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THE BIOCHEMICAL DIFFERENTIATION BETWEEN *SALMONELLA* AND *CITROBACTER*

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ABSTRACT

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A number of bacterial isolates which could not be identified as either *Salmonella* or *Citrobacter* by conventional biochemical tests and could not be typed as *Salmonella* with available antisera, were further examined biochemically and by lysis with phage Felix 0,1. Glycerol-positive salmonellae and lysine-positive citrobacters were encountered, which could be confused with the other genus, but when the reactions of such strains were examined in the other tests, accurate identifications could be done. Of the tests examined, glycerol fermentation, the β-galactosidase test, lysine decarboxylation, sorbose fermentation, galacturonate fermentation and lysis by the phage could be used in the differentiation. These tests in combination, rather than 1 or 2 single tests gave reliable and conclusive differentiation.

INTRODUCTION

The family Enterobacteriaceae can be divided in a lactose fermenting group and a lactose non-fermenting group. During the isolation of an organism from a sample, most of the media employed like MacConkey's agar, Drigalski lactose agar, desoxycholate agar and brilliant green agar, classify the isolate either as a lactose fermenter or a lactose non-fermenter. The lactose-negative members *Salmonella, Citrobacter, Hafnia, Proteus* and *Edwardsiella* are further differentiated by a small number of biochemical tests (Table 1) routinely employed by this laboratory. However, the differentiation between *Citrobacter freundii* and *Salmonella* by these tests poses problems.

The classical biochemical differentiation between *Salmonella* and *Citrobacter freundii* is based on the KCN test and the lysine decarboxylation test (Edwards & Ewing, 1962; Mac Faddin, 1981; Edwards, Fife & Ewing, 1956; Richard, 1981). The KCN test, however, is not used widely as the age of the medium and the size of the inoculum influences the result. The medium is also difficult to prepare and must be incubated anaerobically. To rely on the lysine decarboxylation medium alone to differentiate between *Salmonella* and *Citrobacter* poses problems in our experience, as lysine decarboxylation-positive strains have been encountered, which could not be serologically identified as being *Salmonella* with available antisera, and would thus appear as lysine-positive citrobacters.

Other biochemical tests which can be relied on to differentiate between these 2 organisms are lactose, sucrose, and salicin fermentations (Edwards *et al.*, 1956). These reactions can however, be negative, late or irregularly positive. Inositol fermentation and malonate utilization can also be used (Brenner, 1984).

In addition to this biochemical similarity between *Salmonella* and *Citrobacter,* these two organisms have somatic antigens (Edwards & Ewing, 1962) which cross-agglutinate with the other organism's antisera. A *Citrobacter freundii* isolate which appears biochemically similar to a *Salmonella,* could thus be agglutinated with some of the *Salmonella* O-antisera, but as no flagellar antigens would be detected on such an isolate, a conclusive identification of such an isolate would not be possible. Such a culture is examined with all available antisera with no conclusive evidence that it is a specific serovar. Often, based on this inconclusive evidence alone, such an isolate is classified as a *Citrobacter freundii.* A number of strains with such reactions have been encountered and were examined in this study.

There are presently 67 O-antigens of *Salmonella* known. In our laboratory, serovars with O-antigens of the lower numbers of 0,2 to 0,30, are the most often encountered (Annual Reports, Bacteriology, Veterinary Research Institute). The full range of O-antigen antisera are stocked up to 0,59, but as they are infrequently used their titre and specificity may deteriorate over a period of time without the user being aware of it. It may then happen that an isolate, whose O-antigens are of the higher numbers, is correctly identified biochemically as a *Salmonella,* but cannot be typed as a result of the low potency of the antisera. In such a case the presence of specific flagellar antigens can still indicate that the isolate is a *Salmonella.* However, the whole range of H-antigen antisera of polyvalent HF and HG groups are not stocked, with the result that all the isolate's H-antigens cannot be identified, if the isolate possesses an H-antigen belonging to one of these groups. The complete and satisfactory identification of such a strain poses the same problems as that of a *Citrobacter freundii* isolate.

The nomenclature of *Salmonella* and its more than 2 000 serovars are followed as described by Le Minor, Véron & Popoff (1982b) and Le Minor, Popoff, Aurent & Hermant (1986). The differentiation of the six subspecies of *Salmonella* is based on biochemical reactions (Table 2). It is important to determine to which of the subspecies an isolate belongs, because often serovars of subspecies I and II possess the same antigenic formulae and the only way to determine to which of the two subspecies such an isolate belongs, is by biochemical differentiation.

As a result of the need to be able to differentiate the subspecies biochemically as well as the problems encountered with *Citrobacter* and some *Salmonella* isolates possessing the same O-antigens, a number of biochemical tests as well as lysis by phage Felix 0,1, were carried out on a number of strains which had been difficult to identify. The aim was to see whether a battery of tests could be compiled which would give practical, conclusive as well as reproducible differentiation of *Citrobacter* from *Salmonella* subspecies. The tests described by Le Minor, Véron & Popoff (1982a) to differentiate *Salmonella* subspecies were used, as well as the classical test of lysine decarboxylase to differentiate *Salmonella* from *Citrobacter,* and a number of other tests like

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TABLE 1 Enterobacteria often mistaken for *Salmonella* and their biochemical differentiation

Biochemical tests	*Salmonella* I	*Salmonella* II	*Salmonella* III	*Salmonella* IV	*Salmonella* V	*Salmonella* VI	*Citrobacter freundii*	*Citrobacter diversus*	*Citrobacter amalonaticus*	*Hafnia alvei*	*Proteus* spp.	*Edwardsiella* spp.
Lactose fermentation	–[1]	–	d[3]	–	–	–	d	d	d	–	–	–
Dulcitol fermentation	+[2]	+	–	–	+	d	d	d	–	–	–	–
Sucrose fermentaton	–	–	–	–	–	–	d	d	d	–	d	d
Maltose fermentation	+	+	+	+	+	+	+	+	+	+	d	+
Inositol fermentation	d	d	–	d	NK[4]	NK	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	+	+	–	d	d
Phenylalanine deamination	–	–	–	–	–	–	–	–	–	–	+	–
H_2S production in TSI	+	+	+	+	+	+	d	–	–	–	d	d
Lysine decarboxylation	+	+	+	+	+	+	–	–	–	+	–	+
Growth in KCN	–	–	–	+	+	–	+	+	+	NK	NK	NK
Malonate utilization	–	+	+	–	–	–	d	+	d	d	–	d

[1] – = 90 % or more negative reactions
[2] + = 90 % or more positive reactions
[3] d = different reactions between strains
[4] NK = not known

TABLE 2 Biochemical subdivision of the *Salmonella* species into 6 subspecies*

Biochemical reactions	Subspecies						
	I	II	IIIa	IIIb	IV	V	VI
Dulcitol fermentation	+[1]	+	–	–	–	+	d
ONPG production	–[2]	–	+	+	–	+	d
Malonate utilization	–	+	+	+	–	–	–
Gelatinase production	–	+	+	+	+	–	+
Growth in KCN	–	–	–	–	+	+	–
d-+1-tartrate production	+	–	–	–	–	–	–
Galacturonate production	–	+	–	+	+	+	+
γ-glutamyltransferase production	+	+	–	+	+	+	+
β-glucuronidase production	d[3]	d	–	+	–	–	d
Growth in mucate	+	+	+	70%[4]–	–	+	+
Salicin fermentation	–	–	–	–	+	–	–
Sorbitol fermentation	+	+	+	+	+	+	–
Lactose fermentation	–	–	75%[4]–	75%[4]+	–	–	–
Lysis by phage Felix 0,1	+	+	–	+	–	d	+

* Le Minor *et al.*, 1982a
[1] + = 90 % or more positive reactions
[2] – = 90 % or more negative reactions
[3] d = different reactions according to the serovar
[4] % = reaction of % of strains

sorbose fermentation (Stenzel, 1976) and glycerol fermentation (Richard & Popoff, 1985) were also examined.

MATERIALS AND METHODS

Serotyping

The standard slide agglutination method for *Salmonella* was used, as described by Kauffmann (1972). Difcol[1] antisera were used to identify serovars according to the Kauffmann-White Scheme. New additions to the scheme were followed as in a supplement to the scheme (Le Minor & Bockemühl, 1986).

Susceptibility to phage Felix 0,1

Propagation of the phage: Salmonella phage, Felix 0,1, as well as the propagating strain, *Salmonella* ser Paratyphi B, were kindly supplied by L. Le Minor. The method followed for the propagation of the phage was partly as described by Carlton & Brown (1981).

Phage susceptibility tests: The organism to be examined was grown in L-broth (Carlton & Brown, 1981) to mid log phase, 0,1 mℓ was inoculated over the surface of a LC agar plate (Carlton & Brown, 1981) and kept for 30 min at 37 °C. A volume of 10 μℓ of the routine test dilution of the phage was dropped on the agar plate and incubated overnight at 37 °C. The plaques were described as showing clear lysis (CL) if a clear area of no growth occurred, semi-confluent lysis (SCL) if small areas of lysis with growth in between appeared and opaque lysis (OL) if the area of lysis was opaque. Where no lysis occurred, the lawn of growth of the organisms was smooth.

Biochemical tests

Glycerol fermentation: The medium employed was as described by Richard & Popoff (1985). The

[1] Laboratory & Scientific Equipment Co. (Pty) Ltd, P.O. Box 45125, Mayfair, Johannesburg 2108

medium consisted of peptone 10 g, NaCl 5 g, glycerol 10 g, 5 mℓ of a 1 % phenol red solution and 1 ℓ distilled water. The pH was adjusted to 8,2 and the medium was filter sterilized into tubes. The medium was inoculated from a broth culture and examined after 1, 3 and 7 days. The fermentation of glycerol was marked by the colour change of red to yellow.

Lysine decarboxylation: This characteristic was examined in Moller's decarboxylation broth as described by Mac Faddin (1981). The media were inoculated from broth cultures and were read after 24 h.

Carbohydrate fermentation: Fermentation of sorbose, dulcitol, salicin, lactose and sorbitol was examined in a peptone water fermentation broth with 1 % Andrade's indicator and 0,5 % of a carbohydrate (Cruickshank, Duguid, Marmion & Swain, 1975). The tubes were inoculated from broth cultures and incubated at 37 °C and observed at 1, 3 and 7 days and up to 30 days for sorbose fermentation.

Organic acid utilization: The method was as described by Kauffmann (1961). The basal medium consisted of peptone 10 g, 0,1 N NaOH 8,5 mℓ and distilled water 1 ℓ. A volume of 12 mℓ of a 1:500 bromothymolblue solution and either 1 % d-tartrate[2], 0,5 % l-tartrate[2] or 1 % mucate[2] was added. The pH was adjusted to 7,4. The media were inoculated from a broth culture and incubated at 37 °C for up to 5 days. Mucate utilization was marked by the colour change from green to deep blue. For the d- and l-tartrates, the colour of the medium changed to a light blue and, after the addition of 3 mℓ of a saturated solution of aqueous lead acetate, a small sediment formed while the medium was clear.

Malonate substrate was as described by Cruickshank *et al.*, (1975). The medium was inoculated from a broth culture and incubated at 37 °C for up to a week.

Galacturonate fermentation: The ability of an isolate to ferment galacturonate was examined in peptone water containing 0,0125 % phenol red indicator and 0,5 % D-galacturonate[3] (Le Minor, Buissière & Brault, 1979). The medium was inoculated from a broth culture and incubated at 37 °C for 2 days. The colour change from red to yellow was taken as a positive reaction.

β-glucuronidase and γ-glutamyltransferase examinations: Examination for the presence of γ-glutamyltransferase was done as described by Giammanco, Buissière, Toucas, Brault & Le Minor (1980). GLUPA[3] (γ-L-glutamine-ρ-nitroanilide acid) (28 g) was dissolved in 100 mℓ acetone-distilled water (50:50, v/v). Discs (0,5 cm diameter) were cut from blotting paper and saturated with the GLUPA-acetone-water mixture. The discs were dried at 37 °C and stored at 4 °C. From a phosphate buffer ($Na_2HPO_4.12H_2O$ 7,165 g, KH_2PO_4 1,807 g, toluene 2 mℓ and distilled water 500 mℓ) (C. Richard, personal communication, 1986) 0,5 mℓ was used to make a heavy suspension of growth from the culture in a small test tube. A disc of GLUPA was added and the tube incubated at 37 °C for 24 h. The colour change to yellow indicated a positive reaction.

The β-glucuronidase activity was examined in the same way (Le Minor, Buissière, Novel & Novel, 1978). A mass of 0,3 g ρ-nitrophenyl-β-D-glucuronide[3] (pNPGlcU) was dissolved in 100 mℓ distilled water and 0,5 cm blotting paper discs were saturated with the pNPGlcU-water. Dried discs were added to 0,5 mℓ culture suspension in the buffer and kept for 2 h at 37 °C. A positive reaction was marked by a colour change to yellow.

ONPG test: For the detection of β-galactosidase activity, O-nitrophenyl-β-D-galactopyranoside (ONPG) paper discs[4], were used. Growth from the culture was suspended in 0,1 mℓ saline in a test tube, a disc was added and incubated for up to 6 h for the colour change to yellow, which indicated lactose fermentation.

The 1 % and 5 % lactose fermentations: In addition to the usual lactose fermentation test with 0,5 % lactose in the peptone carbohydrate fermentation broth, the fermentation of 5 % lactose in 0,1 peptone water and 1 % lactose in 1 % peptone water (Lapage & Jayaraman, 1964) with Andrade's indicator, were also examined. Tests were inoculated from broth cultures incubated at 37 °C and examined for lactose fermentation.

RESULTS

Cultures

The cultures examined in this study, their origin and identification are given in Table 3. Most of the samples originated from the environment and by-products, a few from animals, 2 from reptiles and 1 from a dairy product (Table 3). Organisms from which no reaction with the O-antisera could be obtained, were classified as *Citrobacter,* and the others where O-antigens could be determined, were classified as either subspecies I or II, based on biochemical reactions. In none of the organisms listed in Table 3 flagellar antigens could be determined. The two *Salmonella* subspecies III isolates did not pose problems with identification, but these strains, as well as a culture collection subspecies IIIa strain, were included in the study as reference strains.

Lysis by phage Felix 0,1

Of the strains isolated, $\frac{14}{27}$ salmonellas were lysed by the phage (Table 4), and none of the strains identified as *Citrobacter.*

Glycerol fermentation

The result of the test was read after 24–48 h as it was observed that longer incubation resulted in difficulty in determining the exact colour of the medium. Fermentation of glycerol resulted in a yellow colour which was easily differentiated from the negative red colour. All strains identified as *Citrobacter* fermented glycerol promptly. All 3 strains of *Salmonella* subspecies IIIa and the subspecies II strains were repeatedly negative. However, of the subspecies I salmonellae, a number ($\frac{3}{7}$) of strains fermented glycerol but were lysed by the phage and decarboxylated lysine, characteristics of the *Salmonella* group.

Lactose fermentation

All of the *Salmonella* subspecies I and II strains, were lactose-negative as determined by the 4 tests (Table 4). Only 1 strain, 188, was ONPG- as well as 5 % lactose-positive, characteristics of the subspecies IIIa + b. All 3 subspecies IIIa strains were

[2] Merck (SA) (Pty) Ltd, P.O. Box 3497, Johannesburg 2108

[3] Serva, Holpro Analytics (Pty) Ltd, P.O. Box 7868, Johannesburg 2000

[4] Oxoid, Protea Laboratory Services, P.O. Box 784978, Sandton 2146

TABLE 3 The origin, serotyping and biochemical identification of the strains examined

Isolates	Origin	O-antigen	Biochemical classification
92	Animal	OE+	*Salmonella* I
179	Environment	OE+	*Salmonella* I
259	Byproduct	30	*Salmonella* I
266	Byproduct	30	*Salmonella* I
271	Byproduct	30	*Salmonella* I
270	Byproduct	18	*Salmonella* I
275	Byproduct	15	*Salmonella* I
190	Environment	OE+	*Salmonella* II
194	Animal	30	*Salmonella* II
89	Environment	6,7	*Salmonella* II
125	Animal	OC+	*Salmonella* II
172	Byproduct	OB+	*Salmonella* II
28	Environment	OE+	*Salmonella* II
131	Environment	OE+	*Salmonella* II
150	Animal	OE+	*Salmonella* II
188	Animal	6,7,14	*Salmonella* II
55	Reptile	51	*Salmonella* IIIa
198	Animal	13,22	*Salmonella* IIIa
186	Environment		*C. freundii/Salmonella* II
90	Environment		*C. freundii/Salmonella* II
170	Environment		*C. freundii/Salmonella* II
268	Byproduct		*C. freundii/Salmonella* I
237	Byproduct		*C. freundii/Salmonella* I
88	Byproduct		*C. freundii/Salmonella* I
279	Byproduct		*C. diversus*
235	Animal		*C. diversus*
269	Animal		*C. freundii*
225	Animal		*C. freundii/Salmonella* I
229	Animal		*C. freundii/Salmonella* I
156	Animal		*C. freundii/Salmonella* I
145	Animal		*C. freundii/Salmonella* I
97	Reptile		*C. freundii/Salmonella* I
123	Dairy		*C. freundii/Salmonella* I
104	Environment	OD+	*C. diversus*

ONPG-positive, 2 fermented lactose and 1 fermented lactose in the 5 % lactose broth.

Of the *Citrobacter* strains, $\frac{5}{8}$ fermented lactose (conventional test) and were also positive in the 1 % and 5 % tests and ONPG test (Table 5). Strain 229 did not ferment lactose using the conventional test, but was positive in the 1 %, 5 % lactose and ONPG test. Strain 170 was only ONPG-positive, while strain 279 was negative for all the lactose tests. All salmonellae were lysine decarboxylation-positive and all *Citrobacter* strains were lysine decarboxylation-negative (Table 4).

Fermentation of sorbose, salicin, sorbitol

Only a small number of isolates examined, $\frac{6}{35}$, fermented sorbose, all of them within 3 days. All these strains were citrobacters. None of the other isolates fermented sorbose, even after prolonged incubation.

Most of the strains fermented sorbitol, while only a small number of salmonellae ($\frac{3}{26}$) fermented salicin and $\frac{2}{8}$ *Citrobacter* strains fermented this sugar (Table 4). The fermentation of salicin by the *Salmonella* isolates were not subspecies related.

Utilization of mucate and malonate

Most of the strains utilized mucate (Table 4), which appeared not to be species or subspecies specific. Of the salmonellae, malonate utilizers were classified as either *Salmonella* subspecies II or III. A number of *Citrobacter freundii* strains ($\frac{3}{6}$) used malonate. H_2S-negative citrobacters which used malonate were classified as *C. diversus* and the other H_2S-negative strains were *C. amalonaticus* (Table 5).

d- and 1-tartrate utilization

All *Salmonella* subspecies I isolates were positive while subspecies II and III and *Citrobacter* strains examined were negative (Table 4).

Galacturonate fermentation

There was a clear differentiation between the red negative reaction and the bright yellow of the positive reaction.

The *Salmonella* subspecies I isolates, strains 123 and K10 were negative (Table 4). Strain 172, a subspecies II, was positive, strain 55, the subspecies IIIa isolate, was negative, strain 198 was positive and the 2 examined *Citrobacter* isolates were positive.

γ-glutamyltransferase and β-glucuronidase examinations

For the *Salmonella* isolates belonging to subspecies I or II and for *Citrobacter* isolates, this test was either positive or negative. All 3 *Salmonella* subspecies III isolates were negative. γ-glucuronidase was either positive or negative in all the groups (Table 4).

Discussion

Lysine decarboxylation, the classical test to differentiate *Salmonella* and lactose-negative *Citrobacter freundii* strains, was again shown according to the results in Table 4, to be the test which clearly differentiated these 2 organisms. Additional tests valuable in the differentiation of the 2 organisms were glycerol fermentation, lactose fermentation (the β-galactosidase test) and lysis by the phage. However, a battery of tests consisting of all above-mentioned tests, gave a more satisfactory differentiation of the 2 organisms, rather than to rely on the lysine decarboxylation test alone.

Apart from lysine decarboxylation, no single test could be relied on for differentiation as: (i) a number of glycerol-positive ($\frac{3}{7}$) *Salmonella* subspecies I isolates were encountered, (ii) although no *Salmonella* subspecies I strains fermented lactose, a *Salmonella*

TABLE 4 The biochemical reactions, lysis by phage 0,1 of the examined strains and their subsequent classificaton

Isolates	Glycerol fermentation	Lactose fermentation				Lysine decarboxylation	Fermentations						γ-glutamyltransferase	β-glucuronidase	d- + 1-tartrate	Lysis by phage 0,1	Classification
		Lactose	1 % lactose	5 % lactose	ONPG		Salicin	Sorbitol	Mucate	Sorbose	Malonate	Galacturonate					
179	−[1]	−	−	−	−	+[2]	−	+	−	−	−	ND[4]	+	ND	+	+	*Salmonella* I
275	−	−	−	−	ND	+	−	+	+	−	−	ND	ND	ND	+	+	*Salmonella* I, immobile
237	−	−	−	−	ND	+	−	+	−	−	−	ND	ND	ND	+	+	*Salmonella* I
268	−	−	−	−	ND	+	+	+	+	−	−	ND	ND	ND	+	−	*Salmonella* I
251	−	−	−	−	ND	+	−	+	+	−	−	ND	ND	ND	+	−	*Salmonella* I
123	−	−	−	−	−	+	−	+	+	−	−	−	ND	ND	+	−	*Salmonella* I
K10	−	−	−	−	ND	+	−	+	+	−	−	−	ND	ND	+	−	*Salmonella* I
271	+	−	−	−	ND	+	−	+	−	−	−	ND	ND	ND	+	+	*Salmonella* I ser Landau
279	+	−	−	−	−	+	−	+	+	−	−	−	ND	ND	+	−	*Salmonella* I ser Schwarzengrund
156	+	−	−	−	−	+	−	+	+	−	−	−	−	−	+	+	*Salmonella* I
172	−	−	−	−	ND	+	−	+	−	−	+	ND	+	−	−	+	*Salmonella* II
28	−	−	−	−	ND	+	−	+	+	−	+	ND	ND	ND	−	+	*Salmonella* II
125	−	−	−	−	−	+	−	+	−	−	+	ND	+	−	ND	+	*Salmonella* II
194	−	−	−	+	ND	+	−	+	+	−	+	ND	ND	ND	ND	+	*Salmonella* II
150	−	−	−	−	ND	+	ND	ND	ND	−	+	ND	ND	ND	ND	+	*Salmonella* II
90	−	−	−	−	−	+	−	+	+	−	+	ND	ND	−	ND	+	*Salmonella* II
131	−	−	−	−	ND	+	ND	ND	ND	−	+	ND	ND	ND	ND	−	*Salmonella* II
269	−	−	−	−	ND	+	−	+	−	−	+	ND	ND	ND	ND	−	*Salmonella* II
MI4	−	−	−	−	ND	+	ND	ND	ND	−	+	ND	ND	ND	ND	+	*Salmonella* II
190	−	−	−	−	+	+	+	−	+	−	+	ND	−	+	−	+	*Salmonella* II
89	−	−	−	−	ND	+	ND	ND	ND	−	+	ND	ND	ND	ND	−	*Salmonella* II
104	−	−	−	−	−	+	−	+	+	−	+	+	(+)[3]	−	+	+	*Salmonella* II, H_2S−
186	−	−	−	−	+	+	−	+	−	−	+	ND	−	+	−	−	*Salmonella* II
170	−	−	−	−	−	+	−	+	+	−	+	+	−	+	−	−	*Salmonella* II
188	−	−	−	+	+	+	+	+	+	−	+	ND	ND	ND	ND	−	*Salmonella* II
55	−	d[5]	−	−	+	+	−	+	+	−	+	−	−	−	−	−	*Salmonella* IIIa
198	−	−	−	+	+	+	−	+	+	−	+	+	−	+	ND	−	*Salmonella* IIIa
T200	−	(+)	−	−	+	+	−	+	+	−	+	−	−	+	ND	−	*Salmonella* IIIa
97	+	+	+	+	+	−	−	+	+	+	+	+	−	(+)	−	−	dulcitol, sucrose, ornithine, *C. freundii*
92	+	+	+	+	+	−	−	+	+	+	−	ND	−	+	ND	−	ornithine *C. freundii*
145	+	+	+	+	+	−	+	+	+	+	−	+	−	+	−	−	*C. freundii*
225	+	+	+	+	−	−	−	+	−	+	−	ND	ND	ND	−	−	sucrose+, ornithine− *C. freundii*
229	+	−	+	+	+	−	−	+	+	+	+	ND	ND	ND	ND	−	dulcitol−, ornithine− *C. freundii*
235	+	+	+	+	+	−	+	+	+	−	+	ND	ND	ND	ND	−	H_2S −, indole+ *C. diversus*
279	+	−	−	−	−	+	−	+	+	−	−	ND	ND	ND	ND	−	H_2S − *C. amalonaticus*
170	+	−	−	−	+	+	−	+	−	+	+	ND	−	+	−	−	*C. freundii*

[1] − = negative
[2] + = positive
[3] (+) = weak positive
[4] ND = not done
[5] d = late positive

subspecies II and all 3 subspecies IIIa strains were positive in the β-galactosidase test and (iii) although no citrobacters were lysed by the phage, not all salmonellas were lysed by the phage (Table 4).

In Table 5, a complete battery of tests are set out which can be used to differentiate *Citrobacter* from *Salmonella,* as well as the differentiation of the different *Salmonella* subspecies from each other (Le Minor *et al.,* 1982a). Lysis by phage, glycerol fermentation, lysine decarboxylation, the ONPG test and sorbose and galacturonate fermentations are the major tests. These tests, in combination, facilitated the biochemical differentiation of lactose-negative, H_2S-positive *Citrobacter freundii* from *Salmonella* subspecies I and II. These tests will also help in the differentiation of *Salmonella* IIIa and IIIb and in the identification of subspecies IV, V and VI. It must be stressed that the identification of *Salmonella* of subspecies I, II and III which have antigens for which potent typing antisera are available in a laboratory is not difficult, nor is it difficult to differentiate them from *Citrobacter freundii.* The identification of *C. freundii* which has biochemical reactions different from *Salmonella,* also pose no problems. The identification of *C. amalonaticus* and *C. diversus* also poses no problems, as they are H_2S-negative, indole-positive, ferment lactose and sucrose (Brenner, 1984).

The phage lysis test was only of significance in the differentiation when the reaction is positive (Tables 4 and 5). When no lysis occurred, such a strain can either be a *Salmonella* or a *Citrobacter.* Not all of the *Salmonella* serovars are lysed by the phage, and different isolates of the same serovar can also differ in their susceptibility to lysis (Cherry, Davis, Edwards & Hogan, 1954; Fey, Bürgi, Margadant & Boller, 1978; Gunnarsson, Hurvell & Thal, 1977; Bockemühl, 1972; Fey, Margadant & Lozano-Gutknecht, 1971). A very small percentage of other bacteria like *Escherichia coli* and *Citrobacter,* can also be lysed by

TABLE 5 Tests useful in the differentiation between the biochemical subspecies of *Salmonella enterica* and *Citrobacter freundii*

Tests	*Citrobacter freundii*	*S. enterica* subspecies						
		I *enterica*	II *salamae*	IIIa *arizonae*	IIIb *diarizonae*	IV *houtenae*	V *bongori*	VI *indica*
Lysis by phage	−	+	+	−	+	−	+	+
Glycerol fermentation 24 h	+	d	−	−	−	−	−	−
Lysine decarboxylase 24 h	−	+	+	+	+	+	+	+
Lactose fermentation	d	−	−	−	−	−	−	−
β-galactosidase	d	−	d	+	+	−	+	d*
Dulcitol fermentation	d	+	+	−	−	−	+	d
Malonate fermentation	(−)	−	+	+	+	−	−	−
Sorbose fermentation 72 h	(+)	−	−	−	−	−	−	−
Gelatinase production	−	−	(+)	+	+	+	−	+
d- + 1-tartrate fermentation	NK	+	−	−	−	−	−	−
Mucate fermentation	+	+	+	d	+	−	+	+
Salicin fermentation	d	(−)	(−)	−	−	d	−	−
Sorbitol fermentation	+	+	+	+	+	+	+	−
Galacturonate fermentation	+	−	+	−	+	+	+	+
γ-glutamyltransferase	+	(+)*	(+)*	−	(+)*	(+)*	+	+
β-glucuronidase	−	d*	d*	−	d*	−	−	d
KCN growth	+	−	−	−	−	+	+	−

\+ = 90–100 % of strains positive
(+) = 76–89 % of strains positive
d = 26–75 % of strains positive
NK = not known
(−) = 11–25 % of strains positive
− = 0–10 % of strains positive
* = depends on the serovar

this phage (Fey *et al.*, 1971; Takàcs & Nagy, 1973). All of this should be kept in mind when this test is read.

A number ($\frac{3}{7}$) of *Salmonella* isolates fermented glycerol (Table 4), but could be typed as salmonellas and were lysed by the phage. According to Richard & Popoff (1985), *Citrobacter* used this substance in 3 days and *Salmonella* were negative or positive only after 3–7 days. Glycerol fermentation is a valuable test in the differentiation of *Salmonella* and *Citrobacter,* but should be read after 24 h, to exclude the salmonellas which can ferment this substance after longer incubation.

Lactose fermentation (as determined by all 4 tests) was negative for all *Salmonella* subspecies I isolates. Of the 18 subspecies II isolates, only 1 strain was lactose-positive as determined by the ONPG and 5 % lactose tests. A positive ONPG reaction can be observed for a small number of subspecies II isolates (Le Minor *et al.*, 1982a). *Salmonella* of subspecies III are ONPG-positive and can be late or irregular lactose fermenters, as was the case with 2 strains in Table 4.

Citrobacter can show prompt, late or irregular lactose-positive reactions and may even be negative. In our study $\frac{1}{8}$ citrobacters did not attack lactose during the examination period, using the 4 tests. Lapage & Jayaraman (1964) also, using the same tests, found citrobacters which did not ferment lactose. These lactose-negative citrobacters are problematic to differentiate from *Salmonella,* when this characteristic alone is used. It is believed that the ONPG test is more sensitive than lactose fermentation (Le Minor, Coynault & Guiso, 1977; Lapage & Jayaraman, 1964), because the ONPG test tests only for the presence of the enzyme which splits lactose. Of the $\frac{7}{8}$ citrobacters which were lactose-positive (as determined by any of the 4 tests), 1 strain was only ONPG-positive and another isolate was ONPG-positive and late positive in the ordinary lactose fermentation test.

The use of the 1 % and 5 % lactose fermentation tests as opposed to the usual lactose test or the ONPG test did not result in more positive reactions (Table 4). These results were also found by Lapage & Jayaraman (1964). The most sensitive method for lactose fermentation would be the use of the ONPG test.

Sorbose is not a sugar generally used in the differentiation of Enterobacteria. It can be valuable in differentiating *Salmonella* and *Citrobacter.* Stenzel (1976) found in his study that 75 % of citrobacters fermented this sugar in 24 h, while most salmonellas were negative after 3 days. In this study no salmonellas used this sugar, while $\frac{6}{8}$ citrobacters fermented this sugar in 24 h (Table 5). This test, when positive, can thus be used in the differentiation between *Salmonella* and *Citrobacter.* However, as in the case of phage lysis, a negative reaction is of little value.

According to Brenner (1984), 90–100 % of *Citrobacter freundii* and *Salmonella* subspecies I, II and III do not ferment salicin. In this study (Table 4), a small number of isolates belonging to these groups (except for *Salmonella* subspecies III isolates) fermented this sugar. Salicin fermentation can be used to identify *Salmonella* subspecies IV isolates (Table 5). Only salmonellae of subspecies VI do not ferment sorbitol (Table 5) and this sugar can thus be used in that differentiation.

In this study, a high number of *C. freundii* isolates used malonate ($\frac{3}{6}$). This is contrary to the findings of Brenner (1984), where 0–10 % of *C. freundii* strains were found to be positive. These malonate-positive citrobacters could easily be differentiated from the malonate-positive *C. diversus.* The malonate-positive *C. freundii* had been confused with *Salmonella*

subspecies II isolates when biochemical differentiation as set out in Table 1 was used. Using the criteria in Table 5, this confusion did not result.

Mucate cannot be used to differentiate *Citrobacter* and *Salmonella* (Tables 4 and 5), but in the genus *Salmonella* it can be used in the case of subspecies IV isolates. The use of d- and 1-tartrates can be used to identify *Salmonella* subsepecies I (Tables 4 and 5), but not in *Citrobacter* identification.

Galacturonate fermentation is a very valuable test in the differentiation of *Salmonella* subspecies I and IIIa from *Citrobacter*. *Citrobacter* and *Salmonella* subspecies II, IIIb and IV ferment this sugar, while subspecies I and IIIa do not (Le Minor *et al.,* 1979). These same results were obtained from the strains examined in Table 4. γ-glutamyltransferase and β-glucuronidase can be used to differentiate *Citrobacter* from *Salmonella,* and to differentiate the subspecies of *Salmonella* (Table 5). These tests have, however, limitations in these differentiations, as the results of these tests are often based on the serovar (Table 5) (Giammanco *et al.,* 1980; Le Minor *et al.,* 1978). These 2 tests can be very helpful in the differentiation of subspecies IIIa and IIIb serovars, when the antisera necessary for typing the flagellar antigens are not available.

By using the tests set out in Table 5, it is possible to get satisfactory differentiation between *Salmonella* and *Citrobacter freundii,* not based on the lysine decarboxylation test alone but on a number of other tests too. Glycerol, galacturonate and sorbose fermentations, the ONPG test and phage lysis together with lysine decarboxylation are the most valuable tests to use. Glycerol fermentation by *Salmonella* and a possible sensitivity of *Citrobacter* to the phage, should be taken into account, as the results of these 2 tests are important in the differentiation.

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