Hydrogen autotrophy of *Nocardia opaca* strains is encoded by linear megaplasms

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Several linear megaplasms were detected in the facultatively lithoautotrophic Gram-positive bacterium *Nocardia opaca*. The wild-type strain MR11 contains, in addition to the cccDNA plasmids pHG31-a and pHG31-b, the linear plasmids pHG201 (270 kb), pHG202 (400 kb) and pHG203 (420 kb). The wild-type strain MR22 contains, in addition to the cccDNA plasmid pHG33, the linear plasmids pHG204 (180 kb), pHG205 (280 kb) and pHG206 (510 kb). After preparation of DNA from cells embedded in agarose, the linear plasmids were demonstrated by pulsed-field electrophoresis. By means of DNA probes for genes of soluble hydrogenase and ribulose-bisphosphate carboxylase, the conjugative plasmids pHG201 and pHG205 were shown to be the carriers of the genetic information for these enzymes. A restriction map of pHG201 for the enzymes *AsnI*, *SpeI*, *XbaI* is presented.

Introduction

The Gram-positive bacterium *Nocardia opaca* 1b is a strict aerobe able to grow lithoautotrophically on hydrogen and carbon dioxide and heterotrophically on organic substrates such as fructose, gluconate or hydrocarbons. This bacterium provided the first system for the conjugational transfer of the genetic information for hydrogen-autotrophic growth (Aut character) to non-autotrophic mutants or species of the genus *Rhodococcus* (Reh & Schlegel, 1975). The lack of transfer of auxotrophic markers and the instability of the Aut character in the donor wild-type strain and in transconjugants indicated the location of the genes responsible for the Aut character on a plasmid (Reh & Schlegel, 1981). Enzyme studies confirmed that the genetic information for hydrogen-autotrophic growth is transferred *en bloc* from *N. opaca* to Aut⁻ mutants of the same strain or to *Rhodococcus erythropolis* (Ecker *et al.*, 1986). Three cccDNA plasmids were detected in *N. opaca*; however, these plasmids were detected in the wild-type Aut⁺ donor as well as in its Aut⁻ variants (Reh, 1981). Further studies on the plasmids of the original strain of *N. opaca* MR11 and a newly isolated wild-type strain MR22 revealed that at least one of the plasmids of each strain

carried the genetic information for thallium resistance but not that for the Aut character. Even plasmid-free Aut⁺ strains turned out to be able to function as donors of the Aut⁺ character (Sensfuss *et al.*, 1986). Thus the Aut character had to be supposed to be chromosomally located as a special, frequently transferable element or as an extrachromosomal element present in a special form.

The present study was aimed at the detection of a DNA element which conferred upon transconjugants the ability to grow autotrophically with hydrogen. The study took advantage of pulsed-field electrophoresis techniques combined with a new method of DNA preparation and resulted in the detection of linear plasmids in both wild-type strains and autotrophic transconjugants of *N. opaca*.

Methods

Bacterial strains and growth conditions. Sources and references of the bacterial strains used are listed in Table 1. Strains of *Nocardia opaca* were grown heterotrophically as described previously (Sensfuss *et al.*, 1986). For isolating autotrophic transconjugants an agar mating procedure was used (Reh & Schlegel, 1981).

Preparation of cccDNA. cccDNA was prepared by an alkaline lysis procedure as described by Sensfuss *et al.* (1986).

Preparation of DNA for detection of linear megaplasms. Cultures of *N. opaca* were grown and treated with sucrose and glycine as described by Sensfuss *et al.* (1986). After harvesting, the pellet was resuspended in 15 µl EET (0.1 M-EDTA; 10 mM-EGTA; 10 mM-Tris; pH 8.0) per mg

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Abbreviations: ccc, covalently closed circular; CHEFE, contour-clamped homogeneous-field electrophoresis; Rubisco, ribulose-bisphosphate carboxylase.

Table 1. *Nocardia opaca* strains and their plasmids

Strain	Relevant phenotype*	Plasmids (sizes in kb)		Parent strain(s)	Source or reference
		Form of plasmid DNA: ccc	linear		
MR11	Aut ⁺	pHG31-a (140) pHG31-b (17)	pHG201 (270) pHG202 (400) pHG203 (420)	Wild-type	DSM 427; Aggag & Schlegel (1973)
MR22	Aut ⁺	pHG33 (110)	pHG204 (180) pHG205 (280) pHG206 (510)	Wild-type	DSM 3346; Sensfuss <i>et al.</i> (1986)
MR222	Aut ⁻		pHG206	MR22	Sensfuss <i>et al.</i> (1986)
MR2226	Aut ⁻ Str ^r			MR222	Sensfuss <i>et al.</i> (1986)
MR2246	Aut ⁺ Str ^r		pHG201	MR11 × MR2226	Sensfuss <i>et al.</i> (1986)
MR2247	Aut ⁺ Str ^r	pHG31-a	pHG201	MR11 × MR2226	Sensfuss <i>et al.</i> (1986)
MR2248	Aut ⁻ Str ^r	pHG31-a		MR11 × MR2226	Sensfuss <i>et al.</i> (1986)
MR2252	Aut ⁺ Str ^r	pHG33	pHG204 pHG205	MR22 × MR2226	This study
MR2253	Aut ⁺ Str ^r		pHG205Δ (210)	MR22 × MR2226	This study

*Aut, lithoautotrophic growth; Str, streptomycin.

wet weight. Embedding of cells and preparation of DNA by lysing the cells with lysozyme, SDS and proteinase K was performed according to McClelland *et al.* (1987).

Electrophoresis. For conventional electrophoresis, 1% agarose gels in TBE (89 mM-Tris; 89 mM-borate; 2 mM-EDTA; pH 8.0) were used. Electrophoresis was performed at 2.5 V cm⁻¹ for 6 h. Contour-clamped homogeneous-field electrophoresis (CHEFE) was carried out using the Pulsaphor system (Pharmacia LKB). For all separations, 1% agarose slab gels (15 × 15 × 0.5 cm) in 0.5 × TBE were used. Buffer was kept at 7 °C, and gels were run at 6 V cm⁻¹. DNA bands were stained with ethidium bromide and visualized by means of a UV-transilluminator. Concatemers of λ DNA (λ ladder) were used as high-molecular-mass DNA standard (Anand, 1986).

Isolation and manipulation of linear megaplasmid DNA. For isolation of pHG201 DNA, agarose plugs from MR2246 and MR11 were prepared as described above, with the modification that the pellet was resuspended in 1 µl EET per mg wet weight. Linear plasmid DNA was obtained either by electroelution of MR2246 DNA agarose plugs or by a preparative CHEFE gel. Electroelution was performed with an apparatus (HSB-Elutor) obtained from Biometra, Göttingen, FRG. After electroelution the DNA solution was dialysed against water. If necessary the solution was concentrated by treating the dialysis bag with Sephadex G-150. For isolation of plasmid DNA from preparative gels, agarose plugs of MR11 were loaded onto a 0.7% low-melting-point agarose gel. CHEFE was performed for 24 h in 0.5 × TBE. The pulse time was steadily increased from 10 s at the beginning to 80 s during the run time. After separation the band of pHG201 was cut out and stored in 0.5 × TBE at 4 °C.

Restriction digestion. To 1 µg dissolved plasmid DNA, 0.1 vol. 10-fold concentrated appropriate restriction buffer and 10 U of restriction endonuclease were added. Digestion was performed at 37 °C for 3 h. After digestion, the reaction was terminated by addition of 0.25 vol.

stop solution [0.1 M-EDTA, pH 8; 40% (w/v) sucrose; 0.15% (w/v) bromophenol blue]. Agarose plugs containing approximately 0.5 µg plasmid DNA were equilibrated twice with 200 µl of restriction buffer for 0.5 h. Then the buffer was replaced by 30–50 µl fresh restriction buffer. Plasmid DNA was digested with 10 U of the endonuclease. After 2 h an additional 10 U of endonuclease was added. After 5 h at 37 °C the reaction was terminated by adding 10–20 µl of stop solution.

Southern hybridization. For fragmentation of large DNA fragments, the agarose gel was incubated twice in 0.25 M-HCl for 15 min (Wahl *et al.*, 1979). The DNA was then denatured with 0.1 M-NaOH, 1.5 M-NaCl for 45 min. For neutralization, the gel was incubated for 45 min in 0.2 M-Tris/HCl, 2 × SSC (1 × SSC: 0.15 M-NaCl, 15 mM-trisodium citrate; pH 7.5). DNA was transferred to nitrocellulose filters (BA85, Schleicher and Schuell) with 10 × SSC by the Southern blot procedure (Southern, 1975) and fixed by UV-irradiation (Khandjian, 1987). All hybridization reactions were performed at 42 °C with gentle shaking in heat-sealed plastic bags. Prehybridization was carried out for 2–5 h with 0.1 ml cm⁻² of a solution consisting of 45% (v/v) deionized formamide, 5 × SSC, 5 × Denhardt's solution, (1 × Denhardt's solution: 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.5% (w/v) SDS, 20 mM NaH₂PO₄, and 250 µg sonicated salmon testes DNA ml⁻¹. After prehybridization, the buffer was replaced by hybridization buffer, containing the same ingredients plus biotinylated probe (100 ng ml⁻¹), denatured by boiling. Hybridization probes were labelled with biotin-16-dUTP by using a nick-translation kit as specified by the manufacturer (Gibco-BRL). The filter was hybridized at 42 °C for 16–18 h. Washing of the filter and detection of biotinylated probes were performed as described by Chan *et al.* (1985).

Source of DNA probes. Detection of soluble hydrogenase structural genes of *N. opaca* was performed with a cloned 7.8 kb *Bgl*/II fragment from MR11 DNA. The fragment was cloned into the cloning vector λL47 and was shown to carry the soluble hydrogenase structural genes

by heterologous hybridization with an appropriate DNA probe from *Alcaligenes eutrophus* H16 (U. Seitzer & M. Reh, unpublished results). Plasmid pCH139 served as source for this probe (A. Tran-Betcke, U. Warnecke, C. Böcker, C. Zaborosch & B. Friedrich, unpublished). As the probe for the structural gene of the large subunits of ribulose-bisphosphate carboxylase (Rubisco) a cloned 5.1 kb *Eco*RI fragment from MR11 DNA was used. This fragment showed homology to the structural gene of Rubisco from *Rhodospirillum rubrum* (C. Schlüter & M. Reh, unpublished results) which is localized on a 1.3 kb *Ava*II/*Bgl*II fragment of pRR2119 (Nargang *et al.*, 1984).

Fragment DNA was prepared from agarose gels using the GeneClean kit according to the instructions of the manufacturer (Bio 101 Inc., San Diego, USA).

Results

The two hydrogen-autotrophic wild-type strains MR11 and MR22 of *N. opaca*, *Aut*⁻ derivatives of MR22 and various *Aut*⁺ transconjugants (Table 1) were examined by CHEFE for the presence of linear DNA fragments. To prevent physical shearing of the DNA the cells were embedded in low-melting-point agarose plugs and then treated with lysozyme, SDS and proteinase K. CHEFE analysis revealed various distinct bands (Fig. 1*a*). Strain MR11 showed three DNA elements: pHG201 (270 kb), pHG202 (400 kb) and pHG203 (420 kb). The sizes were estimated by comparison with linear standards which

were concatemers of λ DNA (λ ladder). Strain MR22 showed three DNA elements also; their sizes were different from those of the MR11 elements: pHG204 (180 kb), pHG205 (280 kb) and pHG206 (510 kb). The *Aut*⁻ mutant MR222, derived from MR22, contained only pHG206 and the secondary mutant MR2226 contained none of the elements present in the parental cells. The *Aut*⁺ transconjugant pHG2252 derived from MR2226 with MR22 as donor showed the bands of pHG204 and pHG205. Further transconjugants MR2246 and MR2247 derived from MR2226 with MR11 as donor showed bands of pHG201. Finally, the *Aut*⁺ transconjugant MR2253 showed a band of 210 kb, most probably a defective plasmid, pHG205 Δ . The thallium-resistant *Aut*⁻ transconjugant MR2248 showed no linear element. When the agarose plugs with lysed cells of MR2226 and MR2248, which did not give rise to bands on CHEFE, were treated with endonuclease the typical restriction patterns appeared. This indicates that during CHEFE analysis the chromosomal DNA remained at the origin.

The DNA elements pHG201 to pHG206 are linear extrachromosomal DNA molecules, thus linear plasmids. This conclusion is derived from the method used to release the DNA from the cells and from the electrophoretic behaviour. In DNA samples prepared by the

Fig. 1. Detection of linear DNA molecules in lysed cells of *N. opaca*. (a) CHEFE separation. Pulse time 10–80 s. Lanes: L, λ ladder as molecular size standard; 1, MR22; 2, MR222; 3, MR2226; 4, MR2252; 5, MR2253; 6, MR11; 7, MR2246; 8, MR2247; 9, MR2248. (b) Localization of hydrogenase genes by Southern blot analysis. Hybridization was carried out with a biotinylated 7.8 kb *Bgl*II fragment of *N. opaca* MR11 and a labelled λ DNA fragment as probe.

Fig. 2. Conventional agarose gel electrophoresis of *N. opaca* DNA. Lanes: H, λ HindIII; L, λ ladder; 1, MR22; 2, MR2226; 3, MR2252; 4, MR11; 5, MR2246; 6, MR2247.

method of Marmur (1961) these linear plasmids were not detectable; this may be due to their sensitivity to shearing forces. On conventional agarose gel electrophoresis the linear plasmids formed a broad band located slightly above the largest λ HindIII fragment (Fig. 2).

In lysates of *N. opaca* obtained by alkaline treatment, only cccDNA plasmids became visible (Sensfuss *et al.*, 1986). Whereas the mobility of the linear plasmids was dependent on the pulse time, that of cccDNA was not affected when CHEFE was employed (Fig. 3). Figs. 3(a) and 3(b) show the same samples after CHEFE applying a constant pulse time of 40 s and 100 s, respectively. The positions of the elements relative to the λ ladder in the two experiments differed by about 10–20%. The reason for the deviation is not known.

In all the strains mentioned, the presence of the linear plasmids pHG201 or pHG205 correlated with the Aut⁺ character. Southern blotting and hybridization with a DNA probe (7.8 kb *Bgl*II fragment) coding for the genes of the soluble hydrogenase (*hoxS*) was applied to identify Aut genes on the plasmids. Strong positive signals appeared on the bands of pHG201 and pHG205 (Fig. 1b). Furthermore, the linear plasmid from MR2253, which is smaller than pHG205, gave a signal.

Fig. 3. CHEFE separation of isolated cccDNA in comparison to linear plasmid DNA from *N. opaca*. Lanes: L, λ ladder; 1 and 4, pHG31-a; 2, MR11; 3, MR22. (a) Pulse time 40 s. (b) Pulse time 100 s.

Table 2. Restriction fragments of pHG201

	Sizes of fragments (kb)* produced with:					
	<i>AsnI</i>	<i>AsnI/SpeI</i>	<i>SpeI</i>	<i>SpeI/XbaI</i>	<i>XbaI</i>	<i>XbaI/AsnI</i>
	190	100	200†‡	170†‡	230†‡	160
	55‡	60	60	50	25	55‡
	21†	55‡	16	16(2 ×)	16	25
	7.2†	21†		13	6.6†	16
		16		6.6†		5.8†
		7.2†				5.2†
						1.4†
Total:	273	259	276	272	278	268

* Fragment sizes are indicated in kilobase pairs as determined by CHEFE.

† Fragments hybridizing to the 7.8 kb *Bgl*II (hydrogenase) probe.

‡ Fragments hybridizing to the 5.1 kb *Eco*RI (Rubisco) probe.

To determine the restriction pattern, the linear plasmid pHG201 was prepared by electroelution of agarose plugs containing treated cells of MR2246. This strain contains only plasmid pHG201. The prepared plasmid DNA was subjected to restriction analysis using *Xba*I, *Asn*I and *Spe*I as cleavage enzymes. These endonucleases recognize rarely cutting sites in GC-rich DNA (McClelland *et al.*, 1987). As during electroelution of cells embedded in agarose plugs a few chromosomal DNA fragments were co-eluted, the restriction pattern showed a faint background. This artefact was finally avoided when plasmid DNA was isolated by preparative CHEFE in low-melting-point agarose gels and was digested *in situ*. The fragments and their sizes are listed in Table 2.

Table 2 also presents the results of hybridization experiments using DNA probes for the detection of the gene of the large subunits of Rubisco (*cfxL*) which had been cloned as a 5.1 kb *Eco*RI fragment from *N. opaca*. The 7.8 kb *Bgl*II fragment was again used to detect the genes for soluble hydrogenase. The results compiled in Table 2 led to the restriction map of plasmid pHG201 as shown in Fig. 4. Plasmid pHG201 is a linear molecule of 270 kb with defined ends. The genes for soluble

hydrogenase were located on the 7.2 kb *Asn*I and the 6.6 kb *Xba*I fragments. The gene of the large subunits of Rubisco was located on the closely adjacent 55 kb *Asn*I fragment.

In order to distinguish between the Aut plasmids pHG201 and pHG205, pHG205 was also examined by restriction enzyme analysis. The restriction patterns of isolated pHG205 DNA treated with *Spe*I and *Asn*I showed some similarity to those of pHG201. The number of fragments was the same; however, the sizes of the corresponding fragments were significantly different (data not shown). Thus, pHG201 and pHG205 are not identical.

Discussion

A relationship between the Aut character of *N. opaca* and its conjugative cccDNA plasmids was excluded by a previous study (Sensfuss *et al.*, 1986). Therefore, two possible locations of the Aut element were taken into consideration: (1) on a mobile DNA fragment integrated into the chromosome, and (2) on an extrachromosomal DNA molecule which was so far not detectable. For both possible cases the new pulsed-field gel electrophoresis techniques devised to separate large linear DNA fragments lent themselves for further studies. The integration of a large fragment would have been detected by the cleavage of the bacterial chromosomal DNA into only a few fragments and by the comparison of an Aut⁺ and an Aut⁻ strain. The differences of the restriction patterns would have provided information on the presence as well as the size of such a large element. In the present study the use of a pulsed-field electrophoresis technique resulted in the discovery of three linear

Fig. 4. Restriction map of pHG201. *hox*, fragments containing the hydrogenase structural genes; *cfx*, fragment containing the gene of the large subunit of Rubisco.

megaplasmiids in each of the two wild-type strains of *N. opaca* studied. This success is also due to the application of an alternative method for preparation of DNA in agarose plugs containing the bacterial cells. For unknown reasons this method was found not to be suited to detecting the cccDNA plasmids of *N. opaca*.

Only one among the three linear plasmids of each wild-type strain contained genetic information for the key enzymes of hydrogen autotrophy, as detected by means of Southern hybridization using a *hoxS* and a *cfxL* DNA probe. The other four linear megaplasmiids so far remain cryptic; efforts are being made to assign functions to them. The amount of DNA which is present in both wild-type strains of *N. opaca* in the form of extrachromosomal elements, i.e. circular and linear plasmids, is notable: about 1100 kb in MR22 and 1250 kb in MR11. These values correspond to 23% and 27%, respectively, of the *E. coli* genome. The detection of a mutant (MR2226) which lacks these extrachromosomal elements indicates the absence of genetic information for essential metabolic properties.

The presence of linear plasmids as extrachromosomal carriers of genetic information in micro-organisms is not unusual. Within the genus *Streptomyces* several linear plasmids have been described. Linear plasmids designated pSLA1 and pSLA2 (17 kb each), were first discovered in *Streptomyces rochei* (Hayakawa *et al.*, 1979). In *S. azureus*, plasmid pSA1 (9 kb) and the closely related pSA2 were found to be linear plasmids (Ogata *et al.*, 1983). In *S. clavuligerus*, plasmid pSCL (12 kb) (Keen *et al.*, 1988) was found, and in *S. rimosus*, pSRM (42 kb) (Chardon-Loriaux *et al.*, 1986). Outside this genus, in *Borrelia hermsii* and *B. burgdorferi* linear plasmids of 28 kb and 49 kb, respectively, were detected (Plasterk *et al.*, 1985; Barbour & Garon, 1987). *Thiobacillus versutus* contains the linear plasmid pTAV2 (3.7 kb) (Włodarczyk & Nowicka, 1988). In addition to the relatively small linear plasmids mentioned, large ones have been found, for example, in *S. coelicolor* (SCP1) and in *S. lasaliensis* (pKSL, 520 kb) (Kinashi & Shimaji, 1987; Kinashi *et al.*, 1987). Megaplasmiid SCP1 is – like the Aut plasmids in *N. opaca* – a conjugative linear element; furthermore it can be integrated into the chromosome and can mobilize chromosomal markers (Hopwood & Wright, 1976). The wild-type *S. coelicolor* A3(2) was found to contain a series of linear plasmids differing from each other by 30 kb. These plasmids (410–560 kb) are derivatives of SCP1 as demonstrated by Southern hybridization. In two SCP1⁺ transconjugants only one linear plasmid of 350 kb was detected (Kinashi *et al.*, 1987). The linear plasmids of *N. opaca* are not of this kind. The cryptic plasmids pHG202, pHG203, pHG204 and pHG206 did not show any homology to the Aut DNA probes used. It is, therefore, improbable that these plasmids are derivatives of the Aut elements pHG201 or pHG205, respectively.

The majority of the aerobic hydrogen-oxidizing bacteria contain large circular plasmids (Gerstenberg *et al.*, 1982). In *Alcaligenes eutrophus* (Andersen *et al.*, 1981; Friedrich *et al.*, 1981) and *Pseudomonas facilis* (Warrelmann & Friedrich, 1989) the capability for autotrophic growth and the presence of megaplasmiids were found to be strictly correlated. In *A. eutrophus* H16 the structural genes coding for H₂-dependent autotrophic growth are located on the conjugative cccDNA megaplasmiid pHG1. The expression of the genes coding for the soluble (*hoxS*) and the membrane-bound (*hoxP*) hydrogenase is, however, dependent on a chromosomal gene which most probably codes for a sigma factor (Römermann *et al.*, 1989). The organization of the genes coding for the Calvin cycle in *A. eutrophus* is worth noting insofar as one cluster of genes is located on the chromosome and a second almost identical set of *cfx* genes is located on pHG1 adjacent to the *hox* genes (Klintworth *et al.*, 1985; Kortlüke *et al.*, 1987; Husemann *et al.*, 1988).

The present study resulting in the detection of linear plasmids coding for H₂-dependent autotrophy in *N. opaca* solves the problem posed by the detection of *en bloc* conjugative transfer of the Aut character at a high frequency between strains and derivatives of this bacterium (Reh & Schlegel, 1975).

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