could not be transferred. Transformation frequency was highest towards the end of the exponential phase of growth of the recipient bacteria and a DNA concentration of 10 µg/ml gave the best results. Optimum temperature for transformation was 25 to 30 °C and agitation and starvation in diluted media improved transformation frequency.

The present studies extend to a larger number of strains and markers and the requirements for transformation are defined. The DNA base composition of most of the strains used was also determined.

METHODS

Organisms. The strains used, their phenotypes and sources are listed in Table 1.

Media. The media used were the same as described by Khan & Sen (1967). Colwell's YSP medium (yeast extract, 3 g; sodium chloride, 5 g; peptone, 10 g; agar, 20 g; water to 1 l; pH 7.2) was used for testing the ability of the cultures to grow at different temperatures, and for the study of egg-yolk reactions and sensitivity to antibiotics. This medium was also used for isolation of large amounts of *Pseudomonas*.

Genetic markers. The following characters were used as markers: gelatin liquefaction, pigment production, capacity to grow at 37 and 42 °C, and chlortetracycline resistance. All the strains of *P. aeruginosa* produced a blue-green diffusible pigment and liquefied gelatin. Of the *P. fluorescens* strains, only NRRL-B10 was capable of gelatin liquefaction. Fish

Table 1. *Strains used*

Strain	Phenotype	Source
<i>Pseudomonas aeruginosa</i> LC1	Gelatin liquefaction, growth at 37 and 42 °C, pigment production	Laboratory culture, originally obtained from Calcutta Hospital
<i>P. aeruginosa</i> 78	Gelatin liquefaction, growth at 37 and 42 °C, pigment production	Dr J. S. Loutit, Dunedin, New Zealand
<i>P. aeruginosa</i> ilvA9	Isoleucine and valine requirement	
<i>P. aeruginosa</i> 215	Gelatin liquefaction, growth at 37 and 42 °C, pigment production	Dr J. H. Darrell, London
<i>P. fluorescens</i> Darrell	Lacking gelatin-liquefying capacity	
<i>P. fluorescens</i> ATCC12633	Lacking gelatin-liquefying capacity, chlortetracycline resistance	Dr G. L. Bullock, Leetown, W. Va, U.S.A.
Fish <i>Pseudomonas</i> 1	Lacking growth at 37 and 42 °C, capable of liquefying gelatin	
Fish <i>Pseudomonas</i> 21	Lacking gelatin-liquefying capacity	
<i>P. fluorescens</i> NRRL-B10	Gelatin-liquefying capacity	Dr W. C. Haynes, Peoria, Illinois, U.S.A.
<i>P. denitrificans</i> NRRL-B775	Lacking gelatin-liquefying capacity and incapable of growing at 42 °C	
<i>P. putrefaciens</i> NRRL-B950	Lacking gelatin-liquefying capacity and incapable of growing at 42 °C	
<i>P. stutzeri</i> NRRL-B927	Lacking gelatin-liquefying capacity and incapable of growing at 42 °C	
<i>P. mangiferaeindicae</i> P24	Lacking gelatin-liquefying capacity and incapable of growing at 42 °C	Indian Type Culture Collection, New Delhi, India
<i>P. solanacearum</i> P25	Lacking gelatin-liquefying capacity and incapable of growing at 42 °C	
<i>Bacillus subtilis</i> MB294	Gelatin liquefaction and growth at 37 °C	Dr H. B. Woodruff, Rahway, New Jersey, U.S.A.
<i>Sarcina lutea</i>	Gelatin liquefaction and growth at 37 °C	Dey's Medical Stores (Manufacturing) Pvt. Ltd, Calcutta, India

Pseudomonas 21 and *P. solanacearum* P25 also possessed this property. Only strains of *P. aeruginosa* and *P. stutzeri* could grow at 42 °C. In addition to these strains, *P. mangiferaeindicae* P24 and *P. solanacearum* P25 also grew well at 37 °C. As the strains of *P. fluorescens* varied considerably in their sensitivity towards chlortetracycline, chlortetracycline resistance was used as a marker for intraspecific transformation involving *P. fluorescens*.

Isolation of transforming DNA. The methods of isolation, purification and estimation of DNA were described by Khan & Sen (1967).

Transformation. The transformation procedure, including the control experiments, was as described by Khan & Sen (1967). The sterility of the DNA preparation was checked before each experiment. To test for the presence of contaminant spore formers, DL-alanine (20 µg/ml) and glucose (0.5 %, w/v) were added to the DNA solutions, which were incubated at 30 °C for 24 h to permit germination of spores and then plated out on nutrient agar (Lab

Lemco, 10 g; peptone, 10 g; sodium chloride, 5 g; glucose, 1 g; agar, 20 g; water to 1 l; pH 7.2).

The method of scoring gelatin-liquefying transformants has been described by Khan & Sen (1967). To isolate antibiotic resistant transformants, dilution plates of the transformation mixture were incubated at 30 °C for 1 h and then slowly overlaid with 5 ml of nutrient agar containing the antibiotic, followed by incubation at the same temperature. When heat tolerance was the marker, the experimental and control plates were incubated at 37 and 42 °C.

To study the requirement for metal ions for transformation, competent cells were washed twice with 0.05 M-tris-HCl buffer (pH 7.2) containing 0.5 % glucose, before incubation with and without the salts dissolved in the same buffer. The salts used were: NaCl, KCl, MgSO₄, CaCl₂ and BaCl₂, each at a concentration of 10⁻³ M, and MnCl₂, FeSO₄, ZnSO₄ and CuSO₄, each at a concentration of 10⁻⁴ M.

The requirement for DNA-dependent RNA synthesis for transformation was studied by incorporating actinomycin D, a well known inhibitor of DNA-dependent RNA synthesis, in the transformation mixture. Fish *Pseudomonas* 1 was the donor and *P. fluorescens* ATCC12633 the recipient, with gelatin liquefaction as the marker. The recipient was grown in nutrient broth for 18 h, and 0.45 ml of a suspension containing 2.7×10^7 organisms/ml was mixed with 3.6 ml saline, and 0.45 ml fresh nutrient broth was added to it. For pre-treatment with actinomycin D, 1 ml of the cell suspension was treated with the antibiotic at a concentration of 10 µg/ml (lethal concentration 20 µg/ml) and incubated for 5 min. The cells were then centrifuged, washed and resuspended in 1.0 ml of transformation medium followed by the addition of 1.0 ml DNA dissolved in saline (10 µg/ml). The control tubes included 1 ml cell suspension and 1 ml transforming DNA. The tubes were shaken for 30 min at 30 °C in a rotary shaker (200 rev./min), treated with DNase (10 µg/ml) containing 5 µmol MgCl₂/ml, and the number of transformants formed was calculated by dilution plating in nutrient agar containing 0.5 % gelatin.

The decay of transforming ability due to DNase treatment. The effect of DNase on the transforming ability of donor DNA was studied using *Pseudomonas aeruginosa* *ilvA9* as the recipient strain. Before transformation the DNA of *P. aeruginosa* 78 was digested with DNase (10 µg/ml) for 0, 8, 15, 60, 120, 240 and 300 s at 37 °C. The DNase was inactivated by the addition of trisodium citrate to a final concentration of 10 µmol/ml. Preliminary experiments had revealed that trisodium citrate itself had no inhibitory effect on transformation. The DNase-treated preparation was then applied to the recipient for 10 min. Dilutions of the incubation mixture were plated out in (i) Morihara's (1964) medium alone, (ii) Morihara's medium + valine, and (iii) Morihara's medium + isoleucine, and incubated at 37 °C. Transformation frequency was calculated as described previously.

DNA base composition. DNA base composition was calculated from the thermal denaturation temperature (T_m) of DNA following the method of Marmur & Doty (1962). The thermal denaturation profile was studied with a Hilgers' Spectrophotometer fitted with a temperature control device.

RESULTS

Intraspecific transformation

Intraspecific transformation in *Pseudomonas fluorescens* has been observed with respect to two markers, gelatin liquefaction and chlortetracycline resistance (Table 2). The transformation frequency varied from 0.07 to 0.71 %. *Pseudomonas fluorescens* ATCC12633 and *P. fluorescens* Darrell were transformed by *P. fluorescens* NRRL-B10 DNA. *Pseudomonas*

Table 2. *Intraspecific transformation in Pseudomonas*

Recipient strains	Source of donor DNA	Phenotype selected	$10^{-4} \times$ trans- formants/ ml	$10^{-6} \times$ viable organisms/ ml	Trans- formation frequency (%)
<i>P. fluorescens</i> ATCC12633	<i>P. fluorescens</i> NRRL-B10	Gelatin liquefaction	5.0	7.0	0.71
	<i>P. fluorescens</i> NRRL-B10 (DNase treated)	Gelatin liquefaction	0	7.0	Nil
	<i>P. fluorescens</i> ATCC12633	Gelatin liquefaction	0	6.8	Nil
	<i>S. lutea</i>	Gelatin liquefaction	0	7.0	Nil
	<i>B. subtilis</i>	Gelatin liquefaction	0	7.1	Nil
	<i>P. fluorescens</i> ATCC12633	Chlortetracyclin resistance	0.5	6.4	0.077
	<i>P. fluorescens</i> ATCC12633 (DNase treated)	Chlortetracyclin resistance	0	6.4	Nil
<i>P. fluorescens</i> Darrell	<i>P. fluorescens</i> NRRL-B10	Gelatin liquefaction	1.25	7.5	0.16
	<i>P. fluorescens</i> NRRL-B10 (DNase treated)	Gelatin liquefaction	0	7.5	Nil
	<i>P. fluorescens</i> Darrell	Gelatin liquefaction	0	7.3	Nil
	<i>S. lutea</i>	Gelatin liquefaction	0	7.0	Nil
	<i>Bacillus subtilis</i>	Gelatin liquefaction	0	7.1	Nil
	<i>P. fluorescens</i> ATCC12633	Gelatin liquefaction	0	7.0	Nil
	<i>P. fluorescens</i> ATCC12633 (DNase treated)	Gelatin liquefaction	0	7.0	Nil

Table 3. *Interspecific transformation in Pseudomonas*

Recipient strain	Source of donor DNA	Phenotype selected	$10^{-4} \times$ trans- formants/ ml	$10^{-6} \times$ viable organisms/ ml	Trans- formation frequency (%)
<i>P. fluorescens</i> Darrell	<i>P. aeruginosa</i> 215	Gelatin liquefaction	5.0	6.8	0.7
	<i>P. aeruginosa</i> 215 (DNase treated)	Gelatin liquefaction	0	6.8	Nil
	<i>P. fluorescens</i> Darrell	Gelatin liquefaction	0	6.8	Nil
	<i>P. aeruginosa</i> LCI	Growth at 37 °C	0	7.5	Nil
	<i>P. aeruginosa</i> 78	Gelatin liquefaction and growth at 37 °C	0	7.5	Nil
	<i>P. aeruginosa</i> 215	Growth at 42 °C	0	6.8	Nil
	<i>P. solanacearum</i> p25	Gelatin liquefaction	0	7.4	Nil
	<i>P. stutzeri</i> NRRL-B927	Growth at 42 °C	0	7.4	Nil
	<i>P. aeruginosa</i> LCI	Growth at 37 °C	0	6.4	Nil
	<i>P. aeruginosa</i> 78	Gelatin liquefaction and growth at 37 °C	0	7.1	Nil

Table 3 (cont.)

Recipient strain	Source of donor DNA	Phenotype selected	$10^{-4} \times$ trans- formants/ ml	$10^{-6} \times$ viable organisms/ ml	Trans- formation frequency (%)
<i>P. fluorescens</i> ATCC12633	<i>P. aeruginosa</i> 215	Growth at 42 °C and pigment production	0	6.4	Nil
	<i>P. solanacearum</i> P25	Gelatin liquefaction	0	5.8	Nil
	<i>P. stutzeri</i> NRRL-B927	Growth at 42 °C	0	7.5	Nil
Fish Pseudo- monas 1	<i>P. aeruginosa</i> 215	Growth at 42 °C	0	7.0	Nil
Fish Pseudo- monas 21	<i>P. aeruginosa</i> 78	Growth at 37 °C	5.0	6.2	0.8
	<i>P. aeruginosa</i> 78 (DNase treated)	Growth at 37 °C	0	6.2	Nil
	Fish Pseudomonas 21	Growth at 37 °C	0	6.4	Nil
	<i>P. aeruginosa</i> LC1	Gelatin liquefaction	0	6.4	Nil
	<i>P. aeruginosa</i> 215	Gelatin liquefaction, growth at 42 °C and pigment production	0	6.9	Nil
	<i>Sarcina lutea</i>	Growth at 37 °C	0	7.0	Nil
	<i>Bacillus subtilis</i>	Growth at 37 °C	0	7.0	Nil
<i>P. mangi- feraeindicae</i> P24	<i>P. aeruginosa</i> LC1	Gelatin liquefaction	0	8.2	Nil
	<i>P. aeruginosa</i> 215	Gelatin liquefaction	0	5.1	Nil
	<i>P. fluorescens</i> NRRL-B10	Gelatin liquefaction	0	7.0	Nil
	<i>P. fluorescens</i> 215	Gelatin liquefaction and growth at 42 °C	0	5.2	Nil
<i>P. denitrificans</i> NRRL-B775	<i>P. fluorescens</i> NRRL-B10	Gelatin liquefaction	0	6.1	Nil
	<i>P. aeruginosa</i> 215	Growth at 42 °C and gelatin liquefaction	0	6.1	Nil
<i>P. putrefaciens</i> NRRL-B10	<i>P. aeruginosa</i> 215	Growth at 42 °C and gelatin liquefaction	0	6.1	Nil
	<i>P. fluorescens</i> NRRL-B10	Gelatin liquefaction	0	5.7	Nil

fluorescens Darrell could also be transformed by *P. fluorescens* ATCC12633. None of the recipient strains could be transformed when the transforming DNA was degraded by DNase before transformation. No transformation was observed when the recipients were treated with their own DNA or with the DNA of an unrelated organism such as *Sarcina lutea* or *Bacillus subtilis*.

Interspecific transformation

The results of interspecific transformation involving 23 pairs of strains and four markers are given in Table 3. Only two experiments yielded positive results and no transformants

Fig. 1. Decay of transforming ability of DNA due to DNase treatment. Formation of transformants when medium was supplemented with only isoleucine (○), only valine (●), and no amino acid (△). Donor, *P. aeruginosa* 78; recipient, *P. aeruginosa* *ilvA9* (4×10^6 organisms/ml).

could be detected in the control experiments. *Pseudomonas fluorescens* Darrell acquired the property of gelatin liquefaction when treated with the DNA from *P. aeruginosa* 215, with a frequency of 0.7 %. Neither the Darrell nor the ATCC12633 strain, however, was transformed for gelatin liquefaction from *P. aeruginosa* 78. Fish *Pseudomonas* 21, which is probably a strain of *P. fluorescens*, was transformed at a frequency of 0.8 % when growth at 37 °C was used as a marker. This strain, however, did not acquire the property of gelatin liquefaction from *P. aeruginosa* LC1 DNA.

The decay of transforming ability in the presence of DNase

The observation that the transforming ability of the DNA preparations was lost when treated with DNase suggests that transformation was DNA mediated. To examine this more closely, *P. aeruginosa* *ilvA9* and the prototrophic strain *P. aeruginosa* 78 were chosen. Transforming DNA of the latter strain was treated with DNase for different periods to degrade the DNA to varying degrees, and the DNA preparations thus treated were applied to a competent culture of *P. aeruginosa* *ilvA9* cells (4×10^6 organisms/ml) and incubated for 10 min at 30 °C. Frequency of transformation to prototrophy was then determined. The results (Fig. 1) reveal that 50 % of the activity was lost within 15 s.

DNA base composition

The GC contents of the DNA of the strains used are given in Table 4. The range of GC contents of the strains is rather narrow, varying between 61.0 and 64.4 mol %.

Table 4. Guanine + cytosine (GC) contents of *Pseudomonas* strains

Strain	GC content (mol %)
<i>P. aeruginosa</i> LCI	64.0
<i>P. aeruginosa</i> 215	63.3
<i>P. aeruginosa</i> 78	64.4
<i>P. fluorescens</i> ATCC12633	61.0
<i>P. fluorescens</i> Darrell	62.0
<i>P. fluorescens</i> NRRL-B10	61.0
Fish <i>Pseudomonas</i> 1	62.4
Fish <i>Pseudomonas</i> 21	63.3
<i>P. solanacearum</i> P25	63.3
<i>P. mangiferaeindicae</i> P24	63.0

Table 5. Effect of metal ions on the transformation frequency of *Pseudomonas*

Donor, Fish *Pseudomonas* 1; recipient, *P. fluorescens* ATCC12633; selected marker, gelatin liquefaction.

Condition	$10^{-4} \times$ trans- formants/ ml	$10^{-6} \times$ viable organisms/ ml	Trans- formation frequency (%)
No salt	0	2.4	Nil
BaCl ₂ (10^{-3} M)	7.1	2.3	3.1
CaCl ₂ (10^{-3} M)	5.0	1.4	3.5
MgSO ₄ (10^{-3} M)	9.3	2.1	4.4
NaCl (10^{-3} M)	0.2	1.7	0.12
KCl (10^{-3} M)	0.5	2.0	0.25
MnCl ₂ (10^{-4} M)	0	1.7	Nil
FeSO ₄ (10^{-4} M)	0	1.7	Nil
ZnSO ₄ (10^{-4} M)	0	1.7	Nil
CuSO ₄ (10^{-4} M)	0	1.7	Nil

Requirement of metal ions for transformation

It was observed that addition of NaCl or KCl results in transformation of only a small fraction of recipient cells (Table 5). But addition of such divalent metal ions as Mg²⁺, Ca²⁺ or Ba²⁺ markedly stimulated transformation and the frequency was enhanced several fold. Other divalent metal ions, which are usually included in culture media in relatively small quantities (e.g. Fe⁺⁺, Mn⁺⁺, Zn⁺⁺ and Cu⁺⁺ at a concentration of 10^{-4} M), however, had no beneficial effect and not a single transformant was detected. Since sulphate ions do not appear to be useful for transformation, at least at a concentration of 10^{-4} M, the beneficial effect due to MgSO₄ is probably due to magnesium ions.

Requirement of DNA-dependent RNA synthesis during transformation

Actinomycin D was found to reduce transformation. When *P. fluorescens* ATCC12633 was transformed with the DNA of Fish *Pseudomonas* 1, the reduction was about 90 %. In the absence of actinomycin D, 4.6 % of recipient cells were transformed to the property of gelatin liquefaction; after pretreatment with actinomycin D, only 0.46 % were transformed.

DISCUSSIONS

The investigations described here provide further evidence on the transformability of pseudomonads. One of the major factors controlling transformation is the degree of homology between the transforming and the recipient DNA. According to the recent hybridization studies of Palleroni, Ballard, Ralston & Doudoroff (1972), *P. aeruginosa* and *P. fluorescens* belong to a single DNA homology complex. Apart from experimental conditions the homology of donor and recipient DNA determines the results of transformation experiments. The DNA base composition data of the strains used here suggest considerable genetic homology among the strains which have produced transformants. Five of the eleven combinations of *P. fluorescens* examined for transformation in the present and previous investigations have yielded positive results. But in spite of the close similarity in GC content of the strains used in the present investigation, only 2 out of 23 interspecific combinations produced transformants. The close GC contents of the two phytopathogens *P. mangiferaeindicae* P24 and *P. solanacearum* P25 are in good agreement with the results of interspecific experiments involving these two strains reported previously (Khan & Sen, 1967); however, neither of these pathogens could transform or be transformed by the DNA of any other pseudomonad. The marker phenotype chosen is an important determinant of success in transformation experiments. Two of the three transformants obtained in the intraspecific transformation relate to gelatin liquefaction as a marker; in interspecific transformation the same character was transferred from *P. aeruginosa* DNA to *P. fluorescens* Darrell, but it could not be introduced from the DNA of any other strain of *P. aeruginosa* to any pseudomonad. To assess the taxonomic significance of the results of transformation experiments it is essential that several markers are employed.

Observations on the requirements for transformation in *Pseudomonas* provide some interesting information. In its metal ion requirements, *P. fluorescens* ATCC12633 considerably resembled *Bacillus subtilis* (Young & Spizizen, 1963; Spizizen, Reilly & Evans, 1966) and *B. licheniformis* (Leonard, Matheis, Matheis & Housewright, 1964). Bivalent ions, particularly Mg^{2+} , have been found to be highly beneficial for transformation. Transformation is known to depend on several metabolic processes and several inhibitors of respiration, nucleic acid and protein synthesis; for example, 2,4 dinitrophenol, mitomycin C, chloramphenicol and puromycin have been shown to inhibit transformation (Young & Spizizen, 1961; Balassa, 1963; Suzuki, Yamagami & Shimazu, 1965; Kammen, Wajner & Canellakis, 1966). In the present investigation it has been observed that actinomycin D at a concentration of 10 $\mu\text{g/ml}$ inhibits transformation by 90 %, indicating a requirement for DNA-dependent RNA synthesis.

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