On the Antigenic Relationships of Certain *Citrobacter* and *Hafnia* Cultures

BY J. SEDLÁK AND MARIE ŠLAJSOVÁ

*Institute of Microbiology, Medical Faculty of Hygiene, Charles
University, Prague, Czechoslovakia*

(Received 26 October 1965)

SUMMARY

The biochemical and serological characters of 36 unclassified cultures are described, and according to these characters the organisms belong to the genera *Citrobacter*, *Aerobacter* and *Hafnia*. While studying the antigenic relationships of the O antigens the known antigenic relationship between some serotypes of *Citrobacter* and *Salmonella* was confirmed; new antigenic relationships with *S. locarno*, *S. kentucky*, *S. uccle* and *S. tranoroa* are described. The antigenic relationships of the *Hafnia* cultures with *Shigella flexneri* 4a and with two cultures with a provisional O group of *Citrobacter* were confirmed.

INTRODUCTION

The antigenic relationship between the genus *Citrobacter* and the genera *Salmonella*, *Arizona*, *Escherichia* and *Shigella* have been relatively thoroughly described, mainly in the monographs of Kauffmann (1941, 1954, 1961), Edwards & Ewing (1955, 1962), Sedlák & Rische (1961), Kampelmacher (1959). Much less is known about the antigenic relationship between the other genera of this family, Enterobacteriaceae.

The present work aims to improve our knowledge about the antigenic relationships of some of the less common enteric bacteria. We believe that knowledge of these relationships and their further systemic study will lead to a decrease of false diagnoses made on biochemical grounds. The antigenic diagnostic schemes for the groups *Salmonella* (Kauffmann, 1964), *Arizona* (Edwards, West & Brunner, 1948), *Citrobacter* (West & Edwards, 1954), as well as *Escherichia coli* (Kauffmann, 1954) form the basis for a further systematic study of relationships that are important both practically and theoretically.

METHODS

Cultures studied. The biochemical and antigenic properties of 36 cultures of *Citrobacter* were studied. All these strains were isolated from human pathological material during the period 1936–65. Their designations and origin are presented in Table 1.

Strains *Salmonella coli* 1–5, together with *S. ballerup* (Vi, z₁₄) and *S. arizona* (xxxiii: z₄z₂₃z₂₈) were originally listed by Kauffmann (1941) in the 1940 edition of the Kauffmann–White scheme and only later removed. *S. arizona* has become the test culture for serological group O1 of the present genus *Arizona* (Edwards, West &

Brunner, 1948) and *S. ballerup* one of the test cultures of the former 'Ballerup group' (Brunner, Edwards & Hopson, 1949), and is at present the test culture for the serological group O 29 of *Citrobacter* (West & Edwards, 1954).

Table 1. *Designation and origin of the cultures used*

Origin. designation of cultures	Originally described or identified as	Received from
2624/36	<i>S. coli</i> 1 (XXXI Vi...1, 5)	Prof. Dr F. Kauffmann, International Salmonella Centre, Statens Serum Institut, Copenhagen, Denmark
5396/38	<i>S. coli</i> 1 (XXXI...1, 5)	
491/36	<i>S. coli</i> 2 (XXXII...1, 5)	
5821/38	<i>S. coli</i> 2	
'Zurich'	<i>S. coli</i> 3 (IV, V, XII...Z ₂₀)	
'ka'	<i>S. coli</i> 4 (IV, XXVII, XII...Z ₂₁)	Prof. Dr E. van Oye, National Salmonella- Shigella Centre, Brussels, Belgium
'B'	<i>S. coli</i> 4	
LG 1099	<i>Citrobacter</i> (Sa O 3, 10, 19)	
BR 184/6	<i>Citrobacter</i> (Sa O 8, 20)	
Ci 47007	<i>Citrobacter</i>	Dr Lányi, State Institute of Hygiene, Budapest, Hungary
Ci 47009, 47010		
Ci 47012, 57015		
Ci 47018, 47020		
H 8997, H 9004,	<i>Hafnia</i>	Dr E. Aldová, National Shigella Centre, Institute of Epidemiology and Micro- biology, Prague, Czechoslovakia
H 9011, H 9012,		
H 9013, H 9009,		
H 9020, H 9022,		
H 9024, H 9025,		
H 9008, H 9027		
P 6b	<i>E. coli</i> O 67	Dr F. Ørskow, International Escherichia Centre, Statens Serum Institut, Copen- hagen, Denmark
P 11c	<i>E. coli</i> O 72	
H 810a	<i>E. coli</i> O 94	
W 40	<i>E. coli</i> O 122	

Cultures P 6b, 11c, H 810, W 40 are provisional test cultures of *Escherichia coli* serological groups O 67, O 72, O 94 and O 122 (Kauffmann, 1954), but have been characterized by Kauffmann as '...atypical in that they are indole negative, H₂S positive (ferric chloride gelatin) and ammonium citrate positive, i.e. they belong to the Bethesda group (or to *Escherichia freundii*)' (Kauffmann, 1954, p. 178). The other cultures were isolated by us much later (1963–65) and have not been previously described. The antigenic relationship of strains LG 1099 and 184/6 with *Salmonella anatum* and *S. kentucky* was shown by E. van Oye.

For the biochemical identification of the cultures the methods and culture media recommended by the *Enterobacteriaceae Subcommittee* (Reports, 1958) or by Kauffmann (1941, 1954), Edwards & Ewing (1955, 1962), or Kauffmann & Petersen (1956) were used.

Serological examination was done by the methods of slide agglutination and agglutination in test-tubes. Antigenic relationships were studied by using cross-agglutination and in some cases also absorption tests with the respective anti-O or anti-H sera of our own make.

After determination of the O antigens of our *Citrobacter* strains and their allocation to one of the serological O groups, we had at our disposal all the necessary

Table 2. Biochemical properties of the organisms studied

[illegible]

The results of the biochemical tests are indicated in degree by +, +, (+) —. The numbers e.g., +⁷, indicate the day on which delayed reactions became positive.

anti-O sera according to the original antigenic *Citrobacter* scheme of West & Edwards (32 serological O groups); in addition to these we had a further eight of our own anti-O sera (provisional new groups O 33–O 40; Table 4).

RESULTS

All the cultures corresponded to the basic taxonomic definition of the family Enterobacteriaceae in *Bergey's Manual* (1957) and to the definition of one or other of two genera of this family, i.e. *Citrobacter* or *Enterobacter*.

Table 3. *Antigenic relationships*

Designation of cultures	Serological O groups			
	<i>Citrobacter</i>	<i>Salmonella</i>	<i>Shigella</i>	<i>E. coli</i>
	West & Edwards O 1–O 32 prov. 033–041	Kauffmann–White A–O 60		(O 1–O 144)
<i>Citrobacter</i>				
2624/36 } (<i>S. coli</i> 1)	—	O 48 (ident.)	—	—
5396/38 }		(<i>S. djakarta</i>)		
491/36 } (<i>S. coli</i> 2)	—	H 1, 5	—	—
5821/38 }				
'Zürich', (<i>S. coli</i> 3)	O 22	O 4, 5 (<i>S. paratyphi</i> B)	—	—
'B', 'ka' (<i>S. coli</i> 4)				
Ci 47004				
Ci 47001 }	O 12	O 57 (<i>S. locarno</i>)	—	—
Ci 47018 }				
Ci 47019 }				
LEO 429 }	Prov. O 36 (151)	—	—	O 122
w 40 }				
Br 184/6	Prov. O' 88 (138 A)	O 8, 20 (<i>S. kentucky</i>)	—	—
LG 1099 }	Prov. O 37 (153)	O 3, 10, 19 (<i>S. anatum</i>)	—	—
Ci 47013 }				
Ci 47012	—	O 54 (<i>S. uccle</i>)	—	—
Ci 47010	—	O 55 (<i>S. tranoroa</i>)	—	—
Ci 47002	—	VI	—	—
P 6b	—	—	—	O 67 (ident.)
P 11c	—	—	—	O 72 (ident.)
H 310a	—	—	—	O 94 (ident.)
<i>Hafnia</i>				
H 8997 }	Prov. O 37 (153)	—	<i>S. flexneri</i> 4a	Ec (O 1), O 13
H 9009 }				
H 9008, H 9011 }	—	—	<i>S. flexneri</i> 4a	—
H 9004, H 9020 }				
H 9025, H 9027 }				
H 9012, H 9013 }				
H 9024 }				

The biochemical characteristics of all the cultures are shown in Table 2. It is obvious from this Table that 24 of the cultures can, by their biochemical properties, be identified as typical members of the genus *Citrobacter*. Small deviations from the existing definition of the genus *Citrobacter* (Edwards & Ewing, 1962) were shown by

the indole-positive variant 5896/38 *Salmonella coli* 1, and the anaerogenic variants 'Zürich' *S. coli* 3, 491/36 *S. coli* 2, 'Ka' *S. coli* 4, Ci 47004 and Ci 47018. Only five cultures fermented sodium malonate and five cultures salicin.

Production of hydrogen sulphide (H_2S) was detected in all cultures after 24 hr incubation on lead acetate agar and in 14 cultures also on TSI agar.

All the other cultures belong to the genus *Aerobacter* (*Bergey's Manual*, 1957; Edwards & Ewing, 1962). Twelve of them could be included in the *Hafnia* group (Møller, 1954; Edwards & Ewing, 1962) and one of these (Ci 47014) corresponds to the definition of the species variously known as *Aerobacter cloacae* or *Cloaca cloacae* (Kauffmann, 1954), *Enterobacter* (Hormaeche & Edwards, 1959) or *Aerobacter* group A (Edwards & Ewing, 1962). In none of the cultures was H_2S production detected on TSI agar, not even after prolonged incubation. The production of small quantities of H_2S could be proved only on lead acetate agar after prolonged incubation at 22°. After incubation at 37° the MR and VP tests did not give clear positive or negative results. The results of these tests reported in Table 2 were after incubation at 22°.

Table 4. *Antigenic properties of 10 new provisional O-type cultures*

Original designation of studied cultures	O-type culture of provisional serological groups O 33–O 42 (relationship of O antigen)	Further serologically identical strains
P 6b	O 33 (<i>E. coli</i> O 67—)*	—
P 11c	O 34 (<i>E. coli</i> O 72—)*	—
H 310a	O 35 (<i>E. coli</i> O 94—)*	—
Leo 429	O 36 (<i>E. coli</i> O 122)*	KW 151, w 40
2624/36 (<i>S. coli</i> 1)	O 37 (<i>S. djakarta</i> —O 48)	5896/38 (<i>S. coli</i> 1)
BR 184/6	O 38 (<i>S. kentucky</i> —O 8, 20)	KW 138 A
LG 1099	O 39 (<i>S. anatum</i> —O 3, 10)	KW 153
Ci 47007	O 40 (<i>S. locarno</i> —O 57)	—
Ci 47010	O 41 (<i>S. tranoroa</i> —O 55)	—
Ci 47012	O 42 (<i>S. uccle</i> —O 54)	—

* These cultures have in the meantime been excluded from *E. coli*-type cultures.

The results of serological examinations are summarized in Table 3. In this table the antigenic relationships of all the cultures are expressed and in the case of *Citrobacter* cultures also their respective serological O groups according to the scheme of West & Edwards. All the *Citrobacter* strains were tested with *Salmonella* O sera 1–60, *Shigella* sera A I, A II, B, C I, C II, D, and *coli* sera O 1–144, as well as *Citrobacter* O sera 1–32. Some of the strains did not agglutinate in any of them and are not included in Table 3. The results shown in this Table are in complete agreement with the earlier known antigenic relationships of the genera *Citrobacter* and *Salmonella* (Edwards & Ewing, 1962; Kauffmann, 1941) as well as with the original statement of Kauffmann (1941) concerning the so-called *Salmonella coli* 1–4 cultures.

The antigenic relationship of the further *Citrobacter* cultures with *Salmonella locarno*, *S. kentucky*, *S. anatum*, *S. uccle*, *S. tranoroa*, *Shigella flexneri* 4a and *Escherichia coli* O 122 have, according to literature available to us, not yet been described. One of the *Citrobacter* strains (Ci 47002) with a Vi antigen is interesting. Its somatic antigen is entirely different from all other known *Citrobacter* O antigens including those of groups O 5 and O 29 (Ballerup group), which usually possess

Vi antigen (Kauffmann, 1954; West & Edwards, 1954). The antigenic relationship of another *Citrobacter* culture (47018, provisional O 37), with *S. flexneri* 4a and the very near relationship of *Citrobacter* strain Leo 419 (provisional O 36) with test strain *E. coli* O 122 also deserves mention.

In 11 strains of the *Hafnia* group very close antigenic relationship could be proved with *Shigella flexneri* 4a as was pointed out by van Oye & Ghysels (1961). Apart from that, antigenic relationship of two other *Hafnia* strains with *Citrobacter* of the provisional O group 37 was found.

DISCUSSION AND CONCLUSION

We have tried to show that by using suitable biochemical and serological methods differentiation of *Citrobacter* as well as *Hafnia* and *Aerobacter cloacae* strains within the framework of the family Enterobacteriaceae is feasible. Further, that systematic study of the antigenic relationships of the less-frequently isolated species of enteric bacteria has its theoretical as well as practical importance. In this connexion we met a well-known and often discussed problem, the taxonomic value of the MR and VP tests and H_2S production, which are of basic significance and importance in the taxonomy of the family Enterobacteriaceae.

It is well known that the results of MR and VP tests are significantly influenced by many factors; for example, the relative proportion and purity of the reagents, the size of the inoculum and the temperature and duration of the incubation (Kauffmann, 1956; Suassuna, Suassuna & Ewing, 1961; Hugh, 1965).

In the H_2S test there is a considerable difference in the sensitivity depending on the medium used (TSI agar, ferrous chloride gelatin, lead acetate agar, lead acetate test papers). The most sensitive indicator is lead acetate paper, but because of this quality it loses its diagnostic value, and even some *Shigella* strains will give slight and late positive results when grown on suitable media. Lead acetate agar is less sensitive, but with this it is possible to exclude the weakest H_2S ; for example, some strains of *Salmonella typhi*, *S. choleraesuis*, *S. gallinarum-pullorum*, *Citrobacter*, *Enterobacter* and *Hafnia*. The sensitivity of ferrous chloride gelatin and TSI agar and all their known modifications is practically equal; they show only the production of relatively larger quantities of H_2S , as is usually produced by the majority of *Salmonella*, *Arizona*, *Citrobacter*, *Proteus vulgaris*, *P. mirabilis* cultures and perhaps by the newly described *Edwardsiella* strains (Ewing, Worther, Escobar & Lubin 1965). Kauffmann (1954) used ferrous chloride gelatin; Edwards & Ewing (1962) used TSI agar and stated: '... this medium corresponds very closely to the classical descriptions of the various groups of enteric bacteria...'. It is essential to know what culture medium was used for H_2S detection, and the results obtained by two methods (TSI agar, and lead acetate agar) are shown in Table 2.

In the genus *Citrobacter* we consider the extension and completion of the existing antigenic scheme of West & Edwards (1954) to be a logical aim. From our experience we are convinced that this is the only way to reach the correct conclusions on the part played by the different serological types of *Citrobacter* in the diarrhoeal diseases of man and animals (Sedlák & Rische, 1961). Our view of the *Hafnia* group is the same, and we agree with Dr E. van Oye (personal communication) who considers these cultures to be a likely cause of human dysentery.

The conclusions of our taxonomic study of 36 cultures of less common of Enterobacteriaceae can be summarized as follows:

The cultures described originally by Kauffmann (1941) as *Salmonella coli* 1-4 are typical *Citrobacter* cultures. Strains 'S. coli 3 and 4' belong to O group 22 of the antigenic scheme of *Citrobacter*. Also a further four strains of *Escherichia coli* (O 67, O 72, O 94 and O 122) belong the genus *Citrobacter* and the test strain w 40 (O 122) is very closely related to the *Citrobacter* provisional test strain O 37.

An antigenic relationship has been described for the first time between *Citrobacter* group O 12 and *Salmonella locarno*, *Citrobacter* provisional group O 38 and *S. kentucky*, and *Citrobacter* provisional group O 37 and *S. anatum*; in two further *Citrobacter* strains their relationship with *S. uccle* (O 54) and *S. tranoroa* (O 55) has been newly described.

An antigenic relationship first described by van Oye in 11 *Hafnia* strains with *Shigella flexneri* 4a has been confirmed and a new relationship between two *Hafnia* strains and *Citrobacter* provisional O group 37 described.

The authors are indebted to Dr S. T. Cowan and Dr Joan Taylor for their helpful advice concerning the final shape of this manuscript.

REFERENCES

- Bergey's Manual of Determinative Bacteriology* (1957), 7th ed. Baltimore: Williams and Wilkins.
- BRUNNER, D. W., EDWARDS, P. R. & HOPSON, A. S. (1949). *J. infect. Dis.* 85, 290 (cited by Edwards & Ewing, 1962).
- EDWARDS, P. R. & EWING, W. H. (1955). *Identification of Enterobacteriaceae*, 1st ed. Minneapolis: Burgess Publ.
- EDWARDS, P. R. & EWING, W. H. (1962). *Identification of Enterobacteriaceae*, 2nd ed. Minneapolis: Burgess Publ.
- EDWARDS, P. R., WEST, M. G. & BRUNNER, D. W. (1948). *J. Bact.* 55, 711 (cited by Edwards & Ewing, 1962).
- EWING, W. H., WORTHER, A. C., ESCOBAR, M. R. & LUBIN, A. H. (1965). *Edwardsiella*, a new genus of Enterobacteriaceae based on a new species *E. tarda*. *Int. Bull. Bact. Nomencl. Taxon.*, 15, 33.
- HORMAECHE, E. & EDWARDS, P. R. (1959). Observations on the genus *Aerobacter* with a description of two species. *Int. Bull. bact. Nomencl. Taxon.* 8, 111.
- HUGH, R. (1965). A comparison of the proposed neotype strain and 258 isolates of *Vibrio cholerae* Pacini. *Int. Bull. bact. Nomencl. Taxon.* 15, 13.
- KAMPELMACHER, E. H. (1959). On antigenic relationships between the groups *Salmonella*, *Arizona*, *Escherichia* and *Shigella*. *Antonie van Leeuwenhoek*, 25, 289.
- KAUFFMANN, F. (1941). *Die Bakteriologie der Salmonella Gruppe*. Copenhagen: E. Munksgaard.
- KAUFFMANN, F. (1954). *Enterobacteriaceae*, 2nd ed. Copenhagen: E. Munksgaard.
- KAUFFMANN, F. (1956). *Zentbl. Bakt. Parasit. Kde, I. Orig.* 165, 344 (cited by Sedláč & Rische, 1961).
- KAUFFMANN, F. (1961). *Die Bakteriologie der Salmonella Species*. Copenhagen: E. Munksgaard.
- KAUFFMANN, F. (1964). Das Kauffmann-White Schema. In *The World Problem of Salmonellosis*. Ed. by E. van Oye. Haag: Dr W. Junk.
- KAUFFMANN, F. & PETERSEN, A. (1956). The biochemical group and type differentiation of Enterobacteriaceae by organic acids. *Acta path. microbiol. scand.* 38, 481.
- MÖLLER, V. (1954). Diagnostic use of Braun KCN test within the Enterobacteriaceae. *Acta path. microbiol. scand.* 34, 115.

- VAN OYE, E. & GHYSELS, G. (1961). Antigenic relationship between *Hafnia* and *Shigella flexneri*. *J. Bact.* **82**, 813.
- Reports of the Internat. Subcommittee on Enterobacteriaceae (1958). *Int. Bull. bact. Nomencl. Taxon.* **8**, 1.
- SEDLÁK, J. P & RISCHÉ, H. (1961). *Enterobacteriaceae Infektionen*. Leipzig: Georg Thieme Verlag.
- SUASSUNA, I., SUASSUNA, I. R. & EWING, W. H. (1961). The methyl-red and Voges-Proskauer reactions of Enterobacteriaceae. *Bull. Conf. St. prov. publ. Hlth Lab. Dir.* **19**, 67.
- WEST, M. G. & EDWARDS, P. R. (1954). *Publ. Hlth Monogr.* **22**, (cited by Edwards and Ewing, 1962).