Interspecific Transformation in *Azotobacter*

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SUMMARY

This paper describes the results obtained in experiments on interspecific transformation in *Azotobacter*. When *Azotobacter chroococcum* was transformed with the DNA of *A. vinelandii* 6-10% of the colonies differed from the parent strain in colour, colony character and biochemical characteristics. Some of the transformant strains had higher nitrogen-fixing capacities than had the receptor strain. Transformants obtained by treatment of *A. vinelandii* with the DNA of *A. chroococcum* also had different colour, colony characteristics and capacity to grow in nutrient broth and peptone, but their nitrogen-fixing capacity was decreased.

INTRODUCTION

Genetic transformation in micro-organisms has numerous possibilities apart from its utility in genetic analysis. The characters transferred hitherto by this process include resistance to antibiotics and certain enzymes concerned with the production of capsule, specific polysaccharides, antigens and several cellular metabolites. Ravin (1961) listed some 20 bacterial species which have been found to be transformable. We record here observations concerning interspecific transformation between two species of the free-living nitrogen-fixers *Azotobacter chroococcum* and *A. vinelandii*.

METHODS

The strain of *Azotobacter vinelandii* used in this work was originally obtained through the courtesy of Professor R. H. Burris (University of Wisconsin). In most of the work described here a dark brown non-segregating strain AZCF of *A. chroococcum* was used. This was isolated from local soil samples by the enrichment plate method. The cultures were usually grown on Ashby's nitrogen-free mannitol medium having the following composition: mannitol, 10.0 g.; K_2HPO_4 , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; NaCl, 0.2 g.; $MnSO_4$, trace; $FeCl_3$, trace; distilled water, 1000 ml.

DNA extraction. In preliminary experiments several methods of DNA extraction were tried. The methods of McCarty & Avery (1946), Anagnostopoulos & Spizizen (1961), Marmur (1961) and Colter, Brown & Ellem (1962) all yielded good results. Lysozyme, sodium deoxycholate and sodium lauryl sulphate were useful for the disruption of the bacteria. The method finally adopted was as follows: the bacteria were ground with alumina in 50 ml. of saline + EDTA (containing 0.15 M-NaCl and 0.1 M-EDTA, pH 8.0); 1.5 g. sodium citrate and 2.5 ml. of a 1% (w/v) solution of

sodium deoxycholate were added. After 15 min. 2 ml. of 25 % (w/v) lauryl sulphate were added and the mixture warmed on a water bath at 60° for 10 min. This was followed by addition of solid NaCl to a final concentration M. An equal volume of water-saturated phenol was added followed by centrifugation at 4° for 30 min. The upper aqueous layer was removed and re-extracted with water-saturated phenol for 10 min. in the cold, the mixture being stirred with a magnetic stirrer. The liquid was again centrifuged, the upper layer extracted with ether 4 or 5 times for 10–20 min., traces of ether removed from it and left at 0° for several hours, centrifuged and the supernatant fluid added to an equal volume of 95 % (v/v) ethanol in water. The DNA which was collected on a glass rod was dissolved in 9 ml. dilute saline citrate (0.15 M-NaCl + 0.015 M-sodium citrate) and shaken with an equal volume of chloroform + isoamyl alcohol (24 + 1 by vol.) for 15 min. and centrifuged. This operation was repeated twice and the DNA precipitated by addition of 95 % (v/v) ethanol in water. After centrifugation the DNA was dispersed in half the volume of supernatant 0.15 M-NaCl + 0.015 M-sodium citrate. RNA was removed by incubation with ribonuclease (50 µg./ml.) at 37° for 30 min., followed by centrifugation and further washing with chloroform + isoamyl alcohol. The DNA was re-precipitated with ethanol, dissolved in dilute saline citrate buffer and 0.54 volume isopropyl alcohol added dropwise with stirring. The DNA threads were collected on a glass rod, and placed in 70 % (v/v) ethanol in water and then absolute ethanol was added slowly to a concentration of 95 % (v/v) ethanol. After centrifugation the DNA was finally dissolved in 0.85 % NaCl solution (normal saline).

Genetic markers. The characters of the *Azotobacters* which were used as genetic markers were the brown melanin pigment, colony characteristics, fermentation reactions and nitrogen-fixing capacity. Ashby's nitrogen-free mannitol agar media in Petri dishes were seeded with a suspension of the donor strain, incubated, and the bacteria harvested 72 hr later by gently scraping-off the gummy growth from the surface of the agar. The organisms were then suspended in normal saline and their DNA isolated by the procedure described above.

Transformation. For transformation a loopful of a 22 hr growth of a receptor strain was suspended in 2 ml. normal saline to provide a concentration of 10^7 organisms/ml. and incubated with DNA of the donor strain at 37° for 30 min. to 1 hr. At the end of this incubation period dilutions of the bacterial suspension were plated on Ashby's mannitol agar and incubated at room temperature (28–30°) for 7–14 days. Prolonged incubation was useful for the development of the melanin pigment. Colonies bearing resemblance to any character of the donor strain which was absent from the recipient strain were transferred to agar slopes for further study.

Pigment formation. The melanin-type pigment was extracted according to the procedure of Dawes (1941) with slight modifications: a bacterial suspension was boiled for 10 min. and the insoluble residue digested for 48 hr with 100 ml. of 1 % pepsin in KCl + HCl buffer (pH 2.0); a little toluene was added and the mixture shaken. The sediment was washed with distilled water, centrifuged and hydrolysed with 4 % (w/v) NaOH, the boiling being continued for 15 min. The brown pigment was estimated with a Unicam absorptiometer at 400 mµ (E_{400}). For qualitative comparisons of amounts of pigment, suspensions of young cultures of similar bacterial concentrations were used directly for spectrophotometry.

Nitrogen-fixing capacity was estimated by a micro-Kjeldahl determination of the total-N of the crop of organisms obtained on Ashby's nitrogen-free mannitol medium.

RESULTS

*Transformation of Azotobacter chroococcum with
A. vinelandii DNA*

When *Azotobacter chroococcum* was transformed with the DNA of *A. vinelandii* 6–10 % of the colonies obtained through dilution plating were found to be different from the parent colonies. Colonies of the original parent strain of *A. chroococcum* were dark coloured, smooth and glistening but never raised. Colonies of the donor strain of *A. vinelandii* were colourless and raised. The 'transformants' showed much lighter shades of brown, some being completely colourless and others raised and very

Table 1. *Characters of transformants obtained by treatment of
Azotobacter chroococcum with A. vinelandii DNA*

Organisms	Colony characters	Growth in		Utilization of sucrose
		Nutrient broth	Peptone water	
Parent strains				
<i>A. chroococcum</i> (strain AZCF)	Deep brown, gummy	—	++	++
<i>A. vinelandii</i> (strain AZV)	Colourless, very gummy, raised	++	±	+++
Transformant isolates				
AZ CV1	White, gummy	—	+	++
AZ CV2	White, gummy	—	++	++
AZ CV3	White, gummy	++	+	+
AZ CV4	Faint brown	—	—	+++
AZ CV5	White, gummy	++	++	+++
AZ CV6	White, gummy	++	—	++

±, +, ++, +++ indicate doubtful, slight, good and vigorous growth, respectively.

gummy. In several colonies which produced pigment the rate of pigment production was much slower than by the parent strain. Colonies which were faintly brown or white and gummy were used for detailed investigation. The biochemical characteristics of six selected isolates together with a brief description of the features are given in Table 1. All the transformants fermented mannitol, sucrose and xylose like the parent strain, but they differed to some extent in their capacity to grow on peptone or nutrient broth. The AZCF strain of *A. chroococcum* used in this work was unable to grow in nutrient broth, whereas the isolate of *A. vinelandii* grew well in this medium. The transformants cv3 and cv5 utilized peptone and nutrient broth whereas the isolate cv4 did not grow in either of these media. The isolate cv6 grew well on nutrient broth but not in peptone water.

The pigment content and nitrogen-fixing capacities of these isolates are shown in Table 2. It will be seen that all the transformants shown in this table had very little of the melanin pigment, whereas the receptor strain of *Azotobacter chroococcum* AZCF was quite rich in the pigment. The actual pigment content of the parent strain was much higher than the value shown in the table because the method of extraction used could solubilize only a part of the total pigment, and the pigment which

was tightly bound could not be separated by any method. The pigments of the transformants however were almost completely extracted. The strain of *A. vinelandii* used had a higher capacity for fixing nitrogen than the receptor AZCF strain of *A. chroococcum*. The improvement observed in nitrogen-fixing capacity was quite remarkable and all the transformants studied fixed at least twice as much nitrogen as the receptor AZCF strain of *A. chroococcum*.

Table 2. *Pigment content and nitrogen-fixing capacity of transformant Azotobacter isolates*

Pigment present in 1 mg. dry wt. organism dissolved in 1 ml. 4% (w/v) NaOH.

Organism	Pigment E_{400}	Total N (%)
<i>A. chroococcum</i>	0.10	1.5
<i>A. vinelandii</i>	0.015	3.6
<i>A. chroococcum</i> transformed with <i>A. vinelandii</i> DNA		
AZ CV 1	0.02	3.0
AZ CV 2	0.01	3.1
AZ CV 3	0.02	—
AZ CV 4	0.02	3.0
AZ CV 5	0.02	3.6
AZ CV 6	0.02	3.4

Table 3. *Characters of transformants obtained by treatment of Azotobacter vinelandii with A. chroococcum DNA*

Organism	Colony characters	Growth in		Utilization of sucrose
		Nutrient broth	Peptone water	
Parent strains				
<i>A. chroococcum</i> (strain AZCF)	Deep brown, gummy	—	++	++
<i>A. vinelandii</i> (strain AZV)	White, very gummy, raised	++	±	+++
Transformant isolates				
AZ VC1	Deep brown	—	+	+++
AZ VC2	Deep brown	—	±	++
AZ VC3	Deep brown	—	—	++
AZ VC4	White, raised, slightly wrinkled	—	—	+++

±, +, ++, +++ indicate doubtful, slight, good, vigorous growth, respectively.

Transformation of Azotobacter vinelandii with A. chroococcum DNA

Attempts to transform *Azotobacter vinelandii* to *A. chroococcum* have also been successful. Several transformant strains have been isolated which have the property of melanin production as indicated by the formation of brown pigment. The biochemical characteristics, pigment production, and nitrogen-fixing capacities are given in Tables 3 and 4. While the strain of *A. vinelandii* used grows well on nutrient broth but poorly, if at all, in peptone and the converse is true for *A. chroococcum*, the transformants varied considerably in their capacity to utilize these substrates. All of them, however, could metabolize sucrose, mannitol and xylose,

though in varying degrees. The three transformants vc1, vc2 and vc3 increased considerably in pigment production. The nitrogen-fixing capacity was, however, lowered in all the cases and intermediate values were obtained.

Table 4. *Pigment content and nitrogen-fixing capacity of transformant Azotobacter isolates*

Relative amounts of pigment present in 1 mg. dry wt. organism dissolved in 1 ml. 4 % (w/v) NaOH.

Strain	Pigment E_{400}	Total N (%)
<i>A. chroococcum</i>	0.10	1.5
<i>A. vinelandii</i>	0.01	3.6
<i>A. vinelandii</i> transformed with <i>A. chroococcum</i> DNA		
AZ VC1	0.06	2.4
AZ VC2	0.08	2.7
AZ VC3	0.05	3.4

DISCUSSION

The investigations described here indicate the transformability of *Azotobacter chroococcum* and *A. vinelandii*. The property of the production of brown melanin pigment, alteration in the consistency of the gum produced and the shape of the colony can be acquired by the receptor strain when treated with the DNA of the donor strain. The strains of both the species of *Azotobacter* studied were fairly sensitive to penicillin and streptomycin, and the property of resistance to these antibiotics could not be studied.

Genetic implication of the findings will be presented in a subsequent paper. It is, however, of considerable interest that when *Azotobacter chroococcum* is treated with the DNA of the non-pigment-producing *A. vinelandii* a large number of transformants failed to produce the melanin pigment. Whether this is due to the acquisition of the capacity to elaborate a substance which interferes with the production of melanin, or there was some position effect, cannot be decided at present. The capacity to fix nitrogen can apparently be modified and this observation also merits careful consideration and further biochemical studies.

The significance of interspecific transformation in *Azotobacter* in speciation cannot be assessed at present. Variations in the shape of the colony, pigment content and gum production of the transformants are, however, indicative of such a possibility since strains of *Azotobacter* isolated from a given soil are very often found to vary considerably in their morphological features and biochemical characteristics, as has been found in the case of the transformants described here. The significance of genetic transformation in speciation and variability of soil micro-organisms has been discussed elsewhere (Biswas, Sen & Sen, 1964).

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