Hydrogen Autotrophy as a Transferable Genetic Character of *Nocardia opaca* 1b

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A mating system to transfer the ability of growing autotrophically as a hydrogen bacterium (*aut*⁺ marker) has been developed in *Nocardia opaca* strain 1b. Following filter mating between an *aut*⁺ *trp*⁺ *str*^r strain and an *aut* strain, transconjugants of the phenotype *Aut*⁺ *Trp*⁺ *Str*^s were obtained. The number of recombinant colonies exceeded the number of revertants by a factor of 10⁵. This and other crosses indicated that only the *aut*⁺ marker is transferred. Among other markers tested (growth on lactate and benzoate) no new combinations were found. A few strains of the *Rhodococcus* taxon were tested for their ability to act as recipients. Transconjugants of *Rhodococcus erythropolis* and *Corynebacterium hydrocarboclastus* able to grow autotrophically were isolated. Most transconjugants were able to act as *aut*⁺ donors, but with different recipient spectra. As indicated by the observation of sectorized colonies after autotrophic growth, by the measurement of the frequency of loss of autotrophy compared to auxotrophic markers, and by the effect of mitomycin C treatment, the *aut*⁺ marker does not become integrated in the recipient chromosome in a stable state. It is probably located on a plasmid.

INTRODUCTION

The ability to grow as a hydrogen-oxidizing bacterium, i.e. with gaseous hydrogen as the only electron donor and carbon dioxide as carbon source, occurs among various bacteria belonging to different taxonomic groups. The list of hydrogen-oxidizing bacteria comprises 23 species of 9 genera (Bowien & Schlegel, 1981). Hydrogen-oxidizing bacteria are not obligately bound to chemolithoautotrophic growth and usually grow at higher rates under chemoorganotrophic conditions. Growth as a hydrogen bacterium requires three enzyme functions not normally present in a chemoorganotrophic bacterium: hydrogenase makes hydrogen accessible as a source of energy and reducing power for biosynthesis, and phosphoribulokinase and ribulosebisphosphate carboxylase supplement the enzymes of gluconeogenesis and of the pentose phosphate pathway.

Nocardia opaca strain 1b represents a special type among the hydrogen-oxidizing bacteria for the following reasons. (1) Its doubling time during autotrophic growth is 7.5 h (Aggag & Schlegel, 1973), significantly longer than that of other hydrogen-oxidizing bacteria, e.g. the doubling time of *Alcaligenes eutrophus* is 2.5 h. (2) In contrast to the majority of hydrogen bacteria, which contain only a membrane-bound hydrogenase, and to a minority of species, which contain both a membrane-bound and a soluble NAD⁺-reducing hydrogenase (e.g. *Alcaligenes eutrophus*), *N. opaca* contains only a soluble, NAD⁺-reducing hydrogenase (Aggag & Schlegel, 1974; Schneider & Schlegel, 1977). Furthermore, in *N. opaca* isoenzymes of fructose-bisphosphatase are present, and form A is detectable only in autotrophically grown cells (Amachi & Bowien, 1979). The isoenzymes may be involved in both physiological functions, gluconeogenesis and the Calvin cycle. This type of functional separation has not

been found in *A. eutrophus* (Abdelal & Schlegel, 1974) which is the most intensively studied hydrogen bacterium so far.

Taxonomical considerations prompted us to include *N. opaca* in the conjugational system discovered in *Rhodococcus erythropolis* (Adams & Bradley, 1963; Brownell, 1978) and to make the autotrophic character accessible to genetic analysis (Reh & Schlegel, 1975). A further special feature of the autotrophic properties of this bacterium became evident. Autotrophy was the only character which was transferred by conjugation, and it was never integrated into the recipient's genome in a stable state. The present study was aimed at examining the transfer and loss of the autotrophic character and at detecting a conjugative plasmid.

METHODS

Bacterial strains and bacteriophages. The bacterial strains used are listed in Table 1. *Nocardia opaca* strain 1b (Aggag & Schlegel, 1973) was kept in the culture collection of the Institut für Mikrobiologie, Göttingen. *Nocardia opaca* (ATCC 17039, DSM 364) and *Corynebacterium hydrocarboclastus* (ATCC 15592, DSM 311) were provided by the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen. *Rhodococcus erythropolis* cE2 and Ce3, and the bacteriophages ϕ EC, ϕ C and MNP7 were received from Dr G. H. Brownell (Medical College of Georgia, Augusta, Ga. U.S.A.). The bacteriophage ϕ B1 appeared as a contaminant in a culture of *Nocardia opaca* 1b growing autotrophically in a fermenter.

Media, growth conditions, and maintenance of strains. The mineral medium (MM) was that of Schlegel *et al.* (1961). It was solidified by the addition of 15 g agar l⁻¹. For autotrophic growth, cultures were incubated under an atmosphere of H₂/O₂/CO₂ (80:10:10; by vol.). For heterotrophic growth, 0.5% (w/v) fructose (FM) or 0.5% (w/v) fructose plus 0.1% (w/v) tryptose and 0.1% (w/v) yeast extract (FTY) or 0.5% (w/v) of other carbon sources were added, and incubation was under air.

Antibiotics were used at the following concentrations: streptomycin sulphate, 300 µg ml⁻¹; rifampicin, 20 µg ml⁻¹. The temperature for growth was 30 °C. The cell suspensions were diluted in saline containing 9 g NaCl, 0.2 g MgSO₄·7H₂O and 1 g Triton X-100 l⁻¹. For maintenance, the heterotrophic strains were kept on agar slants (1.6% nutrient broth), and the autotrophic strains were grown on mineral agar slants under the gas mixture mentioned. The cultures were kept for many months at 4 °C without loss of viability.

Plaque test. Droplets of diluted lysates were placed on soft agar plates of peptone/yeast extract medium (Brownell *et al.*, 1967) containing the dispersed bacteria. Formation of single plaques indicated the phage sensitivity of the bacteria.

Isolation of mutants. Mutation was induced by u.v. irradiation or by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMG). For u.v. treatment, heterotrophically growing cells were harvested in the stationary phase, washed and resuspended in saline (Triton-free). A 10 ml portion of bacterial suspension (2–5 × 10⁷ cells ml⁻¹) in an open Petri dish was exposed to a dose of u.v. irradiation (254 nm) which inactivated 99.9% of the cells. For NMG mutagenesis, growing cell suspensions were transferred to 4 vol. fresh FTY medium, and 800 µg NMG ml⁻¹ was added. After shaking at 30 °C for 30 min the suspension was diluted with saline and plated out. Spontaneous mutants resistant to streptomycin or rifampicin were selected from FTY agar containing 300 µg streptomycin ml⁻¹ or 20 µg rifampicin ml⁻¹.

With the aim of isolating Aut⁻ variants the following curing agents were applied: mitomycin C, acridine orange, nalidixic acid, novobiocin and hydroxyurea. Flasks containing FTY medium and various concentrations of these agents were inoculated with autotrophically grown bacteria (10⁴–10⁵ cells ml⁻¹). The suspensions without curing agents were diluted after 3 d, and the cultures with the highest concentrations of curing agents just allowing growth were diluted after 6 d; the diluted suspensions were spread on FTY agar. In addition, two mutagenic agents – ethyl methanesulphonate (EMS) and 8-methoxypsoralen – were applied. The former was applied at a concentration of 40 µg ml⁻¹ for 3 h. The latter was added to suspensions of the washed cells at a concentration of 50 µg ml⁻¹ for 20 min. Then the suspension was diluted tenfold and exposed to u.v. irradiation resulting in an inactivation of 99.9% of the cells. The colonies that appeared were transferred by replica plating on to mineral agar plates, which were subsequently incubated under H₂/O₂/CO₂, and on to FM agar plates. In some cases the Aut⁻ derivatives occurred in sectorized colonies. These were not studied further.

Crosses. Two mating methods were used. (1) 'Agar mating'. Colonies (3 d old) of the various mating partners were taken from FTY agar and streaked at right angles to each other. Usually the autotrophic partner was streaked first, and the potential recipients were applied subsequently. After incubation for 3 d, the colony pattern was transferred by replica plating to selective media. The donor strain was eliminated by selection on MM/streptomycin agar or by selection against an auxotrophic marker. Colonies appearing in the area of the crossing streaks after 1 or 2 weeks indicated successful matings.

Table 1. *Bacterial strains*

Species	Strain	Relevant genotype*	Parent strain(s)	Method of production†
<i>Nocardia opaca</i> 1b	MR11	<i>aut</i> ⁺	Wild type	
	MR147	<i>aut</i>	MR11	S
	MR1450	<i>aut str</i>	MR147	SA
	MR1463	<i>aut ben lac rif str</i>	MR1450	UV, SA, UV
	MR1857	<i>aut</i> ⁺ <i>ben lac rif str</i>	MR11 × MR1463	C
	MR1859	<i>aut</i> ⁺ <i>ben lac rif str</i>	MR11 × MR1463	C
	MR113	<i>aut</i> ⁺ <i>phe</i>	MR11	NMG
	MR1439	<i>aut lac str</i>	MR147	NMG, SA
	MR1852	<i>aut</i> ⁺ <i>lac str</i>	MR113 × MR1439	C
	MR1861	<i>aut</i> ⁺ <i>str</i>	MR11 × MR1450	C
	MR1469	<i>aut trp str</i>	MR147	UV, SA
	MR1863	<i>aut</i> ⁺ <i>trp str</i>	MR11 × MR1469	C
	MR1864	<i>aut</i> ⁺ <i>str</i>	MR151 × MR1450	C
<i>Rhodococcus erythropolis</i> Ce3	MR12		Wild type	
	MR1235	<i>str</i>	MR12	SA
	MR1621	<i>aut</i> ⁺	MR113 × MR12	C
	MR1625	<i>aut</i> ⁺ <i>str</i>	MR151 × MR1235	C
<i>Rhodococcus erythropolis</i> cE2	MR13		Wild type	
	MR132	<i>str</i>	MR13	SA
	MR1623	<i>aut</i> ⁺ <i>str</i>	MR11 × MR132	C
<i>Nocardia opaca</i> ATCC 17039	MR15		Wild type	
	MR152	<i>str</i>	MR15	SA
	MR151	<i>aut</i> ⁺	MR1863 × MR15	C
<i>Corynebacterium hydrocarboclastus</i> ATCC 15592	MR17		Wild type	
	MR171	<i>str</i>	MR17	SA
	MR206	<i>aut</i> ⁺ <i>str</i>	MR11 × MR171	C

* Genotype symbols: *aut*, autotrophy; *str*, streptomycin resistance; *rif*, rifampicin resistance; *ben*, benzoic acid; *lac*, lactose; *phe*, phenylalanine; *trp*, tryptophan.

† S, spontaneous occurrence after growth on complete medium; SA, spontaneous, selection on agar plates containing antibiotics; UV, u.v. irradiation; NMG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis; C, after conjugation.

(2) 'Filter mating'. For quantitative experiments the mating procedure of Matney & Achenbach (1962) was used. Donor and recipient cells were grown in FTY medium until the stationary phase was reached, and then 0.1 ml of the recipient culture and 0.2 ml of the donor suspension diluted to 10⁻² were mixed in 2.5 ml FTY medium and sucked on to a membrane filter (pore diameter 0.45 µm; 200 mm² effective filter area). The filters carrying the uniformly mixed cells were incubated on FTY agar for the time periods indicated. Subsequently, the bacterial lawn was collected by rinsing with saline, homogenized, and diluted, and samples were plated on to selective media.

RESULTS

Loss of autotrophy

Variants of *Nocardia opaca* strain 1b unable to grow chemolithoautotrophically could be isolated from heterotrophic cultures without applying mutagenic agents (Table 2). These variants occurred more abundantly than each of the auxotrophic mutants. Treatment with u.v. or mitomycin C increased the frequency of Aut⁻ variants sixfold and ninefold, respectively. Surprisingly, mutagenic agents such as methoxypsoralen, NMG and EMS, as well as nalidixic acid, novobiocin and hydroxyurea, resulted in an even greater increase in the frequency of Aut⁻ variants. Growth in the presence of sublethal concentrations of acridine orange (0.5 µg ml⁻¹), acriflavin (0.5 µg ml⁻¹), ethidium bromide (0.5 µg ml⁻¹), and rifampicin (1 µg ml⁻¹) did not result in an increased frequency of Aut⁻ variants. Aut⁻ variants, whether occurring spontaneously, or isolated after mutagenic treatment, were completely stable; after plating 10⁹ cells on mineral agar and incubating under H₂/O₂/CO₂,

Table 2. *Frequency of Aut⁻ variants and auxotrophic mutants*

Treatment	Number of colonies scored	Frequency of Aut ⁻ variants*	Frequency of auxotrophic mutants† (%)
None‡	1497	0.3	<0.07
Acridine orange (0.5 µg ml ⁻¹)	2875	0.4	0.07
U.v. light (254 nm)	956	1.8	1.2
Mitomycin C (0.1 µg ml ⁻¹)	519	2.7	0.8
Methoxypsoralen (100 µg ml ⁻¹) plus u.v. light (366 nm)	720	8.3	1.0
NMG (450 µg ml ⁻¹)	1440	3.0	2.0
EMS (40 µg ml ⁻¹)	1816	7.5	0.2
Nalidixic acid (60 µg ml ⁻¹)	2792	8.1	0.07
Novobiocin (3 µg ml ⁻¹)	2010	7.7	<0.05
Hydroxyurea (150 µg ml ⁻¹)	368	16.3	0.8

* Aut⁻ variants were selected by their ability to grow on FM agar but inability to grow chemolithoautotrophically on MM agar.

† Auxotrophic mutants were selected by their ability to grow on FTY agar plates but inability to grow on FM agar.

‡ Spontaneous occurrence after growth on FTY medium (15 generations).

Table 3. *Mating of Nocardia opaca strains MR1863 (aut⁺ trp str) × MR147 (aut)*

About 10⁸ cells of each strain were sucked on to a membrane filter either without or after mixing; the filters were incubated on FTY agar for 48 h. The bacterial lawn was collected by rinsing with saline, homogenized and diluted, and samples were plated on selective media.

Filter	Viable counts on selective agar plates				
	MM H ₂ /O ₂ /CO ₂	MM + Str H ₂ /O ₂ /CO ₂	FM + Str Air	MM + Trp + Str H ₂ /O ₂ /CO ₂	FM Air
MR1863 × MR147	1.5 × 10 ⁷	7.2 × 10 ¹	2.4 × 10 ²	6.0 × 10 ⁸	4.2 × 10 ⁹
MR1863	1.4 × 10 ²	1.4 × 10 ³	4.8 × 10 ²	3.7 × 10 ⁹	2.6 × 10 ³
MR147	10	10	10	10	6.4 × 10 ⁹

Aut⁺ revertants were never detected. Loss of autotrophy is, therefore, a frequent and apparently irreversible event.

Transfer of autotrophy between strains of Nocardia opaca

As shown previously, the aut⁺ marker can be transferred from Aut⁺ strains of *N. opaca* 1b to Aut⁻ strains (Reh & Schlegel, 1975). Since matings in liquid cultures led to non-reproducible results, the mating partners were mixed on membrane filters which were laid on FTY agar.

The results of a three-factor cross between an aut⁺ trp str (MR1863) and a aut (MR147) partner are presented in Table 3. The selective marker was autotrophic growth. The only combination found was that of aut⁺ with trp⁺ and str⁺ (Table 3, column 1). The number of recombinant colonies exceeded the number of revertants by a factor of 10⁵. The recombinants were stable with respect to the trp and str markers, but they segregated Aut⁻ variants; this is discussed below. The two other possible marker combinations, which should have resulted from chromosomal transfer, were not detectable.

The transfer frequency was not affected by incubating the membrane filters on agar plates containing DNAase I, RNAase or proteinase K. No transfer occurred in agar matings if the autotrophic partner had been killed by chloroform vapour prior to the addition of the heterotrophic partner. Furthermore, electron microscopic inspection of concentrated

Fig. 1. Kinetics of *aut*⁺ transfer. About 2.6×10^6 cells of *Nocardia opaca* 1b MR11 (*aut*⁺) and 10^8 cells of MR1463 (*aut* *lac* *ben* *rif* *str*) were mixed and sucked on to membrane filters, and the filters were incubated on FTY agar plates at 30 °C. At the times indicated the bacterial lawn was rinsed from one of the filters, evenly dispersed in suspension and plated out for counting. ○, Turbidity (A_{436}); ●, viable count of MR1463 on FTY plates containing streptomycin; □, viable count of MR11 on lactose agar; ■, transconjugants growing on MM agar containing streptomycin.

supernatants of cell suspensions did not reveal the presence of phage particles (unpublished observation). In liquid media the transfer appeared to be casual; evidently intimate cell contact on solid surfaces was required for the transfer. From these controls and observations the conclusion was drawn that in *Nocardia opaca* genetic transfer is due to conjugation.

The kinetics of the transfer of the *aut*⁺ marker was followed in matings of wild-type *N. opaca* 1b with the mutant MR1463 (*aut* *ben* *lac* *rif* *str*) (Fig. 1). The increase of cell density during growth on membrane filters, measured as the turbidity (A_{436}) of the evenly resuspended cells, revealed two phases of exponential growth, a phase of rapid growth and – starting at about the 15th hour – a phase of slow growth. During the first phase, the cell morphology was characterized by hyphae and long rods, during the second phase by irregular short rods. The morphological transition was reflected by the biphasic curve of the viable cell count. It was during this transitional period that the number of *aut*⁺ *str* transconjugants increased by five orders of magnitude.

Non-selected markers were examined in 50 transconjugants. All of them were Lac[–] Ben[–] Rif^r. Attempts to isolate transconjugants of the type Lac⁺ Str^r and Ben⁺ Str^r failed. Plating on lactose/streptomycin and benzoate/streptomycin agar resulted in colony counts which were not higher than the number of revertants. These observations indicate that transfer of complete genomes did not occur; the genetic transfer appeared to be restricted to a part of the genome which harbours the genetic information for autotrophic growth. The function of the wild type was that of the donor strain, and the mutant MR1463 was the recipient.

The yield of transconjugants proved to be dependent on the ratio of donor cells and recipient cells in a linear fashion within two orders of magnitude (Fig. 2). When, however, the mixing ratio of donor and recipient cells exceeded 1 : 100, no further increase of the number of transconjugants occurred, for unknown reasons. The growth rates of the donor and recipient cells in the bacterial lawn were almost the same. If, at the favourable donor : recipient ratio, the cell density on the membrane filters was varied, the yield of transconjugants varied only slightly.

Fig. 2. Dependence of *aut*⁺ transfer on the number of mating partners per filter. For each assay a constant number of cells of MR1463 (recipient) was mixed with a varied number of cells of MR11 (donor) on membrane filters. After 48 h the bacterial lawns were rinsed from the filters, and the cells were evenly resuspended, diluted, and plated for incubation under selective conditions. The initial numbers of recipient and donor cells are indicated by the dashed lines; ○, cells of MR1463 after 48 h; ●, cells of MR11 after 48 h; □, autotrophic transconjugants after 48 h.

Table 4. Influence of medium on the yield of transconjugants formed in the cross MR11 (*aut*⁺) × MR1463 (*aut ben lac rif str*)

Additions to MM agar	Growth (<i>A</i> ₄₃₆)*	Number of transconjugants (c.f.u. per filter)†
Fructose (2%) + tryptose (0.1%) + yeast extract (0.1%)	43.9	9.4 × 10 ²
Fructose (0.5%) + tryptose (0.1%) + yeast extract (0.1%)	24.1	4.6 × 10 ⁵
Fructose (0.1%) + tryptose (0.1%) + yeast extract (0.1%)	13.7	2.4 × 10 ⁴
Fructose (0.5%)	27.4	2.0 × 10 ⁴
Nutrient broth (0.8%)	11.6	4.1 × 10 ³
Pyruvate (0.5%)	11.9	1.6 × 10 ³
Hexadecane (in gas phase)	7.3	2.6 × 10 ⁵
Tryptose (0.1%) + yeast extract (0.1%)	5.7	5.0 × 10 ³
Sodium citrate (0.5%)	5.1	1.1 × 10 ³
H ₂ /O ₂ /CO ₂ gas phase	2.5	9.6 × 10 ³

* Turbidity of the bacterial suspension after collecting cells from the membrane filter.

† The number of colony-forming units (c.f.u.) was determined on MM/streptomycin plates incubated under H₂/O₂/CO₂.

The yield of transconjugants was remarkably influenced by the medium on which conjugation occurred (Table 4). FTY agar resulted in the highest yield of transconjugants. As the table indicates, there was a slight correlation between the cell density reached within 48 h growth and the number of transconjugants; substrates that allowed only low cell yields also resulted in low yields of transconjugants. There were two exceptions to this. (i) Hexadecane, which is an excellent substrate for growth of *N. opaca* in liquid media, made a high yield of transconjugants possible, but supported only a low cell yield. The limited cell yield was probably due to the fact that hexadecane was supplied to the bacterial lawn on the membrane filters via the gas phase, and thus limited growth. (ii) An increased fructose concentration (2%) resulted in a high cell density, but in a low yield of transconjugants. Under these

Table 5. Interstrain transfer of *aut*⁺ from *Nocardia opaca* 1b wild type and from transconjugants into streptomycin-resistant strains

The results were obtained by agar mating (see Methods).

Species	Recipient strain	Number of transconjugants*					Properties of recipients and transconjugants†					
		Donor strain:					Lac	Ben	φEC	φC	MNP7	φB1
		<i>N. opaca</i> MR11	<i>N. opaca</i> MR1863	<i>R. erythro-</i> <i>polis</i> MR1621	<i>N. opaca</i> MR151							
<i>N. opaca</i> 1b	MR1450	+	—	—	+		+	+	—	—	—	+
<i>N. opaca</i> 1b	MR1463	++	+	—	—		—	—	—	—	—	+
<i>R. erythropolis</i> Ce3	MR1235	++	—	—	++		—	—	+	+	+	—
<i>R. erythropolis</i> cE2	MR132	+	—	—	—		—	—	+	—	+	—
<i>C. hydrocarbonolastus</i>	MR171	++	—	+	—		—	+	+	+	—	—
<i>N. opaca</i> ATCC 17039	MR152	—	+	—	++		+	+	—	—	—	—

* —, No transconjugants; +, 1–20 colonies in the crossing area; ++, confluent growth of 20 or more (up to several hundred) colonies.

† +, Growth or plaques; —, no growth or plaques.

Table 6. *Curing frequencies of transconjugants*

FTY liquid media with and without mitomycin C were inoculated with bacteria from single autotrophically grown colonies as described in Methods. Samples of these cultures were diluted after 6 d (after 3 d for controls) and spread on FTY agar. After incubation, 2000–4000 colonies from each strain were transferred by replica plating on to mineral agar plates and scored for autotrophic growth. The frequency of Aut⁻ colonies is expressed as a percentage of the total colonies.

Species	Strain	Origin	Spontaneous frequency (%)	Curing by mitomycin C (%)
<i>N. opaca</i> 1b	MR11	Wild type	0.3	3.7
	MR1857	MR11 × MR1463	2.6	16.5
	MR1859	MR11 × MR1463	6.0	12.3
	MR1861	MR11 × MR1450	2.4	15.9
	MR1864	MR151 × MR1450	8.4	52.6
<i>R. erythropolis</i> Ce3	MR1621	MR113 × MR12	19.8	73.1
	MR1625	MR151 × MR1235	100	100
<i>R. erythropolis</i> cE2	MR1623	MR11 × MR132	73.1	100
<i>N. opaca</i> ATCC 17039	MR151	MR1863 × MR15	29.4	97.0
<i>C. hydrocarboclastus</i>	MR206	MR11 × MR171	100	100

conditions fragmentation of the cell hyphae and long rods did not occur; this again indicates a correlation between morphogenesis and genetic transfer.

The transfer of autotrophy to other species

Nocardia opaca belongs to the *Rhodococcus* taxon (Goodfellow & Alderson, 1977). Among the strains and species of this genus genetic transfer and recombination are well known. Several species of this taxon were therefore included as potential recipients of the autotrophic marker (Table 5). Autotrophic transconjugants were obtained in most cases, provided the wild-type of *N. opaca* 1b (MR11) was used as the donor strain.

Transconjugants isolated from the membrane filters were examined for the non-selective markers and proved to have the markers of the heterotrophic partners. Some transconjugants were examined for their ability to function as donors of the *aut*⁺ marker. All of them had this ability; however, the infertility of certain matings indicated that apparently each donor has a different spectrum of recipients with which it can mate successfully.

Instability of transconjugants

Autotrophically grown colonies of various transconjugants showed sectorized colony growth; the sectors were areas of diminished growth. Sectorized growth was especially pronounced in colonies of transconjugants of heterologous matings. The loss of autotrophy occurred at a high rate even among transconjugants resulting from crosses between *N. opaca* 1b partners derived from colonies which did not show obvious sectorized growth (Table 6). The frequency of loss was at least one order of magnitude higher than in the wild type.

Aiming at the selection of a more stable strain, the transconjugant MR1621, characterized by an especially low stability, was cultivated under autotrophic conditions for four transfers. Neither this procedure nor the growth of this strain under autotrophic conditions for several years for maintenance in the culture collection resulted in an increase of stability. Recombination of the autotrophic genes into the bacterial chromosome therefore appears rather improbable.

DISCUSSION

The genetic exchange between *Nocardia opaca* 1b and *Rhodococcus erythropolis* described in this paper is evidently different from the mating system in *R. erythropolis* as described by Brownell (1978). (i) The genetic exchange concerns only a single marker, namely autotrophic

growth. Evidence for the transfer of further markers has been found neither by selection for other markers nor by analysis of the autotrophic transconjugants. On the basis of these results the involvement of two complete genomes seems improbable. (ii) The instability of the transconjugants is consistent with the extrachromosomal location of the *aut*⁺ marker. Even the transconjugants formed in homologous matings of *Nocardia opaca* 1b lost the autotrophic character at a frequency ten times higher than did the wild type. In the transconjugants resulting from heterologous matings the loss frequency was a further order of magnitude higher.

There is a faint similarity of our mating system to the mating types of *Nocardia opaca* (Brownell, 1978) and of *Mycobacterium smegmatis* (Mizuguchi *et al.*, 1976) with respect to the spectra of recipients giving fertile mating with the wild type and the transconjugants as donors (Table 5). These phenomena can not yet be explained.

There are two convincing criteria for the location of the genetic information for autotrophic growth on a plasmid: (i) the transfer of the autotrophic character occurs independently of chromosomal markers, and (ii) the autotrophic character maintains its autonomy in the recipient cell and can be irreversibly lost. These were the original criteria for the resistance factors of the *Enterobacteriaceae* (Watanabe, 1963). Specific arguments opposing the plasmid hypothesis of the autotrophic character in *N. opaca* 1b concern the failure to 'cure' the bacterium by means of intercalating dyes or rifampicin.

The location on a plasmid of the *aut*⁺ marker, comprising the determinants at least for hydrogenase and the component enzymes of the Calvin cycle, is an especially attractive hypothesis; it could explain the vast distribution of the ability to grow autotrophically among diverse taxonomic groups of soil and water bacteria, including nitrogen-fixing bacteria such as *Rhizobium japonicum*, *Derxia gummosa* and *Azospirillum lipoferum*. In this respect hydrogen autotrophy may be envisaged as only one of the various peripheral catabolic pathways present in soil bacteria in which plasmid-coded enzymes are involved (Wheelis, 1975).

During the isolation of mutants of *Alcaligenes eutrophus* H16, no observations indicative of plasmids coding for the whole set of autotrophic enzymes were made (Schink & Schlegel, 1978). Mutants derived at high frequencies from *A. eutrophus* type strain, from *A. eutrophus* strain H1 and from *Pseudomonas facilis*, by mitomycin C treatment (K. Andersen, W. R. King & R. C. Tait, personal communication; Pootjes, 1977), lacked hydrogenase activities, but contained the information for ribulosebiphosphate carboxylase and phosphoribulokinase. Physical evidence was provided for the existence in the wild-type strains of a large plasmid (mol. wt approximately 200×10^6) which is absent from the hydrogenase-negative mutants. In contrast, treatment of *A. eutrophus* type strain by classical mutagenic agents resulted in various classes of mutants having only low specific activities of ribulosebiphosphate carboxylase (Andersen, 1979). Agar mating of *A. eutrophus* H16 with hydrogenase-negative mutants of various strains of this species resulted in transconjugants which had recovered the ability to grow autotrophically and to form hydrogenase. When using spontaneous hydrogenase-negative mutants of *A. eutrophus* type strain as recipients, the transfer frequency was particularly high. These strains were plasmid-free, while donors, transconjugants and mutagen-treated hydrogenase-negative strains contained a large plasmid (mol. wt 260×10^6) designated pHG1. It is concluded that a determinant for hydrogenase activity is carried by this self-transmissible plasmid (Friedrich *et al.*, 1981).

A plasmid with a molecular weight of about 190×10^6 carrying a determinant for hydrogenase activity beside a determinant for nodulation activity has been found in *Rhizobium leguminosarum* strain 128C53. Although it is not self-transmissible it could be transferred to other strains of *R. leguminosarum* after recombination with a transmissible *R. leguminosarum* plasmid (Brewin *et al.*, 1980). Whether the structural genes or other determinants for hydrogenase expression are carried by the plasmids has so far not been revealed.

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