Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-Like Organism Causing Porcine Necrotic Pleuropneumonia to the Genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* comb. nov.) on the Basis of Phenotypic and Deoxyribonucleic Acid Relatedness

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The *Pasteurella*-like organism of porcine necrotizing pleuropneumonia described by Bertschinger and Seifert in 1978 was shown to be phenotypically similar to the V factor-requiring strains hitherto classified as *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964. Deoxyribonucleic acid-deoxyribonucleic acid hybridization experiments in which the optical method was used yielded 91% binding of the genomes of the type and reference strains of these two taxa, thus indicating that they belong to the same species. The levels of deoxyribonucleic acid binding between these strains and the type strain of *Haemophilus influenzae* indicated no measurable relatedness. Therefore, these organisms do not belong to the genus *Haemophilus* sensu stricto. On the other hand, they are closely related to *Actinobacillus lignieresii*, as determined by both phenotypic characteristics and deoxyribonucleic acid base sequence relatedness (72 to 75% binding). To take into account the different host ranges of these organisms, we propose to classify them as a species distinct from, but closely related to, *A. lignieresii*, under the new combination *Actinobacillus pleuropneumoniae*. The emended species *A. pleuropneumoniae* is composed of V factor-requiring and V factor-independent biovars. The type strain (strain Shope 4047 [= CCM 5869 = ATCC 27088]) requires V factor; the V factor-independent biovar is represented by strain Bertschinger 2008/76 (= Frederiksen P 597 = HIM 677-3/4).

In 1978 Bertschinger and Seifert (H. U. Bertschinger and P. Seifert, Abstr. 5th Int. Pig Vet. Soc. World Cong. Hyol. Hyiatr., 13-15 June 1978, Zagreb, Yugoslavia, abstr. no. M19) described a *Pasteurella haemolytica*-like organism as the causative agent of porcine necrotizing pleuropneumonia in Switzerland. This disease could not be distinguished from the disease caused by *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964 by clinical or histological means (J. Pohlenz, H. U. Bertschinger, F. Ehrensperger, and P. Seifert, Abstr. 5th I.P.V.S. World Cong. Hyol. Hyiatr., abstr. no. M18). The new organism was placed in the close vicinity of *Actinobacillus lignieresii* on the basis of conventional phenotypic characteristics (G. R. Carter, personal communication; W. Frederiksen, unpublished data). In the tissues of the host, however, *A. lignieresii* produces characteristic granulomas that are interspersed with bacterial colonies called sulfur

granules or actinomycotic granules. Such granules were never observed in porcine infections with the new organism. Furthermore, the organism isolated by Bertschinger and Seifert (designated the BS organism) resembled *Haemophilus pleuropneumoniae* in many physiological properties; an exception was the V factor requirement (10). In this paper we describe the genetic relationships among the organisms mentioned above, as revealed by deoxyribonucleic acid (DNA)-DNA hybridization, and propose a classification of these organisms.

MATERIALS AND METHODS

Microorganisms. The bacterial strains which we studied are listed in Table 1. They were kept lyophilized. Before use, they were propagated on chocolate agar (tryptic soy agar [Difco Laboratories, Detroit, Mich.] supplemented after sterilization with 5% [vol/vol] sterile defibrinated sheep blood and kept at 80°C for 15 min before pouring), purified by subculturing

TABLE 1. Bacterial strains investigated and DNA base compositions of the strains used for hybridization

Name as received	Strain ^a	Source	Guanine-plus-cytosine content (mol %) ^b
<i>A. lignieresii</i>	NCTC 4189 ^T (= L. Thompson 1 ^T)	Cattle, glands	44.2 (0.4) ^c
<i>A. lignieresii</i>	NCTC 4976 (= P. H. Martin CM2)	Cattle, glands	43.4 (0.1) ^c
<i>H. influenzae</i>	NCTC 8243 ^T (= A. B. Rosher 680 ^T)	Human	38.5 (0.2)
<i>H. pleuropneumoniae</i>	CCM 5869 ^T (= R. E. Shope 4074 ^T = ATCC 27088 ^T = HK 405 ^T)	Pig, lung	43.2 (0.4) ^c
<i>H. pleuropneumoniae</i>	NCTC 10976 (= R. Nielsen 9711)	Pig, lung	42.1 (0.2) ^c
<i>Pasteurella haemolytica</i>	NCTC 9380 ^T (= J. A. Watt 1266/56 A&B ^T)	Cattle	43.6 (0.1)
<i>Pasteurella haemolytica</i> -like	H. U. Bertschinger 2008/76 (= P 597 = HIM 677-3/4)	Pig, lung	42.2 (0.4) ^c
<i>Pasteurella haemolytica</i> -like	H. U. Bertschinger 339/76 (= P 598 = HIM 635-6/7)	Pig, lung	NT ^d
<i>Pasteurella haemolytica</i> -like	H. U. Bertschinger 1004/77 (= P 600 = HIM 639-7/8)	Pig, lung	NT
<i>Pasteurella haemolytica</i> -like	H. U. Bertschinger 2288/77 (= P 599 = HIM 634-7/8)	Pig, lung	42.5 (0.2) ^c
<i>Pasteurella haemolytica</i> -like	H. U. Bertschinger 204/78 (= P 601 = HIM 636-3/4)	Pig, subcutaneous lymph node	NT

^a ATCC, American Type Culture Collection, Rockville, Md; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; HIM, Bacteriological Culture Collection, Zentrum für Hygiene und Medizinische Mikrobiologie, Marburg, Federal Republic of Germany; NCTC, National Collection of Type Cultures, London, England; P, *Pasteurella-Actinobacillus* Culture Collection, W. Frederiksen, Statens Seruminstitut, Copenhagen, Denmark.

^b Determined by the thermal denaturation method.

^c Data in part from reference 10. The values in parentheses are standard deviations.

^d NT, Not tested.

typical single colonies, and reidentified by conventional methods (6, 9, 10) (Table 2). Mass cultures were grown in Difco Proteose Peptone medium (15) supplemented with 0.25% (wt/vol) Phytone (BBL Microbiology Systems, Cockeysville, Md.) for the BS organism and with 0.25% (wt/vol) Phytone and 1 µg of β-nicotinamide adenine dinucleotide (E. Merck AG, Darmstadt, Germany) sterilized by filtration per ml for *H. pleuropneumoniae*; 300-ml batches in 2-liter Fernbach flasks were incubated at 35°C with moderate shaking, harvested in the early stationary phase, washed in 1× SSC (0.15 M sodium chloride plus 0.015 M trisodium citrate, pH 7.0), and then suspended in 1× SSC containing 0.1 M ethylenediaminetetraacetic acid (disodium salt). The analytical grade reagents used were obtained from E. Merck AG.

Isolation of DNA and determinations of DNA base composition, compositional nucleotide distribution, and degree of DNA binding. Isolation of DNA and determinations of DNA base composition, compositional nucleotide distribution, and degree of DNA binding were done essentially by the methods of De Ly and co-workers (4, 5), using *Escherichia coli* B NCTC 10537 DNA (guanine-plus-cytosine content, 52.0 mol%) as a reference for determining the DNA base composition. Between three and nine reassociation experiments were carried out with each pair of strains, including two different DNA preparations of the type or reference strains used.

RESULTS AND DISCUSSION

As shown in Table 1, all of the DNA preparations used except that of *Haemophilus influen-*

zae were similar in guanine-plus-cytosine content; *H. influenzae* DNA contained significantly less guanine plus cytosine. These DNA preparations also exhibited comparable hyperchromicities (increase in absorbance at 260 nm, between 34 and 38%) and distributional nucleotide variations (ranging from 4.7 to 6.2 mol% guanine plus cytosine). Nearly identical reassociation rates in homologous reactions (data not shown) also suggested that the genomes of *A. lignieresii*, the BS organism strains, and *H. pleuropneumoniae* are very similar in size. The DNAs of the two *A. lignieresii* strains studied have known molecular weights of 1.8×10^9 , whereas the DNAs of *H. influenzae* strains have molecular weights ranging from 1.2×10^9 to 1.4×10^9 (S. Pohl, Ph.D. thesis, Philipps-Universitt, Marburg, Federal Republic of Germany, 1979).

The results of heterologous reassociation reactions (Table 3) agree well with the levels of phenotypic relatedness of the organisms investigated (Table 2). The highest binding ratios were observed among the homonymous strains. The level of DNA relatedness between *H. pleuropneumoniae* and the BS organism was markedly higher than that between *A. lignieresii* and either *H. pleuropneumoniae* or the BS organism. Nevertheless, the *H. pleuropneumoniae*-BS organism group and *A. lignieresii* are closely related. In contrast, the levels of DNA reassociation

TABLE 2. Some characteristics of the bacterial strains studied^a

Character	<i>A. lignieresii</i> (n = 2) ^b	<i>H. pleuropneumoniae</i> (n = 2)	BS organism (n = 5)	<i>Pasteurella haemolytica</i> (n = 1)	<i>H. influenzae</i> (n = 1)
Colonial adherence	v ^c	v	—	—	—
Hemolysis (sheep blood)	—	+	w	+	—
TMPD oxidase ^d	+	+	+	+	—
Catalase ^e	—	—	—	+	+
Requirement for NAD ^f	—	+	—	—	+
Requirement for hematin	—	—	—	—	+
Growth on MacConkey agar	+, (+)	— ^g	v, (w)	+	—
Stimulation of oxygen-limited growth by fumarate	+	—, w	+	+	+
Production of ubiquinone	+	+	w	+	—
Indole	—	—	—	—	+
Urease	+	+	+	—	+
Acid from:					
D(+)-Galactose	+	w	(+)	(+)	+
D(–)-Fructose	+	+	+	+	—
D(+)-Mannose	+	+	+	—	—
L(+)-Arabinose	—	—	—	(vw)	—
D(+)-Xylose	+	+	+	+	(+)
Raffinose	—	—	—	(+)	—
β-Galactosidase (ONPG test) ^h	+	+	+	—	—
Acid from:					
D(+)-Lactose	(+)	—	(+)	—	—
D(+)-Sucrose	+	+	+	+	—
Maltose	+	+	+	+	—
D(–)-Mannitol	+	+	+	+	—
D(–)-Sorbitol	—	—	—	+	—
meso-Inositol	—	—	—	(+)	—

^a Data in part from reference 10, with supplements and corrections. For the methods used, see reference 10. All strains produced smooth, greyish white colonies on chocolate agar and gave positive reactions for fermentation of D(+)-glucose, production of demethylmenaquinone, reduction of nitrates to nitrites, alkaline phosphatase, and susceptibility to benzylpenicillin and colistin. Uniformly negative reactions were observed for phenylalanine deaminase, lysine and ornithine decarboxylases, arginine dihydrolase, gelatinase, production of H₂S in peptone media containing FeCl₂, growth in Simmons citrate, formation of gas from glucose, fermentation of L(–)-sorbitol, L(+)-rhamnose, starch, cellobiose, melibiose, trehalose, salicin, adonitol, and dulcitol, and hydrolysis of esculin.

^b Number of strains tested (the strains listed in Table 1).

^c +, Positive reaction within the first 48 h of incubation; –, negative for 30 days; v, variable reaction; w, weak reaction; (), delayed reaction between 3 and 30 days of incubation.

^d Tested by adding 1% (wt/vol) aqueous *N,N,N',N'*-tetramethyl-1,4-phenylene-diaminehydrochloride (TMPD; Fluka AG, Buchs SG., Switzerland) to 48-h-old colonies in situ (7); different results may be obtained with the Kovacs method (9, 13).

^e Reactions of cells grown on tryptic soy agar (Difco) supplemented with growth factors were necessary.

^f NAD, β-Nicotinamide adenine dinucleotide.

^g According to M. Kilian (personal communication), this organism is positive on MacConkey agar supplemented with β-nicotinamide adenine dinucleotide.

^h ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

between the type strain of *H. influenzae* on the one hand and the type strains of *H. pleuropneumoniae* and *A. lignieresii*, as well as a reference strain of the BS organism, on the other were extremely low. Despite unusually high standard deviations in some of the DNA hybridization experiments, the differences in DNA binding between the three related taxa proved to be statistically significant ($P < 0.05$, Student's *t* test).

The DNA base sequence relatedness data

clearly indicate that both the BS organism and *H. pleuropneumoniae* belong to the genus *Actinobacillus* and that these organisms are not significantly related to the type strain of *H. influenzae*, the type species of the genus *Haemophilus*. This observation was perhaps more surprising in the case of *H. pleuropneumoniae* than in the case of the BS organism strains since the requirement for V factors traditionally has been considered a generic feature of *Haemophilus*. Similarly, *Haemophilus avium* (8) and *Pas-*

TABLE 3. Results of DNA-DNA hybridizations

Source of DNA	Degree of binding (%) with DNA from strain:					
	<i>A. lignieresii</i> NCTC 4189 ^T	<i>A. lignieresii</i> NCTC 4976	Bertschinger 2008/76	Bertschinger 2288/77	<i>H. pleuropneumoniae</i> CCM 5869 ^T	<i>H. pleuropneumoniae</i> NCTC 10976
<i>A. lignieresii</i> NCTC 4189 ^T	100					
<i>A. lignieresii</i> NCTC 4976	95 (4.7) ^a	100				
Bertschinger 2008/76	75 (13)	58 (18)	100			
Bertschinger 2288/77	56 (20)	77 (4.4)	99 (3.0)	100		
<i>H. pleuropneumoniae</i> CCM 5869 ^T	72 (18)	70 (15)	91 (4.0)	78 (18)	100	
<i>H. pleuropneumoniae</i> NCTC 10976	65 (12)	NT ^b	81 (7.0)	NT	93 (2.0)	100
<i>H. influenzae</i> NCTC 8243 ^T	6 (3.0)	NT	NT	4 (14)	7 (17)	NT

^a The values in parentheses are standard deviations.

^b NT, Not tested.

teurella gallinarum are phenotypically related (10) and belong to the same DNA homology group, irrespective of their different V factor requirements (Pohl, Ph.D. thesis; unpublished data).

Apart from the requirement for V factors, the phenotypic similarity between *H. pleuropneumoniae* and the BS organism is striking (Table 2), and, as mentioned above, these organisms display the same host specificities and pathogenicities. Undoubtedly the very high levels of DNA binding among these bacteria justify their classification as members of the same species, and only a much more extended DNA hybridization study could determine whether the V factor-dependent and -independent strains are phylogenetically distant enough to be recognized as stable subspecies. However, the needs of practical diagnostic bacteriology and the ease of maintaining valuable epidemiological information are strong arguments for recognizing two provisional biovars of this species of swine pathogens according to their V factor requirements. In a natural classification system of the genus *Actinobacillus*, the species in question (*H. pleuropneumoniae*-BS organism) undoubtedly should be situated close to *A. lignieresii*, yet one could argue that our data allow these organisms to be united in one species. Although our DNA binding data suggest larger phylogenetic distances between *A. lignieresii* and the *H. pleuropneumoniae*-BS organism group than within the two taxa, the number of strains which we tested admittedly is not sufficient to determine precisely the extent of genomic variation that may occur among these organisms. However, since the strains which we investigated represent well-defined phenotypic groups (6, 9), our data are

sufficiently informative to rearrange these bacteria in a more natural order. Unfortunately, the taxonomic ranks in bacteriology are still very arbitrary. The problem of species discrimination in the range from 70 to 80% DNA binding (under stringent conditions) has been resolved with different results in different areas of the bacteriological system, such as the *Escherichia-Shigella* overlapping (3), the recognition of *Zymomonas* species (14), and the structure of the *Mycoplasma mycoides* group (1). Clearly, the practicability of taxonomic changes based on molecular data and the principle of nomenclatural continuity have to be taken into account. There are good reasons for recognizing the *H. pleuropneumoniae*-BS organism group as a species that is distinct from *A. lignieresii*; these bacteria can be differentiated by their host ranges and pathogenicities and can be identified by phenotypic characteristics (mainly hemolysis and V factor requirement) in diagnostic laboratories. Furthermore, the discrimination of swine and cattle pathogens is important in veterinary practice, and well-known specific epithets can be conserved. Undoubtedly, the classification of the so-called hemolytic strains of *A. lignieresii* that have been described previously (2, 11) should be reconsidered.

We propose to include the BS organism in the species *H. pleuropneumoniae* (Matthews and Pattison) Shope and to transfer *H. pleuropneumoniae* to the genus *Actinobacillus* Brumpt, as follows.

Actinobacillus pleuropneumoniae (Matthews and Pattison) comb. nov. *A. pleuropneumoniae* contains two provisional biotypes; biotype 1 consists of V factor-dependent strains hitherto classified as *H. pleuropneumoniae* (Matthews

and Pattison) Shope, as described in detail by Kilian et al. (9), with strain Shope 4074 (= CCM 5869 = ATCC 27088) as the type strain (12), and biotype 2 consists of V factor-independent strains and is represented by strain Bertschinger 2008/76 (= Frederiksen P 597 = HIM 677-314). An emended description of *A. pleuropneumoniae* is given below.

Cell characteristics: gram-negative, nonmotile, nonsporeforming, small, coccoid to rod-shaped cells occurring singly, in pairs, or short chains; not acid-fast; cells contain demethylmenaquinone and ubiquinone.

Surface colonies: colonies on chocolate agar are smooth and greyish white and reach 3 mm in diameter after 48 h in a candle jar at 37°C; colonies on sheep blood agar produce a narrow zone of complete hemolysis, which is enlarged by staphylococcal β -lysin.

Mesophilic; chemoorganotrophic, with a pronounced growth response to fermentable carbohydrates in peptone media; the minimal nutritional requirements are not known.

Growth factor requirements: most strains are V factor (β -nicotinamide adenine dinucleotide) dependent (biotype 1); some are not (biotype 2). X factors (prothemin or protoporphyrin IX) are not required.

Atmospheric requirements: aerobic, microaerophilic in primary isolation, facultatively anaerobic; no additional carbon dioxide is required for growth in subcultures.

Biochemical reactivity: some biochemical reactions are shown in Table 2.

DNA base composition: the genome DNA of strain Shope 4074^T (type strain) contains 43.2 or 42.0 (9) mol% guanine plus cytosine; the genome DNA of strain Bertschinger 2008/76 contains 42.2 mol% guanine plus cytosine, as determined by the thermal denaturation method.

Source: the sources of biotype 1 strains have been described by Kilian et al. (9). The biotype 2 strains described so far have been isolated from pneumonic lesions of swine; one strain was isolated from a cervical lymph node, and one was isolated from a joint.

Pathogenicity: the pathogenicity of biotype 1 strains has been described by Kilian et al. (9). Since 1975, biotype 2 strains have been repeatedly isolated as the etiological agent of enzootic porcine pleuropneumonia in Switzerland. Bacteremic generalizations and arthritis have occasionally been observed. Morbidity has been low-

er and the severity of clinical infections has been less in outbreaks caused by biotype 2 strains than in outbreaks caused by V factor-requiring strains.

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