Plasmid-determined H₂S Character in *Escherichia coli* and its Relation to Plasmid-carried Raffinose Fermentation and Tetracycline Resistance Characters

Examination of 32 H₂S-positive Strains Isolated During the
Years 1950 to 1971

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SUMMARY

The transmission of the ability to produce hydrogen sulphide was demonstrated in 11 out of 32 H₂S-positive *Escherichia coli* strains collected during the years 1950 to 1971 at the International *Escherichia* Centre (WHO) in Copenhagen. The ability to ferment raffinose was co-transferred in some cases but not in others. Those of the 11 strains which were resistant to tetracycline also transmitted that determinant.

Three strains carrying the H₂S, raffinose and tetracycline resistance characters were examined especially as regards characterization of the transfer factors. It was suggested that one strain contained two compatible *fi*⁻ transfer factors, one carrying the H₂S and raffinose markers, and the other the resistance determinant. In the second strain, an *fi*⁻ factor transferred the resistance to tetracycline and an *fi*⁺ F-like factor was responsible for transfer of the H₂S-raffinose markers. The third strain harboured two compatible *fi*⁺ F-like factors, one transferring the H₂S and raffinose markers and the other the tetracycline resistance. In one case, only the raffinose character was transferred, and the factor responsible for that transfer was incompatible with the factor carrying the resistance determinant. It was thus inferred that in this case the raffinose character had been substituted for the resistance determinant on the factor that normally transfers this latter character.

INTRODUCTION

The finding in Denmark during a period of 14 months of 26 *Escherichia coli* isolates producing hydrogen sulphide has been reported by Lautrop, Gaarslev & Ørskov (1971). Since the strains were of different origin and belonged to different serotypes and fermentation types, it was concluded that they originated from many clones of *E. coli*. Tests in a series of defined H₂S-indicating growth media, each containing one of five sulphur compounds, namely sulphate, sulphite, tetrathionate, thiosulphate or cysteine, showed that the H₂S-positive *E. coli* variants formed hydrogen sulphide from thiosulphate and tetrathionate, in contrast to typical *E. coli*. None of the strains reduced sulphate and sulphite, whereas both variants and typical strains formed H₂S from cysteine. One of the 26 strains was able to transmit the ability to produce H₂S to a recipient in circumstances suggesting that H₂S production was determined by an infectious plasmid. The occurrence of tetrathionate reductase-positive *E. coli* strains has been mentioned by Le Minor (1967). Layne, Hu & Balows (1970) and Layne, Hu, Balows & Davis (1971) reported on two strains behaving

like *E. coli*, apart from their ability to produce H_2S from thiosulphate and to grow in KCN broth and on the finding of extrachromosomal DNA in one of the strains but not in the other. Attempts to transmit the H_2S character were unsuccessful.

In the light of these reports, it was considered worth while to examine the strains collected from many different sources and countries at the International Escherichia Centre (WHO) in Copenhagen during the last 20 years. According to the records, about 250 strains had shown some sort of a blackening in the tubed ferrous chloride gelatine medium, which is the medium used routinely at the Statens Seruminstitut for the detection of H_2S production and gelatin liquefaction. Most of the strains showed only a faint blackening after several months, but a small number had been registered as positive within a period of 1 to 14 days. The strains were retested on a thiosulphate medium, and this paper gives an account of those strains which produced H_2S , and details of the co-transmission of this ability together with fermentation of raffinose and resistance to tetracycline.

METHODS

Bacterial strains are listed in Table 1.

Demonstration of hydrogen sulphide production. The tubed ferrous chloride medium was used for the routine test, the placenta infusion medium containing thiosulphate and ferric citrate for demonstration of H_2S formation in individual surface colonies, and a mineral-base tubed medium with ferrous chloride and one of five sulphur sources (sodium sulphate or sulphite, potassium tetrathionate, sodium thiosulphate and L-cysteine) for characterization of the H_2S production. The media were prepared according to Lautrop *et al.* (1971), except that 25 ml liver autolysate/500 ml placenta broth was added to the placenta infusion plate medium (K. Gaarslev, personal communication). The medium is designated TFPL.

Fermentation tests. These tests were as described by Kauffmann (1966). Bromothymol blue (BTB) plates, with the addition of either 1 % raffinose or lactose, were used when the fermentation of these carbohydrates was examined on solid medium.

Sensitivity to antibiotics. Sensitivity tests were performed on solid medium containing 100 μ g sulphathiazole, 10 μ g tetracycline, 25 μ g chloramphenicol, ampicillin or kanamycin, and 15 μ g streptomycin/ml medium. For sulphathiazole, only blood agar base without peptone was used. The other compounds were added either to the blood agar base medium or to the BTB medium, unless otherwise stated.

Examination for haemolysis. This capacity was tested on 5 % sheep blood agar plates.

Test for colicine production. The method used was that given by Frédéricq (1957).

Serotyping. O and H antigen determinations were performed according to standard procedures (Kauffmann, 1966).

Transfer of plasmids. The method applied was as described by Lautrop *et al.* (1971). Overnight donor (1 ml) and 0.1 ml of recipient broth cultures were mixed in 60 ml broth in a 500 ml Erlenmeyer flask and incubated for 24 h at 37 °C. After dilution, the mixture was plated on appropriate media as indicated by Lautrop *et al.* The percentage of positive recipient colonies was calculated.

Transfer of a chromosomal marker from donor strains containing plasmids. Only a strain derived from test strain of *Escherichia coli* O antigen 100 requiring tryptophane, W3460, was used as recipient in these crosses, which were carried out as described by Ørskov & Ørskov (1966). The sediment of a centrifuged broth culture, which had grown overnight at 37 °C, was suspended in 0.5 ml distilled water and 0.05 ml of each parent was mixed directly on minimal medium (EMS with galactose; Lederberg, 1950).

Table 1. *Escherichia coli* strains used in experiments to characterize transfer factors

D1160	K12	Hfr P4 × 6 <i>met</i> S ^r	D1148	H ₂ S ⁺ raf ⁺ T ^r
D1248	K12	Hfr P4 × 6 <i>met</i> Nal ^r	D1165	w1607 H ₂ S ⁺ raf ⁺ T ^r (D1148)
D1255	K12	HfrC <i>met</i> K ^r	D1169	w1607 H ₂ S ⁺ (D1148)
w1607	K12	F ⁻ <i>met</i> lac S ^r	D1230	D726 H ₂ S ⁺ (D1165)
w3460*	try		D1242	D726 T ^r (D1165)
D726*	<i>his ile</i> S ^r Nal ^r		D1122	H ₂ S ⁺ raf ⁺ T ^r
D1021	H ₂ S ⁺ raf ⁺ T ^r		D1186	w1607 H ₂ S ⁺ raf ⁺ T ^r (D1122)
D1265	D1021 T ^r		D1188	w1607 raf ⁺ (D1122)
D1271	D1021 H ₂ S ⁺ raf ⁺		D1191	w1607 H ₂ S ⁺ raf ⁺ (D1122)
D1261	w1607 T ^r (D1265)†		D1192	w1607 T ^r (D1122)
D1273	w1607 H ₂ S ⁺ raf ⁺ (D1271)		D1212	D726 raf ⁺ (D1188)
			D1221	D726 H ₂ S ⁺ raf ⁺ (D1186)
			D1238	D726 T ^r (D1186)

Indicator strain for phage MS2:

D591 *Escherichia coli* K12 Hfr P4 × 6 *met*

Indicator strains for phage Ifl:

M533 *Salmonella typhimurium* LT2 *cys* D col Ela⁺ Idrd 16D728‡ *Escherichia coli* O40 col Ela⁺ Idrd 16

Indicator strain for colicine producing strains:

Escherichia coli rowS^r, Nal^r, K^r and T^r = resistance to streptomycin, nalidixic acid, kanamycin and tetracycline, respectively.* Derived from test strain of *E. coli* O antigen, 100, H509a.

† Strain designation in parentheses means that this strain had acted as donor strain of the transferred character(s).

‡ Test strain of *E. coli* O antigen 40, H316, to which the col Ela factor and derepressed sex factor Idrd 16 had been transferred.

Sensitivity to sex phages. The F-specific phage MS2 and the I-specific phage Ifl were both supplied by E. Meynell. Phage stocks were prepared from plates with confluent lysis, and preparation and assay of MS2 antiphage serum were carried out according to Adams (1950). Phage sensitivity was tested by the cross brushing (Lederberg, 1947) and agar overlay methods. When no MS2 sensitivity was demonstrated by these methods, the technique described by Meynell & Datta (1966) was used. Bacteria (2.5 to 5 × 10⁸/ml) and phage MS2 at a multiplicity of 2 to 100 were mixed and kept at 37 °C for 8 min. Antiphage serum with high K value was added for neutralization of unabsorbed phage for a period of 10 min at 37 °C, and residual antibody was removed. The culture was assayed for phage and reassayed for increase in phage titre after incubation for 2 h. The K12 Hfr strain P4x6 was used as indicator strain. When no increase in MS2 titre was found and no Ifl sensitivity was observed by the plaque method, examination was carried out for ability of the strain with plasmid to support multiplication of the I-specific phage Ifl according to the method described by Grindley & Anderson (1971), except that the bacteria/phage mixture was not centrifuged after overnight incubation at 37 °C before the phage content was titrated in agar layer. This was because it had been found that too many phages were lost by centrifugation of certain Ifl phage-sensitive strains. The indicator strain used was either the *Salmonella typhimurium* strain M533 col Ela⁺ carrying the derepressed sex factor Idrd 16 or the test strain of the *Escherichia coli* O40 antigen infected with the col Ela factor.

Compatibility test. From donor strains with different characters (H₂S production and raffinose fermentation, tetracycline resistance or only raffinose fermentation) transfers were carried out in the usual manner to the K12 strain w1607 or the O100 strain D726 with and without an already-transferred character from the same donor line. The percentages of

positive recipient colonies were determined to see whether the effect called entry exclusion (Novick, 1969) was demonstrable. Recipient colonies were purified and tested for resident and newly acquired characters, to discover whether both or only one of them were preserved. This phenomenon is designated plasmid incompatibility by Novick (1969) and immunity proper by Meynell & Datta (1969).

Media. In addition to those mentioned above, liquid TYECa medium and solid tryptone plates were used for phage tests, as described by Meynell & Datta (1966). The overlayer for phage MS2 was an 0.6 % tryptone agar, while the Ifl plaques were better developed if 0.35 % agar was used in the overlayer.

Assay for repressor function of the transferable plasmids. The influence on the F factor was examined by testing the MS2 sensitivity of K12 Hfr P4 x 6 or HfrC cultures to which the various plasmids had been transmitted.

Elimination of plasmids by acriflavine. Treatment with acriflavine was performed according to Hirota (1960).

RESULTS

General description of the hydrogen sulphide-producing strains

Two hundred and fifty-three strains were examined on TFPL medium. Thirty-two formed colonies with a central black precipitate, thus demonstrating formation of H₂S from thiosulphate. Twenty-six of the 32 strains exhibited all the characters of *Escherichia coli*, but six strains deviated in one or two respects. Thus, four strains did not ferment lactose on the first day but on the fourth to the eighth day. All these strains were isolated from swine and received from two different laboratories in West Germany (Dessau and Stuttgart). In addition, the strain from Stuttgart did not produce indole. The remaining two deviating strains fermented inositol, and one of them was also indole-negative. Both these strains were isolated from horses in Australia. These deviations did not influence the labelling of all 32 strains as H₂S-producing *E. coli* strains.

The 32 strains originated from six countries outside Denmark and 28 of them were isolated from animals. They belonged to 22 different serotypes (combination of O and H antigen types) and two were non-motile and could not be typed as to O antigen. When tested in the medium with different sulphur compounds, all 32 strains formed H₂S from thiosulphate and all but three from tetrathionate (see Table 2). Sulphate was not reduced by any of the strains, but 12 of 15 strains originating from hens in India (representing eight serotypes), and three strains from hens in Sweden (representing three serotypes), reduced sulphite under the conditions used. The black precipitate was not as heavy in the sulphite as in the thiosulphate and tetrathionate media and was not formed until the fifth to the seventh day. None of the strains which did not originate from a hen showed this reaction in sulphite. Ordinary *E. coli* strains form H₂S in the cysteine medium (Lautrop *et al.* 1971) and so did all the 32 strains described here, with the exception of one strain isolated from man.

Transfer of the hydrogen sulphide-producing capacity

Twenty-eight of the 32 H₂S-producing strains were sensitive to streptomycin and were tested for donor ability of the H₂S character in matings with the K12 strain, W1607 *met*^{str}^r, as recipient. With the four streptomycin-resistant strains, the recipient, D726, was a streptomycin- and nalidixic acid-resistant mutant of the test strain of *Escherichia coli* o antigen 100 requiring histidine and isoleucine. A transfer experiment was not recorded as negative unless up to about 5000 single recipient colonies had been examined for the presence of a black precipitate on the TFPL medium containing streptomycin or nalidixic acid. Eleven of the

Table 2. *Production of H₂S in media with different sulphur compounds*

H ₂ S strains			Sulphur source				
Country	Number	Origin	Sulphate	Sulphite	Tetra- thionate	Thio- sulphate	L-Cysteine
India	12	Hen	—	(+)	+	+	+
	2		—	—	+	+	+
	1		—	—	—	+	+
Sweden	3	Hen	—	(+)	+	+	+
	2	Pig	—	—	+	+	+
	1	Man	—	—	+	+	—
	2	Man	—	—	—	(+)	+
West Germany	4	Pig	—	—	+	+	+
Bulgaria	2	Pig	—	—	+	+	+
Australia	2	Horse	—	—	+	+	+
Great Britain	1	?	—	—	+	+	+
Normal <i>Escherichia coli</i>			—	—	—	—	+

(+) A weak black precipitate.

Table 3. *Strains transmitting H₂S and other characters*

Donor strains		H ₂ S ⁺ recipients after mixed growth with donor (%)	Raffinose		Tetracycline		Sucrose fermenta- tion
Country, designation, year of isolation	Serotype		Fermenta- tion	Transfer	r or s	Transfer	
India							
D1025-1966	0?:H7	0·1	+	—	s	.	+
D1021-1966	020:H ⁻	70·0	+	+	r	+	(+)
D1022-1966	020:H ⁻	95·0	+	+	r	+	(+)
D1152-1966	020:H ⁻	87·0	+	+	r	+	—
D1024-1966	0115:H7	0·1	+	—	s	.	+
D1032-1968	070:H11	0·02	—	.	s	.	—
D1148-1970	036:H34	67·0	+	+	r	+	+
Sweden							
D1019-1952	(09):H ⁻	0·2	(+)	—	s	.	+
D1073-1962	0?:H14	15·0	+	+	s	.	+
D1122-1962	02:H31	0·2	+	+	r	+	+
D1121-1962	073:H18	6·0	+	—	s	.	—

(+) In connexion with fermentation of raffinose or sucrose means delayed fermentation.

32 strains, or nine of the 24 serotypes, were able to transmit the capacity to form H₂S, and when a H₂S-positive recipient culture was tested in the tubes with different sulphur sources they behaved in the same way as the corresponding donor strain. Since no inhibition of non-H₂S-forming recipient colonies was possible, transfer of a lower frequency than 1 out of 5000 might have been undetected.

The 11 donor strains belonged to nine serotypes, and could be considered as representing at least nine clones, since three of the strains of the same serotype, 020:H⁻ (all originating from hens) were sent to us by the same person, Dr M. S. Sethi, India, at the same time and thus might be epidemiologically related. One H₂S-donor strain had been isolated from man, but all the remaining ten originated from hens (or the yolk sac of a developing chick

embryo). The percentage of H_2S -positive recipient colonies varied from about 0.02 % to 95 % (see Table 3). After purification of H_2S -positive recipient colonies one or, where possible, two cultures from each experiment were examined for all the usual fermentation characters, for sensitivity to antibiotics, and for the auxotrophic and serotype markers possessed by the recipient.

Transfer of the ability to ferment raffinose and resistance to tetracycline

Nine of the 11 H_2S donor strains (or seven of the nine serotypes) fermented raffinose promptly and one on the third day. Six of the nine strains had transmitted this character to w1607 with selection for the H_2S marker, while three strains representing three serotypes had not done so (see Table 3). The test for raffinose fermentation had only recently been introduced as routine test in this laboratory, but after examination of the first 500 strains it was obvious that fermentation of raffinose was correlated with fermentation of sucrose. Raffinose is a trisaccharide which could also be designated an alpha-galactoside of sucrose. Twenty-one per cent of the 500 strains were negative and 75 % were promptly positive in both tests. Only 3 % were positive in raffinose and negative or later positive in sucrose, and two strains (0.4 %) were negative in raffinose and positive in sucrose. Among the H_2S -donor strains, five of the seven raffinose positive serotypes fermented sucrose on the first day. Three of the five raffinose-sucrose positive serotypes (or strains) transferred the raffinose character, but an ability to transmit the sucrose character was not demonstrated.

Test for sensitivity to antibiotics showed that five of the 11 H_2S -donor strains (or three of the nine serotypes) were resistant to tetracycline and transferred this character (Table 3). With the exception of the four streptomycin-resistant strains they were sensitive to the other antibiotics tested (see Methods). None of the H_2S -donor strains produced haemolysin or any colicine to which strain ROW was sensitive.

Loss of transmissible characters

Platings from the stab cultures in which the H_2S -donor strains had been maintained for several years (since their arrival) showed spontaneous loss of the H_2S character in a few cases. D1019, which fermented raffinose on the third day, kept this ability when the H_2S character was lost. Another strain, D1121, was promptly raffinose-positive and this ability was lost together with the H_2S character. In spite of this simultaneous loss, the strain did not transmit the ability to ferment raffinose with the H_2S marker. One hundred w1607 cultures which had received the H_2S markers from D1211 were all raffinose negative.

All H_2S donor strains were examined for elimination of the transmissible characters by acriflavine, but none was eliminated. From one strain, D1021, which was H_2S -raffinose-positive and resistant to tetracycline, colonies were isolated which had lost either the H_2S -raffinose character or the resistance determinant. The colonies were found on plates cultured from both the acriflavine and the control broth.

Further investigation of three donor strains

Three donor strains, all carrying transferable H_2S and raffinose- and tetracycline-resistant characters, were investigated further to see whether segregation occurred during transfer and, if so, whether more than one transfer factor could be demonstrated by testing for differing ability to inhibit the F factor (f_i^+ or f_i^-) (Watanabe, Nishida, Ogata, Arai & Sato, 1964), and whether they determined the production of F-like or I-like fimbriae (for a review, see Meynell, Meynell & Datta, 1968).

The three strains D1021, D1148 and D1122 were mated with w1607, and selection of re-

Table 4. *Transfer of determinants for production of H₂S, fermentation of raffinose and resistance to tetracycline from three different donor strains to the recipient W1607*

Donor strain	Selected character	No. of colonies	Unselected characters					
			H ₂ S ⁺	H ₂ S ⁻	raf ⁺	raf ⁻	T ^r	T ^s
D1021	H ₂ S ⁺	100	.	.	100	0	33	67
	raf ⁺	100	100	0	.	.	35	65
	T ^r	100*	100	.	100	.	.	.
D1148	H ₂ S ⁺	100	.	.	7	0	7	0
	.	.	.	.	0	11	11	0
	.	.	.	.	35	0	0	35
	.	.	.	.	0	47	0	47
	raf ⁺	100	100	0	.	.	27	73
	T ^r	100*	100	0	43	57	.	.
D1122	H ₂ S ⁺	104	.	.	104	0	88	16
	raf ⁺	39	38	0	.	.	32	6
	.	.	0	1	.	.	0	1
	T ^r	100	3	0	3	0	.	.
	.	.	.	97	0	97	.	.

* In another transfer experiment with D1021 as donor the tetracycline-resistance marker was found transmitted without the H₂S marker to 71 of 1308 tetracycline-resistant W1607 colonies. In another experiment with D1148 as donor, tetracycline resistance without the H₂S marker was transmitted to 184 of 894 W1607 recipient colonies.

cipients was carried out on plates containing either thiosulphate, raffinose or tetracycline. Where possible, 100 recipients which had received the selected donor marker were examined further for the two non-selected markers (Table 4).

From strain D1021, the H₂S and raffinose characters were transferred jointly in all cases, irrespective of the marker selected. Transfer of H₂S-raffinose characters was not necessarily accompanied by transfer of tetracycline resistance, but when selection was made for transfer of the latter, all exconjugants were also H₂S-raffinose-positive. Thus transfer of resistance was always accompanied by the H₂S-raffinose transfer factor. However, from the spontaneous segregant of D1021, D1265, from which the H₂S-raffinose markers had been lost, tetracycline resistance was still transferable. In order to test the *fi* character of the D1021 factors, transfer was carried out to K12 HfrP4X6*str*^r (D1160). When selection was made of H₂S-positive Hfr colonies, segregation took place as H₂S⁺ raffinose⁺ tetracycline^r, and H₂S⁺ raffinose⁺ tetracycline^s D1160 colonies appeared as in the mating mentioned above. Sensitivity to phage MS2 was maintained in both types of Hfr cultures. The same was true when only the resistance character was transferred from the D1021 mutant, D1265. Only *fi*-factor(s) could be demonstrated.

Strain D1148 behaved differently from D1021. When selection was made for transfer of either the raffinose or the resistance character, segregation occurred between these two markers, but each was transferred jointly with the H₂S character when these tests were first made. Selection for transfer of the H₂S marker resulted in the appearance of colonies with all four possible combinations of transferred characters. The majority had inherited the H₂S-forming ability only and the minority all three characters. When strain D1165, which had acquired all three characters from D1148, was used as donor with D726 as recipient, transmission of tetracycline resistance occurred without the H₂S marker. Descendants of such a clone (D1242) and of one which had received only the H₂S marker (D1230) were used to test the *fi* character of the transfer factors. MS2 sensitivity tests were carried out on

Table 5. *Characters of the factors from donor strains D1021, D1148 and D1122*

Origin of the factors	Characters carried by the factors	<i>fi</i>	Sensitivity to phages		Compatibility	No. of transfer factors demonstrated
			MS2	If 1		
D1021	H ₂ S, raf	—	—	—	+	2 (?)
	T	—	—	—		
D1148	H ₂ S, (raf)*	+	+	N.D.	N.D.	2
	T	—	—	—		
D1122	H ₂ S, raf	+	+	N.D.	+	2
	T	+	+	N.D.		
	raf	+	+	N.D.		

N.D., not done.

* Both a strain carrying only the H₂S marker and one carrying also the raffinose marker were examined.

K12HfrP4X6 or HfrC cultures (D1160, D1255, D1248) which had inherited D1148 factors either directly from D1148 or after passage through W1607 and D726 cultures (D1165, D1230, D1242, see Table 1). Hfr cultures which were either H₂S⁺ raffinose⁺ or H₂S⁺ tetracycline^r or only H₂S⁺ were inhibited in their MS2 sensitivity (*fi*⁺), while transfer of tetracycline resistance only caused no inhibition (*fi*⁻). Thus the resistance determinant was transferred by its own transfer factor. No culture was found which had inherited the raffinose character alone or together with the resistance determinant from D1148. Thus, the question as to whether the raffinose character was transferred by its own *fi*⁺ or *fi*⁻ factor or by the H₂S factor, as the segregation pattern might indicate, is not yet answered. The third strain, D1122, showed, in the same way as D1021, joint transfer of the H₂S and raffinose markers. In contrast to D1021, the majority of H₂S-raffinose positive recipients were tetracycline-resistant, whereas the resistance determinant was transmitted independently of the H₂S-raffinose characters; 97 of 100 colonies selected for transfer of resistance inherited that marker only. Transfer of the raffinose marker as a single transmitted character occurred in one case.

After transfer of D1122 factors to HfrP4X6 or HfrC (D1160, D1248 and D1255) either directly from D1122 or after passage through W1607 and D726 cultures (D1188, D1192 and D1238, see Table 1), no plaques with phage MS2 were formed, irrespective of whether the Hfr strain had received all three characters, the H₂S and raffinose characters, or only the resistance or raffinose marker. As in strain D1021, it was impossible to differentiate in this way between different transfer factors.

Compatibility tests with D1021, D1148 and D1122 factors

In order to ascertain whether cultures, all harbouring *fi*⁻ (or *fi*⁺) factors but possessing different characters inherited from the same donor line, contained identical transfer factors, a compatibility test was carried out (Table 5). When this test was performed with the D1021 strain (D1265) which had lost the H₂S-raffinose markers but kept tetracycline resistance, as donor, the following outcome was obtained. Tetracycline resistance was transferred to D1273, a H₂S-raffinose-positive W1607 culture (see Table 1) at the same frequency as in the control experiment in which the recipient, W1607, contained no transmitted characters. Both superinfecting and resident genomes were expressed. This result suggests the presence of two transfer factors of the *fi*⁻ type.

In D1122, only *fi*⁺ factors were found. The compatibility test was made primarily with

a H₂S⁺ raffinose⁺ and with a raffinose⁺ strain as recipient in mating experiments with a tetracycline-resistant strain as donor. The resistance determinant from W1607 tetracycline^r (= D1192) was just as easily transferred to D726 H₂S⁺ raffinose⁺ (= D1221) as to D726 'wild-type'. Twenty recipient colonies of D1221 were purified on raffinose plates and spotted on to different media containing the appropriate indicators. After purification, all the recipient colonies selected for transfer of the resistance determinant were still H₂S⁺, raffinose⁺ and resistant to tetracycline. The same result was obtained when the resistance marker was transferred from a tetracycline-resistant D726 culture, D1238, to a H₂S⁺ raffinose, W1607 culture (D1191). Both resident and incoming superinfecting genomes were expressed. These tests indicated that even though both the H₂S-raffinose markers and the resistance determinant are transmitted by *fi*⁺ factors, these are probably not identical.

During the whole series of experiments with the three donor strains, transfer of the raffinose character was accompanied by transfer of the H₂S character in all cases except one. This exception was when selection was made for transfer of the raffinose marker from D1122 to W1607, in which case one W1607 clone inherited only the ability to ferment raffinose and this character was further transferable to the D726 strain. When an attempt was made to introduce the resistance marker of D1122 (from D1238 or D1192) to the raffinose⁺ D1188 and D1212, the percentage of tetracycline-resistant recipients was reduced at least 100-fold as compared to transfer to the 'wild-type' strains. This result indicates entry exclusion of the superinfecting genome. In addition, incompatibility (immunity proper) was demonstrated in both experiments, since those of the recipient colonies selected as resistant to tetracycline which showed stable inheritance of the resistance determinant invariably lost the raffinose character after one to four re-isolations on media containing raffinose but not tetracycline. Those which remained raffinose⁺ became sensitive to tetracycline. The fact that entry exclusion and incompatibility were shown between the raffinose factor and the resistance factor, but not between this latter factor and the H₂S-raffinose factor (all originating from D1122), indicated that the raffinose marker was normally associated with the same transfer factor as the H₂S character. However, in the single case where only the raffinose character was transferred, the compatibility test indicated that the raffinose determinant had become linked to the factor which usually transferred the resistance determinant and appeared to have taken the place of that determinant.

Examination for production of F-like or I-like fimbriae

All 11 H₂S-donor strains were tested for sensitivity to phages MS2 and Ifl by the cross-brushing method with negative result. W1607 cultures and one D726 culture which had inherited different characters from the three strains D1122, D1148 and D1021, irrespective of whether *fi*⁺ or *fi*⁻ factors were involved, were mixed with phage MS2 and tested for phage increase (Table 5). An increase was demonstrated in all cases where the characters had been transferred by *fi*⁺ factors, whereas cultures with *fi*⁻ factors failed to show any increase in phage titre. Three *fi*⁻ cultures were examined in the Ifl phage increase test. Of these, two had inherited tetracycline resistance from either D1148 or D1021 (D1242 and D1261) and the third had inherited the H₂S-raffinose characters from D1021 (D1273). No increase was demonstrated.

Transfer of chromosomal marker

W1607 cultures possessing one to three of the three different transferable characters from the 11 H₂S donor strains were grown in mixed cultures with a *try*⁻ strain belonging to the 0100 line (W3460). The tryptophane marker was restored in W3460 when the resistance

determinant from the three H₂S-positive Indian strains belonging to serotype 020:H⁻ was possessed by the w1607 culture, but the frequency of recombination was very low, namely one recombinant/10⁷ to 10⁸ donor cells. When only the H₂S-raffinose markers from one of these three strains and not the resistance marker were present in w1607, no recombinants were formed with w3460. As regards one of the three 020:H⁻ cultures, D1021, it was mentioned above that this might contain two different *fi*⁻ factors. If so, it is the resistance factor that brings about gene transfer leading to restoration of the *try* marker in the F⁻ strain. With all the other w1607 strains, no or so few colonies were found on the recombinant plate that it was questionable whether these were recombinants and the crosses were therefore considered as infertile.

DISCUSSION

The present study confirms the existence of H₂S-positive *Escherichia coli* strains. Thirty-two such strains were demonstrated, the oldest being from the beginning of the examination period covering the last 20 years. Most of the H₂S-positive strains came from animals. However, the material available does not permit an answer to the question whether such strains are more common in animals than in man.

Most of the strains formed a black precipitate after growth in media containing tetrathionate and thiosulphate, and all the strains isolated from hens (or yolk sac of chick embryo) produced H₂S to some extent when grown in the sulphite medium. The nature and number of enzymes involved in these reductions were not investigated and few reports exist which deal with H₂S production in Enterobacteriaceae.

In a previous report (Lautrop *et al.* 1971) one of 11 H₂S⁺ strains was found to transfer the H₂S character. In the present study, 11 of 32 strains (or nine of 24 serotypes) transmitted the H₂S character. The strains which were tetracycline resistant also transmitted that marker. Transfer of the capacity to ferment raffinose was demonstrated in six out of nine strains which fermented raffinose promptly. Transfer of the ability to ferment raffinose has not been reported previously and this character can now be added to the list of those determined by transferable plasmids.

Transfer of biochemical characters is important from a taxonomical point of view. The Enterobacteriaceae are classified according to their biochemical, mostly fermentative, characters, but if several of the characters used can be spread by plasmids, classification may be hampered, if not made impossible. This is more true of H₂S-production than of raffinose fermentation as regards classification of *Escherichia coli*. The species *E. coli* has hitherto been described as unable to produce H₂S, whereas the ability to ferment raffinose may or may not be present. An examination of 500 H₂S-negative *E. coli* strains showed that the fermentation of raffinose, an alpha-galactoside of sucrose, was highly correlated with sucrose fermentation. This correlation was not obvious among the 11 H₂S donor strains, probably because of the small number of strains. Five strains representing five serotypes were raffinose- and sucrose-positive. Three of these transmitted the raffinose marker but no transfer of the sucrose marker was demonstrated. Any attempts to explain pathways in these fermentation reactions and the genetics involved we think will be speculative until further studies have been performed.

In order to characterize the transfer factors in three strains that transferred all three characters, H₂S, raffinose and tetracycline i.e., tests were made for inhibition of the F factor, for F and I phage sensitivity, and for compatibility. In D1021 the raffinose determinant appeared to be linked to the H₂S marker, since they were always transmitted together, and so seemed to constitute a 'co-integrate' *fi*⁻ plasmid (Clowes, 1972). Tetracycline resistance

was never transferred without the H₂S-raffinose characters, which suggests that it was non-self-transmissible, being mobilized by the H₂S-raffinose plasmid. This seemed to be an example of a plasmid 'aggregate' (Clowes, 1972). However, tetracycline resistance was still transmissible and still *fi*⁻ from the strain, D1265, which had lost the H₂S-raffinose markers. Perhaps self-transfer of the tetracycline resistance plasmid was inhibited by the presence of the H₂S-raffinose marker, or possibly the transfer genes of the H₂S-raffinose plasmid were retained in the spontaneous segregant. Thus we cannot be sure whether one or two *fi*⁻ transfer factors were present in D1021 (Table 5). In D1148, the tetracycline resistance factor was an *fi*⁻ factor, while the factor transferring the H₂S or the H₂S-raffinose characters was an *fi*⁺ factor. The pattern of transfer indicated that the raffinose marker was carried on a non-transmissible plasmid, mobilized by the transmissible H₂S factor. Possibly the raffinose determinant was dependent on the H₂S plasmid for replication, since, unlike the pattern of transfer in the ΔSAT aggregate plasmid system of Anderson & Lewis (1965), raffinose utilization was never transferred without the H₂S character. In another H₂S-raffinose strain, D1121, the H₂S character was transferable whilst the raffinose character was not; both characters were lost simultaneously, which suggests dependence of the raffinose character upon the H₂S plasmid.

In D1122 the H₂S-raffinose characters behaved as a co-integrate transferable plasmid as in D1021, but that of D1222 was *fi*⁺. Tetracycline resistance of D1122 was transferable separately and was also carried on an *fi*⁺ transfer factor. The H₂S-raffinose plasmid of D1122 was not so stable as to preclude the possibility of segregation (during transfer) of the raffinose character.

In the one clone where the raffinose marker had become separated from the H₂S marker it seemed to have become integrated into the transfer factor that had previously carried tetracycline resistance. In this clone the raffinose marker appeared to have taken the place of the resistance marker, since the raffinose plasmid was incompatible with the tetracycline resistance plasmid from D1122. Anderson, Keleman, Jones & Pitton (1968) observed a similar substitution of plasmid markers in a strain of *Salmonella typhimurium* after u.v.-irradiation. The apparent recombinant raffinose plasmid was still *fi*⁺. Thus in D1122 two transfer factors were present, both *fi*⁺. The characters of the various factors examined are given in Table 5.

No examination was carried out to see whether the factors were compatible with the factors of the same *fi* type in the two other strains. Tests with the F-specific phage MS2 showed that the *fi*⁺ factors were F-like, whereas in no instance could sensitivity for the I-specific phage be demonstrated. This latter test did not include use of anti-I phage serum and therefore it cannot be precluded that so few I-like fimbriae were present that non-absorbed phage had to be removed for demonstration of an increase in phage titre. However, it is very likely that the *fi*⁻ factors did not belong in the I-like group, particularly because several compatibility groups of *fi*⁻ R factors unrelated to I, such as N, P, W, T and J, have been described recently (Datta & Hedges, 1971; Datta, Hedges, Shaw, Sykes & Richmond, 1971; Hedges & Datta, 1971; Coetzee, Datta & Hedges, 1972).

In contrast to the H₂S and tetracycline characters, the raffinose character in all the cultures examined did not segregate as a separate transferable or non-transferable marker. It was never transferred without the ability to produce H₂S, except in one recipient colony with D1122 as donor, as described above.

Note added in proof. After the present paper was ready for submission for publication, Stoleru, Gerbaud, Bouanchaud & Le Minor (1972) reported that a H₂S-positive *Escherichia coli* strain which had been isolated from a pig contained an infectious plasmid which trans-

mitted H₂S and T resistance characters simultaneously. T resistance and the H₂S character were co-transmitted by phage Pl-Kc, thus indicating that in this case T resistance and H₂S determinants were found on the same replicon. We have not carried out transduction experiments, but analysis of transfer experiments showed that in some of our strains H₂S and tetracycline resistance characters were not carried by the same transfer factor; there is no doubt that this is true for two of the three strains studied in detail. Thus, the H₂S character when found as a plasmid is in some cases transmitted by the same transfer factor as the tetracycline-resistance determinant and in other cases it is not.

This paper is dedicated to Professor Otto Westphal, Max-Planck-Institut für Immunbiologie, Freiburg, Germany, on the occasion of his 60th birthday, 1 February 1973.

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