

Effects of transformation on the hemagglutinins of *Haemophilus paragallinarum*

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ABSTRACT

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Strain 0083 and two field isolates of *H. paragallinarum* were previously converted into NAD-independent organisms by the use of crude DNA extractions from naturally occurring NAD-independent *H. paragallinarum* isolates. Two of these transformed isolates [0083(T) and A745(T)] were used as DNA donors in another transformation experiment in which another field isolate (M85) was used as the DNA recipient. Transformation was confirmed by lack of NAD requirement for growth, by carbohydrate fermentation patterns and by a comparison of the monoclonal antibody patterns of the isolates before and after transformation. Previously, antigenic differences were observed when DNA from an NAD-independent isolate was introduced into strain 0083. Antigenic differences were also seen in the transformed M85 organisms prepared in this work, and these differences were dependent on the antigenic patterns of the DNA donors.

It was established by haemagglutination (HA) and haemagglutination inhibition (HI) that the hemagglutinins of 0083, A745/92 and M85 were not affected by transformation. The use of strains transformed to NAD independence for vaccine production appears to be a valid approach, as the transformation appears not to affect the hemagglutinins of the transformed organisms. The major advantage would be the alleviation of the requirement for chicken serum or NAD in the bacterial growth medium used for infectious-coryza-vaccine production.

Keywords: *Haemophilus paragallinarum*, hemagglutinins, infectious coryza

INTRODUCTION

Infectious coryza is a disease of chickens (Yamamoto 1991) causing substantial financial losses in countries where it occurs (Chen, Zhang, Blackall & Feng 1993). *Haemophilus paragallinarum*, the causative agent of infectious coryza, has also been isolated in South Africa and causes severe problems in this country (Bragg, Coetzee & Verschoor 1993b).

H. paragallinarum was first isolated in the 1930s (De Bleeck 1932) when it was thought to require both NAD (V factor) and haem (X factor) for growth (Schalm & Beach 1936; Delaplane, Erwin & Stuart 1938). This organism was identified as *H. gallinarum*. The first report that the causative agent of infectious coryza grew without the addition of haem (X factor) was as early as 1932 (McGaughy 1932). Subsequent work (Page 1962; Roberts, Hanson & Timms 1964; Blackall & Reid 1982; Blackall & Yamamoto 1989) confirmed that the organism does not require haem, and these organisms have been termed *H. paragallinarum*. In 1990, NAD-independent isolates causing a disease indistinguishable from infectious coryza, were isolated in South Africa (Horner, Bishop & Haw 1992; Mouahid, Bisgaard, Morley, Mutters & Mann-

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heim 1992; Bragg, Coetzee & Verschoor 1993a). Horner *et al.* (1992) suggested that these organisms were not *H. paragallinarum*, by virtue of the fact that they did not require NAD for growth. On the other hand, Mouahid *et al.* (1992), using DNA/DNA hybridization, and Bragg *et al.* (1993a), using monoclonal antibodies (Mabs), established that these isolates were atypical NAD-independent *H. paragallinarum*.

In the same year (1990), NAD-independent *H. parainfluenza* isolates were made from human patients in South Africa (Gromkova & Koornhof 1990). *H. parainfluenza* normally requires NAD for growth and, according to present taxonomic criteria, bacteria which do not require NAD for growth cannot be classified as *H. influenza* or *H. parainfluenza* (Gromkova & Koornhof 1990). The report by Gromkova & Koornhof (1990) is the first report of naturally occurring NAD independence in *H. parainfluenza*. It was established that the NAD independence of these isolates was plasmid-mediated (Windsor, Gromkova & Koornhof 1991) and that the genes for NAD independence are located on a small 5.25-kb plasmid.

Transformation experiments, with NAD-independent *H. paragallinarum* isolated from chickens in South Africa as a DNA donor, and strain 0083 and South African field isolates (A745/92 and A739/92) as recipients, were successfully carried out (Bragg *et al.* 1993a), thus suggesting that the NAD independence in *H. paragallinarum* is also plasmid-mediated. It was, however, noted that the antigen which is detected by the V1 Mab (Verschoor, Coetzee & Visser 1989), was inexplicably lost from strain 0083 when this organism was transformed into an NAD-independent organism (Bragg *et al.* 1993a). These findings led to the following two questions:

- Do genes for surface antigens of *H. paragallinarum* become irrevocably lost upon transformation?
- Can the genes conferring NAD independence and determining surface antigen expression be retrieved from transformed NAD-independent organisms which have previously been NAD dependent?

In order to investigate these questions, another NAD-dependent field isolate (M85) was transformed with DNA from the previously transformed 0083 and A745/92 (Bragg *et al.* 1993a), and these newly transformed isolates were examined with the Mabs produced by Verschoor *et al.* (1989).

The possibilities of using NAD-independent *H. paragallinarum* in vaccine production provides an attractive commercial application for this work, enabling a reduction in the cost of the media used to grow the bacteria. The antigenic changes occurring when strain 0083 is converted to NAD independence (Bragg *et al.* 1993a) may, however, render the transformed NAD-independent organisms unsuitable for vaccine production if the immune response in the host is compromised as a result of the transformation process.

It is therefore of the utmost importance to investigate the effects of transformation of the expression of the hemagglutinins of *H. paragallinarum*, which play an important role in the protective immunity (Iritani, Iwaki, Yamaguchi & Sueishi 1981; Kume, Sawata & Nakase 1980; Otsuki & Iritani 1974; Iritani, Katagiri & Arita 1980; Takagi, Hirayama, Simazaki, Taguchi, Yamaoka & Ohta 1993).

METHODS AND MATERIALS

Bacterial strains and isolates

H. paragallinarum strain 0083 [serotype A (Page 1962)] was supplied by Dr P.J. Blackall, Animal Research Institute, Australia, as well as by Golden Lay Laboratories (now operating under the name of "Avimune"), Verwoerdburg, South Africa. Freeze-dried samples of 0083 have also been reconstituted from stored samples in the Department of Poultry Diseases, Faculty of Veterinary Science, Onderstepoort. Isolate A1343/91 [serotype A (Bragg 1994, unpublished data)] is an NAD-independent *H. paragallinarum* which was isolated in this laboratory (Bragg *et al.* 1993a). Isolate M85 is an NAD-dependent field isolate obtained in 1985 (Verschoor *et al.* 1989) and was kindly supplied by Golden Lay Laboratories. Isolate A745/91 [serotype A (Bragg 1994, unpublished data)] is an NAD-dependent field isolate, obtained in 1991 by this laboratory. Transformed NAD-independent cultures of 0083 and A745/92 were previously prepared in this laboratory (Bragg *et al.* 1993a) and are referred to as 0083(T) and 745(T).

Strain HP60 [serotype C (Blackall, Eaves & Rogers 1990) which was used for the production of rabbit-raised antibodies was kindly supplied by Dr P.J. Blackall of the Animal Research Institute, Queensland, Australia. Spross [serotype B (Rimler, Davis & Page 1977), which was also used for the production of rabbit-raised antibodies, was kindly supplied by Dr A.A.C. Jacobs of Intervet, Boxmeer, The Netherlands.

Culture conditions

All of the NAD-dependent organisms were grown in modified Casman's medium at a pH of 7.4 (Coetzee, Rogers & Velthuysen 1983) which was supplemented with 10% sterile chicken serum. NAD-independent isolates and transformed isolates were grown in modified Casman's medium, at a pH of 7.4, without the addition of 10% sterile chicken serum. All of the isolates were plated out onto Blood Tryptose Agar (BTA) (supplied by the Onderstepoort Vaccine Factory). In the case of the NAD-dependent isolates, a *Staphylococcus aureus* culture was used to supply the NAD required for growth. A sample of *S. aureus* was inoculated across the NAD-independent isolates. No *S. aureus* feeder culture was required when NAD-independent isolates were inoculated onto BTA plates.

Transformation experiments

Transformation of isolate M85 was carried out according to the methods described previously (Bragg *et al.* 1993a). The DNA donors in these experiments were the organisms which had previously been transformed into NAD-independent organisms (Bragg *et al.* 1993a). The cultures used as DNA donors [745(T) and 0083(T)] were inoculated into Casman's medium without sterile chicken serum and incubated overnight at 37°C. At the same time, the isolate to be used as the DNA recipient (M85) was incubated under conditions which would render the organisms competent to take up DNA (Gromkova & Goodgal 1979). This isolate was inoculated into 5 ml of Casman's medium which had been supplemented with 10% sterile chicken serum, in a sterile petri dish with a diameter of 90 mm and incubated at 37°C for 18 h. After incubation, both the donors and recipients were tested for purity by plating them out onto BTA plates. The donors were lysed by addition of 1-ml volumes of sterile 1 N NaOH until a marked increase in viscosity and a decrease in turbidity was seen. The pH of the lysate was then adjusted to approximately 7 by the addition of equal volumes of sterile 1 N HCl. Samples were collected from the lysate and tested for growth by plating them out onto BTA plates and incubating them at 37°C for 18 h inside a candle jar. This confirmed that no viable, NAD-independent organisms survived in the crude DNA extract. One millilitre of the DNA extract was mixed with 2 ml of the competent-recipient-organism suspension. The mixture was incubated for 60 min at 37°C, to allow uptake of the DNA. Subsequently, 10 ml of Casman's medium supplemented with 10% sterile chicken serum was added to the mixture, and incubated for 6 h to allow for the expression of NAD independence. Samples were then plated onto BTA plates without a *S. aureus* feeder culture, for the detection of NAD-independent transformants. The plates were incubated in a candle jar at 37°C for 18 h. A 1-ml sample was also used to inoculate Casman's medium without NAD or sterile chicken serum. This was incubated at 37°C for 24 h, after which any tubes showing visible growth (as seen by an increase in turbidity) were plated onto BTA plates without NAD or the *S. aureus* feeder culture, for the detection of NAD-independent transformants.

As a control on the transformation process, the NAD-dependent M85 was also used as a DNA donor. All of the steps described above were carried out on the NAD-dependent donor, and crude DNA extracts from this culture were used to attempt transformation of isolate M85 as discussed above.

Biochemical characterizations

The carbohydrate fermentation patterns of all of the isolates, before and after transformation, were ob-

tained. In the case of NAD-dependent isolates, Casman's medium, which had been supplemented with sterile chicken serum, was inoculated with the organisms. In the case of NAD-independent organisms, Casman's medium, which was not supplemented with sterile chicken serum, was used. The organisms were inoculated into the appropriate medium and incubated at 37°C for 18 h. After the cultures had been incubated, their purity was ascertained by plating a sample out onto BTA plates. The different isolates were then identified by the performance of a catalase test and it was determined whether the utilization of glucose was fermentative or oxidative. The ability to utilize galactose, glucose, lactose, mannitol, mannose, sorbitol and sucrose was tested by inoculating 0,1 ml of the overnight culture into tubes containing phenol red broth supplemented with 1% (w/v) of each of the different carbohydrates. In the case of the NAD-dependent isolates, the media was supplemented with 0,5% (w/v) NaCl, 1% (v/v) sterile chicken serum and 0,0025% (w/v) NAD (Blackall & Reid 1982). The tubes were incubated at 37°C and the reactions read 48–72 h post inoculation. The carbohydrate fermentation patterns (either before or after transformation) were repeated four times for each isolate.

Monoclonal antibody characterization

The effects of transformation on the antigenic structure of isolate M85 were evaluated. Isolates which had previously undergone transformation were used as DNA donors, and Mab patterns, both before and after transformation had been obtained by means of the Mabs produced by Verschoor *et al.* (1989) in the ELISA test. The methods used for performing the ELISA by means of the Mabs, have been described previously (Bragg *et al.* 1993a, b) and these methods were followed without modification.

Polyclonal antibodies

Polyclonal antibodies were obtained from three different sources. Samples of rabbit-raised antibodies against serotypes A, B and C were obtained from Dr P.J. Blackall of the Animal Research Institute, Australia.

Rabbit-raised antibodies against *H. paragallinarum* strains HP-60 Spross and 0083 were produced by the South African Institute of Medical Research (SAIMR), Johannesburg. The bacteria to be used for inoculating the rabbits were suspended in 0,5% formal saline and incubated for 24 h at 4°C to allow for inactivation. The bacterial suspensions were adjusted to ten international opacity units before the rabbits were inoculated with 0,5 ml of the bacterial suspension in 5 mg/ml of Quill-A adjuvant (supplied by the SAIMR). After 4 weeks the rabbits were re-inoculated with bacteria plus adjuvant. After another

week, the rabbits were subsequently bled and the serum tested for the presence of neutralizing antibodies. The rabbits were inoculated again after 2 weeks, with 1 ml of bacterial suspension without adjuvant, and exsanguinated 1 week after the final inoculation.

Polyclonal antibodies against strain 0083 were also prepared in 5-week-old White Leghorn chickens. The bacterium was grown in Casman's medium supplemented with 10% sterile chicken serum. The cultures were inactivated with 0,1% formalin, after the purity of the inoculum had been ascertained. The chickens were inoculated intramuscularly with 1 ml of inactivated, washed bacteria. The chickens were re-inoculated 2 weeks later with 1 ml of inactivated bacteria. Each chicken was inoculated with another 1 ml of inactivated bacteria after another 2 weeks, and the birds were bled to death two weeks after the last inoculation.

Haemagglutination (HA) and haemagglutination-inhibition (HI) test

Isolates 0083, A745/90 and M85 were inoculated into Casman's medium which had been supplemented with 10% sterile chicken serum and incubated at 37 °C for 18 h. Isolates A1343/91, 0083(T), 745 (T) as well as the two newly transformed isolates, labelled M85 (T/0083) and M85 (T/745), were inoculated into Casman's medium without chicken serum or NAD and were incubated at 37 °C for 18 h. Samples of fresh 18-h-old cultures of all the organisms were tested in the HA test.

The HA test was performed according to a modification of the method of Eaves, Rogers & Blackall (1989) in which 1% gluteraldehyde-fixed chicken-red-blood cells (GA-fixed RBC) were used. The HA test was performed in Veronal buffer (VB) instead of phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin and 0,001% (w/v) gelatin, as used by Eaves *et al.* (1989). Concentrated VB consisted of 2,3 g of 5-5-diethylbarbituric acid (Merck); 1,5 g of barbitone sodium (BDH), 41,9 g of sodium chloride (Merck); 0,05 g of magnesium chloride (Merck); 0,075 g of calcium chloride and 1,26 g of sodium bicarbonate (Merck) in 1 000 ml of distilled water, at a pH of 7,4. The concentrated VB was diluted 1:5 (v/v) with distilled water before use in the HA test.

HA tests were carried out by adding 50 µl of twofold serial dilutions of the different bacterial cultures to 50-µl volumes of VB in V-bottomed 96-well plates. Each well of the plate then received 50 µl of GA-fixed RBC. The plates were incubated at room temperature for 1 h. The HA titre was read as the highest dilution of the bacterial suspension causing haemagglutination of the GA-fixed RBC.

The HI test was also performed according to the methods of Eaves *et al.* (1989), with some modifi-

cations. The HI test was performed with four HA units (as determined from the HA test) of the different bacterial isolates showing HA activity. The HI tests were performed in 50-µl volumes of twofold serial dilutions of the polyclonal antiserum made either in rabbits or chickens, or antiserum obtained from Dr Blackall, in diluted VB. The serum samples obtained from Dr Blackall were diluted 1:100 (v/v) before use. Each well subsequently received 50 µl of a dilution of the different bacterial suspensions, made up to contain four HA units. The plates were incubated for 15 min, followed by the addition of 50 µl of a 1% solution of GA-fixed RBC to each well and the plates were further incubated for 1 h. The HI titre was read as the highest dilution of antiserum which inhibited haemagglutination of four HA units of the different bacteria.

RESULTS

Transformation experiment and isolate characterization

The history of the NAD-independent donor strains, 0083(T) and 745(T), is depicted in Fig. 1 and 2, respectively. These strains were NAD dependent before initial transformation (Bragg *et al.* 1993a) with A745/91 strongly expressing the F1 antigen (Fig. 2) and 0083 strongly expressing the V1 antigen (Fig. 1). Upon transformation with crude DNA from the NAD-independent strain, A1343/91, which was also strongly expressing the V1 antigen (Bragg *et al.* 1993a) (Fig. 1 and 2), it was remarkable that the expression of the V1 antigen was inhibited in the transformed NAD-independent strains, irrespective of whether the recipient strains were positive or negative expressors of the V1 antigen (Bragg *et al.* 1993a). It could be concluded from these results that NAD independence does not correlate with expression of the V1 surface antigen. The expression of antigens F1 and VF3 did not vary much amongst donor, recipient and transformed strains, although a weaker signal for all of the antigens was noted in the transformed strain, 745(T).

Crude DNA extracts were obtained from each of 0083(T) and 745(T) and used to transform the NAD-dependent M85 in order to investigate whether the newly transformed NAD-independent strains 0083(T) and 745(T) could be used as donors of the NAD-independence trait by means of transformation to another NAD-dependent strain. NAD-independent, transformed isolates (as seen by the ability to grow on BTA plates not supplemented with NAD or with the *Staphylococcus* feeder culture) were obtained in both cases, suggesting that this property remains associated with transferable plasmids. The isolate transformed with a crude DNA extract from 0083(T) was referred to as M85(T/0083) and the isolate obtained when 745(T) was used as a donor, as M85(T/

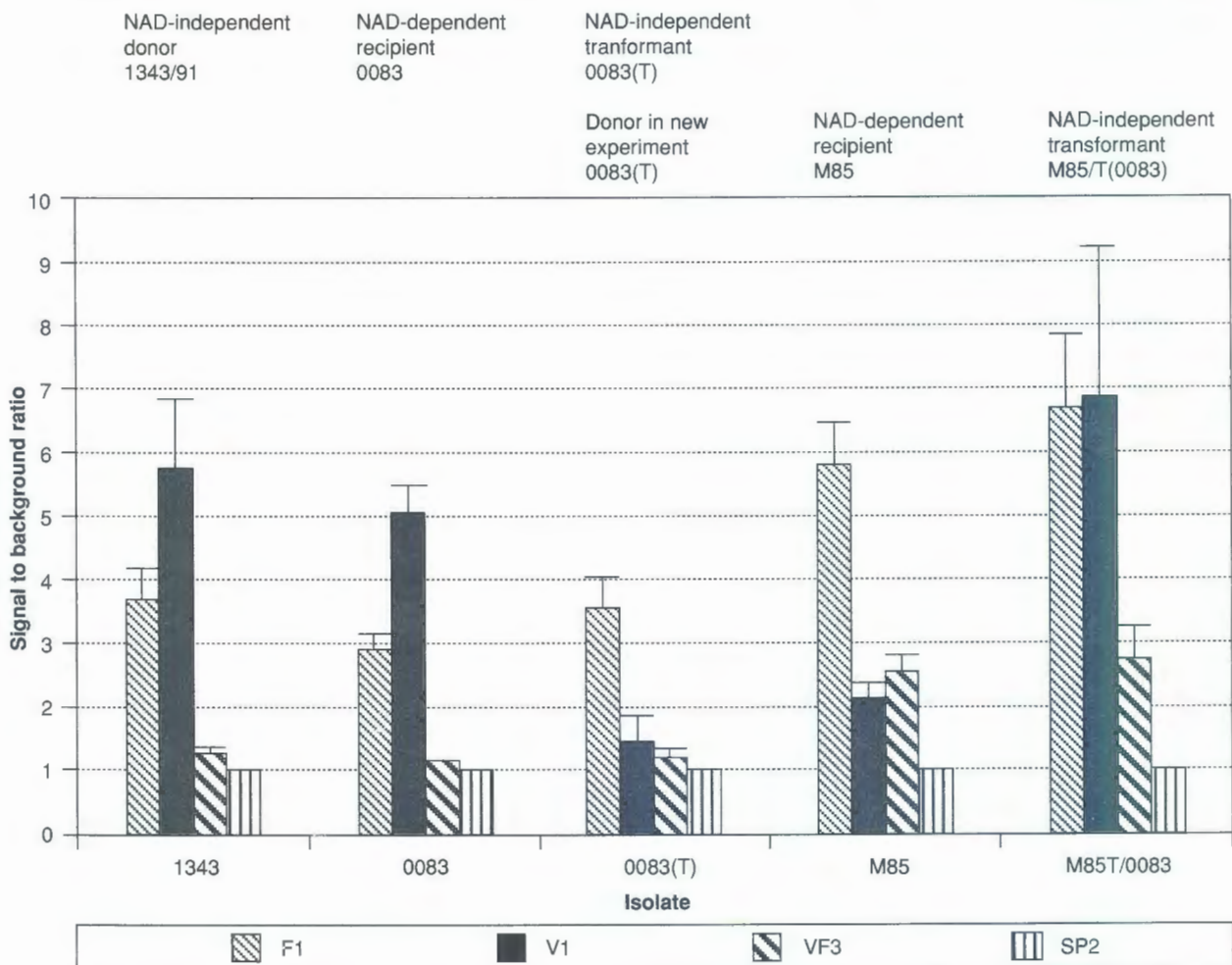


FIG. 1 Monoclonal antibody patterns obtained from a series of transformation experiments involving the reference strain 0083, *H. paragallinarum*. In the first instance the NAD-dependent strain, 0083, was converted into a NAD-independent strain with the use of crude DNA extractions from a naturally occurring NAD-independent field isolate of *H. paragallinarum* (1343/91). The transformed 0083 isolate was termed 0083(T) (graph adapted from Bragg *et al.* 1993a). This transformed NAD-independent 0083(T) was then used as a DNA donor in another transformation experiment in which the NAD-dependent field strain M85 was transformed into NAD independence and labelled M85/T(0083). Note the lack of expression of the V1 antigen in 0083(T) and the expression of the V1 antigen in M85/T(0083). F1, V1 and VF3 denote culture supernatants of Mab directed against different *Haemophilus* antigens, while SP2 is a culture supernatant from a non-antibody-producing hybridoma cell line

745). No NAD-independent transformants were obtained when crude DNA extracts were made from the NAD-dependent M85 and mixed with M85. This confirms that the transformation to NAD independence is dependent on obtaining suitable genetic material from NAD-independent organisms and is not an artifact resulting from the manipulation of the donor or recipient cultures.

No viable bacteria were detected in the crude DNA extracts, as seen by the lack of growth on the BTA plates inoculated with samples of crude DNA extract. This excludes the possibility that viable NAD-independent donor isolates may have been transferred by means of the DNA extract.

The carbohydrate fermentation patterns were found to be identical for all of the isolates which were tested,

both before and after transformation. In all cases, the isolates were catalase negative and fermentative. All of the isolates utilized glucose, mannitol, sorbitol, mannose and sucrose. None of the isolates utilized galactose or lactose.

Biochemical characterization of the transformed isolates, as well as of the wild-type organisms, indicated that the carbohydrate fermentation patterns of both sets of isolates were identical, thus confirming that the transformed isolates were, in fact, *H. paragallinarum*. These biochemical characteristics also correspond to those described by Blackall & Reid (1982).

The monoclonal antibody patterns of all isolates, both before and after transformation, can be seen in Fig. 1 and 2. It is interesting that the V1 antigen is again strongly expressed in the transformed M85(T/0083)

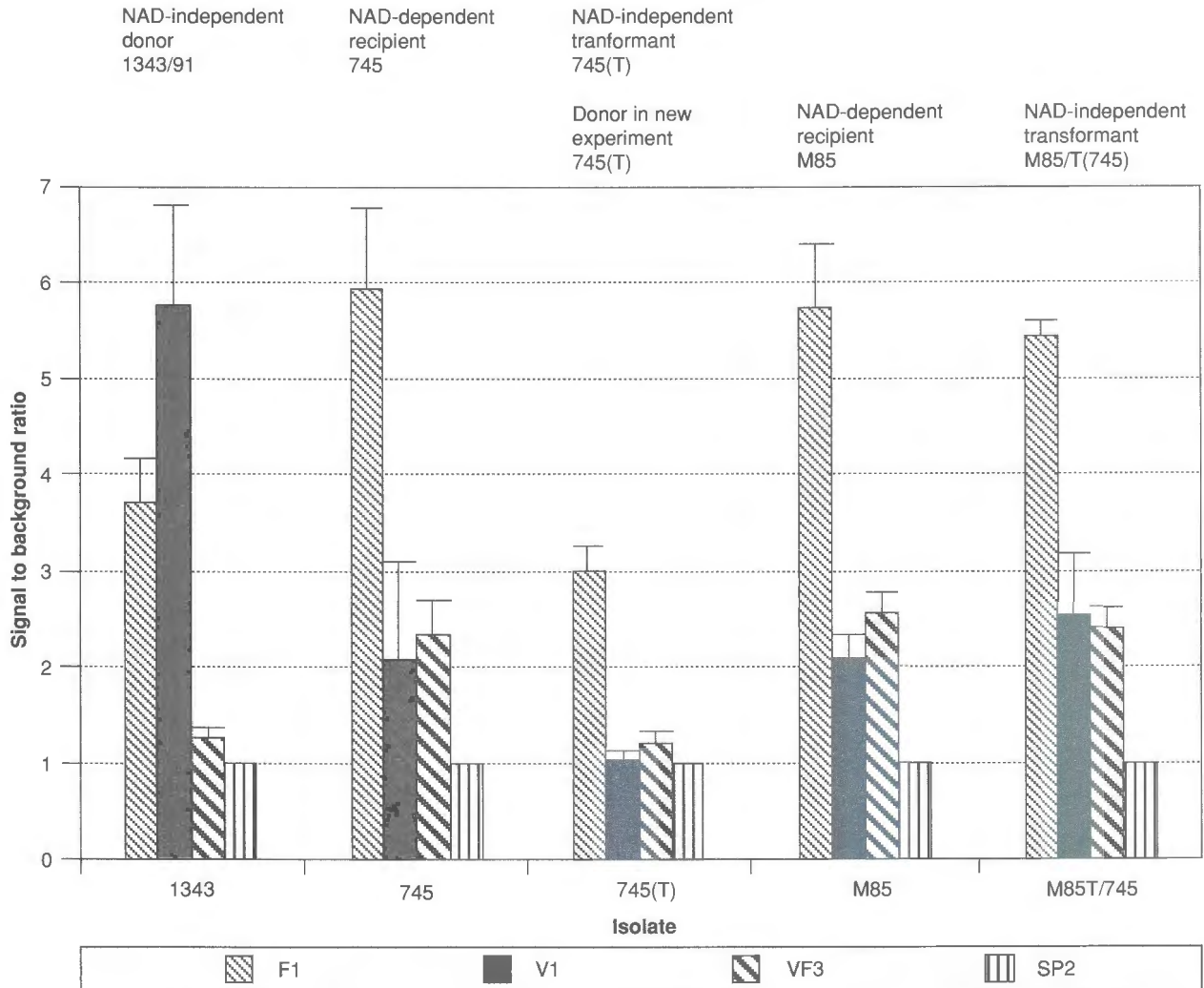


FIG. 2 Monoclonal antibody patterns obtained from a series of transformation experiments involving a field isolate (745/91) of *H. paragallinarum*. In the first instance, the NAD-dependent isolate, 745/91, was converted into a NAD-independent strain with the use of crude DNA extractions from a naturally occurring NAD-independent field isolate of *H. paragallinarum* (1343/91). The transformed 745 isolate was termed 745(T) (graph adapted from Bragg *et al.* 1993a). This transformed NAD-independent 745(T) was then used as a DNA donor in another transformation experiment in which the NAD-dependent field strain, M85, was transformed into NAD independence and labelled M85(T/745)

(Fig. 1), a reoccurring property inherited from the 0083-strain-transformation grandparent (Fig. 1). The 745(T)-transformation donor parent was unable to transcend V1-expression on M85 after transformation (Fig. 2), probably because no V1-expression history exists in the transformation grandparent of strain A745/91 (Fig. 2). Once again, no pronounced variation of expression of the F1 and VF3 antigens occurred, manifesting the non-relatedness of expression of the three antigens recognized by the Mabs with NAD independence.

Haemagglutination and haemagglutination-inhibiton tests

Haemagglutination tests (HA) were performed with both transformed and non-transformed strains of *H.*

paragallinarum to determine whether transformation affects the expression of the protective hemagglutinin antigens.

The HA was successfully carried out on the NAD-dependent strains 0083, A745/92 and M85, and the mean HA titres ($n = 8$) can be seen in Table 1. The HA test was also successfully carried out on the naturally occurring NAD-independent isolate, A1343/91, and the reciprocal of the mean HA titres ($n = 8$) was found to be 28 (Table 1). The HA test was also successfully performed on all four isolates transformed to NAD independence. The isolates tested were 0083(T), A745(T) and the newly transformed M85(T/0083) and M85(T/745). The reciprocal of the mean HA titres ($n = 8$) for these transformed isolates can be seen in Table 1.

TABLE 1 Mean HA titres ($n = 8$) obtained with both naturally occurring NAD-independent as well as transformed NAD-independent strains and naturally occurring NAD-dependent *Haemophilus paragallinarum* strains

Naturally occurring strains			Transformed strains			
NAD dependent		NAD independent	NAD independent			
0083	745/91	1343/91	0083(T)	745(T)	M85(T/0083)	M85(T/745)
31	30	28	17	16	19	20

TABLE 2 HI antibody titres obtained against four HA units of each of the different isolates. These results are the mean titres ($n = 8$) and a titre of 0 represents no HI activity of the antibodies

Serum against (source in brackets)	Naturally occurring strains				Transformed strains			
	NAD dependent			NAD independent	NAD independent			
	0083	745/91	M85	1343/91	0083(T)	745(T)	M85(T/0083)	M85(T/745)
(Blackall)								
A	44	48	0	24	18	17	0	0
B	0	0	0	0	0	0	0	0
C	0	0	2	0	0	0	0	0
(Chicken)								
0083	48	48	0	36	24	20	0	0
(Rabbit)								
0083	48	48	0	48	22	22	0	0
Spross	0	0	0	0	0	0	0	0
HP60	0	0	8	0	0	0	6	6

HI tests were performed with specific polyclonal sera from different sources to determine whether the NAD dependency or the process of transformation affects the antigenicity of the hemagglutinins in any way.

The HI test was successfully carried out with four HA units (as calculated from the HA test) on all of the isolates. Strain 0083 reacted with the polyclonal serum against serotype A (obtained from Dr Blackall) with a mean HI titre ($n = 8$), expressed as the reciprocal of the highest dilution resulting in inhibition (Table 2). Similar results were also obtained for isolate A745/92, with the same polyclonal serum (Table 2). In the case of isolate A1343/92, the reciprocal of the mean HI titres ($n = 8$) was 24 (Table 2). The transformed 0083(T) and 745(T) also reacted with the serotype-A serum obtained from Dr Blackall, with the reciprocal of the mean HI titres ($n = 8$) of 18 and 17, respectively. None of these isolates reacted with either the serotype-B antiserum or the serotype-C antiserum obtained from Dr Blackall.

Very similar results were obtained when four HA units of the above isolates were mixed with either the new-

ly prepared rabbit-raised antiserum against 0083, or the chicken-raised antiserum against 0083. No HI was recorded when any of these isolates were mixed with the rabbit-raised antibodies against Spross (serotype B) or HP-60 (serotype C) (Table 2).

M85 and the transformed M85(T/0083) and M85(T/745) did not show any HI reaction with any of the antibodies against serotype-A or serotype-B *H. paragallinarum*. M85 did show a very weak HI reaction [mean HI titre ($n = 8$) of 2] against the serotype-C antibodies supplied by Dr Blackall (Table 2). There was no HI when the transformed M85 isolates were mixed with the serotype-C antiserum of Dr Blackall. All three of these isolates showed some reaction when mixed with the rabbit-raised antiserum against HP-60 (serotype C) (Table 2).

DISCUSSION

The manufacture of infectious coryza vaccines requires the growth of various strains of *H. paragallinarum* in media which must be supplemented with NAD

or chicken serum—both very costly ingredients. The conversion of strains used in the production of vaccines into NAD-independent organisms could be useful for vaccine manufacture and could result in substantial reduction in the cost of the media, as NAD or chicken serum would no longer be required for growth of the organisms. The indiscriminate loss of surface antigens, exemplified by the finding that the antigen recognized by the V1 Mab was not expressed in the the NAD-independent 0083(T) strain (Bragg *et al.* 1993a), could, however, negatively affect the use of transformed NAD-independent strains in the manufacture of infectious coryza vaccines. It was therefore deemed essential that the effects of transformation on the antigens involved in the immune response be investigated.

Much work has been done on the antigens involved in the immunity of *H. paragallinarum*. The serovar-specific hemagglutinin (Iritani *et al.* 1981; Kume *et al.* 1980) was thought to play a role in the protective immunity due to the correlation between the haemagglutination-inhibition (HI) titre and protection (Otsuki & Iritani 1974; Iritani *et al.* 1980; Kume *et al.* 1980). This was recently confirmed by Takagi, Ohmae, Hirayama & Ohta (1991), who successfully cloned the genomic DNA of strain 221 into the vector plasmid, pBR322, which was introduced into *Escherichia coli*. One of the transformed *E. coli* isolates showed haemagglutinating activity. Chickens were vaccinated with killed *E. coli* expressing the hemagglutinin-produced HI antibodies, and were protected against challenge by 221. There is therefore good evidence that the type-specific hemagglutinins of *H. paragallinarum* play an important role in the protective immunity.

When the previously transformed NAD-independent strains 0083(T) and 745(T) were used as DNA donors to transform another NAD-dependent field isolate (M85) into NAD independence, the phenomenon of the altered expression of outer-membrane antigens detected by the Mabs, was repeated (see Fig. 1). This supports the notion that transformation of NAD-dependent organisms into NAD independence causes changes to the antigenic structure of the bacterium. It also became evident that the genes involved in the expression of selected surface antigens were not lost during transformation. This was illustrated by the reappearance of the antigen detected by the V1 Mab in M85(T/0083). In these experiments, M85, which was transformed by 0083(T), strongly expressed the V1 antigen (Fig. 1), while both the wild-type M85 and the transformed 0083(T) expressed this antigen only very weakly. This antigen was, however, strongly expressed in the wild-type 0083 and was "lost" during transformation. M85(T/745) expressed the V1 antigen weakly (Fig. 2). The wild-type A745/92 and the wild-type M85 also expressed this antigen very weakly. The transformed 745(T)

did not express this antigen at any stage during the experiment. The gene for this antigen in M85(T/0083) must have originated in the wild-type 0083 and was not expressed in 0083(T). At this stage, the mechanisms involved in the regulation of gene expression in the transformed isolates, as compared to the wild-type isolates, are not clear.

From the above, it appears that transformation had some effect on the expression of antigens of *H. paragallinarum*. If these transformed isolates were to be used for the commercial manufacture of vaccines, it was essential to establish what effects the transformation had on the hemagglutinin of the organisms.

Comparable results were obtained with the H_i test on the wild-type 0083, the NAD-independent 0083(T) and the naturally occurring NAD-independent A1343/91, by using polyclonal antiserum raised against 0083, indicating that the type-specific hemagglutinin of these organisms is the same. All three organisms tested could be classified as serotype A by virtue of the HI results. It can be seen from these results that, although the expression of other surface antigens of the transformed 0083(T) organism were affected by the transformation process, the type-specific hemagglutinin appeared to remain expressed constitutively. In the light of the work done by Takagi *et al.* (1991) and Takagi *et al.* (1993), in which it was established that the type-specific hemagglutinin plays an important role in protective immunity, it is postulated that, as the type-specific hemagglutinin of the transformed organisms appeared to be unaffected by transformation, these transformed organisms will be useful in the manufacture of infectious coryza vaccines in medium, without the addition of NAD or chicken serum.

Strain M85 barely reacted with the polyclonal serotype-C antiserum (Table 2), while the transformed M85 isolates did not react at all. The serotype-C antibodies obtained from Dr Blackall were produced against strain H-18 (serotype C) and antibodies against this isolate were shown to have limited cross-reactions (Kume, Sawata, Nakai & Matsumoto 1983) with a uniquely South African serotype (C-3) represented by the strain SA-3 (Eaves *et al.* 1989). Some HI activity was, however, noted with rabbit-raised antiserum against strain HP-60. Blackall, *et al.* (1990) found that unabsorbed rabbit-raised antiserum against HP-60 reacted strongly with strain SA-3. This reaction was lost when the antiserum was absorbed with SA-3. It was noted that higher HI titres were obtained when M85 and the two transformed M85(T/0083) and M85(T/745) reacted with unabsorbed rabbit-raised antiserum against HP-60. It can be suggested from these results that M85 is a serotype-C isolate and is most likely a serotype-C-3 isolate, belonging to the same, uniquely South African (Eaves *et al.* 1989) serotype as strain SA-3.

Further work on the possible use of transformed NAD-independent *H. paragallinarum* in vaccine production is required. It must still be established that the type-specific hemagglutinins of *H. paragallinarum* serotype-B isolates are not affected by transformation to NAD independence. Once it has been established that these hemagglutinins are not affected by transformation either, experimental vaccines based on NAD-independent, transformed organisms could be made and used to evaluate the protective immunity to these organisms in chickens.

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