Spurious Hydrogen Sulfide Production by *Providencia* and *Escherichia coli* Species

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Hydrogen sulfide production was noted in two *Escherichia coli* strains and one *Providencia alcalifaciens* (*Proteus inconstans* A) strain isolated from clinical stool specimens during the summer of 1979. An investigation into this phenomenon revealed the presence of *Eubacterium lentum*, an anaerobe, growing in synergism with the *Enterobacteriaceae* and producing H$_2$S. The implications of this association are discussed with reference to clinical microbiology laboratory practice.

Specific biochemical patterns are blueprints for the microbiologist to use in identifying microorganisms. Biochemical reactions may be so consistent within certain genera that in all reference tables they are listed either as 100% positive or negative. The ability of bacteria to produce hydrogen sulfide (H$_2$S) from sulfur-containing amino acids is generally a fixed and consistent characteristic. Five genera of *Enterobacteriaceae* produce H$_2$S on triple sugar iron agar (TSI) (6). They are *Escherichia*, *Salmonella*, *Arizona* (*Salmonella arizonae*), *Citrobacter*, and *Proteus*. All other genera in the family *Enterobacteriaceae* are generally H$_2$S negative. They are considered incapable of H$_2$S production due to the absence of tetrathionate reductase and thiosulfate reductase (9).

Occasionally, organisms which should not produce H$_2$S appear to spontaneously show H$_2$S production. This aberrant phenomenon was observed as far back as 1945, when Galton and Hess (8) found 5 H$_2$S-producing strains out of 232 strains identified as *Shigella alkaliflavs* (now classified as a biogroup of *Escherichia coli*). Only recently was it discovered that this phenomenon may be due to acquisition of a plasmid by extrachromosomal genetic transfer from another genus (10). The first extensive study of H$_2$S-positive *E. coli* strains was undertaken by Lautrop (8) and co-workers in Denmark. During a period of 14 months beginning in 1969, 26 isolates of H$_2$S-producing *E. coli* were recovered. In the United States, more than 200 isolates of H$_2$S-positive microorganisms resembling *E. coli* were submitted to the Center for Disease Control over a 10-year period beginning in 1962 (4). In 1976, Farmer et al. (6) isolated two H$_2$S-positive *Shigella sonnei* strains, one of which was authentic. The other showed H$_2$S production due to *Eubacterium lentum*, an obligate anaerobe, living in synergism with H$_2$S-negative *S. sonnei*.

Whenever an H$_2$S-producing organism is recovered in our laboratory, it is screened both biochemically and serologically for *Salmonella*. If negative, an identification is made to insure that it belongs to one of the other four genera and is not an unusual *Salmonella* deviating from the common biochemical pattern.

Following are our observations of a *Providencia alcalifaciens* (*Proteus inconstans* A) and three *E. coli* stool isolates producing H$_2$S in association with *E. lentum*.

Media used in the diagnostic laboratory were prepared by formulations described by Ewing (6) or were prepared from dehydrated powders according to manufacturers’ directions. Media used for the anaerobes were prepared aerobically and reduced before use (5).

*P. alcalifaciens* (F65) was obtained from a rack of TSI agar slants tubes being screened for H$_2$S-negative *Salmonella*. It showed no H$_2$S production after 18 h of incubation. H$_2$S appeared only 24 h later, when the first biochemical reactions were being recorded. It did not fit the *Salmonella* biochemical pattern or any other known H$_2$S producer pattern. Tentative identification was made as H$_2$S-positive *P. alcalifaciens*. The TSI slant was then streaked on a Hektoen agar plate to check for purity. The culture appeared to be pure, and yet H$_2$S production was again noted in the confluent growth area, where the medium was stabbled. No H$_2$S production was observed in areas of single, isolated colonies or TSI slants picked from these colonies.

Gram-stained smears made from this area showed gram-negative bacilli and tiny, pleomorphic, gram-positive bacilli. The two organisms were separated by inoculating a blood
agar plate and sodium azide blood agar plate anaerobically and aerobically. The gram-positive organisms were obligate anaerobes, asaccharolytic, and very inert in other biochemical reactions; produced H₂S; and only reduced nitrate to nitrite. They were identified as *Eubacterium lentum* and confirmed by gas-liquid chromatography.

Since H₂S was produced on TSI slants by *E. lentum* alone when incubated anaerobically or when incubated aerobically in association with a pure culture of *P. alcalifaciens*; this demonstrated a synergistic relationship between an aerobe and anaerobe.

While investigating the nature of this spurious H₂S production by *P. alcalifaciens*, a search was started for the occurrence of such associations among other strains. Eighteen-hour H₂S-negative, urea-negative cultures, which were being held pending completion of the results of routine *Salmonella* and *Shigella* screening were observed again after 48 h of incubation. Of 126 cultures observed for delayed H₂S production after 24 h of further incubation, 4 produced delayed H₂S. These isolates were not from consecutive clinical specimens, and, except for H₂S production, showed typical biochemical patterns for *E. coli* (3) and *P. alcalifaciens* (1). In each case *E. lentum* was isolated from the TSI slant showing delayed H₂S production, and it was ascertained in each case that the member of the *Enterobacteriaceae* was indeed not an H₂S producer when pure.

Finally nine other known H₂S-negative strains were checked for synergism with *E. lentum* (P65). These cultures were identified and found to be H₂S negative by API, Minitek, and Inoxel Entero 20 systems, as well as by conventional methods. As before, the anaerobe was stabbed into TSI slants and was then overlayed with *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens*, *Yersinia enterocolitica*, *Klebsiella oxytoca* (indole-positive biogroup of *K. pneumoniae*), *Providencia alcalifaciens*, and *Hafnia alvei*, respectively. Also, recent isolates of *Shigella sonnei* and *Shigella flexneri* O group 2 were tested for synergism with *E. lentum*. Spurious H₂S production was noted with all nine strains when incubated with *E. lentum*.

*E. lentum* is a normal inhabitant of the gastrointestinal tract of humans. Since it is an obligate anaerobe, it does not survive on aerobic selective media such as those used in enteric bacteriology. Its presence, as a contaminant, in a culture growing enteric organisms is seldom considered. The question arising is how did *E. lentum* survive in the four different specimens from which it was isolated?

It is known that *E. coli* can change the redox potential of a culture and provide suitable environment for anaerobes. Bokkenheuser and associates (2) (3) co-cultured *E. coli* and *E. lentum* and observed that *E. coli* not only provided the proper Eh for multiplication of *E. lentum* by reducing the media, but also provided for better functioning of steroid metabolizing enzymes.

Farmer et al. (7) noticed that *E. lentum* survived under *S. sonnei* colonies and produced H₂S in synergistic relationships. They were not certain of the mechanism involved and originally postulated that all H₂S-positive *E. coli* strains reported in the literature might be due to contamination with *E. lentum*, since an anaerobe could thus mimic the behavior of a plasmid. When this hypothesis was tested, all 12 H₂S-positive *E. coli* cultures were examined and found to be authentic H₂S producers. Since other previously submitted H₂S-positive cultures which spontaneously lost H₂S production on subculture were not available to be further examined, no conclusion could be drawn.

It has been postulated that H₂S production is plasmid mediated. However, the likelihood of *E. lentum* contamination of stool specimens is to be considered, as shown in this study. Since it was possible to show H₂S production in TSI slants with known H₂S-negative organisms by co-culturing with *E. lentum*, a question arises as to how many of the H₂S-producing organisms reported in the literature were positive due to contamination with *E. lentum*.

Previous workers have commented on the instability of some of the H₂S-producing isolates (1, 4, 11).

Galton and Hess (8) observed that H₂S production in *S. alkalesscens* was lost on subculture in one of five H₂S-positive isolates. Aikawa and lida (1) in Japan, when studying H₂S-positive *S. sonnei* isolates, described loss of ability to produce H₂S by fermenting mutants. Studying the extrachromosomnal nature of H₂S production by two *E. coli* strains, Layne and co-workers (10) found that the H₂S production trait can be segregated out. Unfortunately, lack of effective screening at that time prevented further work.

Lautrop (9) noted that in H₂S-negative *E. coli*, H₂S-negative colonies occasionally appeared upon seeding from stock cultures. Five H₂S-negative subcultures were isolated from 26 original H₂S-positive strains.

The relationship between anaerobes and aerobes is far from understood. Synergistic associations may be occurring more frequently among different genera than suspected. This complicates the picture obtained by laboratorians who rely heavily on biochemical patterns for
identifying Enterobacteriaceae. In the clinical microbiology laboratory, TSI slants are generally read after 18 to 24 h. Appearance of hydrogen sulfide 24 h later is likely to cause delay in reporting, because all such organisms have to be screened for Salmonella. Therefore, existence of H₂S production due to associations of the type described in the present report is an important observation that might explain some of the atypical reactions and unusual results sometimes encountered in the clinical laboratory.

LITERATURE CITED