Biochemical and Serological Characterization of *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964 and Proposal of a Neotype Strain

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The aim of the present study was to solve the taxonomic problems of haemophili of porcine origin hitherto classified as *Haemophilus parahaemolyticus* Pittman 1953 or *H. pleuropneumoniae* Shope 1964. Forty-seven *Haemophilus* strains isolated mainly from pneumonic lesions in pigs and of different geographic origin were subjected to biochemical and serological examinations. All strains were V dependent and urease positive, and the majority were beta-hemolytic. For comparison, nine beta-hemolytic and V-dependent strains isolated from humans were examined. The porcine strains were divisible into two distinct groups on the basis of their biochemical characteristics, hemolytic activity, and deoxyribonucleic acid base composition. Both groups of porcine organisms were separated from the beta-hemolytic strains of human origin, previously named *H. parahaemolyticus*, by a significant number of biochemical and cultural characteristics and thus should not be classified with these. Following the rule of priority of proposed names, the correct name for the major group of porcine organisms is *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964. The species is described in detail and a neotype strain (Shope 4074) is proposed. The minor group of porcine strains possibly deserves specific recognition, but a recommendation with regard to this matter is deferred pending further studies.

Members of the genus *Haemophilus* have been described as playing significant roles in a variety of serious infectious diseases in swine. A separate entity of *Haemophilus* organisms causing pneumonic lesions and septicemia in swine was isolated independently during outbreaks of disease in swine herds during the early 1960s in Great Britain (10), California (1; H. Olander, Ph.D. thesis, University of California, Davis, 1963), and Argentina (22). Contrary to a previously described *Haemophilus* species from pigs, *H. suis*, the growth factor requirements of this organism were limited to V (nicotinamide adenine dinucleotide). Depending on the hemolytic activity, the isolates were assigned to the already existing species *H. parainfluenzae* Rivers 1922 (10) and *H. parahaemolyticus* Pittman 1953 (Olander, Ph.D. thesis), respectively. Shope (22), however, proposed the name *Haemophilus pleuropneumoniae* for his Argentinian isolate. A similar Swiss outbreak was later described by Nicolet and co-workers (12-14), who found their isolates and that of Shope (22) to be biochemically similar. Like Olander, Nicolet (12) chose the name *H. parahaemolyticus* by the rule of priority of publication. Although a number of distinguishing characters between porcine and

human hemolytic strains could be demonstrated, these were found insufficient to warrant the establishment of a new species for the porcine organism. This decision has been followed in subsequent reports on similar outbreaks in Denmark (15, 16), Great Britain (9), Canada (18, 20, 21), Australia (11), Sweden (2, 3), and the German Democratic Republic (7). However, recently a taxonomic study of the genus *Haemophilus* (5) revealed a number of differences between such porcine strains and human strains for which Pittman (17) established the species *H. parahaemolyticus*. These findings raised serious questions as to the propriety of placing both types of organisms within the one species *H. parahaemolyticus*.

The aim of the present study was to solve these nomenclatural problems and to provide a new description of the porcine organism based on the examination of 47 strains isolated in a variety of geographic areas.

MATERIALS AND METHODS

Bacterial strains. Forty-seven *Haemophilus* strains isolated mainly from pneumonic lesions in pigs were included in the study (Table 1). In addition, nine hemolytic, V-dependent strains of human origin were

TABLE 1. *Strains studied*^a

Strain designation	Synonyms	Isolated from:	Isolated in:	Serovar	Source
CCM 5869	Shope 4074, ATCC 27088, HK 405	Pleuropneumonia, pig	Argentina	1	CCM ← J. Nicolet ← J. J. Callis ← R. E. Shope
2538/4		Pleuropneumonia, pig	Australia	1	R. Mason
3933/3		Pleuropneumonia, pig	Australia	1	R. Mason
4969	HK 351	Pleuropneumonia, pig	Denmark	2	R. Nielsen
2282	HK 352	Pleuropneumonia, pig	Denmark	2	R. Nielsen
2226	HK 353	Pleuropneumonia, pig	Denmark	2	R. Nielsen
5617	HK 354	Pleuropneumonia, pig	Denmark	2	R. Nielsen
5130	HK 355	Pleuropneumonia, pig	Denmark	2	R. Nielsen
2934	HK 357	Pleuropneumonia, pig	Denmark	2	R. Nielsen
2845	HK 358	Pleuropneumonia, pig	Denmark	2	R. Nielsen
4226	HK 359	Pleuropneumonia, pig	Denmark	2	R. Nielsen
2374	HK 360	Pleuropneumonia, pig	Denmark	2	R. Nielsen
9711	HK 361, NCTC 10976	Pleuropneumonia, pig	Denmark	2	R. Nielsen
2224		Pleuropneumonia, pig	Denmark	2	R. Nielsen
Bd 166		Pleuropneumonia, pig	Sweden	2	O. Söderlind
CCM 5870	S 1536, ATCC 27089, HK 406	Pleuropneumonia, pig	Switzerland	2	CCM ← J. Nicolet
L13869		Pleuropneumonia, pig	Switzerland	2	H. Burgisser
ZH 81/65		Pleuropneumonia, pig	Switzerland	2	H. U. Bertschinger
SG 536		Pleuropneumonia, pig	Switzerland	2	R. Schweizer
S 447		Pleuropneumonia, pig	Switzerland	2	J. Nicolet
S 1494		Pleuropneumonia, pig	Switzerland	2	J. Nicolet
S 767		Pleuropneumonia, pig	Switzerland	2	J. Nicolet
HP 8		Pleuropneumonia, pig	Japan	2	M. Ogata
CCM 5871	S 1421, ATCC 27090, HK 407	Pleuritis, abscess, pig	Switzerland	3	CCM ← J. Nicolet
S 851		Lung abscesses, pig	Switzerland	3	J. Nicolet
S 853/70		Lung abscess, pig	Switzerland	3	J. Nicolet
S 1181/70		Vertebral abscess, pig	Switzerland	3	J. Nicolet
S 1178/70		Lung abscess, pig	Switzerland	3	J. Nicolet
S 43/71		Arthritis, pig	Switzerland	3	J. Nicolet
S 1318		Pneumonia, pig	Switzerland	3	J. Nicolet
HLO-108		Pneumonia, pig	England	3	K. Bakos ← Pattison
M 62		Pneumonia, pig	U.S.A.	4	H. Olander
Canada	HK 356	Pleuropneumonia, pig	Canada	5	R. Nielsen ← J. Greenfield
Greenfield		Pleuropneumonia, pig	Canada	5	J. Greenfield
95905		Pleuropneumonia, pig	Taiwan	5	F. S. Hsu
928		Pleuropneumonia, pig	Taiwan	5	R. Nielsen ← F. S. Hsu
12960		Pleuropneumonia, pig	Holland	5	J. P. Akkermans
K 17		Arthritis, lamb	U.S.A.	5	L. Frazier
L 20		Pneumonia, pig	U.S.A.	5	L. Frazier
K 98		Serositis, pig	U.S.A.	5	E. L. Biberstein
266-1920-HAE		Pleuropneumonia, pig	Belgium	5	L. Devriese
Finland		Pleuropneumonia, pig	Finland	5	R. Nielsen ← R. Schildt
Femø		Pleuropneumonia, pig	Denmark	NT	R. Nielsen
202		Pneumonia, pig	Sweden	NT	E. L. Biberstein
343		Pneumonia, pig	England	NT	T. W. A. Little
349		Pneumonia, pig	England	NT	T. W. A. Little
294		Pneumonia, pig	England	NT	T. W. A. Little

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; NCTC, National Collection of Type Cultures, Colindale, London, U.K.; NT, not typable with antisera 1 to 5. HK, collection of strains studied by Kilian (1976); S, collection of J. Nicolet.

examined: *H. parahaemolyticus* NCTC 8479 and NCTC 10794, *H. paraphrohaemolyticus* NCTC 10670 and NCTC 10672, and *H. parainfluenzae* HK 3, HK

23, HK 31, HK 117, and HK 331 (5). All of these human strains will be referred to as *H. parainfluenzae* according to a previous proposal (5).

Biochemical and cultural examinations. The methods applied for the biochemical examinations were those used by Kilian (5). Hemolysis was determined on 5% calf blood agar cross-inoculated with a single streak of *Staphylococcus epidermidis*. The CAMP reaction was tested on 5% sheep blood agar cross-inoculated with a beta-toxinogenic *S. aureus* strain as described previously (6).

Determination of fermentation products. Analysis of glucose fermentation products was performed on a Hewlett-Packard gas chromatograph (model 5830A; Württemberg, Germany). The conditions of analysis were: hydrogen flame detector; oven temperature, 130°C; coiled glass column (1.8 m by 4 mm ID), packed with 80- to 100-mesh Chromosorb G, AW, DMCS (Johns-Manville, Denver, Colo.); stationary-phase 5% FFAP (Jyds Teknologisk Institut, Aarhus, Denmark); and nitrogen flow rate, 60 ml/min. Ether and chloroform extractions of 5-day glucose broth cultures were performed by the method of Holdeman and Moore (4). The glucose broth contained (per liter): neutralized bacteriological peptone (L34, Oxoid Ltd., London, U.K.), 10 g; yeast extract powder (L21, Oxoid Ltd., London, U.K.), 10 g; glucose, 10 g; nicotinamide adenine dinucleotide (Sigma Chemical Co., St. Louis, Mo.), 10 mg; and salt solution as described by Holdeman and Moore (4).

Serological examinations. Serological examinations were performed as previously described (3, 13). The serological scheme was established according to tube agglutination of Formalin-treated whole cells in rabbit sera, comparing homologous and heterologous strains. Reference cultures representing serovars 1, 2, 3 (13), 4, and 5 (3) were those listed in Table 5. Serological diagnosis of field strains was performed by the slide agglutination test.

DNA base compositions. Deoxyribonucleic acid (DNA) base compositions were determined as previously described (5).

RESULTS

Biochemical findings. The results of the biochemical tests are shown in Table 2. It is evident that the strains isolated from swine are divisible into two distinct groups on the basis of their biochemical characteristics. The major group of porcine organisms comprised 43 strains, whereas the minor group included the 4 strains 202, 343, 349, and 294, which were isolated in Sweden and England, respectively (Table 1). The two groups are separated by differences in the fermentation of ribose, xylose, melibiose, soluble starch, and mannitol (Table 3). Both of these groups of porcine strains are differentiated from the hemolytic strains isolated from humans by a number of biochemical reactions (Table 3).

Hemolysis of calf blood was detected in 39 of the 43 strains assigned to the major group (Table 2). None of the four porcine strains included in the minor group on the basis of their biochemical reactivity was hemolytic. Cultures of all 43 strains of the major group produced extended zones of hemolysis on sheep blood agar within

the zone of partial lysis surrounding the beta-toxinogenic feeder strain. The lytic zone produced by all strains of serovar 2, two of the strains of serovar 5 (266-1920-HAE and Finland), and a nontypable strain isolated in Denmark (Femø) had a characteristic triangular shape. All remaining strains of the major group, including the four that were nonhemolytic on calf blood agar, produced a rounded lytic zone in the neighborhood of the feeder strain (6). None of the strains assigned to the minor group or to *H. parainfluenzae* produced a hemolysin that acted synergistically with the beta-toxin of the feeder strain.

The fermentation end products detected by gas chromatographic analysis of the glucose broth cultures were acetic, lactic, and succinic acids.

The guanine-plus-cytosine contents (G+C) of DNA of the three groups were 42.1 ± 0.3 , 39.6 ± 0.2 , and 40.3 ± 0.4 , respectively (Table 4). The results for the individual strains are listed in Table 4.

Serological findings. Forty-two of the 47 porcine strains agglutinated in typing sera 1 to 5. The serological diagnosis of these strains is provided in Table 1. Cross-agglutination tests involving strain Femø revealed no serological relationship with any of the serovars 1 to 5. Cells of the four remaining strains (202, 343, 349, and 294) were also inagglutinable in typing sera 1 to 5.

Upon subcultivation, several strains of serovar 3 lost their capsular antigen. Such strains showed a tendency to agglutinate in sera of the other serovars.

DISCUSSION

It is evident from the results obtained in this and previous studies (1, 2, 5) that a variety of taxonomic groups of bacteria belonging to the genus *Haemophilus* can be isolated from swine. Previously, a positive urease test has been regarded as important for the separation of strains of *H. parahaemolyticus/pleuropneumoniae* from strains of *H. suis/parasuis* (2, 5, 12). All strains included in the present study were urease positive. In addition, their origin, growth patterns, and other biochemical activities strongly suggested their identity as strains of *H. parahaemolyticus/pleuropneumoniae*. However, the data presented in Table 2 show that the 47 porcine strains fell into two separate groups on the basis of their biochemical reactivity and DNA base composition. The 43 strains, comprising the major group, were characterized by the ability to ferment ribose, xylose, and mannitol and by a positive CAMP reaction. The distinguishing characteristics of the remaining four

TABLE 2. Summary of the biochemical reactions of *Haemophilus pleuropneumoniae*, including the type strain, an unnamed *Haemophilus* taxon, and representative beta-hemolytic strains of *H. parainfluenzae*

Test or substrate	Porcine strains			Human strains	Negative strains ^a
	<i>H. pleuropneumoniae</i> "major group" (43 strains)	<i>H. pleuropneumoniae</i> type strain (Shope 4074)	<i>Haemophilus</i> taxon "minor group" (4 strains)	<i>H. parainfluenzae</i> (9 strains)	
V-factor requirement	43 ^b	+	4	9	
ALA ^c → porphyrins	43	+	4	9	
Indole	0	—	0	0	
Urease	43	+	4	9	
Ornithine decarboxylase	0	—	0	5	NCTC 8479, 10794, 10670, and 10672
Lysine decarboxylase	0	—	0	0	
Arginine dihydrolase	0	—	0	0	
Hemolysis, calf blood	39	+	0	9	4226, S851, M 62, 266-1920-HAE
CAMP reaction, sheep blood	43	+	0	0	
Catalase	33	+	4	9	4969, 2282, 5617, 9711, S447, S851, S853, S1318, HLO 108
Oxidase	26	—	4	8	CCM 5869, 2282, 2226, 5130, 2934, 2845, 4226, 2374, 9711, CCM 5870, ZH 81/65, SG536, HLO 108, Greenfield, 95905, L20, 928, NCTC 10670
β-Galactosidase	43	+	4	7	HK 23, NCTC 10794
α-Fucosidase	0	—	1	1	All except 294, NCTC 10672
Alkaline phosphatase	43	+	4	9	
L-Arabinose	0	—	0	0	
D-Ribose	43	+	0	0	
D-Xylose	43	+	0	0	
L-Rhamnose	0	—	0	0	
D-Galactose	43	+	4	7	HK 3, NCTC 8479
Glucose, acid	43	+	4	9	
Glucose, gas	0	—	0	5	NCTC 8479, 10794, 10670, and 10672
D-Fructose	43	+	4	9	
D-Mannose	43	+	4	0	
Sorbose	0	—	0	0	
Cellobiose	0	—	0	0	
Lactose, 1%	4	—	4	0	All except K17, M62, S43/71, and Findland (weak reactions)
Lactose, 5%	6	—	4	0	All except K17, M62, S43/71, Finland, Femø, K98
Maltose	43	+	4	9	
Melibiose	0	—	4	0	
Sucrose	43	+	4	9	
Trehalose	0	—	2	0	202, 349
Melizitose	0	—	0	0	
Raffinose	2	—	4	0	All except M62, Femø
Inulin	0	—	0	0	
Starch, soluble	0	—	4	0	
Esculin	0	—	0	0	
Salicin	0	—	0	0	
Adonitol	0	—	0	0	
Dulcitol	0	—	0	0	
Glycerol	0	—	0	0	
meso-Erythritol	0	—	0	0	
Mannitol	43	+	0	0	
Sorbitol	0	—	0	0	
Inositol	0	—	0	0	
Xylitol	0	—	0	0	
Acid end products: glucose broth	Acetic, lactic, succinic	Acetic, lactic, succinic	Acetic, lactic, succinic	Acetic, lactic, succinic	
G+C content (mol%)	42.1 ± 0.3	42.1 ± 0.3	39.6 ± 0.1	40.3 ± 0.4	

^a Only exceptions within the individual taxa are listed.

^b Number of positive reactions.

^c ALA, δ-Aminolevulinic acid.

strains included: (i) the ability to ferment melibiose and soluble starch but not ribose, xylose, and mannitol; (ii) absence of any hemolytic activity on plain blood agar or in the CAMP test; and (iii) a relatively low G+C content of DNA (39.6 ± 0.2). Both groups of porcine organisms were separated from the strains of human origin by the failure of the latter strains to ferment

TABLE 3. *Diagnostic and differential characteristics of Haemophilus pleuropneumoniae, an unnamed Haemophilus species and H. parainfluenzae (beta-hemolytic strains)*

Test or substrate	<i>H. pleuropneumoniae</i> ("major group")	<i>Haemophilus</i> sp. ("minor group")	<i>H. parainfluenzae</i> (hemolytic strains)
NAD ^a requirement	+	+	+
ALA ^c → porphyrins	+	+	+
Indole	- ^d	-	-
Urease	+	+	+
β-Galactosidase	+	+	d ^e
Ornithine decarboxylase	-	-	d
Hemolysis, calf blood	d	-	+
CAMP reaction, sheep blood	+	-	-
D-Ribose	+	-	-
D-Xylose	+	-	-
Lactose	d	+	-
Melibiose	-	+	-
Starch, soluble	-	+	-
Mannitol	+	-	-

^a NAD, Nicotinamide adenine dinucleotide.

^b +, >95% positive.

^c ALA, δ-Aminolevulinic acid.

^d -, > 95% negative.

^e d, 5 to 95% positive.

TABLE 5. *Serotype reference strains of Haemophilus pleuropneumoniae*

<i>H. pleuropneumoniae</i> serovar	Type strain
1	4074, CCM 5869, (ATCC 27088)
2	S 1536, CCM 5870, (ATCC 27089)
3	S 1421, CCM 5871, (ATCC 27090)
4	M 62
5	K 17

any of the mentioned carbohydrates (Table 2). The major group, in addition, had a significantly higher G+C content of the DNA than both of the other groups ($P < 0.01$) (Table 4).

All strains included in this study produced acetic, lactic, and succinic acids in glucose broth. These findings confirm the previous suggestion (5) that haemophilii are heterofermentative.

Except for one strain (Femø), all 43 isolates of the major group could be accommodated in the serological scheme established by Nicolet (13) and extended by Gunnarsson et al. (3). The existence of a strain untypable with available sera suggests the likelihood of additional, hitherto unrecognized serovars. None of the four strains of the minor group reacted with any of the five major group typing sera (Table 1). An antiserum prepared with minor group strain 202 failed to react with representative cultures of the five serovars of the major group (3).

The major group included authentic strains of both Olander (Ph.D. thesis) and Shope (22). A comparison of the biochemical and cultural characteristics of these strains with those reported by Matthews and Pattison (10) very strongly suggests that the strain described by the latter authors also belongs to this group. Thus, the paper of Matthews and Pattison (10)

TABLE 4. *G+C content of DNA from representative strains*

Taxon	Strain	Mol% G+C ^a	Mean % G+C
<i>H. pleuropneumoniae</i>	CCM 5869 ^b	42.1 ± 0.3	42.1 ± 0.3
	2226	42.4 ± 0.3	
	Canada	41.7 ± 0.3	
	2845	42.0 ± 0.2	
	Femø	42.2 ± 0.1	
	M 62	42.4 ± 0.1	
"Minor group of porcine strains"	202	39.8 ± 0.2	39.6 ± 0.2
	343	39.5 ± 0.1	
	349	39.5 ± 0.2	
<i>H. parainfluenzae</i> (hemolytic strains)	HK 3	40.5 ± 0.2	40.3 ± 0.4
	HK 23	39.8 ± 0.1	
	HK 331	40.7 ± 0.2	
	NCTC 8479	40.2 ± 0.1	
	NCTC 10670	40.2 ± 0.1	

^a Based on three to four determinations. Thermal denaturation point.

^b Proposed type strain = Shope 4074 = ATCC 27088.

appears to be the first published description of this organism.

In most previous reports the names *H. para-haemolyticus* and *H. parainfluenzae* have been used for this organism depending on the hemolytic activity of the described isolates. The present and previous studies indicate that the hemolytic activity is subject to differences in degree between strains and within one strain from passage to passage. The demonstration of hemolytic activity is highly dependent on the specific origin of the blood used (6, 12), and there is growing sentiment that this trait has been given undue importance in classifying haemophili (5). It is evident, however, that the porcine organism, hemolytic or not, is significantly different from the human strains for which the species *H. parainfluenzae* and *H. parahaemolyticus* were established (Table 3) and that it deserves specific recognition. Shope (22) was the first to propose a separate name for this organism, i.e., *H. pleuropneumoniae*. Thus, we consider the correct name of the organisms of our major group to be *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964. Since the authentic strain of Matthews and Pattison (10) is no longer available (Matthews, personal communication) we propose that the strain Shope 4074 be the type (neotype) strain. The cultural and biochemical characteristics of the strain of Matthews and Pattison (10), as far as they were reported, agree closely with those of the proposed neotype strain (Table 2).

The minor group of porcine strains is distinct from the major group of strains assigned to *H. pleuropneumoniae* with regard to hemolytic activity, agglutinating antigens, several biochemical characteristics, and a significantly lower G+C content of DNA. Although these differences possibly justify specific recognition of this group, a recommendation with regard to this matter is deferred pending further studies of these and other haemophili of porcine origin.

Description of *H. pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964.

Cell characteristics. Gram-negative, nonmotile, nonsporeforming, pleomorphic rods, showing a predominance of coccobacillary forms. Only occasional filamentous forms are seen in young cultures. Pathogenic strains are encapsulated.

Surface colonies. Surface colonies on chocolate agar are opaque and reach a diameter of 1 to 2 mm (48 h). Two types of colonies can be seen: a rounded, hard, "waxy" type, which adheres to a platinum loop; or a flatter, soft, glistening type.

Encapsulated strains produce iridescent colonies on Levinthal agar.

Colonies on calf or sheep blood agar usually produce a beta-hemolytic zone. The hemolysin acts synergistically with *S. aureus* beta-toxin on sheep or calf red blood cells giving rise to varying degrees of a positive CAMP reaction.

Growth factor requirements. Requires V-factor (nicotinamide adenine dinucleotide or its riboside precursor) but not X-factor (hemin). Produces porphyrins from δ -aminolevulinic acid.

Atmospheric requirements. Facultatively anaerobic; extra CO₂ is not required for growth in air.

Biochemical reactivity. Chemoheterotrophic. Biochemical reactions are given in Table 2.

Fermentation end products from glucose. Acetic, lactic, and succinic acids are produced. Hydrogen or CO₂ is not produced.

G+C content of DNA. 42.1 $\pm$ 0.3 mol% (thermal denaturation point).

Serology. Five serovars (1 through 5) have been recognized on the basis of surface antigens, which, in the case of serovars 1 to 3, have been identified as capsular polysaccharides.

Source. Have so far been isolated from pneumonic lesions, blood, and various other tissues of swine, a case of arthritis in a lamb, and a brain abscess in a steer.

Pathogenicity. Produces extensive lobar pneumonia with accompanying fibrinous pleuritis in swine; when bacteremic, also causes meningitis and arthritis. The disease is highly contagious under experimental conditions, and the fatality rate may approach 50%.

Type strain (neotype). Shope 4074. Maintained in the American Type Culture Collection as ATCC 27088 and the Czechoslovak Collection of Microorganisms as CCM 5869. Capsular antigen of serovar 1.

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REPRINT REQUESTS

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