Spurious Hydrogen Sulfide Production by Providencia and Escherichia coli Species

BEATRICE E. TRELEAVEN,† ALPHAL A. DIALLO, AND EARL C. RENSHAW, JR.

Bureau of Disease Control and Laboratory Services, Division of Microbiology, Michigan Department of Public Health, Lansing, Michigan 48909

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 H₂S. 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 (H₂S) from sulfur-containing amino acids is generally a fixed and consistent characteristic. Five genera of Enterobacteriaceae produce H₂S on triple sugar iron agar (TSI) (6). They are Edwardsiella, Salmonella, Arizona (Salmonella arizonae), Citrobacter, and Proteus. All other genera in the family Enterobacteriaceae are generally H₂S negative. They are considered incapable of H₂S production due to the absence of tetrathionate reductase and thiosulfate reductase (9).

Occasionally, organisms which should not produce H₂S appear to spontaneously show H₂S production. This aberrant phenomenon was observed as far back as 1945, when Galton and Hess (8) found 5 H₂S-producing strains out of 232 strains identified as Shigella alkalescens (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 H₂S-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 H₂S-producing E. coli were recovered. In the United States, more than 200 isolates of H₂S-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 H₂S-positive Shigella sonnei strains, one of which was authentic. The other showed H₂S production due to Eubacterium lentum, an obligate anaerobe, living in synergism with H₂S-negative S. sonnei.

Whenever an H₂S-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 H₂S 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 H₂S-negative Salmonella. It showed no H₂S production after 18 h of incubation. H₂S 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 H₂S producer pattern. Tentative identification was made as H₂S-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 H₂S production was again noted in the confluent growth area, where the medium was stabbed. No H₂S 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 H$_2$S; and only reduced nitrate to nitrite. They were identified as *Eubacterium lentum* and confirmed by gas-liquid chromatography.

Since H$_2$S 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 H$_2$S production by *P. alcalifaciens*, a search was started for the occurrence of such associations among other strains. Eighteen-hour H$_2$S-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 H$_2$S production after 24 h of further incubation, 4 produced delayed H$_2$S. These isolates were not from consecutive clinical specimens, and, except for H$_2$S 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 H$_2$S production, and it was ascertained in each case that the member of the *Enterobacteriaceae* was indeed not an H$_2$S producer when pure.

Finally nine other known H$_2$S-negative strains were checked for synergism with *E. lentum* (P65). These cultures were identified and found to be H$_2$S negative by API, Minitek, and Inolex 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 H$_2$S 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 H$_2$S in synergistic relationships. They were not certain of the mechanism involved and originally postulated that all H$_2$S-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 H$_2$S-positive *E. coli* cultures were examined and found to be authentic H$_2$S producers. Since other previously submitted H$_2$S-positive cultures which spontaneously lost H$_2$S production on subculture were not available to be further examined, no conclusion could be drawn.

It has been postulated that H$_2$S 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 H$_2$S production in TSI slants with known H$_2$S-negative organisms by co-culturing them with *E. lentum*, a question arises as to how many of the H$_2$S-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 H$_2$S-producing isolates (1, 4, 11).

Galton and Hess (8) observed that H$_2$S production in *S. alkalescens* was lost on subculture in one of five H$_2$S-positive isolates. Aikawa and Iida (1) in Japan, when studying H$_2$S-positive *S. sonnei* isolates, described loss of ability to produce H$_2$S by fermenting mutants. Studying the extrachromosomal nature of H$_2$S production by two *E. coli* strains, Layne and co-workers (10) found that the H$_2$S production trait can be segregated out. Unfortunately, lack of effective screening at that time prevented further work.

Lautrop (9) noted that in H$_2$S-positive *E. coli*, H$_2$S-negative colonies occasionally appeared upon seeding from stock cultures. Five H$_2$S-negative subcultures were isolated from 26 original H$_2$S-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