A Numerical Study on the Relationships of *Listeria* and *Erysipelothrix*

By M. R. STUART* AND PHYLLIS E. PEASE

Department of Bacteriology, University of Birmingham, Birmingham, B15 2TJ

(Received 6 December 1971; revised 2 August 1972)

SUMMARY

The physiological and general characters of 123 strains of *Listeria* were examined, and 33 of these were compared, in a computerized numerical analysis, with *Erysipelothrix*, *Streptococcus*, *Corynebacterium*, *Microbacterium*, *Arthrobacter*, *Jensenia*, *Brevibacterium*, *Cellulomonas* and *Kurthia*. It was concluded that *Listeria* and *Erysipelothrix* are distinct monospecific genera, not closely related.

On physiological grounds, *Listeria* bore the nearest resemblance to faecal streptococci, and *Erysipelothrix* to other streptococci.

INTRODUCTION

The relationships of the two groups of bacteria usually granted the rank of monospecific genera, *Listeria monocytogenes* and *Erysipelothrix rhusiopathiae* (formerly *E. insidiosa*), have been much disputed, and a detailed recapitulation of the stages of the argument is supererogatory. Standard, but divergent, views may be summarized in contrasting the opinions of Wilson & Miles (1955) and of Bisset (1963), that they should be regarded as species in the same genus, with those of Breed, Murray & Smith (*Bergey's Manual*, 1957), that both constitute genera in the family Corynebacteriaceae. The latter opinion was supported by numerical taxonomic studies produced by Davis & Newton (1969), and Davis, Fomin, Wilson & Newton (1969). The purpose of the present study is to provide the same type of evidence, using a larger number of strains.

METHODS

Strains studied. The strains used are listed in Table 1. The 33 listerias were selected from 123 strains for inclusion with the 100 that could be accepted by the computer. They were considered to be representative, and included all recognized serotypes.

Media. TY medium (Davis & Newton, 1969). TA medium (g/l): tryptose, 10; Lab Lemco beef extract, 3; blood agar base no. 2, 40. TYGP medium (g/l): tryptone, 10; yeast extract, 5; NaCl, 5; K₂HPO₄, 5; glucose, 2. All were adjusted to pH 7.2 and sterilized by autoclaving at 15 lb/in² for 15 min.

Biochemical tests

Methyl-red test. Medium (g/l): peptone, 5; glucose, 5; K₂HPO₄, 5, adjusted to pH 7.5 and sterilized by autoclaving at 10 lb/in² for 10 min after distribution into 10 ml amounts. Inoculation was from 24 h cultures on TA agar plates and incubation was for 14 days. A

* Present address: Botany Department, University of Rhodesia, Private Bag 162 H, Salisbury, Rhodesia.

Table 1. *Organisms employed in the computer analyses*

Name	Source*	Strain no.
<i>Arthrobacter</i> sp.	NIRD	1234
<i>A. globiformis</i>	NCIB	8907
<i>A. globiformis</i>	NCIB	8717
<i>A. simplex</i>	Departmental	AS
<i>A. tumescens</i>	Departmental	AT
<i>Brevibacterium linens</i>	NCIB	9909
<i>B. helvolum</i>	NCIB	10352
<i>Listeria monocytogenes</i> (untyped)	Dr B. Gittins	BGI
<i>L. denitrificans</i>	Dr D. Jones, MRC Unit, Leicester University	55134
<i>L. grayi</i>	Dr D. Jones	C214
<i>Corynebacterium bovis</i>	NCTC	3224
<i>C. equi</i>	NCTC	1621
<i>C. haemolyticum</i>	NCTC	8452
<i>C. murium</i>	NCTC	949
<i>C. ovis</i>	NCTC	3450
<i>C. renale</i>	NCTC	7448
<i>C. ulcerans</i>	NCTC	7910
<i>C. pyogenes</i>	NCTC	5224
<i>Corynebacterium</i> sp. H	NCTC	6701
<i>Corynebacterium</i> sp. M	NCTC	7146
<i>Corynebacterium</i> sp. O	NCTC	7420
<i>C. xerosis</i>	Departmental	CXI
<i>Cellulomonas biazotea</i>	NCIB	8077
<i>C. flavigena</i>	NCIB	8073
<i>Corynebacterium tritici</i>	NCPPB	255
<i>C. michiganense</i>	NCPPB	1468
<i>C. poinsettiae</i>	NCPPB	177
<i>C. poinsettiae</i>	NCPPB	854
<i>C. poinsettiae</i>	NCPPB	855
<i>C. betae</i>	NCPPB	363
<i>C. fascians</i>	NCPPB	1488
<i>C. helvolum</i>	NIRD	1192
<i>Erysipelothrix rhusiopathiae</i>	NCTC	807
<i>E. rhusiopathiae</i>	NCTC	4304
<i>E. rhusiopathiae</i>	NCTC	2422
<i>E. rhusiopathiae</i>	NCTC	1224
<i>E. rhusiopathiae</i>	NCTC	1694
<i>E. rhusiopathiae</i>	NCTC	8163
<i>E. rhusiopathiae</i>	NCTC	10383
<i>Jensen canicruria</i>	Departmental	JI
<i>Kurthia zopfii</i>	NCTC	404
<i>Listeria monocytogenes</i> (untyped)	Dr K. B. Rogers	KBRI
<i>L. monocytogenes</i> (untyped)	Dr K. B. Rogers	KBR2
<i>Listeria</i> sp. (Donna Craig) (untyped)	Dr K. B. Rogers	KBR3
<i>L. monocytogenes</i> (serotype 3 a)	Dr J. Donker-Voet	1035
<i>L. monocytogenes</i> (serotype 4 d)	Dr J. Donker-Voet	1501
<i>L. monocytogenes</i> (serotype 6)	Dr J. Donker-Voet	1873
<i>L. monocytogenes</i> (serotype 7)	Dr J. Donker-Voet	1775
<i>L. monocytogenes</i> (serotype 3 c)	Dr J. Donker-Voet	1916
<i>L. monocytogenes</i> (serotype 5)	Dr J. Donker-Voet	Ivanof
<i>L. monocytogenes</i> (serotype 1 a)	Dr W. C. Eveland	1106
<i>L. monocytogenes</i> (serotype 1 a)	Dr W. C. Eveland	1111
<i>L. monocytogenes</i> (serotype 1 a)	Dr W. C. Eveland	1124
<i>L. monocytogenes</i> (serotype 2 a)	Dr W. C. Eveland	2108
<i>L. monocytogenes</i> (serotype 2 a)	Dr W. C. Eveland	2107
<i>L. monocytogenes</i> (serotype 3 b)	Dr W. C. Eveland	3201
<i>L. monocytogenes</i> (serotype 4 b)	Dr W. C. Eveland	4214
<i>L. monocytogenes</i> (serotype 4 e)	Dr J. A. McKiel	14
<i>L. monocytogenes</i> (serotype 4 b)	Dr J. A. McKiel	9258-65
<i>L. monocytogenes</i> (serotype 4 c)	NCTC	4883

Table 1 (cont.)

Name	Source*	Strain no.
<i>L. monocytogenes</i> (serotype 4b)	NCTC	4885
<i>L. monocytogenes</i> (serotype 3a)	NCTC	5105
<i>L. monocytogenes</i> (serotype 4a)	NCTC	5214
<i>L. monocytogenes</i> (serotype 1a)	NCTC	7973
<i>L. monocytogenes</i> (serotype 2)	NCTC	9862
<i>L. monocytogenes</i> (serotype 4b)	NCTC	9863
<i>L. monocytogenes</i> (serotype 1)	Professor H. P. R. Seeliger	881
<i>L. monocytogenes</i> (serotype 4b)	Professor H. P. R. Seeliger	1920
<i>L. monocytogenes</i> (serotype 4b)	Professor H. P. R. Seeliger	1962
<i>L. monocytogenes</i> (serotype 4b)	Professor H. P. R. Seeliger	2015
<i>L. monocytogenes</i> (serotype 4b)	Professor H. P. R. Seeliger	2027
<i>Microbacterium flavum</i>	NIRD	1320
<i>M. flavum</i>	Dr K. Robinson	3
<i>M. flavum</i>	Dr K. Robinson	19
<i>M. lacticum</i>	NIRD	960
<i>M. lacticum</i>	NIRD	747
<i>M. lacticum</i>	NIRD	748
<i>M. lacticum</i>	Dr K. Robinson	6
<i>M. lacticum</i>	Dr K. Robinson	8
<i>M. lacticum</i>	Dr K. Robinson	10
<i>M. lacticum</i>	Dr K. Robinson	12
<i>M. lacticum</i>	Dr K. Robinson	23
<i>M. liquefaciens</i>	Dr K. Robinson	15
<i>M. liquefaciens</i>	Dr K. Robinson	18
<i>M. liquefaciens</i>	Dr K. Robinson	20
<i>M. thermosphactum</i>	NIRD	1676
<i>Streptococcus faecium</i>	NCTC	7379
<i>S. hominis</i>	NCTC	7366
<i>S. bovis</i>	NCTC	8177
<i>S. durans</i>	NCTC	8307
<i>S. faecalis</i>	NCTC	8212
<i>S. faecalis</i>	NCTC	4725
<i>S. agalactiae</i>	NCTC	8181
<i>Streptococcus</i> B	NCTC	9412
<i>Streptococcus</i> F	NCTC	5389
<i>Streptococcus</i> MG	NCTC	8037

* NIRD = National Institute for Research in Dairying; NCIB = National Collection of Industrial Bacteria, Aberdeen; NCTC = National Collection of Type Cultures, London; NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire.

small sample was transferred to an equal volume of methyl red (alcoholic solution) in a haemagglutination tray. A red coloration was scored as positive and yellow as negative.

Voges-Proskauer reaction. After a sample had been removed for the methyl-red test, 3 ml of 5% (w/v) α -naphthol solution in absolute alcohol and 1 ml of 40% sodium hydroxide solution were added to each bottle. These were well shaken, the caps were removed, and they were allowed to stand open on the bench for 2 h. A red coloration was scored as a positive result.

Phosphatase production. Phenolphthalein diphosphate Na salt was made as a 1% (w/v) aqueous solution and filter sterilized. The sterile substrate solution was incorporated at a concentration of 1 ml/100 ml of sterile melted TY agar, and poured into plates. Inoculation was from 24 h TY agar, and incubation was for 6 days at 30°C, 4 days at 37°C and 10 days at room temperature. Results were scored as positive if a pink colour became obvious under and/or around the colonies after flooding the plates with 15% (w/v) aqueous sodium carbonate solution (Davis *et al.* 1969).

Catalase production. Organisms were grown on TY agar base for 24 h, or until growth was evident. A small amount of growth was transferred to a drop of 10 volume hydrogen peroxide on a clean glass slide using the flame-rounded end of a Pasteur pipette, and the system was examined by means of a binocular plate microscope. The production of bubbles of oxygen around the cells within 10 s was scored as positive catalase production.

Nitrate and nitrite reduction. Medium (a) (g/l): tryptone, 10; yeast extract, 5; NaCl, 5; glucose, 2; K_2HPO_4 , 5; KNO_3 , 0.1. Medium (b) (g/l): tryptone, 10; yeast extract, 5; NaCl, 5; glucose, 2; K_2HPO_4 , 5; KNO_2 , 0.1. These media were autoclaved at 15 lb/in² for 15 min, inoculated from 24 h TYGP broth cultures and incubated for 6 days. The cultures were then tested by the addition of 0.1 ml of each of the reagents A (sulphanilic acid, 0.5 g, acetic acid, 30% (w/v), 150 ml) and B (1-naphthylamine, 1 g; distilled water 22 ml; CH_3COOH 30% (v/v), 178 ml). Nitrate: the production of a red coloration was scored as positive reduction of nitrate to nitrate. To tubes not showing a red colour, powdered zinc was added to a weight approximately 5 mg/ml of culture, and after shaking allowed to stand. A red coloration now formed was scored as negative nitrate reduction. In the absence of colour, it was deduced that the nitrate had been reduced to nitrite, which was then further reduced. Such results were confirmed in the nitrite test. Nitrite: after addition of reagents A and B a red colour, indicating presence of nitrite, was scored as negative; absence of colour was scored as positive reduction.

Hydrogen sulphide production. Medium (a) TY broth; medium (b) TY broth + 0.1% (w/v) $Na_2S_2O_4$; medium (c) TY broth + 0.05% (w/v) L-cysteine HCl. The media were dispensed in bijoux bottles autoclaved at 15 lb/in² for 15 min, inoculated from 24 h TY broth cultures and incubated for 10 days. Small strips of sterile filter paper which had been soaked in saturated aqueous lead acetate solution were transferred aseptically to each tube, and held in place above the medium by means of the screw cap. The cultures were examined daily for blackening of the acetate paper; such blackening was scored as a positive result, unless the degree of darkening in the media containing L-cysteine or thiosulphate was not greater than that produced in the TY broth without additions. In this case only the TY broth result was scored as a positive result.

Indole production. TY broth was inoculated from 24 h TY broth cultures, and incubated for 6 days. A 1 ml portion of ether was added to each tube, and after shaking 0.5 ml Ehrlich's reagent was run down the side of the tube. A pink or red colour in the solvent layer was scored as a positive result.

Production of acetic acid from ethanol. Light calcium carbonate (1% w/v) was added to TY agar base, and the medium autoclaved at 15 lb/in² for 15 min. The sterile medium was cooled to 45°C and 2% (v/v) final concentrated ethanol (absolute alcohol or 74 O.P.) in sterile distilled water was added aseptically. Plates were inoculated from 24 h TYGP broth cultures, incubated for 14 days, and examined daily for zones of clearing around the bacterial growth. In the final reading, growth was scraped aside to detect weak reactions under the colonies. Any clearing was scored as positive.

Polysaccharide production. Sterile sucrose solution was added aseptically to melted TY agar base to give a final concentration of 3% (w/v). Plates were inoculated from 24 h TYGP broth cultures. After incubation for 6 days any evidence of mucoid colonies was recorded as positive polysaccharide production.

Aesculin hydrolysis. Medium (g/l): proteose peptone, 10; yeast extract, 5; sodium acetate, 10; aesculin, 5; Tween 80, 1 ml. The broth was autoclaved for 10 min at 10 lb/in², inoculated from 24 h TY broth cultures and incubated for 6 days; any loss of fluorescence and blackening was scored as positive hydrolysis.

Casein hydrolysis. Medium (g/l): tryptone, 10; yeast extract, 5; NaCl, 5; casein, 10; Oxoid agar no. 3, 15. Autoclaving was at 15 lb/in² for 15 min, inoculation was from 24 h TY broth cultures and incubation was for 6 days. Positive results were scored when, after flooding with acid mercuric chloride solution, a clear zone remained around the colonies.

Hippurate hydrolysis. Medium (g/l): Na hippurate, 10; tryptone, 10; yeast extract, 5; K₂HPO₄, 5; NaCl, 5. The broth was autoclaved at 15 lb/in² for 15 min. Inoculation was from 24 h TYGP broth cultures, and incubation was for 6 days. Uninoculated control tubes were also incubated. To 1 ml volumes of the control media, varying amounts (0.2 to 0.5 ml) of acid ferric chloride solution (FeCl₃, 12 g; conc. HCl, 2.5 ml/0.1 l) were added and immediately shaken. The smallest volume to give a clear solution was added to 1 ml samples of clear supernatant fluid from the broth cultures. A heavy precipitate was scored as a positive hydrolysis.

Hydrolysis of chitin and alginate. Chitin: for use in a solid medium it was necessary to obtain chitin in a finely dispersed condition. Granular chitin (10 g) was placed in a 3 l flask, and 1500 ml of distilled water was held in readiness in another flask. A 100 ml sample of 50% (v/v) sulphuric acid was added to the granular chitin, and, as soon as this was seen to dissolve, the acid was rapidly diluted with the 1500 ml of distilled water. The resulting precipitate was allowed to settle overnight and the supernatant then decanted off. The finely divided chitin sludge so formed was dialysed against running water for 2 days to free it from acid. The chitin was resuspended in distilled water to a density such that after diluting 1:10, 5 ml of it dispensed in a Petri dish gave a distinct opalescence. A 50 ml portion of resuspended chitin was added to 450 ml melted TY agar base, the pH adjusted to 7.2, and then it was autoclaved for 15 min at 15 lb/in². Plates were inoculated from 24 h TYGP broth cultures and incubation was for 12 days. A zone of clearing under or around the bacterial colonies was scored as a positive result. Alginate: sodium alginate to a final concentration of 1% (w/v) was added to TY agar, the pH adjusted to 7.0 with HCl, and the medium then autoclaved at 15 lb/in² for 15 min. The sterile melted medium was poured into plates in a layered manner – 10 ml to 10 ml of solidified TY agar in the Petri dish. Inoculation was from 24 h TYGP broth cultures, and incubation was for 12 days. At intervals the plates were examined for clarification of the agar around the colonies, and where this occurred digestion of the alginate resulted in a pitting of the agar. Such clearing was scored as a positive alginate hydrolysis.

Ammonia from arginine. Medium (g/l): tryptone, 10; yeast extract, 5; glucose, 2; NaCl, 5; K₂HPO₄, 5; L-arginine monohydrochloride, 3. The medium was adjusted to pH 7.0, and autoclaved at 15 lb/in² for 15 min. Inoculation was from 24 h TYGP broth cultures, and after 3 days of incubation 0.25 ml Nessler's reagent was added to both the test media, and to a set of control media in which the arginine had been omitted. The formation of a brown colour was scored as positive.

Bile tolerance. Sodium deoxycholate was added to melted TY agar base to give final concentrations of 2% and 5% (w/v). Plates were poured and inoculated from 24 h TYGP broth cultures, and after incubation for 6 days any growth was scored as a positive reaction.

Benzidine reaction. Benzidine dihydrochloride reagent was prepared according to the method of Deibel & Evans (1960). Organisms were grown on TY agar for 3 days, then flooded with the reagent, followed by an equal volume of 5% hydrogen peroxide after 2 min. Development of a blue coloration in the colonies was scored as a positive reaction.

Hydrogen peroxide production. Blood was added to TY agar base to give a final concentration of 5% (v/v). Benzidine solution (Benzidine, 0.25 g; 25% HCl, 0.35 ml/0.05 l) was added to the melted blood agar to a final concentration of 0.01% (w/v). Plates were poured,

Table 2

Computer run A (physiological)	Computer run B (variety of characteristics)
Methyl-red positive	Rods present in 3 day TY agar
Alginate hydrolysis	Cocci
Chitin hydrolysis	Length less than 1.5 μ m
Xanthine decomposition	Length greater than 1.5 μ m
Tyrosine decomposition	Singles
Phenylalanine decomposition	Pairs
Malonate	Chains
Hippurate hydrolysis	Filaments
Starch hydrolysis	Curved
Phosphatase	Club-shaped
Ammonia from arginine	Branched
Voges-Proskauer positive	Motile at 22 or 37 °C
Gluconate	Pigment green, yellow or pink
Gelatine hydrolysis	Gram-positive
Casein hydrolysis	Gram-variable
Tolerance of 2 % bile	Snapping division or angular growth
Arylsulphatase	Evidence of morphological cycle
Ethanol to acetic acid	Origin plant
Dihydroxyacetone from glycerol	Origin animal
Catalase produced	Human pathogen (isolated from human disease)
Calcium lactate to carbonate	Non-pathogenic
Salicylate hydrolysis	Wide distribution
Aesculin hydrolysis	Indole positive
Nitrate reduced	Catalase positive
Nitrite reduced	Voges-Proskauer positive
Hydrogen sulphide from TY broth	Benzidine reaction
Hydrogen sulphide from TY broth plus thiosulphate	Benzidine blood reaction
Hydrogen sulphide from TY broth plus L-cysteine	Methyl-red positive
Benzidine reaction	Sodium azide 0.005 % tolerance
Benzidine blood reaction	Sodium azide 0.05 % tolerance
Polysaccharide produced	Sodium iodoacetate 0.0005 % tolerance
Indole positive	Sodium iodoacetate 0.05 % tolerance
Growth on pyronin	Acid produced from:
Enhancement of haemolysis on azide blood agar	Rhamnose
Pigment produced on TY agar	Glucose
Growth at pH 5.6	Sucrose
Growth at pH 6.2	Mannose
Growth at pH 7.0	Salicin
Growth at pH 8.0	Arabinose
Growth at pH 9.6	Melezitose
Growth in 4 % NaCl	Mannitol
Growth in 6 % NaCl	Erythritol
Growth in 8 % NaCl	Xylose
Growth in 10 % NaCl	Lactose
Growth in KCN broth	Maltose
Growth at 4 °C	Fructose
Growth at 10 °C	Starch
Growth at 30 °C	p-Arbutin
Growth at 37 °C	Cellobiose
Growth at 45 °C	Dextrin
Tolerance of 0.05 % Teepol	Resistance to:
Tolerance of 0.1 % Teepol	Streptomycin
Tolerance of 0.5 % thallos acetate	Bacitracin
Tolerance of 0.1 % thallos acetate	Erythromycin
Reduction of 0.1 % potassium tellurite	Tetracycline
Reduction of 0.1 % potassium selenite	Ampicillin
Tolerance of 0.005 % sodium azide	Resistance to:
	Penicillin G
	Cephaloridine
	Chloramphenicol

Table 2 (cont.)

Computer run A (physiological)	Computer run B (variety of characteristics)
Tolerance of 0.05 % sodium azide	Resistance to
Growth on 0.0002 % Thionin	Chlortetracycline
Growth on 0.01 % Thionin	Neomycin
Reduction of 0.05 % TTC	Oleandomycin
Tolerance of 0.0005 % sodium iodoacetate	Oxytetracycline
Tolerance of 0.005 % sodium iodoacetate	Novobiocin
	Kanamycin
Tolerance of 0.05 % sodium iodoacetate	Detection of amino acids in cell wall:
Growth on 0.01 % Crystal violet	Aspartic acid
Growth on 0.1 % Crystal violet	Alanine
α -Haemolysis	Glutamic acid
β -Haemolysis	Lysine
Cloudiness on Tween 20	Diaminopimelic acid
Cloudiness on Tween 80	Serine
	Glycine

inoculated from 24 h TY broth cultures and incubated for 3 days. Black colony formation was scored as negative peroxide production.

Utilization of carbohydrates. Bacterial suspensions: strains were grown on TY agar for 24 h at 37°C, 3 days at 30°C or 7 days at room temperature, harvested in saline, centrifuged at 2000g for 10 min, washed once in saline and resuspended to a density approximately corresponding to that of Brown's opacity tube no. 3. Carbohydrates tested: the following carbohydrates were made up in distilled water at the concentration stated (% w/v, unless otherwise indicated), and sterilized by steaming for 20 min on 3 consecutive days: arabinose, 5; xylose, 25; rhamnose, 5; glucose, 25; fructose, 15; mannose, 5; galactose, 20; sucrose, 25; maltose, 25; lactose, 25; trehalose, 5; raffinose, 10; melezitose, 5; starch, 5; inulin, 10; dextrin, 10; glycerol, 25 (v/v); erythritol, 5; adonitol, 2.5; mannitol, 5; dulcitol, 5; sorbitol, 5; salicin, 5; aesculin, 2; inositol, 5; cellobiose, 5; melibiose, 5; *p*-arbutin, 2.5. All carbohydrates were adjusted to give a final concentration of 1%. The buffer indicator solution was made up of ethanolic bromocresol purple 1% solution 0.5 ml + phosphate buffer 0.025 M, pH 6.8, 20 ml. Tubes were inoculated with the following test mixture: carbohydrate solution, 0.1 ml; buffer-indicator solution, 0.2 ml; bacterial suspension, 0.2 ml, and incubated in a 37°C water bath and examined at 4, 8 and 24 h. A positive acid production was scored when the colour changed from purple to yellow.

Starch hydrolysis. Soluble starch (5 g) was ground in a mortar with 2.5 ml distilled water to produce a smooth paste. This was added to 500 ml blood agar base no. 2 (Oxoid), mixed, and autoclaved at 10 lb/in² for 10 min. Plates were inoculated from 24 h TY agar cultures and were incubated for 6 days. Lugol's iodine was flooded over the plates, and positive reactions were scored when colourless zones were detected under or around the colonies.

Gelatin hydrolysis. Medium (g/l): tryptone, 10; yeast extract, 5; NaCl, 5; gelatin, 4; Oxoid agar no. 3, 15. Sterilization was at 10 lb/in² for 10 min, and plates were inoculated from 24 h TYGP broth cultures. After incubation for 12 days, the plates were flooded with 10 ml acid mercuric chloride solution. Clear zones around colonies indicating areas of gelatin hydrolysis were scored as positive.

Gluconate oxidation. Medium (g/l): tryptone, 10; yeast extract, 5; K₂HPO₄, 5; NaCl, 5; sodium gluconate, 37.25. The pH was adjusted to 7.0 and the medium was dispensed in 5 ml volumes, autoclaved at 15 lb/in² for 15 min, inoculated from 24 h TYGP broth cultures, and incubated for 6 days. A 1 ml sample of Benedict's solution was then added to each

tube and the tubes boiled for 10 min. The formation of a green, brown or orange precipitate was scored as a positive result.

Miscellaneous tests

Ribonuclease and deoxyribonuclease activity were tested by the method of Jeffries, Holtman & Guse (1957); haemolysis, and the effect upon it of sodium azide, by that of Packer (1943); arylsulphatase, by that of Luppi, Prati & Baretta (1964); malonate decomposition and the production of dihydroxyacetone, according to Harrington (1964); decomposition of tyrosine and xanthine, according to Skerman (1967); phenylalanine deamination, according to Shaw & Clarke (1955); oxidation of calcium lactate to carbonate, according to Shimwell, Carr & Rhodes (1960); and coagulase production, according to Cowan (1938).

Sensitivity and tolerance tests

The various agents and concentrations used in these tests are listed in Table 2. Tolerance of pH was tested in tryptone broth (Oxoid), inoculated from 24 h TYGP broth cultures and incubated for 6 days. Antibiotics were tested on TY agar, by means of Mast sensitivity discs, and incubated at 37 and 30 °C for 5 days. Temperature tolerance was measured on TY or TA medium, in the form of slopes, and incubated for up to 2 weeks. Potassium tellurite (Seeliger, 1961; Kramer & Jones, 1969) and potassium selenite (Mohanty, 1951) were prepared as 2% (w/v) solutions, sterilized by Seitz filtration and added to TY agar medium; sodium azide (Kramer & Jones, 1969) and sodium iodoacetate were prepared in the same manner, but as 10% and 1% solutions respectively. Potassium cyanide (Kramer & Jones, 1969) was prepared in TYGP medium, by adding a 0.5% (w/v) solution in distilled water, to the autoclaved broth. Thallous acetate (Shape, 1952) was prepared as a 10% (w/v) solution, autoclaved at 10 lb/in² for 10 min, and added to TY agar medium. Furacin was prepared according to the technique of McBride & Girard (1960); 2,3,5-triphenyltetrazolium chloride (TTC) by that of Kramer & Jones (1969). Dyes (Pacheco & Santos, 1957; Roberts 1968; and others) were incorporated in TY agar plates.

Cell wall analysis

These observations were made by the methods of Cummins & Harris (1956, 1958) and Davis & Baird-Parker (1959).

Morphology

Gram's stain, cell wall staining by the method of Hale (1953), electron and phase-contrast microscopy were employed to examine the morphology of the bacteria studied. Pigmentation and morphology of colonies was observed.

General characters

Such characters as origin (i.e. from plant or animal infection, or soil) pathogenicity or wideness of distribution are given according to the descriptions of those who isolated or identified and recorded the various strains.

Computer programme

A list of the characters computed for the bacteria in Table 1 is given in Table 2. The programme was essentially similar to that used by Seyfried (1968) in her study of lactobacilli, but modified for use in the Egtran II system employed on the Birmingham University KDF 9 computer. The steps in the programme included the following: calculation of a

Fig. 1. (Physiological.) The strains of *Listeria* including *Listeria grayi* form a compact group consisting of three phenon. *Listeria denitrificans* and *Listeria* sp. (Donna Craig) are quite different. *Erysipelothrix rhusiopathiae* (syn. *E. insidiosus*) forms a compact group of a single phenon. (Ed. note: the commonly used name, *E. insidiosus* for *E. rhusiopathiae*, has been retained for Figs 1-4 of this paper. For the text the ruling of the Judicial Commission in Opinion no. 32 (1970) has been followed.)

Fig. 2. (General characters.) The listeria again form a compact group consisting of two large and two small phena, *Listeria dentrificans* and *Listeria* sp. (Donna Craig), are related only at 66%. *Erysipelothrix rhusiopathiae* (syn. *E. insidiosus*) again forms a single phenon.

Fig. 3. (Physiological.) *Erysipelothrix rhusiopathiae* (syn. *E. insidiosa*) and the true *Listeria* strains (see Fig. 1 and 2) form compact groups; the former in a phenon with haemolytic streptococci, the latter with faecal streptococci. These two phenons are most closely related to a third comprising the corynebacteria of animal origin.

Fig. 4. (General characters.) By these criteria, *Erysipelothrix rhusiopathiae* (syn. *E. insidiosus*) forms a single phenon, whereas true listerias, while still a compact group, appear in a phenon with some corynebacteria, soil diphtheroids and microbacteria.

matrix of similarity coefficients for all possible pairs of strains; grouping of strains with highest similarity into clusters; and calculation of new similarity coefficients between clusters, repeated until all strains were grouped in one cluster. These coefficients (S values) were determined by the method of Sokal & Michener (1958), using the formula $S = NS/(NS + ND)$, where NS was the number of similar features and ND the number of differences.

The program analysed 100 specimens, with 70 characteristics. Because of the large number of characteristics available, these were analysed in two groups: run A, 70 physiological characters; and run B, 70 mixed characters (Table 2). This arrangement was chosen because it is considered that previous conclusions on the classification of *Listeria* have been affected by the types of criteria chosen for analysis. For example, Miller & Silverman (1959) found *Listeria monocytogenes* to be related to lactobacilli on almost purely metabolic grounds, whereas others (Davis & Newton, 1969, and others) concluded that both listerias and *Erysipelothrix rhusiopathiae* resembled the corynebacteria of animal provenance; these views being based on a more general list of characters.

RESULTS AND DISCUSSION

The results of these analyses are given in the form of dendrograms, of which Fig. 1 and 2 show the option 1 analysis (simple matching coefficient) for the listerias and *Erysipelothrix rhusiopathiae* strains only, using the physiological characters (run A) and the general characters (run B) respectively. They show clearly that these two genera are distinct, and that each forms a compact group of strains. *Listeria grayi* is indistinguishable from *L. monocytogenes*, whereas *L. denitrificans* and *Listeria* sp. (Donna Craig) are quite different. As far as *L. denitrificans* is concerned these observations are supported by the difference in its GC ratio from that of other listerias (Welshimer & Meridith, 1971). The genus can reasonably be said to consist of a single species, since the various phenons, into which it is divisible, on each analysis, have no correlation between the two runs, A and B. No correlation was observed between the composition of the strains in the phenon with either serological type or source. *Erysipelothrix rhusiopathiae* may similarly be regarded as a monospecific genus, and in this case it constitutes a single phenon, alike in both runs.

Fig. 3 and 4 show option 1 analysis for all the bacteria studied, on runs A and B respectively. When physiological characters, only, are considered (run A), *Erysipelothrix rhusiopathiae* appears as a compact group, sharing one phenon with the streptococci, other than the faecal ones. The latter share a phenon with the five typical strains of *Listeria* (out of the seven strains, including *Listeria denitrificans* and *Listeria* sp. (Donna Craig), that were chosen as representative of the genus, to balance the seven strains of *E. rhusiopathiae* and comparable numbers from other bacterial genera shown on this graph. Both of these phenons are related to one comprising a group of corynebacteria, mainly of animal origin; at 67% for the listeriae, and 73% for *E. rhusiopathiae*. However, their relationship to the remaining strains is only 2% lower, and on the full range of characters, in Fig. 4, no such relationships appear, above 61%, and the streptococci are distinct. The strains of *Listeria* form a loose group with the microbacteria, and a closer one with a collection of rather ill-defined diphtheroid bacilli of diverse origins.

The one observation of genuine interest provided by these attempts to solve the problem of the relationships of the genera is the close affinity between the listerias and the faecal streptococci, shown in Fig. 3. There is direct serological evidence for such a relationship (Seeliger, 1961), and also indirect evidence, in that Pease (1967*a*) showed certain listeria-like bacteria possessed L-forms that closely resembled *Mycoplasma hominis*, whereas the

latter organism has strong serological cross-relationships with strains of *Streptococcus aecalis* (Pease, 1967*b*). However, the significance of the so-called Rantz antigen common to a wide range of Gram-positive bacteria (Neter, Anzai & Gorzynski, 1960) is not clear and we are currently engaged in work on this problem.

The results of the tests on which the numerical analysis is based may be obtained on application to the authors.

We wish to acknowledge the assistance of Mr B. C. Kershaw, Computer Centre, University of Birmingham.

REFERENCES

- BISSET, K. A. (1963). *Bacteria*, 3rd edn, p. 75. Edinburgh: Livingstone.
- Bergey's Manual of Determinative Bacteriology* (1957), 7th edn, p. 578. Edited by R. S. Breed, E. G. D. Murray and N. R. Smith. Baltimore: Williams & Wilkins.
- COWAN, S. T. (1938). The classification of staphylococci by precipitation and biological reactions. *Journal of Pathology and Bacteriology* **46**, 31-45.
- CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. *Journal of General Microbiology* **14**, 583-600.
- CUMMINS, C. S. & HARRIS, H. (1958). Studies on the cell-wall composition and taxonomy of Actinomycetales and related groups. *Journal of General Microbiology* **18**, 173-189.
- DAVIS, G. H. G. & BAIRD-PARKER, A. C. (1959). The classification of certain filamentous bacteria with respect to their chemical composition. *Journal of General Microbiology* **21**, 612-621.
- DAVIS, G. H. G., FOMIN, L., WILSON, E. & NEWTON, K. G. (1969). Numerical taxonomy of *Listeria*, Streptococci and possible related bacteria. *Journal of General Microbiology* **57**, 333-348.
- DAVIS, G. H. G. & NEWTON, K. G. (1969). Numerical taxonomy of some named coryneform bacteria. *Journal of General Microbiology* **56**, 195-214.
- DEIBEL, R. H. & EVANS, J. B. (1960). Modified benzidine test for the detection of cytochrome containing respiratory systems in micro-organisms. *Journal of Bacteriology* **79**, 356-360.
- HALE, C. M. F. (1953). The use of phosphomolybdic acid in the mordanting of bacterial cell walls. *Laboratory Practice* **2**, 115-116.
- HARRINGTON, B. (1964). *The taxonomy of oral corynebacteria*, p. 75. Ph.D. Thesis, University of Birmingham.
- JEFFRIES, C. D., HOLTMAN, D. F. & GUSE, D. J. (1957). Rapid method for detecting the activity of micro-organisms on nucleic acids. *Journal of Bacteriology* **73**, 590-591.
- KRAMER, P. A. & JONES, D. (1969). Media selective for *Listeria monocytogenes*. *Journal of Applied Bacteriology* **32**, 381-394.
- LUPPI, A., PRATI, L. & BARETTA, G. (1964). Su alcune caratteristiche metaboliche-culturali della *Listeria monocytogenes*. II. Attività arylsolfatase della *Listeria monocytogenes*. *Bollettino dell'Istituto Sieroterapico Milanese* **43**, 209-212.
- MCBRIDE, M. E. & GIRARD, K. F. (1960). A selective method for the isolation of *Listeria monocytogenes* from mixed bacterial populations. *Journal of Laboratory and Clinical Medicine* **55**, 153-157.
- MILLER, I. L. & SILVERMAN, S. J. (1959). Glucose metabolism of *Listeria monocytogenes*. *Bacteriological Proceedings*, 103.
- MOHANTY, U. (1951). *Corynebacterium fascians* (Tilford) Dowson: its morphology, physiology, nutrition and taxonomic position. *Transactions of the British Mycological Society* **34**, 23-34.
- NETER, E., ANZAI, H. & GORZYNSKI, E. A. (1960). Identification of an antigen common to *Listeria monocytogenes* and other bacteria. *Proceedings of the Society for Experimental Biology and Medicine* **105**, 131-134.
- OPINION NO. 32. (1970). Conservation of the specific epithet *rhusiopathiae* in the scientific name of the organism known as *Erysipelothrix rhusiopathiae* Migula 1900, Buchanan 1918. *International Journal of Systematic Bacteriology* **20**, 9.
- PACHECO, G. & SANTOS, M. L. (1957). Bacterioimpedancia de materias corantes sobre listerías. *Revista Brasileira de Medicina* **14**, 316-319.
- PACKER, R. A. (1943). The use of sodium azide and crystal violet in a selective medium for streptococci and *Erysipelothrix rhusiopathiae*. *Journal of Bacteriology* **46**, 343-349.
- PEASE, P. E. (1967*a*). Tolerated infection with the sub-bacterial phase of *Listeria*. *Nature, London* **215**, 936-938.

- PEASE, P. E. (1967*b*). Antigenic relationships between oral group D streptococci, some haemophilus species and *Mycoplasma hominis*. *Journal of General Microbiology* **49**, 433–441.
- ROBERTS, R. J. (1968). Biochemical reactions of *Corynebacterium pyogenes*. *Journal of Pathology and Bacteriology* **95**, 127–130.
- SEELIGER, H. P. R. (1961). *Listeriosis*, pp. 30, 216. Basel: S. Karger.
- SEYFRIED, P. L. (1968). An approach to the classification of lactobacilli using computer-aided numerical analysis. *Canadian Journal of Microbiology* **14**, 313–318.
- SHARPE, M. E. (1952). Group D streptococci in the faeces of healthy infants and of infants with neonatal diarrhoea. *Journal of Hygiene* **50**, 209–228.
- SHAW, C. & CLARKE, P. H. (1955). Biochemical classification of *Proteus* and *Providencia* groups. *Journal of General Microbiology* **13**, 155–161.
- SHIMWELL, J. L., CARR, J. G. & RHODES, M. E. (1960). Differentiation of *Acetomonas* and *Pseudomonas*. *Journal of General Microbiology* **23**, 283–286.
- SKERMAN, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd edn, p. 213. Baltimore: Williams & Wilkins.
- SOKAL, R. R. & MICHENER, C. D. (1958). A statistical method for evaluating systematic relationships. *University of Kansas Scientific Bulletin* **38**, 1409–1438.
- WELSHIMER, H. J. & MEREDITH A. L. (1971). *Listeria murrayi* sp.n.: a nitrate-reducing mannitol-fermenting *Listeria*. *International Journal of Systematic Bacteriology* **21**, 3–7.
- WILSON, G. S. & MILES, A. A. (1955). *Principles of Bacteriology and Immunity*, 4th edn, vol. 1, p. 531. London: Edward Arnold.