Naturally occurring NAD-independent *Haemophilus parainfluenzae*

ROSA GROMKOVA* AND HENDRIK KOORNHOF

Emergent Pathogen Research Unit of the South African Medical Research Council, School of Pathology of the University of the Witwatersrand and the South African Institute for Medical Research, 7 York Road, Parktown, Johannesburg 2193, South Africa

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Four, NAD-independent, clinical isolates of *Haemophilus parainfluenzae* were recovered from a genital ulcer, a purulent skin lesion, a sputum specimen and a throat swab respectively. With the exception of NAD requirement, the strains exhibited the biochemical characteristics of *H. parainfluenzae* biotype II. The genetic relationship between these isolates and a standard strain of *H. parainfluenzae* was determined by testing transforming activities of two chromosomal markers, streptomycin resistance and nalidixic acid resistance. The clinical isolates were efficient donors and recipients in transformation. In addition, we demonstrated transfer of the genes conferring NAD independence to typical, NAD-requiring *H. parainfluenzae* and *Haemophilus influenzae* strains.

Introduction

The laboratory identification and the classification of species of the genus *Haemophilus* is based on their requirement for either NAD (V-factor) or haemin (X-factor) or both. *Haemophilus parainfluenzae* is NAD-dependent, while *Haemophilus influenzae* requires both NAD and haemin. According to the present taxonomic criteria bacteria that are able to grow in the absence of NAD cannot be classified as *H. parainfluenzae* or *H. influenzae*. The NAD dependence is considered to be a genetically stable property because of the inability of these organisms to revert to NAD independence. Attempts to obtain by DNA transformation strains of *H. parainfluenzae* that do not require NAD have been unsuccessful (White & Granick, 1963) and the isolation of such strains from clinical specimens has not been reported. Man is the natural host of *H. parainfluenzae* and *H. influenzae* and these species are usually present in healthy individuals. *H. parainfluenzae* is a known but occasional cause of endocarditis, pneumonia and meningitis while *H. influenzae* is a major pathogen especially in children, causing serious infections such as meningitis, epiglottitis, pneumonia and septicaemia. In this paper we describe the biochemical and genetic properties of four clinical isolates from unrelated sources that had the usual features of *H. parainfluenzae* but were NAD-independent.

Methods

Bacterial strains and growth. Three unusual strains were isolated from paediatric patients at Baragwanath Hospital, Johannesburg whilst examining 20 β -lactamase-producing *Haemophilus*-like bacteria. These strains were isolated from a sputum sample, a specimen of pus and a throat swab, and were designated strains 9, 15 and 19 respectively. In addition, we recovered another isolate, designated strain 78, together with a typical *H. parainfluenzae* strain, designated strain 79, from a genital ulcer in a male patient attending a clinic for sexually transmitted diseases at Carletonville, near Johannesburg. *H. influenzae* Rd and *H. parainfluenzae* 14 (Boss) were provided by G. Leidy, Columbia University, New York, USA. Antibiotic-resistant strains, used as donors in DNA transformation, were obtained by selecting spontaneous mutants on antibiotic-containing plates. *H. influenzae* Rd was grown in Brain Heart Infusion (BHI) broth and BHI agar (Becton Dickinson) supplemented with 10 μ g haemin ml⁻¹ and 2 μ g NAD ml⁻¹ (both from Sigma). For growth of *H. parainfluenzae* the BHI medium was supplemented with 2 μ g NAD ml⁻¹.

Biochemical characterization. The biochemical properties of the four NAD-independent isolates were compared with those of typical strains of *H. parainfluenzae* and *Haemophilus aphrophilus* according to the scheme proposed by Kilian (1976).

β -Lactamase production. The nitrocephin method was used to demonstrate β -lactamase production (O'Callaghan *et al.*, 1974).

DNA transformation. Purified DNA was prepared by the method described by Marmur (1961). Competence was induced by the static aerobic procedure (Gromkova & Goodgal, 1979; Gromkova *et al.*, 1989). For the transformation assay 0.1 ml DNA at a saturating concentration of 1 μ g ml⁻¹ was added to 0.8 ml BHI broth. After the addition of 0.1 ml competent cells, the mixture was incubated for 30 min at 35 °C without shaking and then pancreatic DNAase (Sigma) was added to 10 μ g ml⁻¹. After a further 10 min incubation at 35 °C, the mixture was diluted and each dilution was plated in triplicate for transformants using an overlay method. For expression of the genes

* Correspondence should be sent to PO Box 1038, Johannesburg 2000, South Africa.

conferring NAD independence, the transformation mixture was incubated for 3 h at 35 °C. Each transformation experiment was repeated three times.

Results

Growth and biochemical characteristics

Strains 9, 15, 19 and 78 were able to grow aerobically on chocolate agar and on 5% (v/v) horse blood agar. On the latter medium they produced green discolouration similar to that of α -haemolytic streptococci. The colonies on chocolate agar were small (about 2 mm in diameter) and flat with smooth and shiny surfaces. They exhibited a striking similarity to the colonies of *H. parainfluenzae* 79 and *H. parainfluenzae* 14. They also produced a smell characteristic of *H. parainfluenzae*. However, unlike *H. parainfluenzae*, strains 9, 15, 19 and 78 were able to grow on blood agar and in BHI broth and BHI agar lacking NAD. This property was maintained when three different batches of BHI agar were used and when 20 single colony passages on unsupplemented BHI agar were done. The disc test for V-factor requirement on BHI agar was also negative. The four clinical isolates were all able to produce the satellite effect (Kilian, 1976) when streaked on unsupplemented BHI agar inoculated with a typical NAD-requiring *H. parainfluenzae* strain 14. This suggests that the NAD independence was probably due to the ability of the clinical isolates to synthesize and release into the medium either NAD or some other metabolite that may also serve as a V-factor such as NAD-phosphate, nicotinamide mononucleotide or nicotinamide riboside.

Gram-stained preparations examined microscopically showed small Gram-negative rods arranged in short or long chains.

The properties of the clinical isolates were compared with those of *H. parainfluenzae* and *H. aphrophilus*. The results presented in Table 1 show that the only feature that distinguished the clinical isolates from *H. parainfluenzae* strains was NAD requirement. The remaining biochemical reactions corresponded to biotype II of *H. parainfluenzae*. This pattern was clearly distinguishable from that of *H. aphrophilus*, which does not require NAD for growth. Unlike the clinical isolates, *H. aphrophilus* is characteristically urease-negative, ornithine-decarboxylase- and hydrogen-sulphide-negative and its growth is stimulated by CO₂. The four NAD-independent isolates all produced β -lactamase, a property that typical *H. parainfluenzae* may also acquire.

Transformation studies

Two chromosomal genes conferring resistance to streptomycin and nalidixic acid respectively were used as markers in transformation experiments. The ribosomal streptomycin-resistance (Str^r) gene is considered to be more conserved among species than the non-ribosomal, nalidixic-acid-resistance (Nal^r) gene (Dubnau *et al.*, 1965). Both these genes are located on the chromosome. Double-resistant mutants (Str^r and Nal^r) were selected from each of the four NAD-independent clinical isolates and from the *H. parainfluenzae* strains. The highly transformable strain *H. parainfluenzae* 14 (Leidy *et al.*, 1956) was employed as recipient. The results in Table 2 show that Str^r and Nal^r markers from the NAD-independent clinical isolates were highly efficient in transforming *H. parainfluenzae* 14. There was a two- to threefold difference between the efficiencies of transformation of these markers and the Str^r and Nal^r markers of the isogenic *H. parainfluenzae* 14 donor. The transforming activity of DNA from the NAD-independent donors was equal to that of the *H. parainfluenzae* 79 strain from South Africa. The results were reproducible when repeated three times with different batches of competent cells. The results of a reciprocal transformation experiment are given in Table 3. All strains were good recipients but showed differences in their levels of competence, strain 78 being a more efficient recipient than the other NAD-independent isolates. Strain variation in competence levels has been observed in several transformation systems including *Haemophilus* species (Rowji *et al.*, 1989).

As the successful transfer of genes conferring NAD independence in *Haemophilus* had not been reported previously, we investigated the transfer of such genes using the NAD-independent isolates as the source of donor DNA with both *H. parainfluenzae* 14 and *H. influenzae* Rd as recipients. *H. influenzae* Rd is a highly transformable strain that has been used extensively in transformation studies (Alexander & Leidy, 1956). Since neither the location of the NAD genes nor their transforming ability was known, the transforming efficiency of chromosomal markers was used to determine the level of competence in the recipient cells. We were able to demonstrate transformation to NAD independence in both recipients. The frequency of transformation by the NAD genes in *H. parainfluenzae* 14 (Table 2) was higher than in *H. influenzae* Rd (Table 4), probably due to the degree of genetic homology with donor DNA. The efficiency of transformation of the NAD marker in *H. parainfluenzae* 14 was about 10- to 20-fold lower than that of the chromosomal Str^r marker whereas in the *H. influenzae* Rd recipient these two markers exhibited similar transformation frequencies.

Table 1. *Characteristics of unusual clinical isolates and typical H. parainfluenzae and H. aphrophilus strains*

Tests were done according to the techniques recommended by Kilian (1976).
For designation of strains see Methods.

	<i>H. parainfluenzae</i> strains 14, 79	<i>H. aphrophilus</i> *	Unusual strains 9, 15, 19, 78
NAD requirement	+	—	—
Porphyrin	+	+	+
Indole	—	—	—
Ornithine decarboxylase	+	—	+
Hydrogen sulphide	+	—	+
Urease	+	—	+
Oxidase	+	—	+
Alkaline phosphatase	+	+	+
Nitrate reduction	+	+	+
Lactose, acid	—	+	—
Glucose, acid	+	+	+
Sucrose, acid	+	+	+
CO ₂ (5%) enhances growth	—	+	—

* *H. aphrophilus* according to Kilian (1976).

Table 2. *Transformation of H. influenzae 14 by DNA from NAD-independent strains*

The concentrations of streptomycin and nalidixic acid used for selection of transformants were 100 µg ml⁻¹ and 3 µg ml⁻¹ respectively. For designation of strains see Methods.

DNA donor	No. of transformants (per 5 × 10 ⁸ c.f.u. ml ⁻¹)		
	Str ^r	Nal ^r	NAD independence
Strain 78	2.1 × 10 ⁶	4.5 × 10 ⁶	9.8 × 10 ⁴
Strain 15	1.3 × 10 ⁶	3.3 × 10 ⁶	6.9 × 10 ⁴
Strain 9	1.7 × 10 ⁶	2.4 × 10 ⁶	7.5 × 10 ⁴
Strain 19	2.1 × 10 ⁶	3.9 × 10 ⁶	8.1 × 10 ⁴
<i>H. parainfluenzae</i> 79	2.2 × 10 ⁶	4.1 × 10 ⁶	0
<i>H. parainfluenzae</i> 14	4.1 × 10 ⁶	6.8 × 10 ⁶	0

Table 4. *Transforming activity of the NAD independence and Str^r markers in H. influenzae Rd as recipient*

For designation of strains see Methods.

DNA donor	No. of transformants* (per 5 × 10 ⁸ c.f.u. ml ⁻¹)	
	NAD independence	Str ^r
Strain 78	3 × 10 ³	2.2 × 10 ³
Strain 15	2.2 × 10 ³	2.5 × 10 ³
Strain 9	1.5 × 10 ³	1.9 × 10 ³
Strain 19	1.1 × 10 ³	1.5 × 10 ³
<i>H. parainfluenzae</i> 79	0	2.2 × 10 ³
<i>H. parainfluenzae</i> 14	0	2.8 × 10 ³
<i>H. influenzae</i> Rd	0	3.8 × 10 ⁶

* See Table 2 legend.

Table 3. *Reciprocal transformation of NAD-independent strains and of H. parainfluenzae 14 and 79*

For designation of strains see Methods.

Recipient	No. of transformants* (per 5 × 10 ⁸ c.f.u. ml ⁻¹)	
	<i>H. parainfluenzae</i> 14 donor DNA	Isogenic donor DNA
Strain 78	2.1 × 10 ⁶	3.9 × 10 ⁶
Strain 15	3.1 × 10 ⁵	4.2 × 10 ⁵
Strain 9	2.2 × 10 ⁵	3.1 × 10 ⁵
Strain 19	3.2 × 10 ⁵	4.8 × 10 ⁵
<i>H. parainfluenzae</i> 79	2.2 × 10 ⁶	4.2 × 10 ⁶
<i>H. parainfluenzae</i> 14	4.9 × 10 ⁶	—

* See Table 2 legend.

Discussion

NAD is an essential coenzyme for energy-yielding processes in both eukaryotic and prokaryotic cells. Two major biosynthetic pathways have been described. One involves *de novo* synthesis of NAD and the other is based on the utilization of preformed pyridine bases obtained from the breakdown of intracellular NAD or from exogenous sources (Foster & Moat, 1980). It would be of interest to study which of these pathways is used for the synthesis of NAD in the NAD-independent *H. parainfluenzae* strains. Previous studies have shown that the NAD-independent species *Haemophilus haemoglobinophilus* synthesizes NAD from nicotinamide but is incapable of *de novo* NAD synthesis (Kassarov & Moat, 1973).

Requirements for NAD and haemin have been considered for many years to be essential criteria for the classification of *Haemophilus* species and have also formed the basis for their laboratory identification. This emphasis on growth factors may well explain why NAD-independent *H. parainfluenzae* strains have not been recognized earlier by clinical microbiologists.

The finding that, with the exception of NAD independence, all 12 biochemical reactions were identical to those of biotype II of *H. parainfluenzae* (Table 1) and that two chromosomal genes (one ribosomal and one non-ribosomal) were highly efficient in transforming a standard strain of *H. parainfluenzae* (Table 2) strongly suggests that the isolates are *H. parainfluenzae*. Because of their ability to grow in the absence of NAD, consideration should be given to assign the four isolates to a new biotype. The two- to threefold differences between the transforming efficiencies of isogenic *H. parainfluenzae* 14 DNA and those of DNA from the clinical isolates were probably due to genetic heterogeneity within the species (Table 2). Similar observations have been made previously when the properties of different *H. parainfluenzae* strains have been compared (Pohl, 1981). Recently two former members of the genus *Haemophilus* – *Haemophilus pleuropneumoniae* and *Haemophilus avium* – were transferred to the genera *Actinobacillus* and *Pasteurella*, respectively (Pohl *et al.*, 1983). This was based on genetic homology and phenotypic considerations in which the NAD requirement was shown not to be of overriding importance. Furthermore, Pohl *et al.* (1983) demonstrated that some *H. pleuropneumoniae* strains do not require NAD for growth. Based on these developments and our findings we agree with Pohl (1981) and Pohl *et al.* (1983) that new criteria be formulated for the classification and laboratory identification of this important taxonomic group. An identification scheme based on that proposed by Kilian (1976) would be appropriate. *H. influenzae* strains that are NAD-independent should constitute a new biotype. At present, the only other *Haemophilus* species that does not require either haemin or NAD is *H. aphrophilus*; this organism can be distinguished from *H. parainfluenzae* by its ability to ferment lactose and deoxyribose and to produce oxidase. In addition, unlike *H. parainfluenzae*, *H. aphrophilus* fails to produce H₂S and its growth is stimulated by CO₂. One should also bear in mind that atypical NAD-independent strains of *H. parainfluenzae* are able to grow on media such as blood agar and BHI agar that usually do not support the growth of typical *H. parainfluenzae*. The recovery of NAD-independent strains from natural sources demands that NAD requirement should not be considered a predominant taxonomic criterion for this species.

Previous studies have shown that there is no correla-

tion between marker efficiencies in homospecific as opposed to heterospecific transformation in the genus *Haemophilus* (Goodgal & Gromkova, 1973). In both *H. influenzae* Rd and *H. parainfluenzae* 14 recipients, marker efficiencies in transformation with homologous chromosomal DNA do not generally vary more than by a factor of two or three. The reason for the bigger differences in the transforming efficiencies of the NAD and Str^r markers in *H. parainfluenzae* 14 is unknown. However, several explanations can be offered: (i) incomplete sequence homology between donor and recipient DNA; (ii) poor expression of the NAD genes probably due to changes of some regulatory proteins; (iii) bigger size of the NAD marker relative to that of the Str^r marker.

The similarity of transformation efficiencies of NAD and Str^r markers in heterospecific transformation (see Table 4) does not necessarily indicate that the sizes of these two markers are the same in homospecific transformation (Table 2). Previous studies have shown that DNA fragments that are biologically active in heterospecific transformation may not be able to transform homospecifically, suggesting lack of correlation between the sizes of the biologically active genetic markers (Goodgal & Gromkova, 1973). Furthermore, other factors besides sequence homology and the size of the markers have been shown to play a role in heterospecific transformation in *Haemophilus* species (Steinhart & Herriott, 1968; Goodgal & Gromkova, 1973). The similar frequencies of NAD and Str^r transformation in *H. influenzae*, therefore, does not necessarily indicate similarity between sizes of these two markers. Another explanation of the lower efficiency of NAD transformation of *H. parainfluenzae* recorded here is the possibility of extrachromosomal location of genes conferring NAD independence. It has been previously demonstrated that plasmid transformation in *H. parainfluenzae* is less efficient than by chromosomal DNA (Gromkova & Goodgal, 1979).

The demonstration that typical NAD-requiring strains of *H. parainfluenzae* and *H. influenzae* can be transformed to NAD independence under experimental conditions may have biological significance. *H. influenzae* and *H. parainfluenzae* share a common ecological niche and both species are naturally transformable. Furthermore, they are able to undergo spontaneous autolysis followed by the release of DNA. This suggests that DNA-mediated transfer may occur *in vivo*. One can speculate that the genes conferring NAD-independence to *H. parainfluenzae* have been acquired by genetic exchange with an organism able to synthesize NAD in a shared ecological milieu. The finding that the NAD-independent *H. parainfluenzae* strains were able to develop competence suggests that the NAD genes may

have been acquired by DNA transformation. However, other mechanisms of gene transfer cannot be excluded. Although not impossible, it is unlikely that the NAD-independent clinical isolates have been generated by a mutation. Attempts to obtain such mutants *in vitro* have been unsuccessful (R. Gromkova, unpublished results). Since we were able to demonstrate *in vitro* transfer of the genes conferring NAD independence to *H. influenzae* by DNA transformation, it is possible that the latter, as is the case with *H. parainfluenzae*, may also occur in nature. Because of the greater clinical significance of *H. influenzae*, it is important to be aware of such a possibility and to use diagnostic procedures that cater for the identification of NAD-independent clinical isolates.

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