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SPHAEROPHORUS NECROPHORUS
A STUDY OF 23 STRAINS

By
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The anaerobic, non-sporing, gram-negative rods, for whom the family Bacteroidaceae was advanced by *Breed et al.* (1957), are generally considered to offer difficulties, both concerning cultivation and identification (*Barnes et al.* 1966). Cultivation problems have been largely reduced by introduction of proper media and anaerobic incubation methods (*Beerens* 1953—54). Traditional identification methods being of little differential value (*Barnes et al.*) several new identification methods have recently been developed. The taxonomical problems, caused by several incomplete, contradictory, and multiple descriptions in the literature of the last seven decades, have rendered a simplification inevitable by limiting it to well described groups of comparatively recent date (*Werner* 1968). A confusing nomenclature arises from the current use of several different classifications, viz. that of *Wilson & Miles* (1955), *Breed et al., Prevot* (1966), and *Beerens et al.* (1962). A comparison is given by *Werner* (1965).

The taxonomy of the genus Spaerophorus *Prevot* 1938 was rediscussed by *Sebald* (1962) on the basis of DNA structure studies. A close relationship to the genus Fusobacterium was revealed. Evidence for close bonds between these genera is further supported by the several other characters being shared by them (*Suzuki et al.* 1966). Disagreement exists as to how many species should be accepted within the genus Spaerophorus (*Werner* 1968). The term Spaerophorus was first used for a lichen (*Breed et al.*), but does not fulfill the nomenclatural requirements for lichens and therefore should not be illegitimate for a bacterial genus (*Sebald*). According to the latest classification of the Bacteroidaceae (*Beerens et al.* 1962), the genus Spaerophorus Prevot

1938 (by the authors given the name Bacteroides) is defined from other Bacteroidaceae by the ability of threonine utilization and a non-fusiform morphology. Type species is Spaerophorus necrophorus.

The genus consists of hemolytic and anhemolytic species. By *Prevot* (1966) two hemolytic species are considered: Sphaerophorus necrophorus (*Schmorl* 1891) and Spaerophorus funduliformis (*Hallé* 1898), the former being responsible for animal infections, the latter for human infections. This dualism is not accepted by *Breed et al.*, comprising both of them to one genus: Sphaerophorus necrophorus. The two species of *Prevot* (1966) are distinguished by *Beerens* by a hemagglutinin, present in Sphaerophorus necrophorus and absent in Spaerophorus funduliformis. From his extensive studies of human and animal strains, *Fievez* (1963) was able clearly to distinguish between the two organisms, but suggested the differences to be of minor importance, not justifying division into species, but rather into biotypes of one species: Sphaerophorus necrophorus, type A and type B corresponding to species necrophorus and species funduliformis of *Prevot* (1966), respectively.

MATERIALS AND METHODS

Twenty-three strains of Sphaerophorus necrophorus were isolated from bovine and porcine pathological processes, listed in Table 1. Cultural, morphological, and biochemical characters were examined and compared to those decribed in the literature.

Media. The following media were used: The Rosenow medium of *Beerens* (1953—54) (Bacto-tryptose (Difco) 10 g, beef extract (Lab-Lemco, Oxoid) 3 g, glucose 2 g, NaCl 5 g, cysteine hydrochloride 0.3 g, Andrades indicator (0.5 % aqueous acid fuchsine) 10 ml, distilled water to 1000 ml. pH 7.2. Tubed together with about 0.1 g calcium carbonate and 2 g ox brain tissue). The VL medium (milieu viande levure) of *Beerens* (Bacto-tryptose (Difco) 10 g, beef extract (Lab-Lemco, Oxoid) 3 g, glucose 2 g, NaCl 5 g, yeast extract (Difco) 5 g, cysteine hydrochloride 0.4 g, distilled water to 1000 ml. pH 7.2—7.4), as the VL broth (without agar), as the semisolid VL basal medium (with 0.06 % agar and without glucose), and as the solid VL agar (with 2 % agar). For VL blood agar, 10 % horse blood was added (*Fievez* 1963), ox blood not being hemolysed by Sphaerophorus necrophorus. As selective medium was used the VL agar, supplied with 0.024 g brilliant green, 0.3 g sodium azide, and 30 ml horse blood per l (*Fievez*).

Methods for anaerobic incubation. Dissolved atmospheric gas was removed from all media by boiling for 15 min., the media being inocu-

lated immediately after cooling. Tubed liquid media were then covered with sterile vaseline and incubated aerobically. Tubed, semisolid and solid media were incubated aerobically without cover. Plated media were used freshly poured and anaerobiosis was established immediately after inoculation, utilizing the alcaline pyrogallol method of *Mossel et al.* (1959) in the following modification: Potassium carbonate and terra silicea were dried overnight at 100°C. One part (by weight) of potassium carbonate was grained in a mortar and mixed with one part of pyrogallol and five parts of terra silicea. The mixture was distributed into paper bags (5×10 cm), each containing about 2 g. The bags were kept in a desiccator and were under these circumstances active for more than one month. For anaerobic cultivation a bag was fixed by tape to the inside of the petri dish lid. Another lid was placed under the bottom of the dish, the latter being enclosed between the two lids, which were sealed together with tape.

Isolation technique. Isolation was in most cases performed by surface culture of homogenized material on VL blood agar. In cases with heavy contamination this procedure was supplemented with surface culture on the selective VL brilliant green azide blood agar. In cases, when sparse occurrence was suspected, also a primary enrichment culture in Rosenow medium was made, followed by isolation on solid medium. Pure culture was achieved by passages through Rosenow cultures and VL blood agar surface cultures.

Maintenance of strains. The strains were maintained as Rosenow cultures at room temperature. At three weeks' intervals, the cultures were renewed by transferring a large inoculum. Lyophilization was performed by suspending a VL blood agar surface culture in 3 ml freshly boiled and cooled cysteine milk, which was distributed to two ampoules and lyophilized.

Morphology. In addition to Gram staining, studies on shape, size, and motility were carried out by phase contrast microscopy from young Rosenow cultures. Colony morphology was examined after 72 hrs.' incubation of VL blood agar surface cultures.

The anaerobic property was demonstrated by absence of growth on aerobically incubated VL blood agar and by anaerobic growth type of shake cultures, characterized by a growth-free, upper oxygenated zone, and uniform growth in the rest of the medium.

Carbohydrate fermentation was studied according to *Beerens* in VL basal medium, added 1 % of the carbohydrates (xylose, galactose, arabinose, and sorbitole sterilized by filtration, the others by heat). After four and 14 days of incubation, acid production was demonstrated by adding one drop of bromthymolblue solution (1:500). In the glucose medium pH was measured electrometrically.

Brilliant green test and bile test. The influence of brilliant green (0.0008 %) and ox bile (10 % of rehydrated Bacto-Oxogall Difco) on growth in solid, tubed VL medium was tested according to *Beerens,*

the growth in the test medium being compared to that of the same medium without dye or bile.

Gelatine hydrolysis. Cultures on VL agar, containing 1 % gelatine (*Fievez*) were tested with the acid mercuric chloride reagent of Frazier (*MacDade & Weaver* 1959).

Lecitinase reaction was tested on VL egg yolk agar (*Fievez*).

Indole production. Test for indole production was carried out on four days old VL basal medium cultures, using Kovac's reagent.

Nitrate reduction. Test for nitrate reduction was performed in the VL nitrate medium of *Beerens.*

Action on milk was tested in the cysteine milk of *Beerens* (skim milk, supplied with 0.8 % cysteine hydrochloride and adjusted to pH 7.2–7.4). Further, proteolysis was tested by surface cultivation on VL milk agar (double strength VL agar, added equal parts of cysteine milk).

Hemagglutination. The ability of washed bacterial cells to agglutinate chickens' erythrocytes was tested according to *Fievez.* Chickens' erythrocytes are washed in saline and suspended in saline to reconstitute the original blood volume. The bacteria, cultivated in 10 ml VL broth, are centrifuged, washed, and suspended in 10 drops of saline. A drop of the bacterial suspension and a drop of the erythrocyte suspension are mixed on a slide and the result is read immediately.

Threonine utilization was examined by the nile blue test according to *Beerens & Castel* (1965) and by the ammonia liberation test of *Suzuki et al.* (1966).

The fatty acids, produced in VL broth cultures were detected by a method, developed in collaboration with Benedicte Hald at this department. A culture in 20 ml VL broth, containing 1 % glucose, was incubated four days and centrifuged. The supernatant was acidified with hydrochloric acid (6 N) and cleaned on a column, consisting of terra silicea and anhydrous sodium sulphate (75 + 25). The fatty acids were eluted from the column by 150 ml ethyl ether, which was afterwards evaporated until a volume of 1 ml. The acids were identified by gas liquid chromatography.

RESULTS AND DISCUSSION

Isolation

Isolation of all the Sphaerophorus necrophorus strains, except one, from pathological processes was achieved by primary surface culture on VL blood agar, incubated anaerobically (37°C/72 hrs.) by the alcaline pyrogallol method. In a single case (strain 11), where this method was unsuccesful, isolation was achieved by primary enrichment culture in Rosenow medium, followed by surface culture on the selective medium. The results are in accordance with those of *Fievez* (1963), finding that this latter

method increased the number of succesful isolations with about 10 %, compared to the results obtained by the former method alone. The general superiority of surface cultures for primary isolation of anaerobes, compared to the shake culture method, was demonstrated by *Beerens & Castel* (1958), being ascribed to the disadvantages of the shake culture: rupture of the agar column by gas producing organisms, uncharacteristic colony morphology, difficulties in sampling the colonies. The alcaline pyrogallol method, preferred by *Fievez* for cultivation of Sphaerophorus spp., has worked very reliably. Further, the desiccating effect of the reaction mixture minimizes swarming problems.

Occurrence

As seen from Table 1, Sphaerophorus necrophorus belonging to both the type A and type B of *Fievez* (equivalent to Sphaerophorus necrophorus and Sphaerophorus funduformis of *Prevot*

Table 1. Designation and origin of the strains.

Strain no.	Origin	Type	Occurrence of other organisms
1	bovine hepatitis necrot. c. sequestr.	A	pure culture
2	bovine pneumonia necrot. c. sequestr.	A	pure culture
6	bovine stomatitis necrot. ac.	B	mixed flora
10	bovine hepatitis necrot. c. sequestr.	A	pure culture
11	porcine pneum. emb. necrot. c. sequestr.	B	corynebact. pyogenes + E. coli
12	bovine hepatitis necrot. c. sequestr.	A	pure culture
13	bovine hepatitis necrot. c. sequestr.	A	pure culture
14	bovine hepatitis necrot. c. sequestr.	A	pure culture
15	bovine hepatitis necrot. c. sequestr.	B	pure culture
16	bovine hepatitis necrot. c. sequestr.	A	pure culture
17	bovine hepatitis embol. apostem.	B	corynebact. pyogenes
18	bovine hepatitis necrot. ac.	A	pure culture
19	bovine hepatitis necrot. c. sequestr.	A	pure culture
20	bovine hepatitis necrot. c. sequestr.	A	pure culture
21	bovine hepatitis necrot. c. sequestr.	A	pure culture
22	bovine hepatitis necrot. c. sequestr.	B	corynebact. pyogenes
23	bovine hepatitis embol. apostem.	B	corynebact. pyogenes
24	bovine hepatitis necrot. c. sequestr.	A	corynebact. pyogenes
25	bovine hepatitis necrot. c. sequestr.	A	pure culture
26	bovine omastitis necrot.	B	mixed flora
27	porcine experimental enteritis	B	mixed flora
28	porcine experimental enteritis	B	mixed flora
29	porcine experimental enteritis	A	mixed flora

(1966), respectively), were isolated from cases of animal sphaerophoroses. Type A was found more frequently than type B. Most commonly, pure infections were found. Mixed infections were found with Corynebacterium pyogenes and Escherichia coli. In one case of bovine hepatic necrobacillosis, the two types of Sphaerophorus necrophorus each occurred in pure culture in two different processes (strain 15 and 16). The dominating role of Sphaerophorus necrophorus for the genesis of multiple bovine liver abscesses (*Fievez, Smith* 1963) was found to be valid for the present material too, and constantly numerous viable organisms were present. The results are similar to those of *Fievez*, who found type A more frequently than type B, most commonly found pure infections, and found Corynebacterium pyogenes as the most common accompanying organism in mixed infections. The frequency of Corynebacterium pyogenes in mixed infections with Sphaerophorus necrophorus may be explained by a synergism in pathogenicity, which was experimentally demonstrated by *Roberts* (1967).

Habitat

The few investigations of the habitat indicate more evidence for the human and animal mucous membranes than for the soil as the primary habitat (*Smith*). During investigations of the anaerobic intestinal flora of pigs (unpublished), the author demonstrated small numbers (3×10^3/g) of Sphaerophorus necrophorus in a specimen of colon contents from one out of three normal slaughter pigs.

Colony morphology

After three days' incubation, colonies on VL blood agar of strains nos. 1-2-10-12-13-14-16-18-19-20-21-24-25-29 were flat, grayish, semitransparent, about 2.5 mm in diameter. The margin was irregular (lacerate) and the surface was matt and granular by magnification. The consistency was butyrous. The colonies were surrounded by a sharply demarcated zone of hemolysis, about 6 mm in diameter. During prolonged incubation the agar surface around the colonies developed a dark, metal-like tinge. Colonies of the strains nos. 6-11-15-17-22-23-26-27-28, under identical conditions, were high, conical, yellowish intransparent, about 1 mm in diameter. The margin was macroscopically even, but weakly irregular by magnification. The surface was matt

and weakly granular by magnification. The consistency was firm so the whole colonies could be picked on the inoculation needle. The colonies were surrounded by an unsharply demarcated zone of hemolysis, about 4 mm in diameter.

Clear-cut differences exist in colony morphology and degree of hemolysis. The appearance of the colonies is identical with the description of *Fievez* for Sphaerophorus necrophorus, type A and type B, respectively. The intermediate AB colony type of some strains, having the characteristics of type A in all other respects, described by *Fievez*, was not observed among the present strains.

Representative colonies of the two types are shown in Figs. 1 and 2.

Cell morphology

In preparations from 24 hrs. old Rosenow cultures, the strains nos. 1-2-10-12-13-14-16-18-19-20-21-24-25-29 were Gram-negative rods with a thickness of 0.5 μ and a length varying from 5 to 75 μ. Some cells stained homogenously, others granularly. Swollen forms were rarely observed.

The strains nos. 6-11-15-17-22-23-26-27-28 were Gram-negative rods with a thickness of 0.5 μ and a length varying from 0.5 to 20 μ, the short forms dominating. All cells stained markedly granular, reminding of chains of cocci or short rods. Representative Gram-stained preparations are shown in Figs. 3 and 4. During the several passages, necessary for maintenance of the strain collection for more than one year, the type characters as to colony and cell morphology were found completely stable, a change from one type to the other never being observed.

Based on the morphological observations, the strains were divisible into two groups, the former 14 strains (nos. 1-2-10-12-13-14-16-18-19-20-21-25-29) identical with the description of the Sphaerophorus necrophorus type A, and the latter nine strains (nos. 6-11-15-17-22-23-26-27-28) identical with that of the type B of *Fievez*.

Motility was not observed in any strain.

The anaerobic property

All strains were found to be obligate anaerobes, growing uniformly in shake cultures except in the upper, oxygenated growth-free zone.

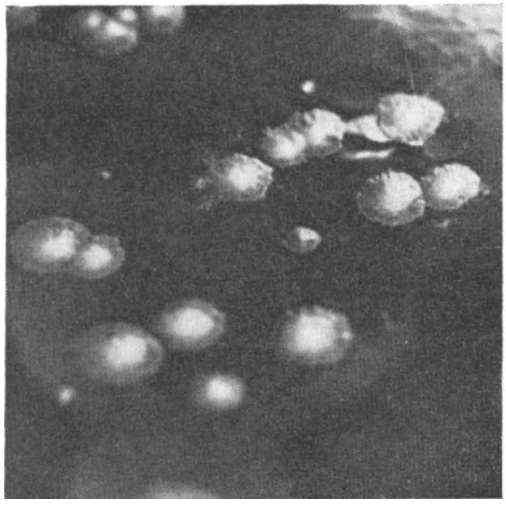

Figure 1.

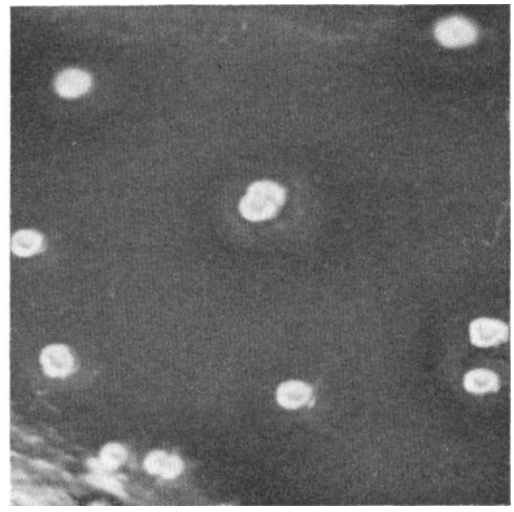

Figure 2.

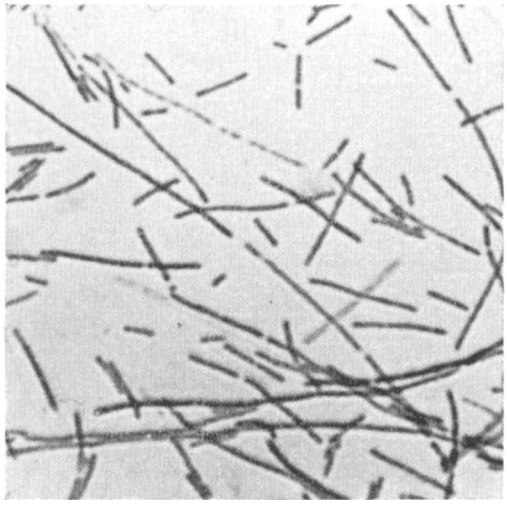

Figure 3.

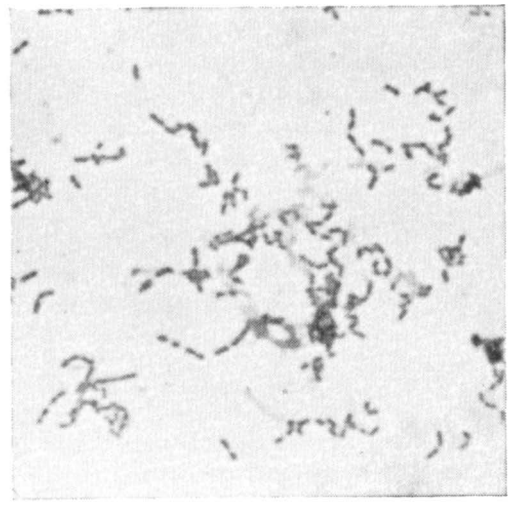

Figure 4.

Figure 1. 72 hrs. surface culture on VL Blood agar of Sphaerophorus necrophorus type A (strain 1).

Figure 2. 72 hrs. surface culture on VL blood agar of Sphaerophorus necrophorus type B (strain 6).

Figure 3. Gram stain of 24 hrs. Rosenow culture of Sphaerophorus necrophorus type A (strain 1). 1200 ×.

Figure 4. Gram stain of 24 hrs. Rosenow culture of Sphaerophorus necrophorus type B (strain 6). 1200 ×.

Table 2. Acid production from carbohydrates.

	The 14 Sphaerophorus necrophorus type A strains			The 9 Sphaerophorus necrophorus type B strains		
	+	(+)	—	—	(+)	—
Monosaccharides						
Pentoses						
Arabinose			14			9
Xylose			14			9
Hexoses						
Fructose	11	3		9		
Galactose	9	3	2	5	2	2
Glucose	14			9		
Mannose		14			9	
Disaccharides						
Lactose			14			9
Maltose	13	1		8	1	
Saccharose			14			9
Cellobiose			14			9
Trisaccharides						
Melezitose			14			9
Alcohols						
Adonitole			14			9
Sorbitole			14			9
Mannitole			14			9
Glycosides						
Salicine			14			9

\+ : Acid production demonstrated by adding indicator after four days.
(+): Acid production absent or weak after four days, but present (generally weak) after 14 days of incubation.
— : Acid production not demonstrated after 14 days of incubation.

Acid production from carbohydrates and related compounds

As seen from Table 2, none of the strains produce acid from the following compounds: adonitole, melezitose, xylose, lactose, saccharose, mannitole, cellobiose, salicine, arabinose, sorbitole. They are variable against galactose (acid produced by most strains). All strains produce acid from mannose (all weak and/or late), from maltose and fructose (a few strains weak and/or late) and from glucose. No difference between type A and type B

strains appears. With the exception that cellobiose is not utilized by the present strains the results agree with those of *Fievez,* finding that Sphaerophorus necrophorus always produces acid from glucose and also from fructose, mannose, and cellobiose, but sometimes weakly, is variable against galactose and maltose, and with few exceptions does not produce acid from other carbohydrates. On the other hand, the results differ from those, cited by *Breed et al.* (1957) and *Wilson & Miles* (1955).

pH in glucose medium

By electrometrical measurement of pH in VL basal medium containing 1 % glucose after four days' incubation an average of 5.77 was found with a standard deviation of ± 0.17. A small difference, found between the average pH of the type A strains and the type B strains, was found to be insignificant ($s > 5$ %). *Lahelle* (1947) found that Sphaerophorus necrophorus, grown in peptone water with 1 % glucose, resulted in a terminal pH of 5.65–6.47, generally about 6.0. *Fievez,* with 1 % glucose in VL basal medium, reported a terminal pH of 5.6–5.7. It is considered a characteristic feature that Sphaerophorus necrophorus in opposition to some other members of the Bacteroidaceae produces a terminal pH of 5.6–6.2 (*Barnes et al.* 1966). The relatively weak acid production of Sphaerophorus necrophorus seems not to be shared by the anhemolytic members of the genus Sphaerophorus. During an investigation of 34 such strains (not published), the author by the same method found an average pH of 5.19 with a standard deviation of ± 0.23. This result was found to be significantly different from that of Sphaerophorus necrophorus strains ($s < 0.1$ %).

Brilliant green test

In the brilliant green test (*Beerens* 1953–54) growth of a shake culture in a medium containing 0.0008 % brilliant green is compared to that in the same medium without dye. All Sphaerophorus necrophorus strains, irrespective of their type, were unaffected by the dye, the growth intensity being identical in test and blind medium, being characterized by strong gas production, decoloration of the dye beginning at the bottom, and a growth-free upper, oxygenated zone. These results are identical with those described by *Beerens* and *Fievez.*

Bile test

The bile test (*Beerens*) is performed as the brilliant green test, the brilliant green being replaced by 10 % ox bile. Compared to the growth intensity in the blind medium, all 14 Sphaerophorus necrophorus type A strains were more or less inhibited by 10 % bile, nine strains being partially inhibited and five strains totally inhibited. Of the nine Sphaerophorus necrophorus type B strains, one grew indifferently (was not favoured and not inhibited), six were partially inhibited and two were totally inhibited. These results are identical with those of *Fievez*, finding that few Sphaerophorus necrophorus strains are indifferent, most are partially inhibited, and few are completely inhibited.

Indole production

All the strains produced indole in the VL basal medium.

Nitrate reduction

None of the strains were found to reduce nitrate.

Hemolysis

On VL horse agar colonies of all the type A strains were after three days' incubation surrounded by a sharply demarcated zone of hemolysis, about 6 mm in diameter. All the type B strains had unsharply demarcated zones of hemolysis, about 4 mm in diameter.

Lecitinase reaction

On VL egg yolk agar (*Fievez*) inoculated with a drop of Rosenow culture and incubated four days, all 23 strains produced opacity under the colony and 1–2 mm outside it, the opacity being surrounded by a clear zone, about 1 mm wide.

The results for indole production, nitrate reduction, hemolysis and lecitinase production all agree with those of *Fievez*.

Growth modus in VL broth

In VL broth, containing 0.2 % glucose, all the 14 Sphaerophorus necrophorus type A strains produced permanent, homogenous cloudiness, whereas all the nine type B strains had a flocculating growth modus. The broth remained clear and the produced flocks were soon sedimented. Both types showed abundant gas production. This characteristic difference between the two types is described by *Prevot* (1966) and *Fievez*. In Rosenow me-

dium both types initially produce colour change of the indicator to red because of acid production from glucose, but a few hours later the indicator is reduced to a colourless state. Gas production is abundant.

Gelatine hydrolysis

On VL gelatine agar (*Fievez*), inoculated with a drop of Rosenow culture, incubated four days and flodded with acid mercuric chloride solution, the 14 type A strains showed clearing of the medium under the colony and 2–3 mm outside it, the margin of the clearing being unsharp. The nine type B strains showed a negligible clearing under the colony.

Reaction in cysteine milk

The 14 type A strains showed a weak gas production, beginning after one or a few days of incubation. The medium was coagulated after four to 14 days of incubation, and the coagulation was followed by peptonization. Eight of the nine type B strains also showed a weak gas production, beginning after one or a few days of incubation. The medium was coagulated after 12–40 days of incubation, and the coagulation was followed by peptonization. One strain (no. 11) caused no change of the medium except a weak gas production.

The gelatine hydrolysis test and the cysteine milk test thus have demonstrated moderate proteolytic properties in the type A strains and faint proteolytic properties in the type B strains (except strain 11). These results do not conform to those of *Fievez* finding that Sphaerophorus necrophorus, although weakly hydrolysing gelatine, exclusively coagulates cystein milk in some days, but never causes peptonization. *Lahelle* reported that Sphaerophorus necrophorus coagulated and peptonized milk. *Thjötta & Jonsen* (1948) found that most strains of Bacteroides funduliformis (Sphaerophorus funduliformis) produced no changes of milk and no clearing of milk agar.

Reaction on milk agar

The proteolytic property was further tested by inoculating the strains on the surface of VL agar containing 50 % cysteine milk. After four days of incubation the 14 type A strains had cleared the medium under the colony and 4 mm outside it, while eight of the nine type B strains had cleared the medium under

the colony and 0.5–1 mm outside it. Strain 11 produced no clearing. After seven days of incubation, the cleared zones were 6 mm around the type A colonies and 2 mm around the type B colonies, except strain 11, which now had cleared the medium under the colony and 0.5 mm outside it. The zones remained clear after flodding the cultures with acid mercuric chloride solution. All the strains thus have proteolytic properties, being less pronounced in the type B strains than in the type A strains.

Hemagglutination test

Beerens described a hemagglutination test, allowing a differentiation between Sphaerophorus necrophorus and Sphaerophorus funduliformis.

The 14 type A strains were all found strongly agglutinating and the nine type B strains were all negative. This result conforms to the results given by *Beerens, Prevot* and *Fievez.*

The threonine metabolism

The threonine metabolism in a single strain of a Sphaerophorus sp. was studied in detail by *Guillaume et al.* (1957). L(-) threonine was found to be transformed to both alpha keto butyrate and ammonia (enzyme:L(-) threonine hydrolyase, deaminating) and to alpha amino butyrate (enzyme: threonine dehydrogenase). The threonine metabolism was studied in several strains belonging to the Bacteroidaceae by *Beerens et al.* (1959). Differences between the genera were revealed, but the methods were too complicated for general identification purposes. The authors developed a simplified test method, in which threonine utilization was demonstrated by gas production in a threonine medium, compared to its absence in the same medium without threonine. This test is unreliable, because some strains utilize threonine without gas production (*Fievez*). Another simplified test method, based on reduction of nile blue as indication of threonine utilization in buffered mixtures of dye, threonine, and washed bacterial cells, was advanced by *Beerens & Castel* (1965). *Suzuki et al.* (1966) devised a test, in which threonine utilization was indicated by liberation of ammonia in buffered mixtures of threonine and washed bacterial cells, compared to a blind test without threonine.

The enzymatical basis for the nile blue test being unknown, whereas the ammonia liberation test reveals the action of a threo-

nine hydrolyase (deaminating), comparable results are not necessarily to be expected from the two methods. However, the nile blue test of *Beerens & Castel* (1965) and the ammonia liberation test of *Suzuki et al.* were both found positive for all the present strains of Sphaerophorus necrophorus.

Production of volatile fatty acids

The volatile fatty acids (C1–C6), which accumulate in broth cultures of anaerobes, were studied by *Guillaume et al.* (1956), demonstrating that differences in fatty acid pattern were usable

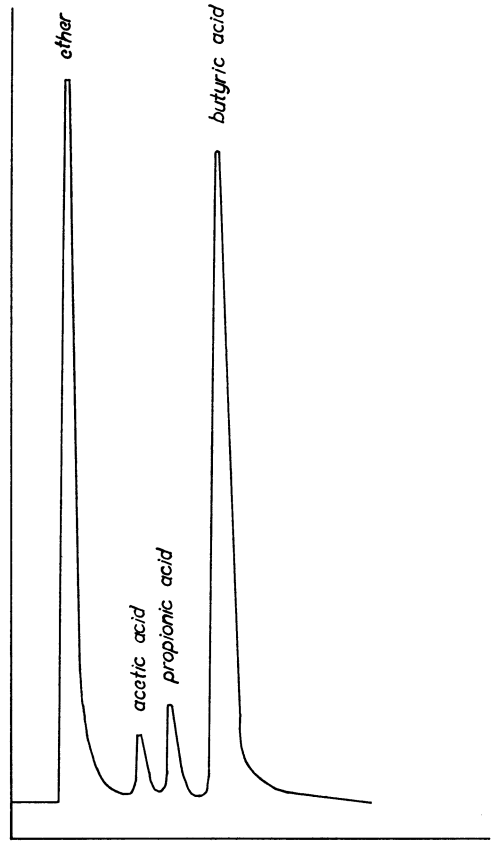

Figure 5. Aerograph Series no. 200. Column chromosorb w (60—80 mesh) + 5 % FFAP, 10′, ⅛″. Carrier gas nitrogen 20 ml/min. Temp. detector 210°C, injection port 210°C, column 135°C. Range 1. Attenuation 8. Sample size 0.1 µl.

Table 3. Individual characters of the 23 Sphaerophorus necrophorus

Strain no.	Colony morphology*	Cell morphology*	Acid from**														
			arabinose	xylose	fructose	galactose	glucose	mannose	lactose	maltose	saccharose	cellobiose	melezitose	adonitole	sorbitole	mannitole	salicine
1	A	A	—	—	+	—	+	(+)	—	+	—	—	—	—	—	—	—
2	A	A	—	—	+	—	+	(+)	—	+	—	—	—	—	—	—	—
10	A	A	—	—	+	(+)	+	(+)	—	+	—	—	—	—	—	—	—
12	A	A	—	—	(+)	(+)	+	(+)	—	+	—	—	—	—	—	—	—
13	A	A	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
14	A	A	—	—	+	(+)	+	(+)	—	+	—	—	—	—	—	—	—
16	A	A	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
18	A	A	—	—	(+)	+	+	(+)	—	+	—	—	—	—	—	—	—
19	A	A	—	—	(+)	+	+	(+)	—	+	—	—	—	—	—	—	—
20	A	A	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
21	A	A	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
24	A	A	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
25	A	A	—	—	+	+	+	+	—	+	—	—	—	—	—	—	—
29	A	A	—	—	+	+	+	(+)	—	(+)	—	—	—	—	—	—	—
6	B	B	—	—	+	—	+	(+)	—	+	—	—	—	—	—	—	—
11	B	B	—	—	+	(+)	+	(+)	—	(+)	—	—	—	—	—	—	—
15	B	B	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
17	B	B	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
22	B	B	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
23	B	B	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
26	B	B	—	—	+	+	+	(+)	—	+	—	—	—	—	—	—	—
27	B	B	—	—	+	(+)	+	(+)	—	+	—	—	—	—	—	—	—
28	B	B	—	—	+	—	+	(+)	—	+	—	—	—	—	—	—	—

*A: Colony and cell morphology identical with that described for Sphaerophorus necrophorus type A.
B: Colony and cell morphology identical with that described for Sphaerophorus necrophorus type B.
**+: Acid production demonstrated after four days.
(+): Acid production weak and/or late.
—: Acid not produced during 14 days.

strains, grouped into the 14 type A strains and the nine type B strains.

pH in 1 % glucose medium	Growth in 0.0008 % brilliant green***	Growth in 10 % bile***	Indole production	Nitrate reduction	Hemolysis	Lecitinase	Growth modus in broth****	Gelatine hydrolysis	Peptonization of cysteine milk	Clearing of milk agar	Hemagglutination	Threonine utilization, Nile blue test	Threonine deaminase, Suzuki's test	Volatile fatty acids produced in broth: acetic acid	Volatile fatty acids produced in broth: propionic acid	Volatile fatty acids produced in broth: butyric acid
5.90	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.70	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.80	+	—	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.65	+	—	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.85	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.35	+	—	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.85	+	—	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.80	+	—	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.80	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.70	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.80	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.60	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.60	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.80	+	(+)	+	—	+	+	H	+	+	+	+	+	+	+	+	++
5.40	+	+	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
6.05	+	(+)	+	—	+	+	F	(—)	—	+	—	+	+	+	+	++
5.70	+	(+)	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
5.80	+	—	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
5.75	+	(+)	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
5.75	+	—	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
5.90	+	(+)	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
6.10	+	(+)	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++
6.00	+	(+)	+	—	+	+	F	(—)	+	+	—	+	+	+	+	++

*** + : Growth in test medium equal to growth in blind medium.
(+): Growth in test medium partially inhibited, compared to blind medium.
— : Growth totally inhibited in test medium.
****H: Homogenous growth.
F: Flocculating growth.

for distinguishing genera of the Bacteroidaceae. A simplified method was advanced by *Charles & Barrett* (1963). By the present gas liquid chromatography technique, all the strains were found to behave markedly uniformly with respect to fatty acid production, producing moderate amounts of acetic acid and propionic acid and large amounts of butyric acid. A representative chromatogram is outlined in Fig. 5. Glucose has been supposed to be involved in the fatty acid production (*Charles & Barrett*). *Werner* (1969), however, demonstrated that Bacteroides fragilis produced fatty acids both in the presence and in the absence of glucose. With five of the present strains, the effect of glucose on fatty acid production was studied in parallel VL broth cultures, containing no glucose and 1 % glucose, respectively. In the cultures without glucose, gas production was weak (0.2–1 ml) in opposition to a gas production of about 10 ml in the glucose medium. pH of the cultures without glucose was on an average 6.5, whereas pH of the cultures with glucose was on the average 5.9. From glucose gas and acid compounds are produced. By titrating ether extracts from the cultures with and without glucose, average amounts of ether extractible acids were found to be 22 μM and 18 μM, respectively, per ml of the original broth culture. By gas liquid chromatography of the ether extracts, no qualitative or quantitative difference was observed between cultures with and without glucose, all resembling the chromatogram, outlined in Fig. 5.

CONCLUSIONS

On basis of the characters examined, the 23 strains were divisible into two groups, differing in: colony morphology, cell morphology, degree of hemolysis, growth modus in broth, and hemagglutination. The strains nos. 1-2-10-12-13-14-16-18-19-20-21-24-25-29 possess the characters of Sphaerophorus necrophorus type A and the strains nos. 6-11-15-17-22-23-26-27-28 possess those of Sphaerophorus necrophorus type B of *Fievez* (1963). The earlier conception of Sphaerophorus necrophorus type A as the agent of animal sphaerophorosis and type B as the agent of human sphaerophorosis can not be maintained. By *Fievez*, the type B was found in 28 % of animal sphaerophoroses, and among the present strains 39 % belong to the type B. In the present strains good agreement was found with the characters described by *Fievez*. However, the present strains could not be demonstrated

to produce acid from cellobiose, and the weak proteolytic properties, demonstrated by *Fievez* against gelatine, in the present strains were demonstrable against caseine too. The production of fatty acids as a character for identification of anaerobes is defined as the volatile and non-volatile aliphatic acids arising from the fermentation of broth with added carbohydrate and remaining at the end of the fermentation. With five of the present strains, the presence or absence of glucose did not interfere appreciably with fatty acid production in the VL medium.

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SUMMARY

Twenty-three strains of Sphaerophorus necrophorus, isolated from animal pathological processes, were characterized. The two biotypes of *Fievez* (1963) were recognized, differing in colony morphology, cell morphology, degree of hemolysis, growth modus in broth, and hemagglutination. The following characters were shared by all strains: Gram-negative, anaerobic, nonsporeforming, immotile rods, producing acid from fructose, glucose, mannose, maltose, and (variably) from galactose, but not from arabinose, xylose, lactose, saccharose, cellobiose, melezitose, adonitole, sorbitole, mannitole, and salicine. Terminal pH in glucose medium was 5.77 ± 0.17. Growth was not inhibited by 0.008 % brilliant green, but was partially inhibited by 10 % ox bile. Indole was produced. Nitrate was not reduced. A lecitinase was present. Proteolytic properties were present. Threonine was deaminated. The fatty acids: acetic, propionic, and butyric acid were produced in medium both with and without glucose.

SAMMENDRAG

Sphaerophorus necrophorus.
En undersøgelse af 23 stammer.

23 stammer af Sphaerophorus necrophorus isoleredes fra animalt patologisk materiale og karakteriseredes. Repræsentanter for begge biotyper (*Fievez* 1963) påvistes. Disse adskilte sig ved kolonimorfologi, cellemorfologi, grad af hæmolyse, vækstype i bouillon og evne til hæmagglutination. Følgende egenskaber fandtes hos alle stammer: gramnegative, anaerobe, non sporogene, ubevægelige stave, som under syredannelse metaboliserede fructose, glucose, mannose, maltose og (variabelt) galactose, men ikke arabinose, xylose, lactose, saccharose,

cellobiose, melezitose, adonitol, sorbitol, mannitol og salicin. Slut pH i glucosemedium var 5,77 ± 0,17. Væksten hæmmedes ikke af 0,008 % brilliantgrønt, men hæmmedes partielt af 10 % oksegalde. Indol produceredes. Nitrat reduceredes ikke. En lecitinase produceredes. Proteolytiske egenskaber påvistes. Threonin deamineredes. Af lavere fede syrer produceredes såvel i glucoseholdigt som i glucosefrit medium eddikesyre, propionsyre og smørsyre.

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