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Plasmid-encoded NAD independence in some South African isolates of *Haemophilus paragallinarum*

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ABSTRACT

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Atypical *Haemophilus paragallinarum* have been isolated from both laying hens and broilers suffering from typical symptoms of infectious coryza in South Africa. Re-inoculation of these bacteria into SPF chickens resulted in similar pathology. The bacteria could be successfully re-isolated from the experimentally infected chickens.

Four of the isolates from layers and 3 of those from broilers were found to be closely related to *H. paragallinarum* serotype A (0083 strain) when tested by the use of a panel of locally developed monoclonal antibodies in the enzyme linked immunosorbent assay (ELISA). A total of 15 isolates from layers and 19 from broilers were found to be more typical of previously collected South African field isolates of *H. paragallinarum*. A 3rd group, consisting of 5 isolates from layers and 15 from broilers, showed no reaction with the panel of monoclonal antibodies.

All the isolates were regarded as atypical because they no longer required V factor (NAD) for growth, whereas strain 0083 and previously collected field isolates M 85 and SB 86 did require it.

Crude plasmid extractions from an isolate serologically related to 0083 was used to convert reference strains of *H. paragallinarum* into NAD-independent isolates, thus indicating that NAD independence is carried on a plasmid.

INTRODUCTION

The first isolation of a *Haemophilus* species from chickens was in the 1930s by De Blieck (1932). This bacterium was later termed *H. gallinarum* (Elliot & Lewis 1934) and apparently required both V (NAD) and X (haem) factors (Schalm & Beach 1936; Delaplane, Erwin & Stuart 1938). As early as 1932, McGaughy (1932) reported that his isolates were dependent on V factor, but not X factor. This work was largely overlooked until the 1960s when various workers reported that the isolates made from chickens were not dependent on X factor (Page 1962; Roberts, Hanson & Timms 1964). All subsequent isolates from chickens with infectious coryza have been found to be dependent on only V factor (Blackall & Reid 1982) and these isolates have been termed *H. paragallinarum*. Blackall & Yamamoto (1989) tested 2 cultures which had been isolated in the 1940s and '50s and were labelled *H. gallinarum*, and they found these isolates to be dependent on

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V factor, but independent of X factor. It is now widely accepted that the pathogenic *Haemophilus* species which can be isolated from chickens with infectious coryza are all dependent on V factor, but not on X factor. Recently, V factor- (NAD)- independent *H. paragallinarum* was isolated from chickens in Natal, South Africa (Mouahid, Bisgaard, Morley, Mutters & Mannheim 1992). Horner, Bishop & Haw (1992) have also reported on the isolation of NAD-independent organisms from chickens suffering from typical infectious coryza symptoms in Natal. Horner *et al.* (1992) suggested that these isolates do not belong to the genus *Haemophilus* by virtue of the NAD independence of the isolates. However, the observation of NAD independence in other species of *Haemophilus* (Gromkova & Koornhof 1990), argues against this.

Gromkova & Koornhof (1990) reported on the isolation of 4 organisms from human patients in South Africa, that were biochemically indistinguishable from *H. parainfluenza*, except for the fact that they were capable of growth without NAD, and as such could not be classified as *H. parainfluenza* according to the current taxonomic criteria. In subsequent work (Windsor, Gromkova & Koornhof 1991), it was established that these isolates carried a small 5,25 kb plasmid which, if lost or removed, rendered the isolates NAD dependent. The isolates could then be classified as *H. parainfluenza*. They also established that a low incidence of spontaneous NAD-dependent revertants occur naturally. It can therefore be concluded from their work that *H. parainfluenza* is capable of acquiring a small plasmid which renders the isolate NAD independent. These isolates must, however, still be classified as *H. parainfluenza* in spite of the naturally occurring NAD independence.

Since 1991, this laboratory has made a number of bacterial isolations from chickens showing typical symptoms of infectious coryza in South Africa. A total of 42 isolates have been identified as *H. paragallinarum* (Bragg 1992, unpublished data) and 61 isolates have been identified as NAD-independent *H. paragallinarum*. The NAD-independent isolates proved difficult to identify with conventional biochemical techniques.

By use of a locally developed panel of monoclonal antibodies (Mab) against *H. paragallinarum* (Verschoor, Coetzee & Visser 1989) in an enzyme-linked immunosorbent assay (ELISA), most of these NAD-independent isolates are shown to be closely related to either the 0083 strain or the local field strain of *H. paragallinarum*.

METHODS AND MATERIALS

Bacterial isolation

Bacteria were isolated from the sinuses of chickens showing clinical signs of infectious coryza. The heads of the chickens were removed in the post mortem room and disinfected with merthiolate before an incision into the sinus was made with a sterile scalpel blade. Samples were then collected from the sinus with a sterile swab. Two blood tryptose agar (BTA) plates were inoculated with the swab taken from the sinus, of which 1 was then streaked across the inoculum with *Staphylococcus aureus*. Both plates were incubated at 37 °C in a candle jar for 18 h.

Experimental infection of chickens

Samples of the isolates made from infected chickens were inoculated into modified Casman's medium (Coetzee, Rogers & Velthuysen 1983) without the addition of sterile chicken serum, or NAD, and were incubated overnight. Ten SPF chickens were inoculated either by the eye-drop method or intra-sinus with 0,1 mℓ of the bacterial suspension. Another 10 SPF chickens were inoculated with sterile growth medium to serve as controls. The chickens were observed daily. Any bird showing symptoms of infectious coryza was killed and bacteria were isolated from the sinus according to the method described above.

Typing with monoclonal antibodies

Bacterial clones from the BTA plates were inoculated into 10 mℓ liquid-modified Casman's medium without the addition of NAD (Coetzee *et al.* 1983) or sterile chicken serum, and incubated at 37 °C for 18 h. The 0083 strain of *H. paragallinarum* and A745/91 (a South African field isolate of NAD-dependent *H. paragallinarum*, isolated in this laboratory) were inoculated into liquid-modified Casman's medium which had been supplemented with 10 % sterile chicken serum in the place of NAD (Coetzee *et al.* 1983). After incubation, purity of the cultures was checked by streaking a sample onto BTA plates. The plates inoculated with the 0083 strain or A745/91 were streaked with *S. aureus*. The liquid cultures were then inactivated with 0,1 % (v/v) formalin and incubated at room temperature for 24 h. Inactivated bacteria from the different isolates were washed and used for the coating of plates and for carrying out the ELISA according to the methods of Verschoor *et al.* (1989). Three Mab were used, i.e. F1, which previously recognized field (F) strains only, V1, which previously recognized vaccine (V) strains only and VF3, which cross-reacted with vaccine and field strains. As negative control, the mature culture supernatant of the non-antibody-producing SP 2/0 myeloma was used instead of monoclonal antibody containing culture supernatant.

Plasmid extraction

Crude plasmid extractions were made according to the methods of Gromkova (personal communication

1992). As plasmid donor, isolate A1343/91 (isolated in this laboratory) was inoculated into 30 mℓ liquid Casman's medium and incubated at 37 °C for 18 h. After incubation, a sample was removed and plated onto BTA plates to check the purity of the culture. A sample of 5 mℓ was removed and inactivated and this sample was used to determine the Mab pattern of the donor isolate. The remainder of the donor culture was lysed by the addition of 1 mℓ volumes of a 1N NaOH solution, until a marked increase in viscosity and a decrease in turbidity of the culture were noted. The sample was neutralized by the addition of a volume of 1 N HCl equal to the volume of 1 N NaOH used to lyse the bacteria. After lysis, a sample was removed and plated out onto BTA plates to ensure that no viable NAD-independent donor isolates remained in the crude plasmid extraction.

Inducing competence in recipient isolates

Strain 0083 and isolate A745/91 were used as recipients of the plasmids in the crude extract. These isolates were inoculated into 10 mℓ liquid Casman's medium and incubated overnight. After incubation, a sample was removed and plated onto BTA plates to check the purity of the culture. To establish a competent culture, the techniques of Gromkova & Goodgal (1979) were followed. Basically the technique entailed spreading bacteria over a large surface area under aerobic conditions by the inoculation of 1 mℓ of the overnight culture of the reference strains into 5 mℓ Casman's medium in a sterile petri dish with a diameter of 90 mm. The samples were incubated at 37 °C for 18 h. After incubation, a sample was removed and plated onto BTA plates (with *S. aureus*) as a check on the purity of the culture.

Bacterial transformation

Each 2 mℓ sample of competent reference strain was mixed with 1 mℓ of the crude plasmid extraction and incubated for 1 h. After incubation, 10 mℓ liquid Casman's medium was added to the mixture of crude plasmid and competent cells. The culture was incubated for 6 h, after which it was inoculated onto BTA plates without the addition of *S. aureus*. The plates were incubated in a CO_2 environment and any colonies which were found to grow without NAD were inoculated into liquid Casman's medium and incubated. These cultures, as well as control non-transformed competent cultures, were inactivated with 0,1 % (V/V) formalin (after incubation and a purity check) and used for the coating of ELISA plates.

RESULTS

Experimental infection

Intra-sinus inoculation of chickens with the atypical *H. paragallinarum* isolates produced symptoms indistinguishable from those of typical infectious coryza 3 d post inoculation (P.I.). The chickens inoculated by the eye-drop method started showing the same symptoms 4 d P.I. No symptoms were detected in the control birds inoculated with sterile growth medium. Bacteria which resembled the original isolates in colony morphology, NAD independence, biochemistry and monoclonal antibody reaction patterns could be re-isolated from the experimentally infected chickens.

Typing with monoclonal antibodies

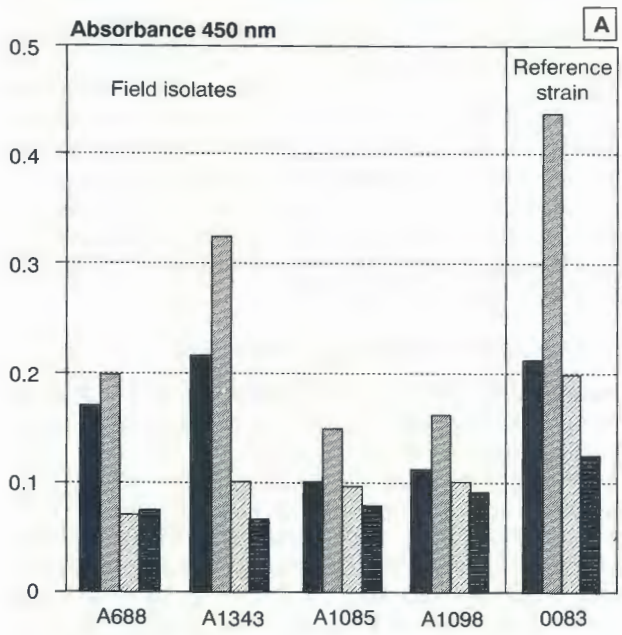
Mab reaction patterns were obtained for 61 isolates of NAD-independent isolates made from both layer and broiler chickens in South Africa between June 1991 and July 1992. Of these, only 4 isolates from layers and 3 from broilers showed a reaction with the V1 Mab. The Mab pattern for these isolates showed considerable homology with the Mab pattern of strain 0083 (Fig. 1). A total of 15 NAD-independent isolates from layers and 19 from broilers showed a Mab pattern which was similar to the South African field isolates of *H. paragallinarum* which were used for the production of the Mab panel (Verschoor *et al.* 1989) (cf. Fig. 2). A 3rd group, made up of 5 isolates from layers and 15 from broilers showed no significant reaction with any of the 3 Mab used (Fig. 3).

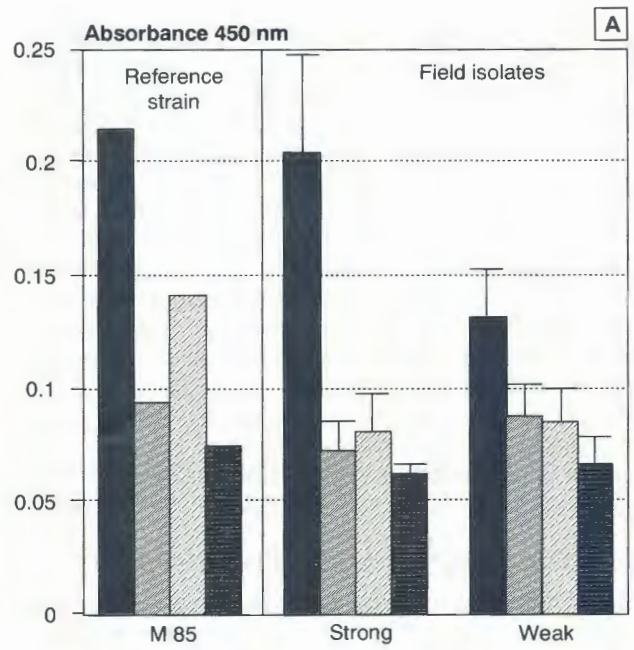
Bacterial transformation

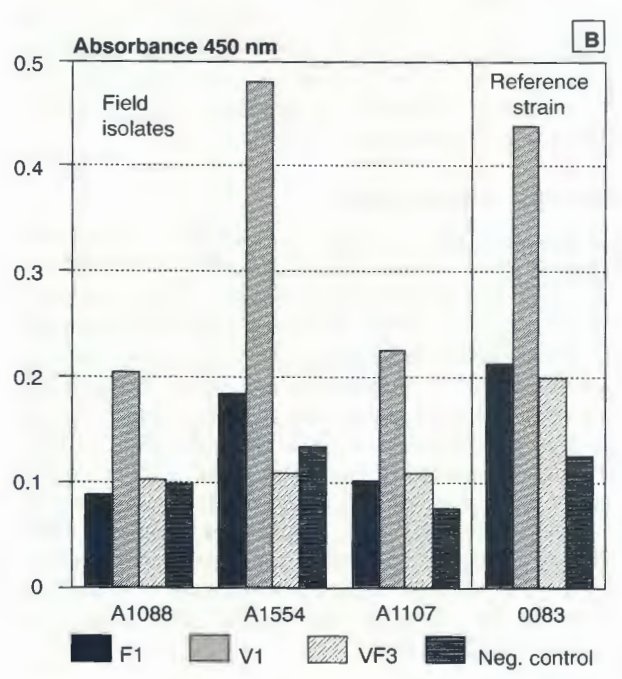
Isolate A1343/91 was selected as NAD-independent plasmid donor. Crude plasmid extract from this isolate was used for transformation of the recipient NAD-dependent strain 0083 and isolate A745/91 after it had been ascertained that no viable bacteria remained in the plasmid extract. NAD-independent bacterial colonies appeared on BTA plates inoculated with the transformed 0083 and A745/91 isolates. The Mab patterns of the 0083 strain and A745/91, before and after transformation can be seen in Fig. 4. Whereas the VF3 signal disappeared from A745/91, the V1 signal disappeared from strain 0083 upon transformation of the bacteria. In neither case did the signal to background ratio of Mab F1 change significantly.

DISCUSSION

The discovery that recent isolates of *H. paragallinarum* from layer hens and broilers could grow independently of NAD or serum, was initially thought to be due to a contamination. The fact that cloned NAD-independent bacteria caused infectious coryza in experimentally infected fowls with symptoms identical to those caused by NAD-dependent *H. paragallinarum* removed any doubt regarding the possiblity of contamination. This strongly suggests that the NAD-independent isolates are atypical *H. paragallinarum* isolates.







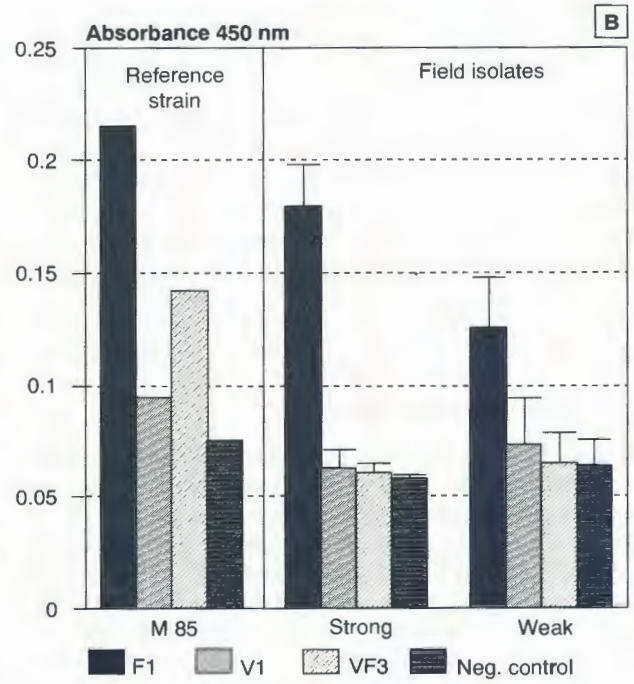


FIG. 1 ELISA patterns of NAD-independent *H. paragallinarum* isolates from layer hens (A) and broilers (B) resembling that of the 0083 reference strain in respect of their reactivity with Mab V1. Data represent the mean of at least 2 tests, with the exception of A 1098 and A 1088, of which only 1 test each was done

FIG. 2 ELISA patterns of NAD-independent *H. paragallinarum* isolates from layer hens (A) and broilers (B) resembling a typical NAD-dependent field isolate (M 85) in terms of their recognition by Mab F1. Strong reactors from layer hens (A) represent the mean of 6 and weak reactors the mean of 9 different isolates. Strong reactors for broilers (B) represent the mean of 7 and weak reactors the mean of 12 different isolates

Monoclonal antibody characterization of the bacteria showed that some of the NAD-independent isolates reacted with the V1 Mab in a manner indistinguishable from that of strain 0083 (Fig. 1). Another group of NAD-independent isolates has been found to show serological similarity to the NAD-dependent South African field strains of *H. paragallinarum* which were used for the development of the panel

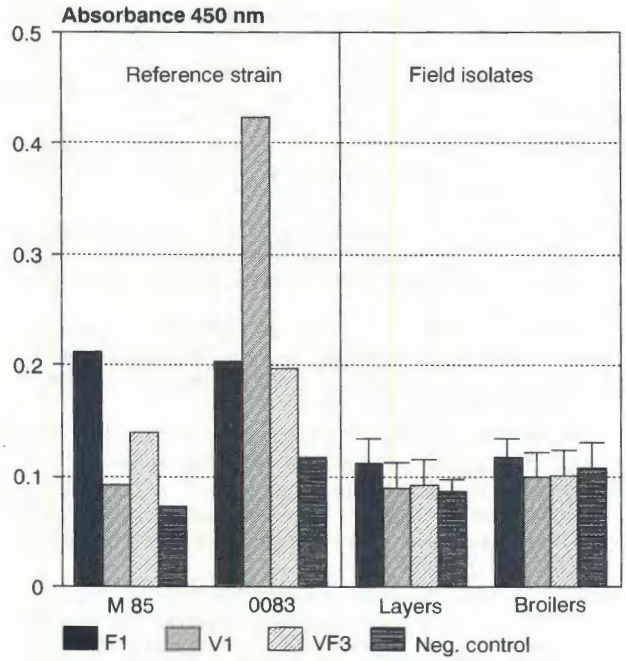


FIG. 3. ELISA patterns of NAD-independent *H. paragallinarum* isolates exhibiting no reaction with the Mab tested. Layers represent the mean of 5 isolates and broilers the mean of 15 different isolates. Each isolate was tested at least in duplicate, except for A555/92 BH2 which was tested only once

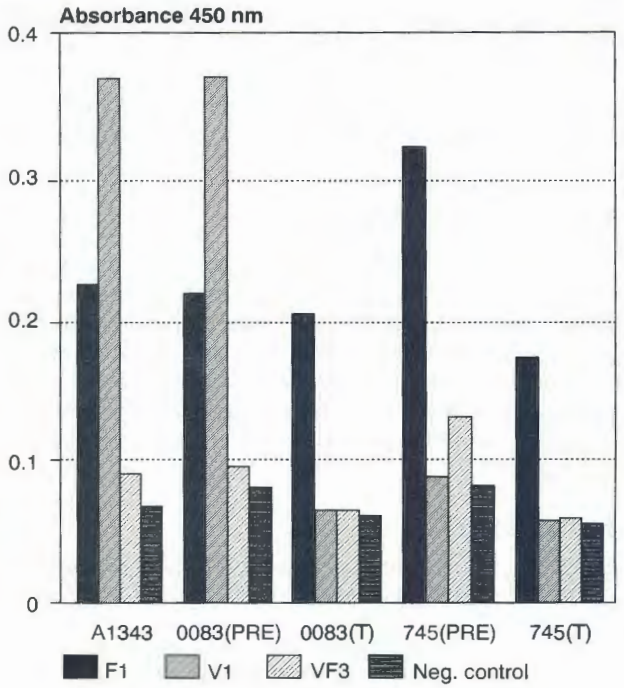


FIG. 4 ELISA patterns of the plasmid donor bacterium (A1343/91) and of the recipient 0083 and A745/91 (labelled 745 in graph) bacteria before (PRE) and after (T) transformation with the donor plasmid extract. Values represent the mean of at least quadruplicate tests

of monoclonal antibodies employed here (Verschoor *et al.* 1989). Moreover, the Mab pattern of these NAD-independent isolates was also similar to a recently collected NAD-dependent South African field isolate, designated A745/91. A minor group of NAD-independent isolates showed no reaction with the monoclonal antibodies. This may be due to incomplete cross-reactivity of the panel of monoclonal antibodies employed here, as it was postulated that all the variants of *H. paragallinarum* can be serologically defined as consisting of 9 different serovars (Blackall *et al.* 1990). Work in this regard is underway and will be reported on in due course. For the main part, however, the monoclonal antibody characterization of our atypical isolates confirms their identity as authentic *H. paragallinarum*.

In bacterial transformation experiments the NAD-independent isolate A1343/91 was used as a plasmid donor. Cultures of NAD-dependent reference strains (0083 and A745/91) were used as plasmid recipients. Transformation of these recipient strains with plasmids from the donor bacteria incurred NAD independence on them. In the light of the observations of Gromkova & Koornhof (1990) and Windsor *et al.* (1991), in which NAD independence of *H. parainfluenza* was shown to be plasmid mediated, we propose that isolate A1343/91 is a naturally occurring NAD-independent *H. paragallinarum*, of which NAD independence was gained by plasmid transformation. As this isolate is serologically related to reference strain 0083 and 6 other NAD-independent isolates from layers and broilers, it is postulated that all 6 of these isolates are most likely of Page's A serotype (Page 1962). All the other NAD-independent isolates which either showed reaction with the F1 Mab, or no reaction with our panel of Mabs, are similarly postulated to be NAD-independent *H. paragallinarum*, albeit of different serovars to the isolates which reacted with the V1 Mab.

This work corroborates the observations of Mouahid *et al.* (1992), who identified NAD-independent South African isolates of *H. paragallinarum*, by means of DNA/DNA hybridization, and argues against the suggestion of Horner *et al.* (1992) that the NAD-independent isolates, made from chickens with symptoms of infectious coryza in Natal cannot be classified as *H. paragallinarum* based purely on the NAD independence of these isolates.

From this work, it may be concluded that at least 3 distinct serovars of NAD-independent *H. paragallinarum* isolates have been identified from chickens in South Africa.

It is interesting to note that when the 0083 strain of *H. paragallinarum* was transformed into an NAD-independent isolate, the Mab pattern of the isolate changed from a predominantly V1 phenotype to predominantly F1 phenotype. This phenomenon can-

not be simplistically ascribed to plasmid-encoded antigen, as the donor bacterium was itself predominantly of the V1 phenotype. The effects of DNA transformation between strains of *H. paragallinarum* on their serological classification needs urgent investigation.

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