

# DNA–DNA hybridization determined in micro-wells using covalent attachment of DNA

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**The present study was aimed at reducing the time and labour used to perform DNA–DNA hybridizations for classification of bacteria at the species level. A micro-well-format DNA hybridization method was developed and validated. DNA extractions were performed by a small-scale method and DNA was sheared mechanically into fragments of between 400 and 700 bases. The hybridization conditions were calibrated according to DNA similarities obtained by the spectrophotometric method using strains within the family *Pasteurellaceae*. Optimal conditions were obtained with 300 ng DNA added per well and bound by covalent attachment to NucleoLink. Hybridization was performed with 500 ng DNA, 5% (w/w) of which was labelled with photo-activatable biotin (competitive hybridization) for 2.5 h at 65 °C in 2 × SSC followed by stringent washing with 2 × SSC at the same temperature. The criteria for acceptance of results were a maximum of 15% standard deviation, calculated as a percentage of the mean for four replicate micro-wells, and that DNA similarities were not significantly different in at least two independent experiments. The relationship between DNA similarities obtained by the micro-well method ( $y$ ) and by the spectrophotometric method ( $x$ ) was  $y = 0.534x + 30.6$ , when these criteria had been applied to 23 pairs of strains of *Actinobacillus* species, avian [*Pasteurella*] *haemolytica*-like bacteria and *Mannheimia* species. The correlation (Pearson) between DNA similarities obtained by interchange of strains used for covalent binding and hybridization was 0.794. Significantly lower DNA similarities were observed by the spectrophotometric compared with the micro-well method for three pairs of hybridizations. After removal of these data, the relationship between DNA similarities obtained by the micro-well and spectrophotometric methods improved to  $y = 0.855x + 11.0$ . It was found that the accuracy and precision of the micro-well method was at the same level as that of the spectrophotometric method, but the labour and analysis time were reduced significantly. The use of hybridization in the micro-well format will allow DNA–DNA hybridizations to be carried out between all strains selected for a particular taxonomic study, in order to construct complete data matrices and improve species definition.**

**Keywords:** DNA–DNA hybridization, *Pasteurellaceae*, micro-well, taxonomy

## INTRODUCTION

Hybridizations between total genomic DNA preparations are fundamental for classification of bacteria at

the species level (Murray *et al.*, 1990; Vandamme *et al.*, 1996; Wayne *et al.*, 1987). The existing spectrophotometric, filter competition, S1 nuclease and hydroxyapatite methods are reliable (De Ley *et al.*, 1970; Grimont *et al.*, 1980; Johnson, 1984) but time-consuming, due to the large amount and high purity of DNA required. Another problem is that these methods

**Abbreviations:** EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; 1-Melm, 1-methylimidazole buffer; PAB, photo-activatable biotin.

are not easy to automate. The present lack of methodological improvement limits the resolution of complex bacterial groups at the species level. One example is the family *Pasteurellaceae*, within which numerous unclassified taxa exist (Christensen & Bisgaard, 1997). The need for rapid, miniaturized, automated and standardized methods for DNA–DNA hybridization was pointed out by Vandamme *et al.* (1996).

A micro format DNA–DNA hybridization protocol was described by Ezaki *et al.* (1988, 1989), in which DNA was fixed to the surface of micro-wells by physical adsorption. However, the low retention efficiency results in a requirement for large amounts of DNA and the immobilized DNA is probably relatively inaccessible because only a small part of the target is available for hybridization. The method was improved by covalent attachment of DNA to CovaLink micro-well plates (Rasmussen *et al.*, 1991) for detection of streptococci and aeromonads (Adnan *et al.*, 1993; Kaznowski, 1995).

In the present study, covalent attachment of DNA to NucleoLink micro-well strips (Nalge Nunc International) was used to develop a micro-well DNA–DNA hybridization assay. NucleoLink strips were selected for the present study because the binding efficiency of DNA to NucleoLink has been shown to be five to 10 times higher than to CovaLink (Oroskar *et al.*, 1996). NucleoLink strips are made of an activated heat-stable polymer and support covalent binding of DNA by carbodiimide-mediated condensation, and these strips can be assembled in micro-well format and used in micro-well plate readers (Chu *et al.*, 1983; Ghosh & Musso, 1987; Oroskar *et al.*, 1996; Rasmussen *et al.*, 1991; Wolf *et al.*, 1987).

The new method was validated with strains of the *Pasteurellaceae* for which DNA similarities have been determined by the spectrophotometric method.

## METHODS

**DNA extraction.** Initial DNA-attachment studies of covalent binding to micro-well strips were performed with DNA (Sigma) that had been isolated from *Escherichia coli* by the procedure of Marmur (1961).

For validation of the method against DNA similarities determined by the spectrophotometric method, strains of *Actinobacillus equuli*, avian [*Pasteurella*] *haemolytica*-like bacteria, '*Actinobacillus salpingitidis*', *Mannheimia* species and strains of the provisional taxon 11 (Table 1) were cultured in 40 ml brain/heart infusion broth (Difco) for 18 h. Cells were harvested by centrifugation for 10 min at 5900 *g*. The pellet was washed in 10 ml 50:10 TE buffer (50 mM Tris/HCl, 10 mM EDTA, pH 8), concentrated by centrifugation and resuspended in 10 ml 50:10 TE buffer. Lysozyme (10 mg) was dissolved in the cell solution and allowed to act at 37 °C for 30 min. Next, 1 ml of 10 mg proteinase K ml<sup>-1</sup> was added to each tube and the tubes were turned 10 times before they were incubated at 56 °C for 2 h. For precipitation of protein and cell debris, 6 ml 1.5 M potassium acetate was added, the tubes were turned 50 times and centrifuged for 15 min at 12 100 *g*. The supernatant was

collected through sterile gauze and mixed with 10 ml 2-propanol by turning the tube 10 times. After 10 min rest, centrifugation was performed at 12 100 *g* for 15 min. The supernatant was decanted and the pellet was washed with 70% (v/v) ice-cold ethanol by centrifugation at 12 100 *g* for 10 min and the pellet was dried. The pellet was subsequently redissolved in 5 ml 10:1 TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and 25 µl of a solution of 10 mg DNase-free RNase ml<sup>-1</sup> was added, with subsequent incubation at 37 °C for 30 min. Five ml phenol:chloroform:isoamyl alcohol (25:24:1) was added and the solution was turned until it was milky-white. The solution was centrifuged at 5900 *g* for 15 min and 4 ml of the upper phase was transferred to a new tube. One vol. chloroform:isoamyl alcohol (24:1) was added, the tube was turned until the content was mixed and centrifugation was carried out at 5900 *g* for 15 min. The upper phase was transferred to a new tube and DNA was precipitated with 0.1 vols 3 M sodium acetate (pH 5.2) and 2 vols 96% (v/v) ethanol. The solution was left for 30 min at –20 °C and centrifuged at 12 100 *g* for 30 min. The supernatant was decanted and the pellet was washed in 70% (v/v) ice-cold ethanol by centrifugation. The pellet was dried and resuspended in 5 ml distilled, filter-sterilized water (MilliQ). DNA was sheared twice in a French pressure cell at 2.3 × 10<sup>8</sup> Pa (15 000 p.s.i.) to fragments of between 400 and 700 bp, as confirmed by agarose gel electrophoresis. As an alternative to the French pressure cell, DNA was sheared by passing samples twice through a cell disrupter operated at 2.3 × 10<sup>8</sup> Pa (Cell Disruption Systems, Constant Systems). If traces of RNA were observed by agarose gel electrophoresis of the DNA preparation, the RNase treatment was repeated, including extractions and precipitations. To remove any flocculent material, DNA was sterile-filtered (0.20 µm, Minisart; Sartorius). To enable filtration, DNA was denatured (100 °C, 10 min) and immediately put on crushed ice before the filtration.

**Binding of DNA to micro-wells.** The first step in the covalent binding process is to generate a phosphorimidazolide by reacting the 5'-phosphoryl groups of nucleic acid with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in 1-methylimidazole buffer (1-Melm) (Ghosh & Musso, 1987). The EDC-activated DNA is unstable and reacts with 1-Melm. The 1-Melm-activated DNA then binds covalently to the NucleoLink surface. Optimal binding was achieved with 300 ng DNA (stock solution 50–150 ng µl<sup>-1</sup>) diluted in ice-cold 10 mM 1-Melm (Sigma), pH 7, distributed with 75 µl in each well. Twenty-five µl 40 mM EDC (Sigma) dissolved in 10 mM 1-Melm was added and the sealed strips (heat-resistant sealing tape; Nalge Nunc International) were incubated at 50 °C for 18 h without shaking. To remove unattached DNA and make the attached DNA single-stranded, wells were washed three times with 0.4 M NaOH plus 0.25% (v/v) Tween 20 at 50 °C. After 15 min at 50 °C with the NaOH solution, the NaOH wash was repeated. Wells were then rinsed three times with MilliQ water, rested for 5 min with water and then rinsed three more times. Wells were washed three times with 0.2 M NaOH and 0.1% (v/v) Tween 20 at ambient temperature, rested with the NaOH solution for 5 min and then washed three more times with the NaOH solution. Finally, wells were washed three times with 100 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20 at ambient temperature, rested for 5 min with the solution and then washed three more times. Strips coated with DNA were stored at 4 °C in sealed plastic bags for a maximum of 5 d before hybridization.

**Table 1.** Bacterial strains used for development of the micro-well method

DNA of the strains listed was used for development of the micro-well method by covalent attachment of DNA to determine DNA similarity and for comparison of DNA similarities with the spectrophotometric method. DNA G+C contents are indicated. References are given to reports of DNA similarities determined by the spectrophotometric method. ND, Not determined.

Taxon	Strain	G + C content (mol %)	Reference
<i>Actinobacillus equuli</i>	NCTC 8529 <sup>T</sup>	42.9	Bisgaard <i>et al.</i> (1984)
Taxon 11	F 154	42.2	Bisgaard <i>et al.</i> (1984)
' <i>Actinobacillus salpingitidis</i> '	CCM 5974 <sup>T</sup>	40.3	Piechulla <i>et al.</i> (1985)
	CCM 5975	41.3	Piechulla <i>et al.</i> (1985)
	CCM 5976	40.4	Piechulla <i>et al.</i> (1985)
	CCM 5995	42.1	Piechulla <i>et al.</i> (1985)
<i>Mannheimia haemolytica</i>	NCTC 9380 <sup>T</sup>	43.6	Angen <i>et al.</i> (1999)
	265	ND	Angen <i>et al.</i> (1999)
<i>Mannheimia glucosida</i>	P 925 <sup>T</sup>	41.6	Angen <i>et al.</i> (1999)
	P 731	43.2	Angen <i>et al.</i> (1999)
	UT 18	41.8	Angen <i>et al.</i> (1999)
	P 737	43.5	Angen <i>et al.</i> (1999)
	P 730	43.9	Angen <i>et al.</i> (1999)
	<i>Mannheimia</i> spp.	UT 27	43.7
R 19.2		ND	Angen <i>et al.</i> (1999)
HPA 121		40.2	Angen <i>et al.</i> (1999)
[ <i>Pasteurella</i> ] <i>haemolytica</i> -like	F 114	40.9	Piechulla <i>et al.</i> (1985)
	Gerl. 3348	42.6	Piechulla <i>et al.</i> (1985)

**DNA labelling with photo-activatable biotin (PAB).** For detection of the degree of DNA–DNA hybridization, DNA was labelled by PAB, which forms a stable aryl nitrene that binds covalently to the aromatic bases of nucleic acids (Ezaki *et al.*, 1988; Forster *et al.*, 1985). Biotin is bound via a spacer arm of poly(U)<sub>11</sub> and one biotin has been estimated to be incorporated per 100–400 bases (Forster *et al.*, 1985). DNA was labelled by PAB (EZ-Link; Pierce) according to the manufacturer's protocol and the protocols of Forster *et al.* (1985) and Clontech. Ten µl PAB dissolved in water to 1 µg µl<sup>-1</sup> and DNA at a concentration of 0.5–1.0 µg µl<sup>-1</sup> in 10–30 µl MilliQ water were mixed under subdued light in micro tubes (1.5 ml). The tubes were illuminated with their lids open, 10 cm below a 400 W Philips sun-lamp (SGR 140) for 30 min on crushed ice. TE buffer (100 mM Tris/HCl, 1 mM EDTA, pH 9) was added to the solution to 100 µl and the solution was extracted twice with 100 µl 2-butanol. The volume was returned to 100 µl with water and DNA was precipitated with 50 µl 7.5 M ammonium acetate and 300 µl ice-cold ethanol for 1 h at –20 °C. DNA was pelleted (15000 g, 15 min) and the pellet was washed once with ice-cold 70% (v/v) ethanol, dried in a speed-vac and redissolved in 20 µl MilliQ water. The DNA concentration was determined fluorometrically with Hoechst 33258 (Paul & Myers, 1982). Quantification of DNA with Picogreen (Molecular Probes) corresponded to the results obtained by the Hoechst 33258 method. The mean recovery of DNA through the PAB-labelling procedure was 62% (four independent experiments).

**Hybridization with PAB-labelled DNA to DNA attached to micro-wells.** PAB-labelled DNA was diluted 1:20 with unlabelled DNA to achieve competitive hybridization. During competitive hybridization, unlabelled DNA fragments

compete with labelled DNA for duplex formation to DNA bound to the well surface (Johnson, 1981). The mixture of PAB-labelled and unlabelled DNA solutions was heated at 100 °C for 10 min and held at 65 °C. The formula  $T_m = 81.5 + 16.6 \log(M) + 0.41(G + C \text{ mol \%})$  (Schildkraut & Lifson, 1965) was used to calculate the hybridization temperature. By insertion of the molarity of monovalent cations,  $M = 0.39$ , in  $2 \times \text{SSC}$  and the molar G + C content of 42 mol% (Table 1), the  $T_m$  was calculated to be 95 °C. The maximum rate of hybridization is obtained 16–32 °C below the  $T_m$  (Wetmur & Davidson, 1968) and a hybridization temperature of 65 °C was selected, 30 °C below the  $T_m$ .

Hybridization was carried out for 2.5 h with 500 ng PAB-labelled DNA solution per well in 100 µl  $2 \times \text{SSC}$ , 0.1% (v/v) Tween 20 and 0.5% (v/v) blocking reagent (cat. no. 1096176; Boehringer Mannheim). Wells were sealed with heat-resistant sealing tape (Nalge Nunc International) and the strips were mounted with gel-clamps between silicone plates and glass plates to hinder evaporation during hybridization. Shaking was not applied during hybridization. Washing was performed three times with  $2 \times \text{SSC}$  containing 0.1% (v/v) Tween 20 at ambient temperature and then wells were heated to 65 °C with the solution and held at that temperature for 15 min without shaking. Washing was then performed three more times with  $2 \times \text{SSC}$  containing 0.1% (v/v) Tween 20 at ambient temperature.

**Detection of DNA hybridization.** The amount of biotin-labelled DNA bound to wells after stringent washing was determined by the addition of streptavidin-conjugated alkaline phosphatase acting on the substrate 4-methylumbelli-

feryl phosphate (4-MUP), which generates a fluorescent signal (Nalge Nunc International).

The detection system recommended for a specific PCR test run in NucleoLink strips was utilized (Nalge Nunc International). Aliquots of 100 µl streptavidin-conjugated alkaline phosphatase (Dako) diluted 1:3000 in 100 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20 and 0.5% (v/v) blocking reagent (Boehringer Mannheim) were added to the wells. The wells were sealed and incubated for 1 h at 37 °C and then washed three times with 100 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20 at ambient temperature. The wells were rested for 5 min and the washing was repeated three more times. For fluorometric detection, 100 µl 1 mM 4-MUP (Sigma) in 1 M diethanolamine, pH 9.8, and 1 mM MgCl<sub>2</sub> was added. The wells were sealed and incubated at 37 °C for 1 h in the dark. The reaction was stopped by the addition of 50 µl 3 M K<sub>2</sub>HPO<sub>4</sub> and fluorescence was read in an LS-50B fluorometer (Perkin Elmer) at 350 nm excitation and 480 nm emission, with a slit width of 3 and 3 s reading time. The relationship between signal and reaction time was found to be linear up to 2 h. Background hybridization was determined in wells without covalently bound DNA. The background hybridization, expressed as the percentage of homologous hybridization (the reference strain with itself), was around 10% for a 2.5 h hybridization period. The degree of background hybridization was determined in each experiment.

**Quantification.** The percentage DNA similarity was calculated as  $100[(I_{\text{test}} - I_{\text{blank}})/(I_{\text{ref}} - I_{\text{blank}})]$ , where  $I_{\text{test}}$  is the intensity of hybridization between the strain to be tested and the reference strain,  $I_{\text{ref}}$  is the intensity of hybridization of the reference strain with itself and  $I_{\text{blank}}$  is the background hybridization. Each experiment was performed with four replicates. The differences of mean DNA similarities between experiments were evaluated statistically by the d-test (Campbell, 1981).

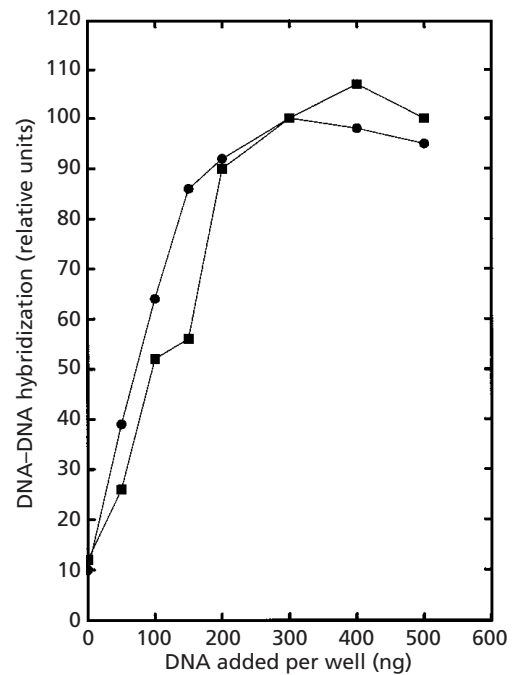
## RESULTS AND DISCUSSION

### Binding of sheared DNA to micro-wells

The optimal level of DNA required for covalent binding was determined by homologous hybridization. From Fig. 1, the hybridization signal is seen to increase linearly with the concentration of unlabelled DNA added up to 200 ng per well. By application of higher DNA concentrations, the signals were gradually reduced, probably because of prevention of DNA binding to the surface. For the micro-well hybridization method, 300 ng DNA per well was chosen for binding to secure an even level of DNA in all wells. DNA of *E. coli* performed similarly to that from *Mannheimia haemolytica*, which showed that the binding is applicable to different bacterial groups and to DNA extracted by different protocols. The binding of DNA to the NucleoLink polymer surface was developed originally with 5'-phosphorylated oligonucleotides (Oroskar *et al.*, 1996). Our results show further that binding of mechanically sheared DNA to the NucleoLink surface is possible.

### Adjustment of the hybridization conditions

The time of hybridization and the strength of SSC in the hybridization buffer were adjusted at a constant



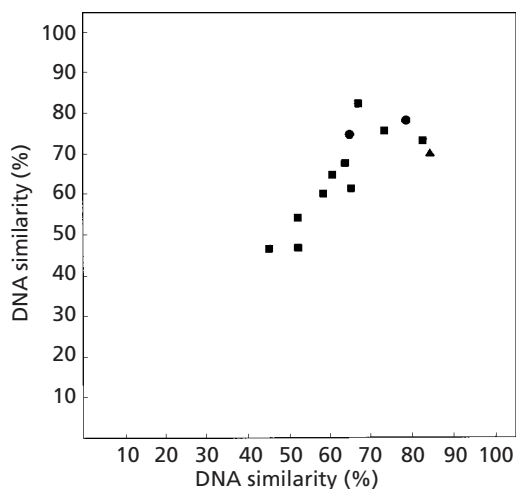
**Fig. 1.** Relationship between degree of DNA–DNA hybridization and amount of DNA bound to NucleoLink wells for DNA of *M. haemolytica* strain NCTC 9380<sup>T</sup> (■) and *E. coli* strain B (●) in homologous hybridization. The DNA–DNA hybridization achieved at 300 ng bound DNA was set to 100 and the other values were normalized to this level. Data points represent single measurements.

hybridization temperature of 65 °C to obtain correspondence to the DNA similarity obtained by the spectrophotometric method.

A hybridization time of 2.5 h was chosen for the method because of the greater precision observed [the coefficient of variation (SD as a percentage of the mean) decreased from 13% at 18 h to 10% at 2.5 h hybridization time] and because the whole assay could be performed within one working day, starting with wells pre-coated with DNA.

It was necessary to optimize the strength of SSC for a hybridization time of 2.5 h. By hybridizing in 1 × SSC and stringent washing in 0.5 × SSC, the DNA similarity was 15%, too low for seven pairs of hybridizations compared with spectrophotometric values (data not shown). The use of 2 × SSC in both the hybridization and washing steps provided correspondence between DNA–DNA hybridization values obtained from the micro-well and spectrophotometric methods (see below).

Lower DNA similarity was obtained for DNA that was extracted to greater purity according to the protocol described by Angen *et al.* (1999) than by the present protocol (data not shown), indicating that variable quality of DNA preparations might affect the DNA similarities obtained. Dialysis of DNA samples overnight in 2 × SSC did not change the DNA simi-



**Fig. 2.** Comparison of DNA similarity determined by interchange of the strains used for binding to micro-wells and addition in solution during hybridization with strains of *Mannheimia* species (●), avian [*Pasteurella*] *haemolytica*-like bacteria/*Actinobacillus salpingitidis* (■) and *Actinobacillus* species (▲). Each experiment included four replicates.

larities obtained by the micro-well method. Some organic compounds are known to increase the reassociation rate or to reduce the thermal stability of DNA hybrids (Johnson, 1991) and impurities in DNA preparations could act in a similar way.

#### Comparison of DNA similarities determined by the spectrophotometric and micro-well methods

In order for results obtained by the micro-well method to be acceptable, two criteria were applied. Fluorometric readings were only used for calculation of DNA similarities if the coefficient of variation did not exceed 15% for four wells. This led to the rejection of nine of

a total of 94 experiments performed (10%). The variation between fluorometric readings of individual micro-wells might be related to an additive effect of variation on binding, hybridization and detection steps, as well as to the many washing steps. The second criterion for acceptance of results was that no significant difference should be observed between DNA similarities calculated from two replications with the same pairs of strains, including interchange of the strains used for covalent binding and hybridization. This criterion led to the rejection of 19 of 85 experiments (22%). The rejected experiments showed deviations of more than 3%, but always less than 38% DNA similarity, from the mean of pairs of strains. For the accepted experiments (66 of 85), individual experiments never showed more than 11% deviation in DNA similarity from the mean. Some of the deviating results were related to the use of unlabelled DNA preparations with concentrations lower than 50  $\mu\text{g ml}^{-1}$  and samples of PAB-labelled DNA with less than 100  $\mu\text{g ml}^{-1}$ . For further use of the method, it is recommended that unlabelled and labelled DNA preparations of high concentration are used.

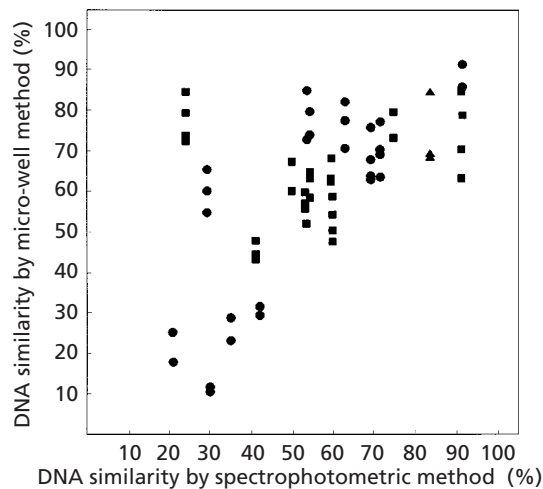
In at least two independent experiments, each including four repeats, the micro-well method was compared to the spectrophotometric method for 23 pairs of strains where DNA similarities ranged from 21 to 91%, as determined by the spectrophotometric method. The correlation between DNA similarities obtained by interchange of strains used for covalent binding and hybridization was significant (Fig. 2, Table 2). Data for individual hybridization experiments are shown in Fig. 3. The mean deviation in DNA similarity between the two methods was only 3% when all data-pairs were compared; however, the linear relationship obtained between DNA similarities was poor (Table 2). The low slope of the regression line is related to significantly lower DNA similarities determined by the spectrophotometric compared with

**Table 2.** Statistical evaluation of DNA similarities determined by the micro-well method

The Pearson correlation coefficient was obtained between DNA similarities, derived by the micro-well method, for pairs of strains when the strains used for covalent binding and hybridization were interchanged. Linear regression was performed for percentage DNA similarities determined by the micro-well method ( $y$ , dependent variable) on similarities obtained by the spectrophotometric method ( $x$ , independent variable).

Data subset	Correlation (Pearson)	Regression
Experiments excluded that gave significant differences from mean DNA similarity	0.794 (significant; $P = 0.05$ )	$y = 0.534x + 30.6$ , $r^2 = 0.327$
Experiments excluded that gave significantly different mean DNA similarities between micro-well and spectrophotometric methods	–	$y = 0.855x + 11.0$ , $r^2 = 0.721$





**Fig. 3.** Comparison between DNA similarities obtained by the micro-well and spectrophotometric methods for strains of *Mannheimia* species (●), avian [*Pasteurella*] *haemolytica*-like bacterial/*Actinobacillus salpingitidis*' (■) and *Actinobacillus* species (▲). For the micro-well method, each experiment included four replicates. For the spectrophotometric method, data represent experiments performed with three replications.

the micro-well method for three pairs of hybridizations. If these data were removed, the relationship was improved tremendously (slope 0.855).

In five pairs of hybridizations (NCTC 9380<sup>T</sup>, P 730; NCTC 9380<sup>T</sup>, P 737; NCTC 9380<sup>T</sup>, UT 27; NCTC 9380<sup>T</sup>, HPA 121; CCM 5995, F 114), the DNA similarities obtained by the two methods were significantly different. We have reason to believe that the DNA-binding values obtained between NCTC 9380<sup>T</sup> and strains P 730 and P 737 by the spectrophotometric method, 29 and 54%, respectively, are too low compared with the micro-well estimates of 60 and 78%. Strains P 730, P 731, P 733, P 737, P 925<sup>T</sup> and UT 18 (Table 1) have been shown previously to represent the same species, *Mannheimia glucosida* (Angen *et al.*, 1999). These strains were all closely linked to *M. haemolytica* by multilocus enzyme electrophoresis, 16S rRNA sequencing and DNA–DNA hybridization, and in the polyphasic taxonomic investigation performed, the DNA-binding values of strains P 730 and P 737 to NCTC 9380<sup>T</sup> were outliers. Both of these hybridizations were performed in the early 1980s (Mutters *et al.*, 1986) and consequently the discrepancy might result from the use of different DNA preparations. By the micro-well method, all strains of *M. glucosida* have a DNA similarity to NCTC 9380<sup>T</sup> of between 64 and 79%, a result which reflects the taxonomic relationship between these strains, as indicated by the polyphasic investigation (Angen *et al.*, 1999).

For the avian [*P.*] *haemolytica*-like bacterial/*A. salpingitidis*' strains CCM 5995 and F 114, 16S rRNA gene sequence comparisons have shown 99.2% simi-

larity (unpublished result), indicating that the spectrophotometric DNA similarity value of only 24% was less likely than the micro-well estimate of 77%. Similarities based on 16S rRNA sequence comparison are insufficient, per se, for species separation (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994) and DNA–DNA hybridization must be repeated with these strains, both by the spectrophotometric and micro-well methods, to solve this problem.

For strains UT 27 and HPA 121 hybridized with NCTC 9380<sup>T</sup>, the estimates obtained by the micro-well method, of 31 and 11%, respectively, underestimated the DNA similarities compared with the spectrophotometric estimates of 42 and 30%. Lower correspondence between methods has been observed when strains were distantly related compared with bacteria belonging to the same hybridization group (Grimont *et al.*, 1980). The major purpose of performing DNA–DNA hybridization experiments is to carry out classification at the species level, and low correspondence between methods for low DNA similarities (<40%) seems to be a minor problem. For discrimination of groups related only at the genus level or higher, phylogenetic comparison of 16S rRNA sequence is the primary method. In taxonomic studies, specific methods have to be used for specific taxonomic levels.

Divergence between DNA similarities obtained by different methods has been reported occasionally (Johnson *et al.*, 1970; Bouvet & Grimont, 1986). In most cases, good correspondence has been obtained, such as a mean variation of around 2% DNA similarity between the S1 nuclease and hydroxyapatite methods (Grimont *et al.*, 1980) and between the spectrophotometric and hydroxyapatite methods (Kurtzman *et al.*, 1980). The spectrophotometric method showed good correspondence to the filter method (variation around 3% DNA similarity) (De Ley *et al.*, 1970). The mean variation between the micro-well method and the spectrophotometric method fell in this low range.

The standard deviation of the DNA similarity determined by the micro-well method, of around 6%, is comparable to other reports of the micro-well method (Goris *et al.*, 1998) and the spectrophotometric method (Angen *et al.*, 1999; Piechulla *et al.*, 1985). However, lower mean standard deviations of only 2.4% for the spectrophotometric method have been reported (Huss *et al.*, 1983). In the present study, linear regression of DNA similarities obtained by the micro-well method against data obtained by the spectrophotometric method showed a poor relationship. Exclusion of data that deviated between the two methods provides a relationship comparable to the study of Yaeshima *et al.* (1996), where the micro-well method showed good correspondence to the S1 nuclease method ( $y = 0.823x + 13.93$ ). In another study, the DNA similarities by the micro-well method were slightly higher than by the spectrophotometric method ( $y = 1.05x + 2.77$ ) (Goris *et al.*, 1998).

The genotypic criteria for including taxa in the same species were set at 70% or greater DNA similarity and a difference in  $T_m$  of less than 5% (Wayne *et al.*, 1987). A comparison of different taxonomic investigations showed this limit to vary from approximately 60 to 80% DNA similarity (Vandamme *et al.*, 1996). For the strains of *Mannheimia* included in the present study, one strain was included in *M. haemolytica* with 91% similarity, whereas strains belonging to other species had 71% or lower similarity to the type strain of *M. haemolytica* on the basis of spectrophotometric data. The corresponding values were 89 and 78% using similarities obtained by the micro-well method. These limits are both in accordance with a species limit of around 85% for this genus (Angen *et al.*, 1999; Mutters *et al.*, 1989).

The same hybridization temperature of 65 °C may be used for many other bacteria with DNA G+C contents of around 40 mol%. Adjustment of the hybridization temperature would be necessary for bacteria deviating by 10 mol% or more in their DNA G+C content. The lowest limit would be for mycoplasmas, with G+C contents as low as 25 mol%, giving an expected  $T_m$  of 85 °C (Schildkraut & Lifson, 1965) and a hybridization temperature of around 50 °C (Wetmur & Davidson, 1968). The upper limit would be for high-G+C-content Gram-positives, with G+C contents as high as 74 mol%, from which a predicted  $T_m$  of 105 °C and a hybridization temperature of around 80 °C can be calculated.

## Conclusion

It is concluded that the present micro-well DNA-DNA hybridization method has reduced the time for analysis considerably, while the high levels of accuracy and precision of the existing methodology have been preserved. Due to savings of labour and time, it is now possible to perform full matrix hybridizations and thereby improve species definitions. Compared with the spectrophotometric method, only about 1% of the amount of DNA is needed when performing micro-well hybridization. Correspondence to results obtained by the spectrophotometric method was obtained for a range of DNA similarities obtained by the micro-well method. In some instances, the data obtained by the micro-well method better reflected the taxonomic relationship between strains, as indicated by a polyphasic approach, than did those obtained by the spectrophotometric method.

In the application of the micro-well DNA-DNA hybridization method to other bacterial groups or using different DNA extraction protocols, the hybridization temperature might need adjustment. In addition, it is important to validate the results against the spectrophotometric method or another traditional DNA-DNA hybridization method until the performance of this new micro-well hybridization method is better known.

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