The penicillin binding proteins of the genus *Haemophilus*

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Summary. We questioned whether the penicillin binding protein (PBP) profiles of representative strains from the 19 species varied within the genus *Haemophilus* and whether these profiles would be of taxonomic value. Seventeen of the 19 representative strains studied had distinct PBP profiles; only those of *H. avium* and *H. paragallinarum* were identical. The data support the inclusion of *H. aegyptius* in the genus as a species related to but separate from *H. influenzae* and could not exclude *H. somnus*, *H. agni*, and *H. equigenitalis* from the genus. Comparative PBP analysis within the genus *Haemophilus* may therefore be useful taxonomically.

Introduction

The genus *Haemophilus* belongs to the family Pasteurellaceae and its 19 members are aerobic or facultatively anaerobic, nonmotile, pleomorphic, gram-negative rods which require complex media for growth (Kilian and Biberstein, 1984). All are obligate parasites of man (10 species) or other animals (9 species) (fig. 1) and may be part of the normal flora (Kilian and Biberstein, 1984; Mendelman, 1987). However, all species isolated from man and 6 of 9 species isolated from animals have been associated with clinical disease (Kilian and Biberstein, 1984; Mendelman, 1987; Albritton, 1982); the pathogenic significance of *H. haemoglobinophilus*, *H. avium*, and *H. paracuniculus* has not been determined (Kilian and Biberstein, 1984).

Penicillin binding proteins (PBPs) are chromosomally determined enzymes that participate in cellwall synthesis and covalently bind penicillin. Every bacterium examined contains at least one, and usually several PBPs and they appear to be species specific (Georgopapadakou and Liu, 1980). The PBPs of other genera have been studied in tests with single representative strains from each species (Ohya *et al.*, 1979). In the present study, we investigated whether the PBP profiles of 19 representative strains varied within the genus *Haemophilus* and if these profiles would be of taxonomic value.

Materials and methods

Seventeen isolates were obtained from the American Type Culture Collection (ATCC), and *H. somnus* strain 8025 and *H. agni* were obtained from Dr T. Inzana, Washington State University, Pullman, WA. The following ATCC strains were evaluated: *H. influenzae* 33391, *H. parainfluenzae* 33392, *H. aegyptius* 11116, *H. paraphrophilus* 29241, *H. haemolyticus* 33390, *H. parahaemolyticus* 10014, *H. segnis* 33393, *H. aphrophilus* 33389, *H. paraphrohaemolyticus* 29237, *H. ducreyi* 33940, *H. haemoglobinophilus* 19416, *H. avium* 29546, *H. paragallinarum* 29545, *H. paracuniculus* 29986, *H. parasuis* 19417, *H. pleuropneumoniae* 27088, and *H. equigenitalis* 35865.

All *Haemophilus* spp. grew on enriched chocolate agar plates (Remel Laboratories, Lenexa, KS, USA) at 37°C in CO₂ 5% in air. *H. ducreyi* required incubation for 4 days in a moist atmosphere and grew better at 33°C. Initial growth of *H. avium* and *H. paragallinarum* from the ATCC stock vials required Brain Heart Infusion (BHI) Broth (Difco) supplemented with yeast extract 2%, nicotinamide adenine dinucleotide (NAD) (Sigma) 0.01% and heat-inactivated chicken serum 5% whereas *H. ducreyi* required the addition of 25% of defibrinated rabbit blood and incubation at 30–33°C.

Strains were tested for β -lactamase production with the chromogenic cephalosporin, nitrocefin, as described by O'Callaghan *et al.* (1972). Penicillin MIC and MBC determinations were performed by the macro broth dilution method (NCCLS, 1985) in 2-ml volumes of Mueller-Hinton Broth (MHB) supplemented with Iso Vitalex (BBL) 1% and haemin chloride/histidine (Sigma) 10 mg/L. Inocula were prepared in MHB to a 0.5 McFarland standard and diluted to give a final inoculum of 10⁵ cfu/ml; tests were incubated in CO₂ 5% in air at 37°C for 18–24 h. All *Haemophilus* spp. were tested in

this manner with the exception of *H. ducreyi* which was tested by an agar dilution method in GC Base medium (Difco) with Iso Vitalex (BBL) 1% and haemoglobin 1% at an inoculum of 10^4 cfu and with incubation in CC_2 5% according to NCCLS guidelines (NCCLS, 1985). The MIC was read as the lowest concentration of penicillin with no visible growth; the MBC was the concentration of penicillin resulting in 99.9% killing. Control strains included a β -lactamase producing strain of *H. influenzae* (RSF 007), the penicillin susceptible *H. influenzae* strain MAP, and *Escherichia coli* strain ATCC 25922.

PBPs were detected with whole cells grown to mid exponential phase ($\text{OD}_{600} = 0.6$) in BHI broth, harvested, washed twice with 50 mM Tris-1 mM MgCl_2 and 1 ml was concentrated ten-fold (resuspended in 95 μl of the above buffer). Five μl of [^3H]-penicillin (0.4 mg/L) was added and incubated at 37°C for 15 min, when 5 μl of unlabelled 1 M penicillin G was added. The reaction was stopped with 12.7 μl of 20% Sarkosyl and 22.5 μl of sample buffer (0.0625% 2M Tris, pH 6.8, SDS 2%, β -mercaptoethanol 5%, glycerol 10%, bromophenol blue 0.004%). After boiling for 5 min, 60 μl was loaded into

each gel lane. *H. aphrophilus*, *H. paraphrohaemolyticus*, and *H. ducreyi* grew poorly in broth culture and, therefore, were taken directly off agar plates after overnight growth and labelled. As a control, *H. influenzae* strain MAP was labelled similarly. Cells were suspended in the above buffer to an OD of 0.6 and 1 ml of each was concentrated ten-fold and labelled as described. *H. ducreyi* was labelled after growth for 4 days. Electrophoresis and processing were performed as previously described (Mendelman and Chaffin, 1985); the autofluorograms were developed after exposure for 14 days.

Results and discussion

No strains produced detectable β -lactamase. The MIC and MBC of penicillin at an inoculum of 10^5 cfu were ≤ 0.5 mg/L for all strains tested except *H. parainfluenzae* (MIC and MBC = 2 mg/L) and *H. paraphrophilus* (MBC = 1 mg/L).

The PBP pattern of *H. influenzae* strain ATCC 33391 grown in broth to exponential phase (figure,

Figure. Autofluorogram of the PBPs of the genus *Haemophilus*. The 19 species currently listed within the genus *Haemophilus* (Kilian and Biberstein, 1984), are indicated at the top of each lane. *Haemophilus* species 1–10 are isolated from man, whereas species 11–19 are isolated (respectively) from dogs, poultry, chickens, rabbits, pigs, pigs, mares, sheep, and cattle (Kilian and Biberstein, 1984; Albritton, 1982; Mendelman, 1987). The numbers to the left of lane 1 indicate the 8 PBPs of the *H. influenzae* strain and the numbers to the right of lane 19 indicate the apparent mol. wt (10^3) of protein standards. Whole cells were incubated with 4 mg/L (final concentration) of [^3H]-penicillin for 15 min before SDS-polyacrylamide gel electrophoresis and processing as described in the text.

lane 1) was identical to the pattern observed with strain MAP taken directly off the agar plate; 8 PBPs were detected (data not shown). Thus, with *H. influenzae* as the control, the PBPs of the 3 strains tested from agar plates appear comparable for analysis with the PBPs of the strains tested from broth cultures. Furthermore, the PBP profile of the *H. influenzae* type strain was identical to that of 10 other susceptible isolates previously described (Mendelman and Chaffin, 1985; Serfass *et al.* 1986; Mendelman *et al.*, 1987).

Identical PBP profiles were observed for *H. avium* and *H. paragallinarum* (figure, lanes 12 and 13); the other 17 species had distinct PBP profiles. The PBP profile of *H. influenzae* and that of *H. aegyptius* were similar but not identical (figure, lanes 1 and 3, respectively) and the PBP profiles of *H. agni* and *H. somnus* (figure, lanes 18 and 19) and *H. parainfluenzae*, *H. parahaemolyticus* and *H. paraphrohaemolyticus* (figure, lanes 2, 6, and 9) were distinct.

There are certain taxonomic controversies within the genus *Haemophilus*. It has been proposed that *H. aegyptius* should be represented within biotype III of *H. influenzae* (Carlone *et al.*, 1985; Martel *et al.*, 1985) because no features unequivocally differentiate *H. aegyptius* from *H. influenzae*. DNA homology studies appear to indicate that separation of the two species is unjustified (Martel *et al.*, 1985; Casin *et al.*, 1986); however, *H. aegyptius* has remained as a separate species because it appears to be clinically distinguishable, causing an acute form of contagious conjunctivitis (Kilian and Biberstein, 1984). More recently, it has been associated with Brazilian Purpuric Fever with a high fatality rate (Brazilian Purpuric Fever Study Group, 1987). The PBP patterns of *H. aegyptius* and *H. influenzae* suggest species relatedness, but are distinct; PBP 5 is a major PBP of *H. influenzae* in the 10 penicillin-susceptible isolates reported in the literature including two biotype III strains (Mendelman *et al.*, 1987). In contrast, the comparable PBP (by apparent M_r) in the *H. aegyptius* strain studied bound significantly less penicillin (figure, lane 3).

Three species, *H. somnus*, *H. agni*, and *H. equigenitalis*, although described as members of the

genus *Haemophilus*, are reported not to qualify for inclusion in this genus (Kilian and Biberstein, 1984). The PBP profiles of these three species are no more different than those of other members of the genus and thus these species cannot be excluded on the basis of their PBP profiles. Although it is generally accepted that *H. somnus* and *H. agni* represent a common species (personal communication, T. Inzana, Washington State University) isolated from cattle or sheep, respectively, our data suggest that these are two distinct species. It has been proposed that *H. parahaemolyticus* and *H. paraphrohaemolyticus* are haemolytic variants of *H. parainfluenzae* (Albritton, 1982); however, their PBP profiles support separate species designations. Of the 19 species tested, only *H. avium* and *H. paragallinarum* had identical patterns which places in question their separation into two species. The latter species has been implicated in respiratory disease of chickens whereas *H. avium*, isolated from the respiratory tract of poultry, has not been associated with disease (Kilian and Biberstein, 1984).

Only the PBPs of *H. influenzae* have previously been characterised; their PBP expression has been shown to be growth-phase dependent and certain variability in binding, especially among ampicillin-resistant non- β -lactamase producing isolates, has been noted (Mendelman and Chaffin, 1985; Mendelman *et al.*, 1987). To minimise this possible variability (if it exists within the other species), we confined our analysis to penicillin susceptible *Haemophilus* isolates examined in the exponential phase of growth. The distinct profiles appear to reflect real PBP differences between species within the genus *Haemophilus*. However, as we have examined only single isolates, PBP variability within a species remains to be determined. Further analysis is needed to determine the exact number of PBPs for each species and the kinetics of penicillin acylation.

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