Biochemical and Serological Characterization of Hydrogen Sulfide-Positive Variants of *Escherichia coli*

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Over 200 H$_2$S-positive, gram-negative rods have been characterized by standard biochemical and serological techniques. The results indicate that the isolates are H$_2$S-positive variants of *Escherichia coli*. Comparison of the variants with biochemically typical *E. coli* suggests that they represent a rather limited subgroup within the species. The H$_2$S-positive strains were more resistant to antibiotics than the typical strains; 54% of the H$_2$S-positive isolates were resistant to three or more antibiotics compared with only 25% of the typical strains. Similar differences were also seen in the distribution of O and H antigens and in the results of certain biochemical tests.

At present, either triple sugar-iron agar (TSI) or peptone-iron agar (PIA) is recommended for the detection of H$_2$S production by members of the family *Enterobacteriaceae* rather than a more sensitive method such as lead acetate strip (1). When the recommended media are used, the inability of *Escherichia coli* to produce H$_2$S is considered axiomatic. In fact, the inability of these organisms to produce detectable amounts of H$_2$S in standard diagnostic media is included in the definition of the genus *Escherichia* (2, 3). The existence of H$_2$S-producing variants is seldom considered. However, a few isolated reports describing such variants have appeared in the literature (6–8). Although the relative frequency with which these variants are isolated appears to be low, clinical laboratories should be made aware of their existence.

In the present study, more than 200 isolates of previously unidentified H$_2$S-producing microorganisms with other characteristics typical of *E. coli* were examined in detail. These isolates have been referred to the Center for Disease Control during the past 7 years. The results clearly indicate that these organisms are *E. coli* variants. In addition, the distribution of O and H antigens in the H$_2$S-positive strains and the results of certain differential tests suggest that the variants comprise a rather distinct subgroup within the species *E. coli*.

MATERIALS AND METHODS

**Bacteria.** The bacterial strains included in this present survey were submitted to the Center for Disease Control (CDC) in Atlanta, Georgia, as "unidentified, H$_2$S positive, gram negative rods." Shortly after arriving in the enteric unit, they were inoculated into TSI; those isolates which were H$_2$S positive and could not be assigned to one of the traditional H$_2$S-positive tribes of the *Enterobacteriaceae* are included in this study.

For comparative purposes, the results of serotyping and biochemical tests on typical *E. coli* were taken from data accumulated at CDC or from previously published sources (5). Attempts were made to insure that the control group of typical *E. coli* and the H$_2$S-positive strains used in this study were received at CDC during the same period of time, i.e., 1966 to 1972.

**Differential tests.** The biochemical tests performed were those routinely used in the enteric laboratories at CDC (4). These included tests for: (i) the production of H$_2$S in PIA, urease, indole, acetyl-methylcarbinol, lysine and ornithine decarboxylases, arginine dihydrolase, and phenylalanine deaminase; (ii) growth on or in Simmons citrate, KCN, malonate, Jordan tartrate, and mucate; and (iii) the fermentation of glucose, lactose, sucrose, mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, arabinose, raf-finose, rhamnose, maltose, xylose, trehalose, cellobi-ose, and glycerol. Also included were tests for motility and the liquefaction of gelatin. In most cases, all 220 H$_2$S-positive isolates were tested on all 32 differential media.

**Antibiotic susceptibility tests.** One hundred forty-four H$_2$S-positive isolates received between 1969 and 1972 were selected at random for screening to susceptibility to ampicillin (50 μg/ml), tetracycline (10 μg/ml), and streptomycin (50 μg/ml). Blood agar base (BAB) plates supplemented with one of the three antibiotics were inoculated from single colonies from BAB by using sterile toothpicks. Growth on the antibiotic-supplemented plates after 24 h at 37°C was assumed to indicate resistance to the antibiotic in question.

Twenty-two H$_2$S-positive *E. coli* strains were
chosen at random from the above-mentioned strains and examined more extensively. Susceptibility to tetracycline, sulfadiazine, streptomycin, ampicillin, kanamycin, erythromycin, chloramphenicol, and colistin was determined by the Kirby-Bauer method (1).

For comparative purposes, 144 strains of typical E. coli were chosen at random from the culture collection at CDC and examined in the same way.

Serotyping. The somatic (O) and flagellar (H) antigens were determined by procedures described by Edwards and Ewing (2). In addition to antisera to O groups 01 through 0150, nine different antisera to provisional E. coli antigens designated OX1, OX2, OX3, OX5, OX6, OX8, OX9, OX12, and OX13 were used. Antisera to Shigella antigens A9, C7, and D were also used, as were antisera to Alkalcescens-Dispar group O3.

To determine the O antigen group, a 5- to 7-h culture in Trypticase soy broth (TSB) was heated for 60 min in an Arnold steam sterilizer and then diluted approximately 1:6 with Formalinized saline (0.5% NaCl + 0.185% Formalin). Antiserum was added to the diluted cell suspension to give an antiserum dilution of 1:500 in a final volume of 1 ml. The suspension was incubated overnight in a 48 C water bath.

H antigen determinations were performed on overnight TSB cultures of actively motile isolates. To insure adequate motility, the isolates were first seri- ally passed at least three times, and in some cases as many as ten times, through motility medium. The overnight TSB cultures were diluted approximately 1:2 with phenolized saline (0.5% NaCl + 0.5% phenol). Antiserum was added to give a final antiserum dilution of 1:500 in a total volume of 1 ml. The mixtures were incubated in a 48 C water bath and checked for agglutination after 15, 30, or 60 min.

Serotype data for typical E. coli was obtained from previously unpublished information on file at CDC. The data is restricted to those E. coli that were serotyped between 1966 and August 1972.

Preparation of O antisera. An attempt was made to characterize the O antigens of those isolates that failed to agglutinate in the above O antisera. Three smooth, untypable isolates, 3580-70, 2614-71, and 2579-72, were used for the preparation of antiserum. Rabbits were immunized by repeated intravenous injection of acetone-extracted cells (see reference 2). The sera were stored as a 1:2 dilution in phenolized saline until used. Antiserum diluted 1:500 were used to test the untypable H,S-positive strains. Agglutination at this antiserum concentration was considered sufficient to establish an antigenic relationship between the test strain and the strain used for immunization.

RESULTS

Source of the isolates. The 220 isolates included in this study were received at CDC over a 10-year period beginning in 1962. However, only six of the strains were received before 1966. Although the cultures were obtained from a variety of sources, 65% were from either stool or urine samples.

The relative frequency with which these isolates were received at CDC averaged 4.8% of the E. coli identified between 1966 and 1972 and was remarkably constant during this interval.

Biochemical characteristics. Casual examination of the results obtained on the commonly used differential tests revealed that, with the exception of the ability to produce H2S on TSI, the isolates could not be distinguished from typical E. coli. Thus, an overwhelming majority of the strains were aerogenic, fermented lactose, produced indole, and decarboxylated lysine. The isolates were uniformly negative for urease and Voges-Proskauer reaction and failed to grow on Simmons citrate.

However, when the results of the differential tests were examined more critically, it was found that the samples of H2S-positive and H2S-negative E. coli differed significantly (P < 0.01) in 9 if the 32 biochemical tests considered (Table 1). Such differences reflect real population differences.

Antibiotic susceptibility. The preliminary screening of the H2S-positive isolates for susceptibility to ampicillin, streptomycin, and tetracycline revealed that considerably more of the H2S-positive variants were resistant to the antibiotics used (Table 2). Over 65% of the H2S-positive isolates were resistant to one or more of the antibiotics as compared with only 36% of the H2S-negative control group. Fifty-four percent of the isolates were multiple resistant as compared with 25% of the typical E. coli. These differences are significant (P < 0.025) and once again indicate that the two groups are not random samples from the same population of E. coli.

The results obtained with high-potency antibiotic disks used according to the method of Bauer et al. (1) were in total agreement with the above data (Table 3). Ten of 22 H2S-positive isolates examined by this technique were resistant to three or more of the antibiotics ampicillin, streptomycin, tetracycline, and sulfadiazine, whereas only four H2S-negative cultures exhibited such multiple resistance.

O antigens. The O antigens of 212 H2S-positive isolates were examined. Only 96 (45.3%) were found to react specifically with one of the 163 routine typing sera used at CDC. Forty-six different O groups were represented, but only two groups were represented by more than three isolates. The two exceptional O groups were O23 (23 isolates) and O68 (9 isolates). Neither of these groups was particularly common in the control population (Table 4).

When the above data were pooled by the method recommended by Edwards and Ewing...
Arginine  Lysine  Ornithine  Sucrose  Lactose  Xylose  Sorbitol  Salicin

**TABLE 1. Results obtained with H$_2$S-positive isolates and H$_2$S-negative E. coli in routine biochemical tests**

| Differential test          | H$_2$S positive | H$_2$S negative | \( \chi^2 \) |
|---------------------------|-----------------|-----------------|-------------|
|                           | +   | -   | + (%) | -   | + (%) | |
| Lysine decarboxylase      | 212 | 8   | 96    | 716 | 156  | 82 | 27.8 |
| Arginine dihydrolase      | 90  | 130 | 41    | 483 | 389  | 55 | 14.2 |
| Ornithine decarboxylase   | 165 | 55  | 75    | 568 | 304  | 65 | 7.3  |
| Jordan tartrate           | 134 | 57  | 70    | 205 | 1    | >99 | 28.0 |
| Sucrose                   | 87  | 132 | 40    | 1,117| 770  | 59 | 29.6 |
| Salicin                   | 170 | 46  | 79    | 905 | 976  | 48 | 71.2 |
| Sorbitol                  | 211 | 7   | 97    | 1,536| 351  | 81 | 31.3 |
| Xylose                    | 202 | 9   | 96    | 462 | 55   | 89 | 6.8  |
| Lactose                   | 198 | 22  | 90    | 1,808| 79   | 96 | 13.3 |

* Includes only those biochemical tests where a significant \( (P < 0.01) \) difference was observed between the two classes.

* Data from Ewing et al. (5).

* \( \chi^2 > 6.63 \) indicates a significant difference between the two classes \( (P < 0.01) \).

**TABLE 2. Isolates of H$_2$S-positive and H$_2$S-negative E. coli that exhibited resistance to three antibiotics**

| Resistance pattern* | No. of isolates* | H$_2$S positive (%) | H$_2$S negative (%) |
|---------------------|------------------|---------------------|---------------------|
| AST                 | 47 (33)          | 19 (13)             |
| AS                  | 2 (1)            | 3 (2)               |
| AT                  | 12 (8)           | 2 (1)               |
| ST                  | 17 (12)          | 12 (8)              |
| A                   | 3 (2)            | 3 (2)               |
| S                   | 2 (1)            | 21 (8)              |
| T                   | 11 (8)           | 5 (4)               |
| None                | 50 (35)          | 92 (64)             |
| Total               | 144              | 144                 |

* A, ampicillin resistant; S, streptomycin resistant; T, tetracycline resistant.

\[ \chi^2 = 18.4 \ (\chi^2 > 16.01 \) indicates significant difference at 2.5% level. Degrees of freedom = 7. \]

**TABLE 3. Isolates of H$_2$S-positive and H$_2$S-negative E. coli resistant to antibiotics with high potency disks**

| Antibiotic         | H$_2$S positive (%) | H$_2$S negative (%) |
|--------------------|---------------------|---------------------|
| Tetracycline       | 73                  | 2                   |
| Sulfadiazine       | 50                  | 2                   |
| Streptomycin       | 45                  | 3                   |
| Ampicillin         | 41                  | 2                   |
| Kanamycin          | <1                  | <1                  |
| Erythromycin       | 86                  | 95                  |
| Chloramphenicol    | 0                   | <1                  |
| Colistin           | 0                   | 0                   |

* There were 22 randomly chosen isolates in each group.

**TABLE 4. Distribution of H$_2$S-positive isolates and H$_2$S-negative E. coli by O group antigens**

| O group | H$_2$S positive | H$_2$S negative* |
|---------|-----------------|------------------|
|         | No.       | O-typable (%) | No.       | O-typable (%) |
| O1      | 0          | 0.0            | 110       | 4.7            |
| O4      | 0          | 0.0            | 190       | 8.1            |
| O6      | 2          | 2.1            | 259       | 11.0           |
| O18     | 1          | 1.0            | 154       | 6.6            |
| O23     | 23         | 24.0           | 21        | 0.9            |
| O68     | 9          | 9.4            | 7         | 0.3            |
| O75     | 0          | 0.0            | 147       | 6.3            |
| All other O groups| 61*        | 63.5           | 1,459     | 62.2           |
| Total   | 96         | 100.0          | 2,347     | 100.1          |
| Rough   | 41         | 406            |
| Untypable | 75        | 486             |
| Total   | 212        | 3,239          |

* Includes four H$_2$S-positive isolates that agglutinated in two different O antisera.

\[ \text{Data from previously unpublished information on file at CDC. Includes all E. coli serotyped between 1966 and August 1972.} \]

\( (P < 0.01) \) from that of typical E. coli (Table 5). It is interesting to note that the five most common O groups in the control population (O1, O4, O6, O18, and O75), which represent about 37% of the 2,347 isolates of E. coli serotyped at CDC between 1966 and 1972, accounted for only 3.1% of the 96 typable H$_2$S-positive strains considered in this study (Table 3).
TABLE 5. Distribution of H$_2$S-positive isolates and H$_2$S-negative E. coli by O antiserum pools

| O antiserum pool* | No. of isolates* |
|-------------------|------------------|
|                   | H$_2$S positive | H$_2$S negative |
| 1A                | 7               | 816             |
| 2A                | 6               | 195             |
| 3A                | 1               | 134             |
| 5A                | 2               | 368             |
| 4A + 6A + 7A'     | 11              | 266             |
| 1B                | 27              | 188             |
| Pools 2B to 6B'   | 23              | 209             |
| Pools 1C to 5C + Pool 8* | 15 | 171 |
| Total             | 92*             | 2,347           |

*Antiserum pools correspond to those recommended by Edwards and Ewing (2).

*Degrees of freedom = 7. $x^2 = 113.04$ ($x^2 > 18.48$ indicates significance at 1% level).

*Data are grouped to insure expected frequencies of at least 5.0.

*The four H$_2$S-positive isolates that agglutinated in two different O antisera are excluded.

**H** antigens. One hundred thirty-seven H$_2$S-positive isolates were motile, and, of these, 94.8% were shown to react with available H antisera (Table 6). Of the H-typable isolates, 33% (43/130) were H16 and 12% (16/130) were H10. All other H antigens were represented by three or fewer isolates. In all, 28 different H antigens were demonstrated. Analysis of the data revealed some interesting differences between the control group of biochemically typical E. coli and the H$_2$S-positive isolates. Table 7 summarizes a statistical analysis of the data based upon the H antiserum pools recommended by Edwards and Ewing (2). The distributions of H antigens differ significantly at the 1% level. Close inspection of the data in Table 5 reveals the source of this difference. The five most common H antigens in the control group (H1, H4, H5, and H6, and H7) account for 46.1% of the H-typable isolates of H$_2$S-negative E. coli but only 8.5% of the typable H$_2$S-positive strains.

**New antisera.** Two isolates, 2614-71 and 2579-72, produced sufficiently high titers to be used for serological analysis of the untypable H$_2$S-positive isolates. The titers were 1:1,280 and 1:2,560, respectively. Antisera to these two strains resulted in the characterization of two additional isolates; one isolate agglutinated with each antiserum.

**DISCUSSION**

There remains little doubt that the isolates described above are, in fact, H$_2$S-positive variants of E. coli. These bacteria share a number of O and H antigens with typical E. coli and resemble the species in respect to overall biochemical properties. Although significant differences are seen in the distribution of O and H antigens and in the relative frequency with which certain substrates are utilized, these differences were detected only after a relatively large number of isolates were examined. These differences are of little value to the microbiologist whose primary objective is to identify clinical isolates. In this sense, all of the isolates fall within the range of typical E. coli (5), and

**TABLE 6. Distribution of H$_2$S-positive isolates and H$_2$S-negative E. coli by H antigens**

| H antigen | H$_2$S positive | H$_2$S negative* |
|-----------|----------------|------------------|
|           | No. H-typable (%) | No. H-typable (%) |
| H1        | 3               | 2.2             | 277 | 11.8 |
| H4        | 6               | 4.4             | 157 | 6.7  |
| H5        | 0               | 0.0             | 192 | 8.2  |
| H6        | 0               | 0.0             | 135 | 5.7  |
| H7        | 2               | 1.5             | 243 | 10.3 |
| H10       | 16              | 11.7            | 77  | 3.3  |
| H16       | 43              | 31.4            | 1064| 45.3 |
| All other H antigens | 60 | 43.8 | 1,064 | 45.3 |
| Untypable | 7               | 5.1             | 172 | 7.3  |
| Total     | 137*            | 100.1           | 2,349| 100.0 |

*From unpublished data on file at CDC. Includes all E. coli typed for H antigen between 1966 and August 1972.

*Motile strains.

**TABLE 7. Distribution of H$_2$S-positive isolates and H$_2$S-negative E. coli by H antiserum pools**

| H antiserum pool* | No. of isolates* |
|-------------------|------------------|
|                   | H$_2$S positive | H$_2$S negative |
| 1                 | 53              | 461             |
| 2                 | 7               | 560             |
| 3                 | 7               | 181             |
| 4                 | 23              | 439             |
| 5                 | 15              | 102             |
| 6                 | 7               | 201             |
| 7                 | 7               | 108             |
| 8 to 10           | 9               | 125             |
| Total             | 128*            | 2,177           |

*Recommended by Edwards and Ewing (2).

$x^2 = 53.79$ ($x^2 > 18.48$ indicates significance at the 1% level). Degrees of freedom = 7.

*Two isolates that agglutinated in two different H antisera are excluded.
there is no reason for considering them as members of some other taxon. Therefore, laboratories isolating such strains should not be reluctant to identify them as \( \text{H}_2\text{S}\)-positive \( E. \text{coli} \).

It is difficult to estimate with any degree of certainty the relative frequency of \( \text{H}_2\text{S}\)-positive \( E. \text{coli} \) in nature. There is no way of knowing, for example, how many such variants have been misidentified as members of genera such as \( \text{Citrobacter} \) or \( \text{Arizona} \) or even how many have remained unidentified. On the other hand, the estimate of 4.8% in the present paper is likely to be high because of the selective manner in which cultures are submitted to CDC from other laboratories. The organisms submitted to CDC for identification are often atypical strains which are difficult to classify. Such samples would introduce considerable bias into the data. However, it seems apparent that \( \text{H}_2\text{S}\)-positive variants may not be as rare as was previously thought (2, 5).

The only disconcerting aspect of this study was the fact that an extraordinarily high percentage of the isolates were either rough or untypable when standard O antisera were used. The reason for this is not clear. The length of time many of the isolates were stored before they were serotyped may have been a contributing factor. However, it should be noted that no obvious correlation existed between O group antigen and the date the culture was received. Furthermore, no significant biochemical differences were found between O-typable and untypable strains by chi-square analysis of 2 × 2 contingency tables.

Although the data obtained in conventional biochemical tests indicate that the isolates are \( E. \text{coli} \), it is equally clear that they represent a rather unique group within the species. In 9 of 32 routine biochemical tests, significant (\( P < 0.01 \)) differences were seen. Differences were also detected in the susceptibility of the two groups to antibiotics. Perhaps the most interesting difference between the two groups was the high percentage of isolates that were resistant to three or more antibiotics. In addition, the distribution of both O and H antigens was not typical of the species as a whole. Statistical analysis indicates that the observed differences are not likely to be the result of sampling errors.

An obvious question concerns the origin of these \( \text{H}_2\text{S}\)-positive variants. The apparent association of the trait with antibiotic resistance is consistent with suggestive evidence that a plasmid, and in particular a resistance transfer factor, may be involved. All three groups of previous workers have commented on the relative instability of this characteristic (6–8). Lautrop and co-workers (7) have demonstrated the transmissible nature of the trait, and Layne et al. (8) have shown that extrachromosomal deoxyribonucleic acid appears to be associated with the ability to produce \( \text{H}_2\text{S} \). Recently, it has been further demonstrated that the gene responsible for \( \text{H}_2\text{S} \) production is closely linked to a gene for tetracycline resistance (10). Experiments are now underway in our laboratory to verify these observations.

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