The Identity of *Proteus hydrophilus* Bergey *et al.* and *Proteus melanovogenes* Miles & Halnan, and their Relation to the Genus *Aeromonas* Kluyver & van Niel

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SUMMARY: *Proteus melanovogenes*, originally isolated from black-rot in eggs, does not differ from *Pr. hydrophilus*, the cause of red-leg in frogs, more than different strains in each species differ from one another; and the two species may be considered as identical.

Both species resemble *Aerobacter liquefaciens* Beijerinck, *Pseudomonas fermentans* von Wohlzogen Kühn and *Ps. ichthyosmia*. The divergences from a common type displayed by these 'species' may be sufficient to warrant the preservation of some as separate species, some as varieties, but all strains of this type with the property of producing 2:3-butanediol from glucose should be allotted to the genus *Aeromonas* in the Pseudomonadaceae, as recommended by Kluyver & van Niel (1936).

In 1937, Miles & Halnan isolated a monotrichate, Gram-negative bacillus which was the cause of black-rot in South African eggs imported into England, and which they named *Proteus melanovogenes*. They found a similar bacillus in English soils and manures, and in the faeces of a patient with chronic colitis. Aitken, Barling & Miles (1936) also found a similar bacillus (Strain M90) in a meat-pie responsible for a fatal *Clostridium botulinum* intoxication in man. From this limited experience it appeared that *Proteus melanovogenes* might be widely distributed and was potentially parasitic in a number of ways.

Miles & Halnan (1937) noted the close resemblance of their bacillus to *Pr. hydrophilus*, the cause of red-leg in frogs, as described by Bergey (1934), but in their view *Pr. hydrophilus* was decisively distinguished by its reported possession of peritrichate flagella, and they accordingly created a new species in the genus *Proteus*. Bergey's description, which they omitted to check, was however incorrect. Of the authorities cited in the 1934 (4th) edition of *Bergey's Manual*, none mentions the number of flagella, though all agree in describing active motility (Ernst, 1890; Sanarelli, 1891; Chester, 1897; Weldin, 1927). Nevertheless, in 1898 Russell had described the bacillus of red-leg as monotrichate. The 1948 edition of *Bergey's Manual* correctly records *Pr. hydrophilus* as monotrichate, and presumably for this reason, transfers the species to the genus *Pseudomonas*. Russell's (1898) and Kulp & Borden's (1942) establishment of *Pr. hydrophilus* as monotrichate clearly indicated a reconsideration of the validity of *Proteus melanovogenes* as a separate species.

SOURCE OF THE STRAINS

Unfortunately most of the original strains of *Pr. melanovogenes* were lost during the 1939-45 war, including the type culture ER 1. Two strains survived, ER 75 from a rotten South African egg, and M90 from the poisoned meat-pie men-

tioned above. Since, however, both strains had been proved capable of producing typical black-rot in hen eggs soon after isolation, they may be considered sufficiently representative of *Pr. melanovogenes*. For comparison, we obtained from the National Collection of Type Cultures, two strains of *Pr. hydrophilus* N.C.T.C. 7810 (=American Type Culture Collection 9071; Kulp & Borden's 1942, strain P2) and N.C.T.C. 7812 (=A.T.C.C. 7965; from M.L. Speck); and a strain of *Pseudomonas ichthyosmia* (N.C.T.C. 8049=A.T.C.C. 7966). From Prof. Kluyver at Delft we obtained: an authentic strain of *Ps. fermentans* isolated by von Wohlzogen Kühr and now named *Aeromonas liquefaciens* (L 417); a strain of *Aeromonas liquefaciens* (L 418) isolated at Delft; one strain of *A. hydrophila* (L 447) sent to Delft by Dr Hitchner. In June 1949, during an epidemic of red-leg in a dealer's batch of *Rana temporaria*, we also isolated four strains from three frogs—DF 1*a* and DF 1*b* from one frog, DF 2 and DF 3. Nine of the twelve strains produced a rapidly fatal bacteraemia in frogs when injected either intraperitoneally or into the lymph sac in doses of 0.4 ml. of an overnight culture in heart extract broth, the exceptions being the strains *Pseudomonas ichthyosmia*, *Ps. fermentans* and *Aeromonas liquefaciens* (L 418). *Pseudomonas ichthyosmia*, which was originally isolated from a tin of milk with a fishy smell, has never been associated with red-leg in any way, and appears to be quite harmless to frogs, although pathogenic for mice. The *Ps. fermentans* produced a severe, haemorrhagic local lesion in the thigh of one of three frogs injected subcutaneously and was isolated in pure culture by direct plating of the heart blood of the animal; the other frogs survived 3 weeks in apparent good health. The *Aeromonas liquefaciens* (L 418) had no effect on frogs, little virulence for mice, and differed in other respects from the rest of the group.

All twelve strains induced a characteristic black-rot in eggs in 9–21 days at 37°, when injected by the method of Miles & Halnan (1937), but ER75, M90 and *A. liquefaciens* L 418 were only feebly saprogenic, though the first two when originally isolated had produced severe black-rot. An attempt to enhance saprogenicity by frog and mouse passage was unsuccessful. L 418 produced a true black-rot only in one of the three eggs inoculated. We were clearly dealing with a group of strains generally similar in their parasitizing capacity.

COMPARISON OF THE TWELVE STRAINS

The following description applies to the twelve strains; divergences are noted in brackets.

Morphology. Gram-negative rods, 0.4–0.8 × 1.5–3.0 μ., with parallel sides and rounded ends. Non-acid fast. No endospores. Filaments common in 'rough' colony forms. Actively motile by a single polar flagellum about 5–6 μ. long (cells of ER75 and DF 3 were either monotrichate, or lophotrichate with two to eight flagella).

Colonial appearance. After 24 hr. on horse blood agar plates, colonies were 2–3 mm. in diameter (L 418, 0.5 mm.), round, entire-edged and raised, smooth moist surface (L 417 slightly granular), semi-translucent greyish white in colour (DF 1*b* had opaque colonies and was originally distinguished from DF 1*a* on this

ground; both DF1*a* and DF1*b* bred true with respect to colony form: L418 was also opaque); haemolysis marked (ER75 moderately haemolytic, L418 non-haemolytic). After 3–5 days at room temperature a dirty brown-yellow coloration appeared.

Growth in broth. After 24 hr. abundant, turbid, with a moderate, readily disintegrable sediment and fine pellicle.

Gelatin stab. Growth good, liquefaction marked and commonly saccate.

Loeffler's serum slope. Growth abundant (for digestion, see Table 1).

Potato slope. Growth abundant, moist and glistening. Six strains produced salmon pink pigment. The rest varied from creamy white to brownish yellow (see Table 1).

Metabolism. Aerobic. Anaerobic growth feeble. Grow well on ordinary laboratory media at 20°, luxuriantly at 37°. In a basal medium containing 1 % glucose and 0.1 % K_2HPO_4 in tap water, utilize 0.1 % $(NH_4)_2SO_4$, uric acid or asparagine as source of nitrogen. In basal medium containing 0.1 % $(HN_4)_2SO_4$ and 0.1 % K_2HPO_4 utilize 0.5 % citrate, but not 0.5 % meso-tartrate as source of carbon (*Pseudomonas ichthyosmia*, N.C.T.C. 8049, and L418 negative). (For utilization of acetate, lactate and malate, see Table 1.)

Biochemical. Glucose, galactose, fructose, mannose, maltose, sucrose, manitol, glycerol and starch fermented at 37° in 1–2 days with production of acid and gas. Arabinose, raffinose, rhamnose, dulcitol and inositol not fermented. (L418 ferments both raffinose and inositol with the production of acid only. It also gives a trace of acid in the Durham's tubes with inulin and arabinose.)

The fermentation of salicin is interesting. At 37° only DF3 and ER75 ferment it. Hammer (1917) observed that *Ps. ichthyosmia* produced more acid from sugars at 22° than at 37°, and that salicin, notably, was fermented at 22° but not at 37°. With two exceptions (N.C.T.C. 7810 and *Ps. fermentans*, L417) all our strains fermented salicin at 22°. We also tested the equivocal lactose fermentation at this temperature, but the results were no better than at 37° for other sugars; see Table 1).

Litmus milk acidified in one day, then clotted and digested. Nitrates reduced to nitrites. Thiosulphate reduced. Indole positive; H_2S , NH_3 , catalase, diastase and methylene-blue reductase positive. Urease negative. (For methyl red, Voges-Proskauer and Koser's citrate tests, and lipase production, see Table 1.)

The formation of 2:3-butanediol from glucose was tested in 3-day cultures in the medium recommended by Stanier & Adams (1944), which was extracted with ethyl ether, and residue after evaporation of the ether tested for acetoin and 2:3-butanediol as recommended by these authors. A strain was regarded as positive if the 2:3-butanediol produced was > 4 % of the starting weight of the glucose. All the twelve strains were positive in this sense. The strains of *Ps. ichthyosmia*, ER75 and DF1*a* were the least active.

Serological. Antigenically heterogeneous by a limited analysis with agglutinating antisera to all but the DF strains and L447 (Table 2). *Proteus hydrophilus* N.C.T.C. 7810 and 7812 were slightly related, and M90 and N.C.T.C. 7812 closely related. L417 *Pseudomonas fermentans* was markedly related to 7810 and DF3, and slightly to all the rest of the group excepting *Ps. ichthyosmia*.

Table 1. *Divergent characters of the twelve strains*

	N.C.T.C. 7810	N.C.T.C. 7812	ER75 m+1	M90 m	DF1a m	DF1b* m	DF2 m	DF3 m+1	N.C.T.C. 8049 m	L417 m	L418* m	L447 m
Flagella	—	—	A†	—	—	A	—	—	—	—	A	—
Fermentation of	—	—	—	—	—	—	—	—	—	—	A	—
Xylose	—	—	—	—	—	—	—	—	—	—	A	—
Raffinose	—	—	—	—	—	—	—	—	—	—	A	—
Lactose	a ₄	a ₄	a ₄	a ₄	Ag	Ag†	a ₄	Ag	a ₄	A ₆	A ₁₄	—
Inositol	—	—	—	—	—	—	—	—	—	—	A ₃	—
Sorbitol	—	A	A	A	—	—	A	—	—	—	A	—
Aesculin†	—	—	A	—	A	—	—	a	nt	nt	nt	nt
Salicin (22°)	—	Ag	Ag	Ag	Ag	Ag	Ag	Ag	a	—	a	AG
Salicin (37°)	—	—	Ag	—	—	—	—	Ag	—	—	—	—
Glycogen and dextrin	A	A	—	A	A	A	A	A	A	A	—	A
Utilization of												
Koser's citrate	—	—	+	+	+	—	—	+	+	+	+	—
Citrate	+	+	+	+	+	+	+	+	—	+	—	+
Acetate } with	—	—	—	—	—	—	+	—	—	—	—	—
Malate } (NH ₄) ₂ SO ₄	+	+	+	+	—	+	—	+	+	+	+	+
Lactate	—	—	—	—	+	+	—	+	+	+	+	+
Lipase	tr	tr	tr	+	±	±	—	tr	±	±	—	+
Digestion of												
Loeffler's serum	+	—	+	+	+	—	+	+	+	+	—	—
Voges-Proskauer	+	+	+	+	+	+	+	+	+	+	?	?
Methyl red	—	—	—	—	—	+	—	—	—	+	—	—
Pigment on potato	SP	SP	GB	SP	C	C	SP	SP	CL	CL	CL	C/SP

m, l = monotrichate, lophotrichate.
a, A = slight, marked acidity. } Without suffixed number = 1 day's incubation; suffixed number = day of first appearance.
g, G = slight, marked gas.
* = colonies atypical.
† = negative on first isolation. ‡ = tested by von Wohlzogen Kühr's method.
nt = no test.
— = no reaction.
C = pale or dark cream. } Pigment on potato.
CL = café-au-lait.
GB = grey-brown.
SP = salmon pink.

L318 markedly related to ER75 and slightly to 7810 and M90, and *Ps. ichthyosmia* markedly to DF1a and slightly to 7812. M90 and ER75 formed unstable suspensions in 0.85 % saline, and both strains grew in two forms, a transparent colony whose constituent cells were long and filamentous, forming suspensions that were precipitated by 0.85 % saline on heating at 100° for 30 min.; and a more opaque colony consisting of saline- and heat-stable short rods; by virulence tests in frogs and mice the 'opaque' form of M90 was slightly more virulent than the 'transparent'; the two forms of ER75 were equally virulent.

Table 2. *Cross-agglutination of twelve strains examined by Proteus hydrophilus and Pr. melanovogenes antisera, and of Pr. hydrophilus and Pr. melanovogenes strains by Aeromonas liquefaciens and Pseudomonas ichthyosmia antisera.*

Bacillus	Strain	Titre with antisera to						
		N.C.T.C.		N.C.T.C.				N.C.T.C.
		7810	7812	ER75	M90	L417	L418	
<i>Pr. hydrophilus</i>	N.C.T.C. 7810	80	10	0	10	80	80	10
	N.C.T.C. 7812	10	320	0	160	20	20	20
<i>Pr. melanovogenes</i>	ER75	0	0	160	0	0	> 160	0
	M90	40	320	10	640	10	10	10
'Red-leg'	DF1a	0	0	0	10	0	0	> 160
	DF1b	10	0	10	10	10	10	0
	DF2	50	50	0	50	20	10	0
	DF3	0	20	0	0	> 160	0	0
<i>Aeromonas liquefaciens</i>	L417	160	20	40	40	320	0	40
	L418	10	0	80	10	10	> 320	0
<i>Aeromonas hydrophilus</i>	L447	40	> 160	0	80	0	10	10
<i>Ps. ichthyosmia</i>	N.C.T.C. 8049	0	20	0	0	0	0	80

0 = titres < 10. Homologous titres in heavy type.

It is possible that we were dealing with S and R states of M90, but a detailed analysis was not possible, because $R \rightarrow S$ and $S \rightarrow R$ occurred readily in overnight culture. The nature of the two stages in ER75 is less clear. Both N.C.T.C. 7812 and N.C.T.C. 7810 were consistently in the Smooth state.

Pathogenicity. With exceptions already noted, fatal bacteraemia within 36 hr. of intraperitoneal injection in mice. In frogs, with exceptions already noted, fatal red-leg with bacteraemia, many animals dying in tetanic spasm. (N.C.T.C. 7810 produced no symptoms of red-leg, perhaps because of its high virulence; the frogs died with a copious bacteraemia in < 18 hr.)

Saprogenicity. Characteristic black-rot produced in hen eggs.

DIFFERENCES OF THE TWELVE STRAINS STUDIED

Table 1 summarizes the outstanding differences in the twelve strains. By itself the lophotrichy of ER75 and DF3 does not necessarily separate them from the rest, because within well-recognized 'monotrichate' species, moderate lophotrichy is not uncommon (cf. Bartholomew, 1949). None of the strains differs consistently from the others in respect even of the majority characters listed.

Three of the DF strains ferment lactose with moderate vigour. Most strains produced acid after 1 day's growth in glucose phosphate medium, but this was neutralized by basic substances produced during the 2-7 days required in the standard M.R. test (see 'Committee', 1947). DF1*b* did not reverse the acidity.

In 1937, Miles & Halnan's strains of *Proteus melanovogenes* readily fermented arabinose. ER75 and M90 no longer do so, but in all other respects they behaved in our hands as described by Miles & Halnan. During the years of maintenance in soft nutrient agar they had lost some of the power to produce rot and some of their virulence for mice, but the virulence was restored by selection of colonies in the Smooth state and by frog and mouse passage. Their saprogenicity was not improved either by egg or mouse passage.

With regard to the authentic *Pr. melanovogenes* and *Pr. hydrophilus* strains, there is, compared with the large numbers of common characters listed in the preceding section, nothing in the differences to justify maintaining the separations of the two species; ER75 and L418 are the most divergent, and are serologically closely related to one another and alone of the group fail to ferment dextrin and glycogen.

Where the results of Koser's citrate test and of cultivating in citrate- $(\text{NH}_4)_2\text{SO}_4$ differed with each strain, the differences were consistently obtained in several repetitions of the tests; due precautions were taken against the carry-over of carbon sources other than citrate when seeding the media. These differences may reflect real differences in biochemical constitution, but as far as the bare fact of citrate-utilization is concerned, the twelve strains are all positive.

If we allow that the divergence displayed by the first four strains listed in Table 1 is insufficient to maintain *hydrophilus* and *melanovogenes* as separate species, and agree to class them as *hydrophilus*, there is little reason for excluding the DF strains from the species, though the ready fermentation of lactose perhaps denotes a noteworthy variety; but even this character was lost by DF3 after a few subcultures. The fact that all the DF strains induced red-leg in frogs and rot in eggs is an added reason for their inclusion with the rest. The fact that they were isolated from red-leg in frogs is also suggestive, but is not in our view decisive, because we deprecate the use of habitat as a taxonomic character of microbes. The limitations of habitat, including hosts that are parasitized, as a distinctive character in bacterial classification, are well illustrated in *Pr. hydrophilus*, which, if *defined* as the cause of red-leg, must be restricted to the microbe found in diseased reptiles and amphibians—a useless restriction when the ecological significance of bacilli of this kind is under consideration. Moreover, haemorrhagic infections in these animals may be caused by microbes other than *Pr. hydrophilus*. Thus a typical outbreak of red-leg in green tree-frogs was produced by *Bacterium alkaligenes* (Miles, 1950).

Bergey's Manual (1948) records the liquefaction of gelatin by *Proteus hydrophilus* as 'napiform', the utilization of citrate as positive and the fermentation of lactose as negative. We are not inclined to regard napiform liquefaction as definitive. In our hands, the liquefaction varied between saccate, infundibuliform and napiform, and one strain could be made to produce

liquefied masses of different shapes in stab cultures by varying the concentration of gelatin and the amount of bacterial growth seeded. All our strains except N.C.T.C. 8049 and L418 grew in a citrate medium with $(\text{NH}_4)_2\text{SO}_4$ as a source of N, and were readily subcultured in the same medium; but we were unable to subcultivate all the strains serially in Koser's citrate, although N.C.T.C. 8049 and L418 grew quite readily in this citrate medium. With regard to lactose, the slight acid produced by most strains in 4 days at 37° in 1 % lactose in peptone water is not frank fermentation; but five strains produced marked acidity, three of them in 1 day at 37° , and it would be as well, pending future work on newly isolated strains, to characterize the action of *Pr. hydrophilus* on lactose as variable, but usually feeble.

THE CLASSIFICATION OF *PROTEUS HYDROPHILUS*

There is no reason to doubt that *Pr. hydrophilus* is the bacillus described by Sanarelli (1891) as *Bacillus hydrophilus fuscus*, and that, if Sanarelli's description is the first, with due avoidance of a trinomial, *B. hydrophilus* Chester (1901) gives the valid specific epithet. One species that resembles *Proteus hydrophilus* was described in 1890 by Zimmerman—*Bacillus punctatus*. Zimmerman's description, however, is very scanty. *B. punctatus* corresponds to *Proteus hydrophilus* in size, rapid motility by one polar flagellum, turbid growth in broth with pellicle, brownish pigment on potato, and saccate liquefaction of gelatin. Its eponymous character, the production of white punctate colonies floating on the surface of liquefied gelatin, may be accidental. None of our twelve strains had it, when tested on 15 and 20 % nutrient gelatin plates. For these reasons we think that *Bacillus punctatus* has no good claim as a recognizable first description of a species, and that the specific epithet *hydrophilus* should be regarded as established for this species.

The generic name, on the other hand, is more doubtful. If with Bergey (1948) we limit the genus *Proteus* to peritrichate bacilli that split urea, clearly *hydrophilus* belongs elsewhere. Its monotrichy suggested the genus *Pseudomonas* to Guthrie & Hitchner (1943), but Stanier (1943) objected on the grounds that the 'true fermentative ability (*sensu* Pasteur)' of *Proteus hydrophilus* was not characteristic of typical *Pseudomonas* spp. With this objection we agree; some other genus is required. Stanier observed in *Pr. hydrophilus* the 2:3-butanediol fermentation described by Beijerinck (1900) for his *Aerobacter liquefaciens*, a Gram-negative bacillus isolated from the dried rhizome of *Althaea officinalis*. Kluyver & van Niel (1936) proposed the genus *Aeromonas* for bacilli in the family Pseudomonadaceae that ferment glucose with the production of 2:3-butanediol. According to Stanier, Beijerinck's microbe should therefore be *A. liquefaciens*. It resembles *Pr. hydrophilus* closely, except that its growth was optimum about 28° and often inhibited at 37° . If we regard optimum growth temperature as an accidental and not specific character, then 'liquefaciens' must give way to the earlier epithet 'hydrophilus'. We also concur with Stanier's view that *Pseudomonas fermentans* von Wohlzogen Kühn (1932) is *Proteus hydrophilus*. We repeated all the tests that von Wohlzogen Kühn describes in his paper, and in all of them our twelve strains, which include two

authentic strains of *Pr. hydrophilus* and an authentic strain of *Pseudomonas fermentans*, behaved alike. The pigment production by *Ps. fermentans* does not appear to have been sufficiently marked to warrant the description 'chromogenic' (Bergey, 1934), though six out of our twelve strains produced a definite salmon pink colour on potato which, however, varied in degree with repetition of the potato culture. The other six strains grew with the production of pigment varying from creamy white to pale café-au-lait (Table 1).

CONCLUSIONS

We suggest that: (a) *Bacillus punctatus* Zimmerman be recognized as insufficiently described, and therefore without status as regards priority in naming; (b) *Aerobacter liquefaciens* Beijerinck may be synonymous with *Bacillus hydrophilus fuscus* Sanarelli and *Proteus hydrophilus* Bergey *et al.*, and certainly belongs to the same genus as these two species; (c) *Pseudomonas fermentans* von Wohlzogen Kühr, *Proteus melanovogenes* Miles & Halnan and *Pseudomonas ichthyosmia* Hammer are synonymous with *Proteus hydrophilus*; (d) if the production of 2:3-butanediol in the fermentation of glucose proves to be a property characterizing an otherwise substantially homogeneous group within the Pseudomonadaceae, the genus *Aeromonas* Kluyver & van Niel should be accepted.

The recognition of the type species and the authenticating of type-cultures must await the establishment of *Aerobacter liquefaciens* and *Proteus hydrophilus* as two species or one. If two, the type species is clearly *Aeromonas liquefaciens*; if one, we suggest that the type species is *A. hydrophila*, and the type culture should be an authentic strain of *Proteus hydrophilus* Bergey *et al.*

We are indebted to Prof. A. J. Kluyver for strains and information about them; and to our colleague Dr S. Jacob for his estimates of 2:3 butanediol production.

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(Received 26 June 1950)