Tryptophan and Hydrogen Sulfide Reaction from Modified Trypticase Soy Agar

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The use of Trypticase soy agar modified by the addition of 0.05% ferric ammonium citrate and 0.01% sodium thiosulfate for the determination of indole, phenylpyruvic acid, anthranilate, kynurenine, and hydrogen sulfide reactions is described.

In an ongoing search for biochemical reactions which will more specifically identify nonfermenting, gram-negative bacilli and thus, we hope, lead to improved methods for the identification of these organisms, we were examining the ability of *Pseudomonas acidovorans* and *P. testosteroni* to form anthranilate and kynurenine from tryptophan. R. Hugh and J. D. Welch (Bacteriol. Proc., p. 148, 1969) had stated that these two intermediates of tryptophan metabolism are responsible for the light yellow-to-orange colors which develop in the alcohol layer when tryptone broth cultures of *P. acidovorans* and *Comamonas terrigena* are tested with Kovacs reagent. When we tested our strains of *P. acidovorans* and *P. testosteroni* on Trypticase soy agar (TSA; Baltimore Biological Laboratory, Cockeysville, Md.) slants with Ehrlich reagent, the growth of *P. acidovorans* strains became orange, the reagent which collected at the base of the slant became increasingly yellow, and the medium in the area of the slant became increasingly and diffusely yellow. These colors developed within 5 to 15 s.

If anthranilate and kynurenine could be detected in TSA slants with an indole reagent, then indole might also be detectable. When we tested strong (*Escherichia coli*) and weak (*Flavobacterium meningosepticum*) indole-positive cultures on TSA slants with Ehrlich reagent, the results were comparable to tests made in tryptone broth.

Since T. Cekoric, P. Yanaton, and G. L. Evans (Bacteriol. Proc., p. 90, 1970) had indicated that an intense brown color is developed by tryptophan deaminase (TDA)-positive organisms in a solid medium containing an iron salt and tryptophan, we added ferric ammonium citrate (0.05%) to TSA and tested with TDA-positive species. A diffuse brown color developed in the area of the slant with all of our strains of *Proteus* and *Providencia*. When the indole- and TDA-positive strains were tested with Ehrlich reagent,

the indole reaction was also detectable. The brown color of the deaminase reaction was bleached by the reagent. Since ferric ammonium citrate had been added to detect TDA, it seemed reasonable to add sodium thiosulfate, the second component of the conventional hydrogen sulfide indicators (Kligler iron agar, triple sugar iron agar, lysine iron agar, xylose lysine agar, Hektoen enteric agar, etc.) to test for hydrogen sulfide production. When strains selected for their abilities to form hydrogen sulfide were tested in TSA containing 0.05% ferric ammonium citrate and 0.01% sodium thiosulfate, the results were as expected; furthermore, formation of the black precipitate did not interfere with the determination of indole and/or indolepyruvic acid. Thus, modifying TSA by the addition of an iron salt and sodium thiosulfate and subsequently testing cultures grown on the modified TSA with an indole reagent (Ehrlich or Kovacs) permitted determination of four reactions developed singly or in combinations, i.e., formation of indole (tryptophanase), indolepyruvic acid (tryptophan deaminase), and anthranilate and/or kynurenine (tryptophan pyrrolase for the initial step) from tryptophan and hydrogen sulfide from thiosulfate.

To test the reliability, sensitivity, and efficiency of the modified TSA, approximately 250 strains of glucose-fermenting and 400 strains of non-glucose-fermenting, nonfastidious gram-negative bacilli were tested in the following way. Modified TSA slants were prepared by adding ferric ammonium citrate (0.05%, wt/vol) and sodium thiosulfate (0.01%, wt/vol) to TSA, tubing 6 ml in 16- by 125-mm tubes, autoclaving at 121°C for 15 min, and then slanting as for other differential slants (e.g., Kligler iron agar). Except for a very slight tinge of brown, the modified medium was almost indistinguishable from conventional TSA. Stock cultures were transferred from cysteine Trypticase agar (Baltimore Biological Laboratories TSA slants and incubated

logical Laboratories) to TSA slants and incubated at 37°C for 24 h. The modified TSA slants were then inoculated by stab and streak from the TSA cultures and incubated at 37°C. After 24 h, the cultures were examined for the deaminase reaction (diffuse browning in the area of the slant) and the hydrogen sulfide reaction (blackening which was confined for the most part to the stab). When both reactions were positive, neither overshadowed the other. The surface of each slant was then rinsed with approximately 0.2 to 0.3 ml of Ehrlich reagent (*p*-dimethyl-aminobenzaldehyde, 1 g; 95% ethanol, 95 ml; concentrated HCl, 20 ml), and the fluid was allowed to collect at the base of the slant. The development of pale pink-to-red in the reagent was positive for the presence of indole. The development of yellow in the medium and the reagent and yellow-to-orange in the growth within 5 to 15 s, with all colors intensifying with time, was taken as positive for the presence of anthranilate and/or kynurenine.

For the glucose-fermenting gram-negative bacilli, the positive reactions for indole, indolepyruvic acid, and hydrogen sulfide were entirely typical of and in accord with the reactions of the species tested. Some strains of *Klebsiella oxytoca* (Bergey's *Manual of Determinative Bacteriology* [5] calls this organism *K. pneumoniae*) produced a slight discoloration in the medium suggestive of a weak TDA reaction; however, since this discoloration was also observed in TSA slants without ferric ammonium citrate, it was not interpreted as a positive deaminase reaction. The weakest indole reactions were those of five strains of *Pasteurella multocida* which were tested. Table 1, based on indole, indolepyruvic acid, and sulfide reactions, shows the patterns that can generally be expected for most strains (80% or more) of the glucose fermenters that are shown. None of the fermenters are positive for the anthranilate/kynurenine reaction.

Most of the nonfermenting gram-negative bacilli are negative for all four of the characteristics; however, these reactions are pertinent for some members of this group. For the nonfermenters, indole formation is characteristic for *F. meningosepticum* and some unnamed Center for Disease Control groups (6, 7); amino acid deaminase activity is characteristic for *Moraxella phenylpyruvica* (7) and possibly some other species and Center for Disease Control groups (2, 4, 7); formation of hydrogen sulfide in the stab is characteristic for *Pseudomonas putrefaciens* (1, 2); and formation of kynurenine and anthranilate is characteristic for *P. acidovorans* and *P. testosteroni* (2; Hugh and Welch, Bacteriol. Proc., p. 148, 1969). Each of these reactions,

TABLE 1. Indole, deaminase, and sulfide reactions of gram-negative fermenters on modified TSA

Reactions ^a			Organisms
I	D	S	
+	+	+	<i>Proteus vulgaris</i> (4/4) ^b
+	+	-	<i>Proteus morganii</i> (10/10), <i>P. rettgeri</i> (2/3), <i>Providencia stuartii</i> (6/8)
-	+	+	<i>Proteus mirabilis</i> (8/11)
+	-	+	<i>Edwardsiella tarda</i> (1/1), <i>E. coli</i> (1/16)
+	-	-	<i>Aeromonas hydrophila</i> (2/2), <i>A. shigelloides</i> (1/1), <i>Citrobacter diversus</i> (3/3), <i>Enterobacter</i> sp. (2/35), <i>E. coli</i> (15/16), <i>K. oxytoca</i> (31/31), <i>P. multocida</i> (5/5), <i>Pectobacterium</i> sp. (1/8), <i>Shigella</i> sp. (2/7), <i>Yersinia enterocolitica</i> (11/15)
-	+	-	<i>P. mirabilis</i> (3/11), <i>P. rettgeri</i> (1/3), <i>P. stuartii</i> (2/8)
-	-	+	<i>Arizona hinshawii</i> ^c (3/3), <i>Citrobacter freundii</i> (13/13), <i>Salmonella</i> sp. (9/11)
-	-	-	<i>Enterobacter</i> sp. (33/35), <i>K. pneumoniae</i> (21/21), <i>Pectobacterium</i> sp. (7/8), <i>Salmonella</i> sp. (2/11), <i>Serratia</i> sp. (18/18), <i>Shigella</i> sp. (5/7), <i>Y. enterocolitica</i> (4/11), <i>Y. pseudotuberculosis</i> (8/8), <i>Y. pestis</i> (7/7)

^a I, Indole; D, deaminase; S, hydrogen sulfide.

^b Number of strains with reaction pattern/number of strains tested.

^c Bergey's *Manual of Determinative Bacteriology* (3) calls this organism *Salmonella arizonae*.

when taken in association with other characteristics being used to identify nonfermenters (2, 4, 6, 7), is distinctive for the organisms mentioned. For example, if shown to form indole, a yellow, oxidase-positive nonfermenter would be a *Flavobacterium* sp. and not *Pseudomonas maltophilia*, *Pseudomonas cepacia*, or a *Xanthomonas* sp. (1, 6); similarly, a nonfermenter which forms hydrogen sulfide anaerobically could only be *P. putrefaciens* (2, 6). If greater attention were paid to these reactions with nonfermenters, identification of some of the less frequently encountered species in this complex group might be facilitated.

When indole reagent was added to pyocyanogenic strains of *Pseudomonas aeruginosa*, the growth and the reagent changed to red as the pyocyanine reflected the acid condition of the reagent. With weakly positive pyocyanogenic strains, care must be taken that the pale red color which develops is not wrongly interpreted as a positive indole reaction. With some strains of *P. aeruginosa*, there was some blackening in the aerobic area of the slant (8), whereas the positive hydrogen sulfide reaction of *P. putrefaciens* was in the stab. The brown, soluble pigment formed by some strains of *P. aeruginosa* was not bleached by the indole reagent and was therefore distinguishable from the TDA reactions.

In the observations for tryptophan pyrrolase,

it was noted that strains of *P. maltophilia* and *Pseudomonas diminuta* and some yellow-pigmented strains (indole negative; therefore, not considered *Flavobacterium*) caused the almost colorless Ehrlich reagent to become yellow. In this reaction, at least for the first five min, the color was limited to the reagent collected at the base of the slant, and the appearance of the reaction was quite distinct from that produced by the tryptophan pyrrolase-positive strains of *P. acidovorans*. Further examination of this type of reaction is needed before an interpretation can be developed.

Procedures which use existing media, such as Kliglers iron agar, triple sugar iron agar, lysine iron agar, motility-indole-ornithine medium, phenyl-alanine agar, indole-nitrite-medium, peptone broth, etc., for determining indole, deaminase, and hydrogen sulfide reactions require some combination of two or more of these media. This study demonstrates that: (i) TSA, a commonly used nutrient agar, can be used to determine the formation of indole or anthranilate/kynurenine from tryptophan; (ii) the addition of iron salts (0.05%) enables the detection of indolepyruvic acid from tryptophan; (iii) the further addition of sodium thiosulfate (0.01%) adds the capability for detecting hydrogen sulfide reactions; and (iv), being devoid of pH indicators and sugar, the modified TSA can still serve as a nutrient agar for use in detecting other characteristics. In cost-conscious laboratories and perhaps in Third-World laboratories where it may not be possible or practical to have available the necessary commercial media, the use of

modified TSA for the functions described could provide some savings in time and money.

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