Rev. Inst. Med. trop. São Paulo
19(5):355-359, *setembro-outubro,* 1977

UDC 616.891
616.34-002

H_2S PRODUCING VARIANTS OF ESCHERICHIA COLI FROM ROUTINE FECAL CULTURES

Marcelo MAGALHÃES and Adelma VÉRAS

SUMMARY

During a survey carried out in Recife, Brazil, for recovering lactose fermenting **Salmonella,** an unexpectedly large number of hydrogen sulfide positive variants of **Escherichia coli** were encountered. Such variants (72) were obtained from 3600 human fecal specimens routinely sent for culturing. Among the isolates 37 different biochemical types were found. Single disc susceptibility testing indicated that H_2S positive strains of **E. coli** were much less drug resistant than the H_2S negative ones. Matings have shown that H_2S production was plasmid mediated, and there was an evident correlation between serotypes 0124 and 0128 and plasmidial transfer of H_2S production and raffinose fermenting ability. Moreover, it was also observed a close linkage between Hys and Raf determinants, but an independent transfer of **tet** from H_2S positive and tetracycline resistant donors.

INTRODUCTION

Since many lactose fermenting variants of **Salmonella typhimurium** and **S. oranienburg** have been found in São Paulo, Brazil [12,13] an attempt was made in Recife to determine the incidence of such variants in clinical specimens. Instead of lactose positive salmonellae, a surprisingly large number of hydrogen sulfide producing strains of **Escherichia coli** were encountered.

Over the last few years, there has been an increased interest in H_2S positive strains of **E. coli**. Fermentative and serological studies have indicated the occurrence of many different clones [6,8,12], and the suggestion that an extrachromossomal genetic element (Hys) might direct the H_2S production [9] has definitely been confirmed [8,12,15]. The Hys plasmid could be transferred alone [8], jointly with the tetracycline (Tc) resistance determinant [15] or linked to raffinose (Raf) fermenting ability [12]. The bacteriological and some genetical characteristics of 72 Brazilian strains of H_2S positive **E. coli** are reported here.

MATERIALS AND METHODS

Organisms — The wild-type H_2S positive strains of **E. coli** included in this study were recovered from 3600 human feces routinely submitted for isolation of pathogenic enterobacteria. In matings, **E. coli** K12 F- rif^r (K12) was used as recipient. For Fi testing **E. coli** K12 HfrH nal^r and the F-specific phage μ^2 were used. These standard microorganisms were kindly supplied by Dr. E. S. Anderson of the Enteric Reference Laboratory, London.

Isolation and identification of cultures — In an attempt to isolate lactose positive salmonellae, the Bacto hecktoen enteric agar (HE) was introduced in the standard routine [4]. The specimens were inoculated on this medium directly and after enrichment in Kauffamanns'tetrathionate. Because many rapid lactose fermenting bacteria produce yellow colonies on HE, any yellow or black colored colony was assumed to be a probable lactose positive salmonella and was picked to triple sugar-iron agar (TSI) and Merck lysine--iron agar (LIA). Isolates which produced H_2S

Faculdade de Medicina, Departamento de Microbiologia, Cidade Universitária, 50000 Recife, Pernambuco, Brasil

in LIA and a yellow slant with or without a black precipitate in TSI were submitted for a conclusive biochemical identification [5]. Enteropathogenic serotypes were screened by the slide agglutination technique employing commercial **E. coli** OK antisera (Difco).

Drug susceptibility testing — Susceptibility tests were done by the single disc method [2]. Ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline, and sulphamethoxazole were the drugs used. For comparative purposes 60 H_2S negative fecal strains of **E. coli** were also examined.

Matings — H_2S-positive strains of **E. coli** that were noncolicinogenic and either fermented raffinose or were resistant to tetracycline were selected for transfer studies. Strains harbouring only the Hys character were not tested due to the lack of a suitable selective marker. Colicinogeny was studied by the agar-overlay method [9], using K12 as the indicator strain. Donor and recipient were grown overnight in brain heart infusion and mixed in a ratio of 1:10 for 6 h crosses. Mating mixtures were washed three times with saline and streaked on selective media. These included plates of MacConkey agar supplemented with tetracycline (20 mg/l) and rifampicin (50 mg/l) or a minimal raffinose rifampicin medium (Simmons' citrate agar with 5 g of raffinose and 50 mg of rifampicin per liter). Seeded plates were incubated at 37C for 48h, and the frequency of transfer was calculated relative to the number of donors. Master plates containing purified transconjugant colonies were prepared and replicated on to appropriate selective media to examine linkage among Hys, Raf and Tc governing genes. To test for the Fi character of the plasmids the method given by ANDERSON & TRELFALL [1] was used.

RESULTS

Incidence of H_2S positive E. coli — During an 8-month period, 3600 fecal specimens were submitted for culturing. Of these, 72 (2%) yielded H_2S positive strains of **E. coli.** All the strains were isolated from HE plates streaked directly.

Cultivation characteristics — On HE agar, individual colonies may resemble certain rapid lactose fermenting strains of **Citrobacter,** that is, they present a pale yellow tone more evident when the plates are observed on the reverse, in contrast to the salmon colored colonies of the H_2S negative **E. coli** strains. In some cases, the central area of the colony was light gray to deep black in color. In LIA all cultures produced H_2S within 24 h, but in TSI only 66.6% of them were able to blacken the medium.

TABLE I

Biotypes of H_2S positive **E. coli**[a]

Lysine	Ornithine	Sucrose	Dulcitol	Salicin	Adonitol	Sorbitol	Arabinose	Raffinose	Rhamnose	Nº of cultures with biotype
+	+	+	+	+	+	+	+	+	+	1
+	+	+	+	+	—	+	+	+	+	9
+	+	+	+	—	—	+	+	+	+	9
+	+	+	—	+	—	+	+	+	+	1
+	+	+	—	—	—	+	+	+	+	1
+	+	—	+	+	—	+	+	+	+	3
+	+	—	+	+	—	+	+	—	—	4
+	+	—	+	+	—	+	—	—	+	2
+	+	—	+	+	—	+	+	—	+	5
+	+	—	+	—	—	+	+	+	+	2
+	+	—	—	+	+	+	—	+	+	1
+	+	—	—	+	—	—	+	—	+	1
+	+	—	—	+	—	+	+	+	+	1
+	+	—	—	+	—	+	+	—	+	1
+	+	—	—	+	—	+	+	—	—	1
+	+	—	—	—	—	+	+	—	+	1
+	+	—	—	—	—	+	+	—	—	1
+	+	—	—	—	—	—	+	—	+	1
+	—	+	—	—	+	+	+	+	+	1
+	—	+	—	+	—	+	—	+	+	1
+	—	+	+	+	—	+	+	+	+	1
+	—	—	+	+	—	+	+	—	+	1
+	—	—	+	+	—	+	+	+	+	1
+	—	—	+	—	—	+	+	+	+	2
+	—	—	+	—	—	+	+	—	+	2
+	—	—	—	+	+	+	—	—	+	4
+	—	—	—	+	—	+	+	—	+	3
—	+	+	—	+	—	+	+	+	+	1
—	+	+	—	—	—	+	+	+	+	1
—	+	—	+	+	—	+	+	—	+	1
—	+	—	—	—	+	+	+	—	+	1
—	+	—	—	—	—	+	—	+	+	1
—	—	+	—	+	+	+	+	+	+	1
—	—	+	—	—	—	+	+	+	+	1
—	—	—	+	+	—	+	+	—	—	1
—	—	—	+	+	—	+	+	—	+	1
—	—	—	—	+	—	+	+	—	+	2

a+ indicates a positive test within 24 — 48 h.

Biochemical tests — All the strains were aerogenic, methyl red positive, produced indol and arginine dihydrolase, and fermented lactose and mannitol promptly. The isolates were uniformly negative in malonate, inositol, cellobiose, urease, phenylalanine and Voges-Proskauer tests, and failed to grow on Simmons' citrate and KCN media. Thirty-seven different biochemical types were found (Table I).

Serotyping — Results of serotyping and the correlation between some serotypes and plasmidial transfer of Hys-Raf determinants are shown in Table II.

TABLE II

Correlation between serotypes and raffinose utilization or the plasmid transfer of Hys-Raf

Entero-pathogenic serotypes	No. of strains examined	No. that utilized raffinose	No. that transferred Hys-Raf	No. of biotypes
0124 B17	6	6	5	3
0128 B12	3	3	3	3
026 B6	3	1	0	3
0127 B8	1	0	—	1
Untypable(a)	59	30	7	34

(a) Five cultures were rough

Drug susceptibility — The results of susceptibility tests of the H_2S positive and the H_2S negative **E. coli** strains are shown in Tables III and IV.

Transfer of plasmids — The results of transfer studies and Fi testing are shown in Table V. The strain PE 1593 was the only culture that transferred both Hys-Raf and Tc plasmids, but at different rates. The resistance plasmid was transmitted at a high frequency (1×10^{-2}) and was Fi-, whilst the "metabolic" plasmid was Fi+ and was transferred at a lower rate (1×10^{-6}).

TABLE III

Results obtained with H_2S positive and H_2S negative isolates in routine susceptibility testing

Drugs	Isolates(a) (%) resistant to	
	H_2S positive	**H_2S negative**
Ampicillin	4.17	11.66
Chloramphenicol	0.00	26.66
Kanamycin	1.40	20.00
Streptomycin	4.17	31.66
Sulpha	5.50	41.66
Tetracycline	18.10	51.66

(a) There were 72 isolates H_2S positive and 60 isolates H_2S negative.

TABLE IV

Isolates of **E. coli** that exhibited resistance to six drugs

Number of R-determinants(a)	No. of isolates (%)	
	H_2S positive	**H_2S negative**
None	56 (77.8)	27 (45.0)
1	11 (15.3)	6 (10.0)
2	2 (2.8)	3 (5.0)
3	2 (2.8)	10 (16.7)
4	1 (1.3)	4 (6.6)
5	0 (—)	7 (11.7)
6	0 (—)	3 (5.0)

(a) Ampicillin, chloramphenicol, kanamycin, streptomycin, sulpha, and tetracycline.

TABLE V

Transfer and Fi grouping of plasmids(a)

Donor groups	Selection on	No. of transfer/No. tested	Markers transferred	Frequency per donor	No. of Fi+/Fi—
Hys-Tc	tetracycline	1/8	Tc	$1x10^{-2}$	1/0
Hys-Raf	raffinose	14/33	Hys-Raf	$1x10^{-6}$ — $1x10^{-2}$	14/0
	tetracycline	1/5	Tc	$1x10^{-2}$	0/1
Hys-Raf-Tc(b)	raffinose	1/5	Hys-Raf-Tc	$1x10^{-6}$	1/0
	raffinose and tetracycline	1/5	Hys-Raf-Tc	$1x10^{-6}$	1/0

(a) Twenty-four strains were not mated because of lack of either Tc or Raf, and 2 because were colicinogenic.
(b) Out of 5 Hys-Raf-Tc strains, only PE 1593 was able to transfer plasmids.

DISCUSSION

Present results have shown that H_2S positive variants of **E. coli** are sufficiently frequent among fecal specimens to merit consideration in enteric bacteriology. Indeed, as previously indicated, it is difficult to estimate how many of such variants are probably being misidentified in clinical laboratories as **Citrobacter** or **Arizona** [6] and, at least in Brazil, as lactose positive **Salmonella.** The large number of H_2S producing strains of **E. coli** encountered in this laboratory was quite fortuitous. In fact, it might be attributed to our interest in lactose positive salmonellae, but the frequency of H_2S positive **E. coli** may increase as a result of a better recognition of their colonies on HE agar. The precipitation of the iron sulfide in TSI, which has been devised as an important screening characteristic for recognizing H_2S positive **E. coli** [3,6,11], was another dubious point taken into account in this survey. Thirty-three percent of the Brazilian isolates failed to blacken TSI, despite the evident blackening in LIA. This apparent discrepancy had been studied before and has been ascribed to solubilization of the iron sulfide as a result of an intense acidification of the medium [16]. Thus, H_2S positive **E. coli,** like **Citrobacter,** may or may not blacken TSI, and often produces yellow colored colonies on HE agar.

The biochemical behaviour of our strains was typical of **E. coli** except for the production of H_2S. Although the rare lysine negative cultures might be taken at first sight for certain indol positive **Citrobacter,** they could be easily differentiated on Simmons'citrate and by fermentation of cellobiose [5]. Indeed, **Citrobacter** is from the biochemical viewpoint the enterobacterium most closely connected to H_2S producing variants of **E. coli,** but preliminary attempts to transfer H_2S production and raffinose fermenting ability from 32 strains of **Citrobacter** into K12 have failed.

Contrasting with a previous report [6] our H_2S positive isolates were unquestionably much less drug resistant than the H_2S negative controls. Perhaps, differences concerning source and collection of cultures are the most probable explanation of the discrepancy, but an occasional incompatibility between the resident Hys and incoming R plasmids, might also occur.

Matings have confirmed the Danish results [12] showing a close association between Hys and Raf. All the isolates that transmitted the Raf character, jointly transferred the ability to produce H_2S. Such markers constitute a single linkage group, since they were inseparable after sub-culturing and were lost simultaneously in spontaneous segregant. As selection for H_2S transfer was not performed, we cannot be sure whether the Hys plasmid would be transmitted alone. A recent paper, however, has indicated that Raf could be transferred in the absence of Hys, often associated with the K88 antigen determinant [14]. On the other hand, a linkage between Hys and **tet** markers in a single conjugative plasmid, like that described elsewhere [15], was not observed. In PE 1593, the only strain which was able to transfer both Hys-Raf and Tc plasmids, results of Fi testing and transfer indicate the occurrence of two distinct and compatible groups of transfer genes; one linked to Hys-Raf and another associated with **tet.** This appears to rule out any influence of tetracycline in selecting H_2S positive strains of **E. coli.** Although the serotypes 0124 and 0128 transferred the Hys-Raf plasmid more efficiently, they presented several biotypes, suggesting that transfer ability is not a characteristic of unique clones within limited serotypes.

RESUMO

Variantes de Escherichia coli produtoras de H_2S isoladas de coproculturas rotineiras

Durante investigação realizada no Recife, Brasil, para determinar a freqüência de linhagens lactose positivas de **Salmonella,** encontrou-se, inesperadamente, grande número de variantes de **Escherichia coli** produtoras de sulfeto de hidrogênio. Essas variantes (72) foram isoladas de 3600 espécimes fecais humanos enviados rotineiramente para cultura. Entre as amostras isoladas, encontraram-se 37 diferentes tipos bioquímicos. Os testes de susceptibilidade às drogas, pelo métodos dos discos, evidenciaram que as linhagens de **E. coli** H_2S positivas são muito menos droga-resistentes que as H_2S negativas. Os cruzamentos mostraram que a produção de H_2S é governada por um elemento extracromossômico, e observou-se marcante correlação entre os sorotipos 0124 e

0128 com a capacidade de transferir o plasmídeo responsável pela produção de H_2S e fermentação da rafinose. Além disso, observou-se íntima ligação entre os genes responsáveis pelos caracteres Hys e Raf, mas transferência independente de **tet** das culturas doadoras H_2S positivas e tetraciclina resistentes.

REFERENCES

1. ANDERSON, E. S. & THRELFALL, E. J. — The characterization of plasmids in the enterobacteria. **J. Hyg.** (Camb.) **72**: 471-487, 1974.

2. BAUER, A. W.; KIRBY, W. M. M.; SHERRIS, J. C. & TURCK, M. — Antibiotic susceptibility testing by a standardized single disc method. **Amer. J. Clin. Path. 45**: 493-496, 1966.

3. BRAUNSTEIN, H. & MLADINEO, M. A. — — **Escherichia coli** strains producing hydrogen sulfide in iron-agar medium. **Amer. J. Clin. Path. 62**: 420-424, 1974.

4. EDEL, W. & KAMPELMACHER, E. H. — **Salmonella** isolation in nine European laboratories using a standardized technique. **Bull. Wld. Hlth. Org. 41**: 297-306, 1969.

5. EDWARDS, P. R. & EWING, W. H. — **Identification of Enterobacteriaceae.** 3rd ed. Minneapolis, Burgess Publishing Co., 1972.

6. DARLAND, G. & DAVIES, B. R. — Biochemical and serological characterization of hydrogen sulfide-positive variants of **Escherichia coli. Appl. Microbiol. 27**: 54-58, 1974.

7. FREDERICO, P. — Colicins. **Ann. Rev. Microbiol. 11**: 7-22, 1957.

8. LAUTROP, H.; ORSKOV, I. & GAARSLEV, K. — Hydrogensulphide producing variants of **Escherichia coli. Acta Path. Microbiol. Scand. (Section B) 79**: 641-650, 1971.

9. LAYNE, P.; HU, A. S. L.; BALOWS, A. & DAVIES, B. R. — Extrachromosomal nature of hydrogen sulfide production in **Escherichia coli. J. Bact. 106**: 1029-1030, 1971.

10. LE MINOR, L.; COYNAULT, C. & PESSÔA, G. — Déterminisme plasmidique du caractère atypique 'lactose positif' de souches de **S. typhimurium** et de **S. oranienburg** isolées au Brésil lors d'épidémies de 1971 a 1973. **Ann. Microbiol.** (Inst. Pasteur) **125A**: 261-285, 1973.

11. MARKER, M. D. & WASHINGHTON II, J. A. — Hydrogen sulfide producing variants of **Escherichia coli. Appl. Microbiol. 28**: 303-305, 1974.

12. ORSKOV, I. & ORSKOV, F. — Plasmid-determined H_2S-character in **Escherichia coli** and its relation to plasmid-carried raffinose fermentation and tetracycline resistance characters. **J. Gen. Microbiol. 77**: 487-499, 1973.

13. PESSÔA, G. V. A. — Sobre a ocorrência de uma variante de **Salmonella typhimurium** fermentadora da lactose. **Rev. Inst. Adolfo Lutz** (São Paulo) **33**: 13-28, 1973.

14. SMITH, H. W. & PARSELL, Z. — Transmissible substrate-utilizing ability in enterobacteria. **J. Gen. Microbiol. 87**: 129-140, 1975.

15. STOLERU, G. H.; GERBAUD, G. R.; BOUANCHOUD, D. H. & LE MINOR, L. — Étude d'un plasmid transférable déterminant la production d'H2S et la résistance a la tétracycline chez «**Escherichia coli**». **Ann. Inst. Pasteur 123**: 743-754, 1972.

16. VÉRON, M. & GASSER, F. — Sur la détection de l'hydrogen sulfuré produit par certains entérobactériacées dans le milieux dits de diagnostic rapide. **Ann. Inst. Pasteur 105**: 524-532, 1963.

Recebido para publicação em 5/7/1976.