BACTERIAL PATHOGENICITY

Occurrence of plasmids in pathogenic strains of *Nocardia*

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The purpose of the present study was to determine the relative distribution of plasmids in 87 clinical isolates of *Nocardia*, belonging to the five major pathogenic species. A correlation between plasmid content and the site of infection within the host, resistance to antibiotics and enzymic profiles was also investigated. The plasmid extraction procedure of Kado and Liu was used. Electrophoretic analysis revealed one-to-four plasmid bands, ranging in size from <8 to >50 kb, in 27 strains (31%). Based on the number of isolates tested, the incidence of plasmid-bearing strains was significantly higher among *N. farcinica* than *N. asteroides* strains. Within *N. farcinica*, the incidence of plasmids was higher among strains isolated in the Paris area than in strains isolated elsewhere, such as in the French provinces or outside France. A statistically significant correlation was demonstrated between the cutaneous localisation of infections and the incidence of plasmid-bearing strains. The presence of plasmids in nocardiae could not be associated with specific phenotypic traits such as resistance to antibiotics or enzymic activity. The fact that the majority of *Nocardia* clinical isolates (60 of 87) did not contain plasmids suggests that plasmids are not involved directly in virulence and that there is no selective pressure for plasmid acquisition.

Introduction

Nocardia spp. are gram-positive aerobic actinomycetes with rudimentary to extensively branched vegetative hyphae that fragment into bacteroid, rod-shaped to coccoid elements. The DNA of these organisms have a G + C content ranging from 64 to 72 mol% and their cell walls contain mycolic acids composed of 46–60 carbon atoms. Nocardiae have a worldwide distribution in soil and some strains are pathogenic for man and animals [1]. They may enter the body *via* inhalation of contaminated dust particles or wounds contaminated with dust or soil. In recent years, pulmonary and systemic human infections have been reported most frequently in immunocompromised patients [2].

Evidence for plasmid carriage may be of special interest because plasmids could encode for important phenotypic properties as well as specific virulence factors. There are very few reports of plasmid carriage within true *Nocardia* spp., and these have referred mainly to *N. asteroides* isolates [3, 4]. However, it is known that strains previously identified simply as *N. asteroides* are in fact members of the *N. asteroides*

general complex, and this group must be differentiated now into at least three species, i.e., *N. asteroides sensu stricto* (here called *N. asteroides*), *N. farcinica* and *N. nova*. Their epidemiological [5], pathogenic [6] and antibiotic susceptibility patterns seem to be different [7, 8], which might lead to distinct clinical outcomes in infected patients [5].

The purposes of the present study were: (a) to screen a large number of clinical isolates of *Nocardia*, including strains belonging to the *N. asteroides* complex, to determine the relative distribution of plasmids; (b) to examine the correlation between plasmid content and phenotypes, resistance to antibiotics and enzymic profiles; (c) to investigate the relationships between plasmid content of clinical isolates and the site of infection within the host; and (d) to evaluate the stability of plasmid carriage.

Materials and methods

Bacterial strains and media

Eighty-seven strains of nocardiae isolated from human infections were used in this study. They included 79 strains of the *N. asteroides* complex (46 *N. asteroides*, 23 *N. farcinica*, 10 *N. nova*), four *N. brasiliensis* and

four *N. otitidiscaviarum*; they were isolated from patients with nocardiosis and cultured from specimens submitted to the National Reference Center for Human Mycosis and Antifungal Agents (Institut Pasteur, Paris, France) over a 5-year period (1990–1994). The strains were isolated from various sites of infection: lung (LU, 36 isolates), brain (BR, nine isolates), primary cutaneous (CU, 11 isolates), other single localisation (SL, six isolates) and multiple localisations (ML, eight isolates). These isolates came predominantly from France (60 strains, including 16 strains isolated in the Paris area and 44 strains isolated in French provinces). Other strains were from Italy (six strains), Switzerland and Martinique (two strains from each country), and Algeria and Portugal (one strain from each country). In addition, 10 isolates from human nocardiosis were received from the University of California, Davis, CA, USA. Type strains of each species obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were also included in this study (i.e., *N. asteroides* ATCC 19247, *N. farcinica* ATCC 3318, *N. nova* ATCC 33726, *N. brasiliensis* ATCC 19296 and *N. otitidiscaviarum* ATCC 14629). Isolates were maintained at 4°C on Bennett's agar slants [9].

Culture conditions and identification

Each strain was identified by standard tests including colony and microscopic morphology, staining characteristics, cell-wall type [10], mycolic acid composition [11] and physiological properties [1].

Plasmid screening

The plasmid extraction procedure of Kado and Liu [12] was used. Briefly, covalently closed circular DNA was released from cells under conditions that denature chromosomal DNA by alkaline sodium dodecyl sulphate (pH 12.6) extraction at 65°C. Proteins and cell debris were removed by extraction with phenol-chloroform and the clarified plasmid DNA extract was used directly for electrophoretic analysis. The strain of *Klebsiella* serotype 35 Dd, harbouring the plasmid pIP55 with a mol. wt of *c.* 130 kb, was used as a control for the extraction of a high mol. wt plasmid [13]. Plasmid DNA extracts (20 µl) were loaded into horizontal Seakem ME (FMC BioProducts, Rockland, ME, USA) agarose 0.5% gels (15 × 15 cm). The samples were electrophoresed at 25 V constant voltage in 1× Tris-acetate EDTA buffer as described by Sambrook *et al.* [14], until the bromophenol blue tracking dye traversed the gel (*c.* 16 h). Subsequently, the gel was stained with ethidium bromide 1 µg/ml. Plasmid DNA bands were viewed with an ultraviolet transilluminator and photographed (Fotodyne Inc., New Berlin, WI, USA) with landscape polaroid negative film (no. 667). The approximate *M_r* of plasmids, in kb, was determined by comparing with a supercoiled DNA ladder and high mol. wt DNA markers (Gibco BRL, Gaithersburg, MD, USA).

Antibiotic susceptibility

In-vitro susceptibility testing was performed on Mueller-Hinton agar by a procedure described previously [15]. Briefly, a homogeneous suspension of organisms was prepared by shaking cultures in Mueller-Hinton broth containing sterile glass beads to decrease aggregation of the nocardial cells and incubating for 24–48 h at 35°C. An inoculum yielding *c.* 10⁴–10⁵ cfu/ml was used for disk susceptibility testing, which was performed on Mueller-Hinton agar and read after incubation for 24–48 h at 37°C. The antibiotics tested (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) were ampicillin (Am), amoxycillin (Amx), cefamandole (Ma), imipenem (Ipm), cefuroxime (Cxm), cefoxitine (Fox), cefotaxime (Ctx), ceftriaxone (Cro), tetracycline (Te), minocycline (Mno), doxycycline (Do), kanamycin (K), tobramycin (Tm), amikacin (An), gentamicin (Gm), chloramphenicol (C), erythromycin (E) and clindamycin (Cm). All strains were tested at least twice without any discrepancy between the results. The strains were scored as susceptible, intermediate or resistant according to the guidelines of the Antibigram Committee of the French Society for Microbiology [16].

Enzymic characterisation

Enzymic profiles of strains were determined by the API ZYM system (API System SA, La Balme les Grottes, France) as previously described [17]. All tests were done with standardised inoculum size, temperature and duration of incubation as described previously.

Plasmid stability

The plasmid-containing strains were subcultured at intervals of 2 weeks and plasmid DNA preparations were repeated three times at intervals of 2 months. This corresponded to four subcultures between the first and the last DNA extractions.

Statistical analysis

The incidences of plasmids in *Nocardia* spp. were compared by the χ^2 test.

Results

Occurrence of plasmid-bearing strains

Eighty-seven strains of *Nocardia* spp. isolated from clinical cases were examined. In 27 strains (31%), electrophoretic analysis with agarose 0.5% gel revealed one-to-four plasmids, ranging in size from <8 to >50 kb (Table 1). Plasmids with mobility faster than chromosomal residues (*c.* 40 kb) were considered low mol. wt plasmids, whereas plasmids with mobility slower than chromosomal residues were considered high mol. wt plasmids. Plasmid DNA, either of low or high mol. wt, was demonstrated in all *Nocardia* spp.

Table 1. Geographic origin, source, plasmid band profile and resistance-pattern of *Nocardia* spp.

Species and strain number	Place	Source	Plasmid profile (kb)	Resistance pattern*
<i>N. asteroides</i>				
Na1	Paris†	LU	15	FoxC
Na2	Paris	LU	29	nd
Na3	Nantes	LU	10, 12, 17	TeCECm
Na4	Italy	BR	10, 12, 24, 38	FoxCroTeMnoDoKC
Na5	Algeria	LU	38, >50	MaCxmFoxCtxTeMnoDoKCECm
Na6	USA	LU	8, 10, >50	AmAmxMaCxmFoxCtxCroTeDoKTmGmCECm
Na7	Bayonne	LU	>50	TeCCm
Na8	Toulouse	CU	>50	AmAmxTeKCECm
Na9	Toulouse	LU	>50	AmAmxMaFoxTeDoKTmGmCECm
Na10	Périgueux	ML	>50	AmMaTeKCECm
Na11	Rodez	LU	>50	AmMaIpmCm
<i>N. farcinica</i>				
Nf1	Paris	LU	15	AmMaCtxTeKTmGmCECm
Nf2	Paris	CU	15, 48	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf3	Paris	CU	15, >50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf4	Besançon	LU	15, >50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf5	Paris	LU	50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf6	Paris	LU	>50	AmAmxMaCxmCtxCroTeMnoDoKTmGmCECm
Nf7	Paris	CU	>50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf8	Paris	LU	>50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf9	Tours	CU	>50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf10	Lille	CU	>50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
Nf11	USA	LU	>50	AmAmxMaCxmFoxCtxCroTeMnoDoKTmGmCECm
<i>N. nova</i>				
Nn1	Amiens	ML	<8	AmxFoxCroTeDoKTmGm
Nn2	Nantes	CU	19, >50	AmMaCxmFoxCtxCroKTmGmCECm
<i>N. otitidiscaviarum</i>				
No1	Toulouse	CU	29, 38	AmAmxMaIpmCxmFoxCtxCroTeCE
No2	St Etienne	CU	<8, 8	AmFoxKAnGmCECmE
<i>N. brasiliensis</i>				
Nb1	Portugal	LU	17	AmAmxMaIpmCxmFoxCtxK

*Strains were susceptible or intermediary to other antibiotics tested.

†Paris area.

nd, not determined (because of severe clumping during growth).

studied, including 11 (24%) of 46 strains of *N. asteroides* (*sensu stricto*), 11 (48%) of 23 strains of *N. farcinica*, two of 10 strains of *N. nova*, one of four strains of *N. brasiliensis* and two of four strains of *N. otitidiscaviarum*. None of the type strains of the nocardial species contained plasmids. Based on the number of isolates tested, the incidence of plasmid-bearing strains was significantly higher among *N. farcinica* than *N. asteroides* strains ($p < 0.025$). The number of strains of other nocardial species investigated was too low for frequency analysis of plasmid-bearing strains.

Table 2 shows the geographic distribution of strains of *N. asteroides* and *N. farcinica*, either with or without

plasmid bands. Within *N. farcinica*, the incidence of plasmids was higher among strains isolated in the Paris area than in strains isolated elsewhere, such as in the French provinces or outside France ($p < 0.1$).

The site of infection was known for 71 strains (Table 3). A statistically significant correlation was demonstrated between the cutaneous localisation of infections and the incidence of plasmid-bearing strains ($p < 0.005$). Thus, all *N. asteroides* and *N. farcinica* strains isolated from cutaneous infection contained a high mol. wt plasmid (≥ 50 kb); however, *N. otitidiscaviarum* strains showed only low mol. wt plasmid bands (<8, 8, 29 and 38 kb). In contrast, *N. brasiliensis* strains isolated from cutaneous lesions did not have any visible plasmid band.

Table 2. Geographic distribution of strains of *N. asteroides* and *N. farcinica*

Species	Number of strains from	
	Paris area	Other areas*
<i>N. asteroides</i>		
Plasmids	2	9
No plasmid	4	31
<i>N. farcinica</i>		
Plasmids	7	4
No plasmid	2	10

*Including French provinces and other countries.

Table 3. Site of infection of strains of *Nocardia* spp.

Species	Number of isolates from				
	LU	BR	CU	SL	ML
<i>N. asteroides</i>	23/8*	7/1	1/1	5/0	3/1
<i>N. farcinica</i>	9/6	2/0	5/5	—	2/0
<i>N. nova</i>	4/0	—	1/1	1/0	2/1
<i>N. brasiliensis</i>	1/1	—	2/0	—	—
<i>N. otitidiscaviarum</i>	—	—	2/2	—	1/0

*Number of strains tested/number of plasmid-bearing strains.

Antibiotic susceptibility tests

Table 1 shows the resistance profiles of plasmid-containing strains against various antibiotics. The incidence of susceptibility amongst *N. farcinica* isolates was similar, showing resistance to all but two antibiotics (Ipm and An). In contrast, resistance was variable amongst *N. asteroides* isolates. The susceptibility of nocardial isolates to antibiotics did not correlate with the presence of plasmids.

Enzymic characterisation

Plasmid-bearing strains showed enzymic (physiological) profiles that were not significantly different from those isolates that had no demonstrable plasmids (data not shown).

Plasmid stability

For all strains, plasmid patterns were identical after at least 4 subcultures with those patterns identified during the first screening (data not shown).

Discussion

There have been only a few reports of plasmids in nocardiae. It should be noted that *N. corallina*, *N. erythropolis*, *N. orientalis* and *N. mediterranei*, from which plasmids have been isolated [18–21], are not members of the genus *Nocardia* but have been re-assigned to the genus *Rhodococcus* for the two former species and *Ammycolatopsis* for the two latter species. Up to the present, plasmids have been demonstrated only in a few isolates of *N. asteroides* [3, 4] which, based on taxonomic difficulties, might have been either *N. asteroides sensu stricto*, *N. farcinica* or *N. nova*.

Plasmid content and plasmid sizes may differ according to the plasmid extraction procedure used [22]; therefore, the procedure of Kado and Liu was selected in this study because it was rapid and permitted extraction of both small and large plasmids. The chromosomal DNA, which could mask plasmids migrating at this level, was reduced significantly by heat treatment at 65°C for 2 h.

A total of 60 strains, belonging to five species of *Nocardia*, appeared to be plasmid-free. The fact that the majority of *Nocardia* strains did not contain plasmids suggests that there is no selective pressure for plasmid acquisition. The lack of sufficient transfer events from strain to strain, or too low a copy number, could also be responsible for not finding plasmids in these nocardial isolates.

Based on the few reports in the literature, the relative frequency of plasmid-containing strains in this study is similar to those described by Kasweck *et al.* [3] and Jonsson *et al.* [23]. Thus, Kasweck *et al.* reported

finding plasmids in four of 12 laboratory isolates of *N. asteroides*, with plasmid sizes ranging from 13 to 36 kb, and Jonsson *et al.* found plasmids varying in size from *c.* 8 to >100 kb, in 11 of 24 clinical isolates of *N. asteroides*.

The significance of plasmids for their host bacteria probably depends on their stability. The present results confirm that plasmids do not seem to be lost readily during laboratory cultivation and maintenance [3]. For this reason, when plasmid(s) is(are) present, plasmid analysis could be a means of strain comparison in an epidemiological evaluation of outbreaks of nocardial infections [24] or in recurrence of infection in an individual case [23].

The significance of the fact that the incidence of plasmid-bearing strains seems higher among *N. farcinica* than in *N. asteroides* strains, particularly among strains isolated in the Paris area, requires further investigation. Thus, restriction endonuclease analysis of these plasmids and DNA-DNA hybridisation studies may indicate similarity among isolates, and suggest common sources of exposure.

From the data presented above, it can be concluded that there may be a correlation between the incidence of plasmid-harboursing strains and primary cutaneous localisation of infection. Surprisingly, plasmid bands were demonstrated in *Nocardia* spp. isolated from this site except *N. brasiliensis*, even though this species is known to be predominant in primary cutaneous nocardiosis [25]. Plasmid bands detected in *N. otitidiscaviarum*, a species also frequently involved in primary cutaneous infection [26], were of lower mol. wt than plasmid bands carried by *N. asteroides* and *N. farcinica* strains isolated from cutaneous sites. For this reason, it is important to investigate more isolates from cutaneous lesions. It would be of interest to determine whether there is a relationship between these plasmids and the ability of nocardiae to cause primary cutaneous disease.

The presence of plasmids in nocardiae could not be associated with specific phenotypic traits such as resistance to antibiotics or enzymic activity. Moreover, these plasmids appear not to be involved directly in virulence, because in man, strains lacking plasmids were shown to be as effective in causing nocardiosis as those with plasmid(s). However, continued investigation for such correlation is needed because the incidence of plasmid-bearing strains appears to be higher among *N. farcinica* isolates than among *N. asteroides* isolates. Other investigators have suggested that *N. farcinica* infection is characterised by higher rates of disseminated disease, antimicrobial resistance and mortality than other *Nocardia* spp. [27].

The information on the plasmid profiles of different strains of *Nocardia* reported here may offer greater

insight into both plasmid and nocardial ecology. Additional prospective studies are necessary to trace the plasmid evolutionary behaviour within each species to understand the functional roles of these extra-chromosomal elements in nocardial biology.

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