Two-Dimensional Thin-Layer Chromatography for the Specific Detection of Hippurate Hydrolysis by Microorganisms

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Glycine, one of the end products of hippurate hydrolysis by microorganisms, was detected by a rapid, specific technique utilizing two-dimensional thin-layer chromatography. A loopful of growth of each organism from its suitable agar medium was washed, suspended, and incubated with 0.1% sodium hippurate for 30 min at 37°C. The supernatant of the incubated suspension from each organism was then dansylated, and the dansyl derivatives were separated by two-dimensional thin-layer chromatography on polyamide sheets. Glycine, a product of hippurate hydrolysis, was detected under UV light. This technique does not require prolonged incubation and was found to be more specific and reliable than the standard ninhydrin reaction. In addition, it is inexpensive and can be easily conducted in a clinical microbiological reference laboratory. By this method, 100% (22/22) of *Campylobacter jejuni* and 0% (0/9) of *Campylobacter coli* reference strains were positive. In addition, 100% (13/13) of group B streptococci, 100% (24/24) of group D streptococci, and 90% (18/20) of *Gardnerella vaginalis* clinical isolates were positive for hippurate hydrolysis. This method is useful for the identification to the species level of *Campylobacter* organisms and the biotyping of *Gardnerella* organisms and for the detection of hippurate hydrolysis by unknown microorganisms.

The hydrolysis of hippurate (*N*-benzoylglycine), used in clinical laboratories for the identification or biotyping of certain microorganisms (3, 19), has been based on the detection of either of the two end products, benzoic acid (1, 8, 11, 15) or glycine (12, 14), after incubation of microorganisms with a high concentration of hippurate. Methods for detecting benzoic acid include precipitation of benzoate with ferric chloride (1, 11), use of chromogenic reagents (8), and gas-liquid chromatography (15). Glycine is detected by the purple produced from the ninhydrin reaction either directly in the incubation mixture (14) or after separation of glycine from the mixture by one-dimensional thin-layer chromatography (TLC) (12).

Detection of benzoate in the spent medium by using ferric chloride requires prolonged incubation and gives an equivocal endpoint determination (1, 11). The colorimetric method introduced by Edberg and Samuels (8) has been tested only for group B streptococci. Although gas-liquid chromatography (15) is the only current method that is specific and applicable to various microorganisms, it involves lengthy derivatization and is labor intensive (7); it is therefore not efficient for testing a large number of microorganisms. The ninhydrin test (14) is probably the most widely used method to detect hippurate hydrolysis in clinical laboratories. However, it detects not only glycine, but also other amino acids, peptides, and biogenic amines such as agmatine, putrescine, and cadaverine (decarboxylated products or arginine, ornithine, and lysine). These amino acids, peptides, and amines may be carried over from the spent agar medium or may also be produced by the microorganisms during incubation, thereby giving false-positive results. Methods for detecting glycine after separation from the spent medium by single-dimensional TLC provide more reliable results (12); however, there has not been a suitable single-solvent system reported to separate glycine from all other amino acids (17).

We previously reported rapid procedures for the simultaneous detection of eight bacterial amino acid decarboxylases and for the detection of the arginine dihydrolase system by using two-dimensional TLC (2-D TLC) on polyamide sheets (4). In the present study, we modified the 2-D TLC procedures for the rapid, inexpensive, and specific detection of glycine resulting from hippurate hydrolysis. Using this method, we were able to differentiate reference strains of *Campylobacter jejuni* from *Campylobacter coli*, group B from group D streptococci, and hippurate-negative from hippurate-positive biotypes of *Gardnerella vaginalis*. Several other microorganisms were also tested for hydrolysis of hippurate by this method.

MATERIALS AND METHODS

Culture conditions. The microorganisms used in this study were from various sources. Stock cultures were obtained from the American Type Culture Collection and the National Collection of Type Cultures. Group B and D streptococci and *G. vaginalis* were recent clinical isolates. *Campylobacter* strains were kindly provided by J. L. Penner.

All *Campylobacter* species tested in this study were grown on selective agar plates containing brucella agar base (Difco Laboratories, Detroit, Mich.), 10% sheep blood, vancomycin (10 µg/liter), polymyxin (2,500 IU/liter), trimethoprim (5 mg/liter), and amphotericin B (2 mg/liter). These plates were then incubated for 24 h at 37°C in a GasPak anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) in which a microaerophilic atmosphere was created by using H₂+CO₂ generator envelopes with the catalyst removed (10).

Streptococci were isolated from vaginal washings and were cultured on primary isolation plates of Trypticase soy agar (BBL) with 5% sheep blood. Group B streptococci were identified by colonial morphology and the Streptex Rapid Latex Test Kit (Wellcome Research Laboratories, Beckenham, England). Group D streptococci were identified by colonial morphology, bile-esculin reaction (9), and the Streptex Rapid Latex Test Kit. *G. vaginalis* strains were

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TABLE 1. Hippurate hydrolysis by representative microorganisms detected by 2-D TLC and by ninhydrin reaction

Microorganisms ^a	Hippurate hydrolysis ^b			
	TLC ^c	Ninhydrin method ^d		
		Test	Control	Results ^e
Group B streptococci JH 1442, 6430, 6442, 6465, 6468, 6469, 6530, 6535, 6541, 8514, 8529, 8570, 8580	+	+	—	+
Group D streptococci JH 3761, 4173, 4179, 4306, 8299, 8302, 8310, 8421, 8524	W	W	—	W
ATCC 12984; JH 4159, 4168, 4207, 4234, 8293, 8312, 8379, 8394, 8402, 8417, 8419, 8437, 8459, 8500	W	—	—	—
<i>Campylobacter jejuni</i> Pen 1, 7, 10, 17, 18, 21, 31, 35, 36, 40, 41, 43; NCTC 11351	+	+	W	+
Pen 2, 3, 4, 6, 11, 13, 16, 32	+	+	—	+
Pen 22	+	W	—	W
<i>Campylobacter coli</i> Pen 14, 20, 24, 25, 26, 39, 47; NCTC 11366	—	W	W	—
Pen 34	—	—	—	—
<i>Gardnerella vaginalis</i> JH 8373, 8380, 8384, 8405, 8413, 8417, 8431, 8485, 8494, 9495, 8497, 8501, 8502, 8503, 8507, 8508	+	+	W	+
JH 8496, 8512	+	W	W	—
JH 8427	—	W	W	—
JH 8376	—	—	—	—
Gram-negative enteric bacteria <i>Citrobacter freundii</i> ATCC 10787	—	W	W	—
<i>Enterobacter agglomerans</i> ATCC 29915	—	W	W	—
<i>Escherichia coli</i> ATCC 27549	—	W	W	—
<i>Proteus mirabilis</i> ATCC 14273	—	W	W	—
<i>Serratia marcescens</i> ATCC 17991	—	W	W	—
<i>Shigella sonnei</i> ATCC 11060	—	W	W	—
Gram-negative nonenteric bacteria <i>Pseudomonas cepaciae</i> BM 2	+	W	—	W
<i>Pseudomonas fluorescens</i> BM 3	—	—	—	—
<i>Pseudomonas maltophilia</i> BM 4	—	—	—	—
Gram-positive bacteria <i>Bacillus licheniformis</i> ATCC 14409	—	—	—	—
<i>Bacillus subtilis</i> ATCC 14410, 14415, 14416, 14807	—	—	—	—
<i>Staphylococcus aureus</i> ATCC 12598, 25923; BM Me19, U17, Su3	+	+	—	+

^a Strain numbers are those of the American Type Culture Collection (ATCC), National Collection of Type Cultures (NCTC), Judith Hale (JH), Barbara H. Minshew (BM), or J. L. Penner (Pen). The growth conditions for each microorganism are described in the text.

^b +, Positive; —, negative; W, weakly positive.

^c The activity of hippurate hydrolysis by microorganisms was detected by evaluating the fluorescence intensity of dansyl glycine after 2-D TLC on polyamide sheets. Activity of hippurate hydrolysis was recorded as weakly positive when the fluorescence intensity of the dansyl glycine was faint; the result was positive when bright fluorescence was observed.

^d The ninhydrin test for hippurate hydrolysis was recorded as weakly positive when a faint purple was observed at the end of incubation and as positive when a deep purple was observed.

^e The results of the ninhydrin reactions were interpreted by comparing the color intensities of the test and control tubes.

isolated from vaginal washings on human blood bilayer agar media with Tween 80 or without Tween 80 (20) in 36°C candle extinction jars and identified by typical microscopic and colonial morphology (20). The growth conditions and sources of gram-negative bacteria (enteric and nonenteric) and other gram-positive bacteria listed in Table 1 were described previously (6).

Hippurate hydrolysis and dansylation. One loopful (2-mm diameter) of growth from microorganisms grown on an agar plate was suspended (by blending in a Vortex mixer) in a Microfuge tube (250 μ l; Stockwell Scientific, Monterey Park, Calif.) containing 60 μ l of phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M sodium phosphate buffer [pH 7.0]) and centrifuged in a Microfuge (model 152; Beckman Instruments, Inc., Fullerton, Calif.) for 1 min. The superna-

tant was discarded. The pellet was washed once again as above to completely ensure the removal of glycine and other amino acids or amines from the spent agar medium. PBS (60 μ l) was then added to the washed pellet and blended in a Vortex mixer. A 20- μ l sample of this bacterial suspension was added to a 250- μ l microfuge tube containing 20 μ l of 0.2% sodium hippurate (Sigma Chemical Co., St. Louis, Mo.). Another 20 μ l of this bacterial suspension was added to a tube containing 20 μ l of PBS as a negative control. Each mixture was blended briefly in a Vortex mixer, incubated aerobically at 37°C for 30 min, and centrifuged in a Microfuge for 1 min to pellet the organisms. The supernatant (20 μ l) from each tube was removed and placed in another microfuge tube containing 20 μ l of 1 M NaHCO₃. This mixture was blended briefly again in a Vortex mixer, and 40

FIG. 1. Summary of the protocol for the specific detection of hippurate hydrolysis by microorganisms. Note that the protocol also includes a control tube in which sodium hippurate is replaced by PBS for the determination of the fluorescence intensity of residual glycine carried from the washed bacterial suspension.

μ l of dansyl chloride solution (5 mg/ml of acetone) was added to the mixture. The microfuge tubes were capped tightly, blended in a Vortex mixer, incubated at 37°C for 1 h, and centrifuged for 1 min after incubation.

Separation of dansyl derivatives by 2-D TLC on polyamide sheets. The dansylated supernatant (2 μ l) from each test microorganism (incubated with sodium hippurate) was spotted on one side (front side) of a Chen-Chin polyamide layer sheet (Accurate Chemical and Scientific Corp., Hicksville, N.Y.), and the dansylated supernatant (2 μ l) from the controls (incubated with PBS) was spotted on the opposite side (back side) of the same sheet. TLC was performed as described by Woods and Wang (21), except that both dimensions of the chromatography were run to only about 60% of the length of the sheet. The position of the glycine was then identified under UV light. The summary of the protocol by using 2-D TLC for the specific detection of glycine resulting from hippurate hydrolysis by microorganisms is shown in Fig. 1.

Detection of hippurate hydrolysis by ninhydrin. The ninhydrin method was performed as described by Hwang and Ederer (14). Negative control organisms (unwashed) were incubated with normal saline, whereas organisms being tested (unwashed) were incubated with 1% sodium hippurate as previously described (14). Both the test and control tubes were incubated at 37°C for 2 h, reacted with ninhydrin (Pierce Chemical Co., Rockford, Ill.) at 37°C for 10 min, and read as previously described (14).

RESULTS

Use of 2-D TLC on polyamide sheets to detect glycine produced by hippurate hydrolysis. The relative positions of dansyl derivatives of glycine, other amino acids, and ammonia after 2-D TLC were determined as described previously by Chen et al. (4). The activity of each microorganism in hydrolyzing hippurate was detected by the fluorescence intensity of dansyl glycine on polyamide sheets. Controls using washed microorganisms incubated with PBS instead of sodium hippurate showed no glycine, other amino acids, or biogenic amines (Fig. 2A and 2C and Fig. 3A and 3C). Ammonia was always present on polyamide sheets after 2-D TLC and served as a marker to help identify dansyl glycine (Fig. 2 and 3). The fluorescence intensity of dansyl glycine was recorded as positive, weakly positive, and negative. For instance, glycine was produced by a strain of group B streptococci (JH 6430) after 30 min of incubation with sodium hippurate at 37°C (cf. Fig. 2A and 2B). Under the same experimental conditions, glycine was produced weakly by a strain of group D streptococci (JH 8421) (cf. Fig. 2C and 2D). Likewise, glycine was produced by a *C. jejuni* strain (Pen 10) after incubation with sodium hippurate (cf. Fig. 3A and 3B). However, glycine was not produced by a *C. coli*

strain (cf. Fig. 3C and 3D). Note that dansyl derivatives from all test and control tubes were spotted on the same side of the sheets shown in Fig. 2 and 3 for easy illustration and comparison.

In this study, 13 strains of group B streptococci, 24 strains of group D streptococci, 22 strains of *C. jejuni*, 9 strains of *C. coli*, and 20 strains of *G. vaginalis* were studied by 2-D TLC to detect their ability to hydrolyze hippurate. The results were then compared with those from the ninhydrin test method (Table 1). All 13 strains of group B streptococci were positive by both 2-D TLC and the ninhydrin test. On the other hand, a weakly positive dansyl glycine was detected by 2-D TLC in all (24/24) strains of group D streptococci tested, but only 38% (9/24) were positive for hippurate hydrolysis by the ninhydrin test. Our study of the Penner reference strains and the National Collection of Type Cultures strain of *C. jejuni* also showed that all 22 strains were positive for hippurate hydrolysis by 2-D TLC and by the ninhydrin reactions. All nine *C. coli* strains used in this study were negative for hippurate hydrolysis by 2-D TLC, but several gave positive reactions in the ninhydrin test (Table 1). These could be identified as false-positives only by comparing them to the control tubes, which are not generally included in clinical microbiology laboratory studies. Of 20 *G. vaginalis* strains studied, 18 were hippurate positive by 2-D TLC.

We also analyzed 19 strains of various other microorganisms by 2-D TLC and compared the results with those from the ninhydrin method (Table 1). The results of our 2-D TLC correlated well with those of the ninhydrin method, provided both the test and control tubes were used to avoid the ambiguity of faint purple in some of the ninhydrin reaction mixtures.

DISCUSSION

The occasional false-positive results encountered in the ninhydrin test without controls (14), as shown in Table 1, may be attributable to amino acids, peptides, and amines, which were carried from the agar medium or produced during incubation. For example, spent broth after the growth of *G. vaginalis* contained a much higher concentration of free amino acids than the uninoculated broth (5), which may explain the faint purple present in the ninhydrin control tube (Table 1). On the other hand, false-negative results from the ninhydrin method may also have been encountered when the inoculum was insufficient or when microorganisms showed weak hippurate hydrolytic activity. Group D streptococci represented this type of microorganism, whose hippurate-hydrolyzing activity was below the sensitivity of the ninhydrin test. Finally, we experienced difficulty in setting a cutoff point by the ninhydrin method, especially when unknown microorganisms were tested. For instance, organisms of the

FIG. 2. Hippurate hydrolysis by streptococci. (A) Group B streptococci incubated with PBS (negative control) showed no dansyl glycine. (B) Group B streptococci incubated with 0.1% sodium hippurate showed strongly positive dansyl glycine under UV light. (C) Group D streptococci incubated with PBS (negative control) showed no dansyl glycine. (D) Group D streptococci incubated with 0.1% sodium hippurate showed weakly positive fluorescence of dansyl glycine under UV light.

family *Enterobacteriaceae* produced biogenic amines such as agmatine, putrescine, and cadaverine (4) and subsequently gave more color intensity in the ninhydrin control tubes than that observed in the weakly positive ninhydrin test tubes produced by group D streptococci.

In this study, we analyzed recent clinical isolates and reference strains. The results of hippurate hydrolysis by 2-D TLC were compared with the ninhydrin results utilizing both the test and control tubes as described in Materials and Methods (Table 1). *C. jejuni* and *C. coli* have been separated into species based on their abilities to hydrolyze hippurate (12, 13, 16). Determination of hippurate hydrolysis among *Campylobacter* species based on the ninhydrin method was generally accurate, but an additional negative control is essential for interpreting the results of the ninhydrin test, particularly when the organisms are grown on an enriched medium (10). In comparison, our 2-D TLC offered a more specific and sensitive test method to detect hippurate hydrolysis by *C. jejuni* and *C. coli*. The National Collection of Type Cultures and Penner reference strains tested by 2-D TLC showed 100% correlation with the ninhydrin test results published by Penner et al. (18) and Harvey (12). However, we also want to stress that although hippurate hydrolysis by *C. jejuni* and *C. coli* correlated well with identification to the species level by DNA homology analysis of the reference strains used in this study, we have identified several hippurate hydrolysis-negative strains of *C. jejuni* in Seattle, Wash. (P. A. Totten and F. C. Tenover, unpublished data). Harvey (12) recently suggested that hippurate-negative strains of *C.*

jejuni might represent a biotype within the species. Thus, the reliability of hippurate hydrolysis testing for the identification to the species level of *Campylobacter* strains may vary with the geographic sampling.

As with the *Campylobacter* strains tested in this study, we also experienced ambiguity in results of tests with the *G. vaginalis* strains (JH 8427, 8496, and 8512) by using the ninhydrin method for detection of hippurate hydrolysis (Table 1). For this reason, we suggest that our 2-D TLC be used instead of the ninhydrin method for the biotyping of *G. vaginalis* organisms, according to the scheme described by Piot et al. (19). Furthermore, the conventional ninhydrin method (14) did not correlate well with the results by 2-D TLC when group D streptococci were tested. The 2-D TLC showed that all 24 strains of group D streptococci isolated from vaginal washings, presumably *Streptococcus faecalis*, were weakly positive for hippurate hydrolysis, whereas only 38% (9/24) were weakly positive by the ninhydrin method (Table 1). A report in the Minitak (BBL) manual (2) indicates that 96% of group D *S. faecalis* organisms were positive for hippurate hydrolysis. All group B streptococci tested were strongly positive for hippurate hydrolysis by 2-D TLC (Table 1); therefore, 2-D TLC may prove useful for distinction between group B and D streptococci based on the different levels of fluorescence intensity of dansyl glycine produced.

The 2-D TLC procedures described in this study allowed rapid and specific detection of glycine, one of the end products of hippurate hydrolysis. Sodium hippurate was stable during 30 min of incubation with PBS and could be

FIG. 3. Hippurate hydrolysis by *Campylobacter* species. (A) *C. jejuni* incubated with PBS (negative control) showed no dansyl glycine. (B) *C. jejuni* incubated with 0.1% sodium hippurate showed strongly positive dansyl glycine under UV light. (C) *C. coli* incubated with PBS (negative control) showed no dansyl glycine. (D) *C. coli* incubated with 0.1% sodium hippurate showed no dansyl glycine under UV light.

stored at -20°C for several months without detectable nonenzymatic hydrolysis (checked by dansylation and 2-D TLC as described under Materials and Methods). This method is inexpensive, applicable to various microorganisms grown on different media, and potentially useful for reference laboratories to characterize strains of *Campylobacter* and *Streptococcus* and for the biotyping of microorganisms such as *G. vaginalis*. Six polyamide sheets (12 specimens applied) can be chromatographed in one glass jar (13 by 16 by 26 cm). Less than 1 h was required to complete both dimensions of chromatography. Polyamide sheets can be washed and reused at least 10 times, as previously described (4). Unlike the ninhydrin method, the detection of dansyl glycine on polyamide sheets by 2-D TLC gave definite evidence of hippurate hydrolysis regardless of the fluorescence intensity of dansyl glycine observed under a long-wave UV light. Therefore, this method is objective, and it can be best used in the analysis or characterization of new strains of microorganisms isolated in clinical reference laboratories.

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