Detection of Arginine Dihydrolase in Nonfermentative Gram-Negative Bacteria by Use of Thin-Layer Chromatography

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Thin-layer chromatography was used to detect the presence of the arginine dihydrolase system in nonfermentative gram-negative bacteria. The test was positive for the fluorescent pseudomonads as well as Pseudomonas maltophilia, whereas other Pseudomonas sp., Mima, and Herellea were negative. This procedure can be completed in a few hours and may be useful in the clinical laboratory.

Many groups of nonfermenting bacteria produce arginine dihydrolase. This has recently been demonstrated by several authors (1, 4, 6) especially for the fluorescent group of Pseudomonas. The methods used by these authors require at least several days. The method of Thornley for arginine dihydrolase as used by Taylor and Whitby (7) requires from 2 to 7 days. If a simpler, more rapid method for the detection of this enzyme were available, this might be useful for the identification and differentiation of nonfermenters in the clinical laboratory.

Soru (5) developed a method for the detection of arginine dihydrolase production in beta-hemolytic streptococci by using circular paper chromatography. Arginine dihydrolase converts arginine to citrulline and finally citrulline to ornithine (7), both of which can be easily detected by chromatography. However, the Soru method requires 24 hr for the development of the paper chromatogram.

We have developed a more rapid procedure by adapting the principles of the Soru method (5) to thin-layer chromatography (TLC) which is much faster than paper chromatography.

MATERIALS AND METHODS

Seventy-eight clinical isolates were obtained from the Diagnostic Microbiology Laboratory of the University of Minnesota Hospitals in 1970 and 1971. They were identified by the method of King (2). The distribution of species is shown in Table 1. Reference strains used were Pseudomonas putida ATCC 12633, P. maltophilia ATCC 13637, P. fluorescens ATCC 13525, P. stutzeri ATCC 11607, P. denitrificans ATCC 19244, P. odorans ATCC 15553, and P. alcaligenes ATCC 14909, obtained from the American Type Culture Collection, and Mima polymorpha var. oxidans (Moraxella) CDC B1882, obtained from the Center for Disease Control. Each organism was tested for arginine dihydrolase production by the method of Thornley according to Taylor and Whitby (7). A control medium without L-arginine was also used. The medium was dispensed in 2-ml amounts in test tubes (13 by 100 mm) and autoclaved at 121 C for 20 min and stored at 4 C. Both tubes were inoculated by stabbing the media one half way to the bottom. They were then covered with a 5-mm layer of sterile liquid paraffin, tightly capped, and incubated at 35 C for 7 days. The tubes were observed on days 1, 2, 5, and 7 for positive or negative results. A positive test for the presence of arginine dihydrolase was indicated by a deep pink to red color whereas a negative test was demonstrated by an absence of color change in the medium or a yellow color.

For the TLC method, organisms were grown for 18 to 24 hr at 35 C on either triple sugar iron or Trypticase soy agar slants. A generous loopful of growth was taken from the slant and inoculated into 0.25 ml of 0.01 M L-arginine hydrochloride (pH 6.4) in distilled water in test tubes (13 by 100 mm) which had been stored at —20 C and then thawed before use. The cell suspensions were incubated for 2.5 hr at 37 C in a heating block, and then 2-µliters were applied on a cellulose precoated sheet (MN Polygram Cel 300, Brinkman Instruments, Inc.) with 2-µlitter spotting capillaries (Brinkman Instruments, Inc.). Care was exercised so that the cells, which had settled, were not agitated when the samples were taken. The samples were applied 1.5 cm from the bottom of the chromatogram at 1.0-cm intervals across it. Half of the sample was applied and dried with a stream of air and the second half was applied and dried in the same way. On each chromatogram, 2-µliter amounts of standard 0.01 M aqueous solutions of arginine, citrulline, and ornithine were applied in the same way. After all samples had been completely dried, the chromatogram was developed in a standard Desaga tank filled to a depth of approximately 0.3 cm by using n-butanol–acetone–
acetic acid–water (35:35:10:20) as a solvent. This solvent was prepared by adding 12 ml of acetic acid–water (1:2) mixture to 28 ml of an n-butanol–acetone (1:1) mixture. The chamber was allowed to become saturated before the chromatograms were placed in the tank. Development to 10 cm took approximately 45 min. After this, the chromatogram was completely dried on a slide drier at 50 C for about 10 min. The chromatogram was then sprayed to saturation with a 0.4 M solution of ninhydrin in n-butanol–acetone (1:1) and then again dried completely on the slide dryer. Development of the purple spots took 15 to 20 min. To demonstrate the effect of an organism with an arginine decarboxylase system would have on the arginine substrate, a strain of Escherichia coli (3) was used in this study. Aqueous solutions of 0.01 M urea and agmatine were prepared as controls for this enzyme system.

RESULTS

The average $R_F$ values from 10 different chromatograms were as follows: ornithine, 0.18; arginine, 0.22; citrulline, 0.24. Agmatine had an $R_F$ of 0.42, based on two determinations.

Organisms positive for arginine dihydrolase converted arginine to ornithine with no evidence of citrulline as an intermediate product. The results of all tests are shown in Table 1. In Thornley's medium, all positive reactions occurred after 2 days of incubation. All organisms which gave positive results with Thornley's test showed some conversion of arginine to ornithine, whereas those which gave a negative Thornley's test showed no detectable conversion of arginine to ornithine, except for P. maltophilia. This organism was arginine dihydrolase-negative with Thornley's method; however, when tested with TLC, it always converted some arginine to ornithine. In addition to producing ornithine, this organism also gave trailing and another well defined spot with the same $R_F$ value as agmatine.

When P. maltophilia and E. coli, which is known to have the arginine decarboxylase system, were compared on TLC, the results for both organisms were nearly identical. E. coli gave the same trailing and spotting that had been previously found with P. maltophilia.

DISCUSSION

TLC is a very simple technique when used for the detection of arginine breakdown among non-fermenting bacteria. If fresh bacterial cultures are available on triple sugar iron or Trypticase soy agar slants, the test can be completed within 4 hr. Results are clear and simple to read.

The arginine dihydrolase systems produce citrulline and ornithine from arginine, all three of which may easily be detected by TLC. The first enzyme system, arginine desimidase, hydrolyzes arginine to citrulline and NH$_3$ (7). The second enzyme of the arginine dihydrolase system converts citrulline to ornithine, NH$_3$, and CO$_2$ (7).

Since citrulline is an intermediate in the reaction, it may have been converted into ornithine too rapidly to be detected. In the case of P. maltophilia, the arginine dihydrolase and arginine decarboxylase systems could be operating simultaneously. However, since P. maltophilia was negative for arginine dihydrolase in Thornley's method, it is possible that this organism produces an arginase which converts arginine to ornithine and urea. This could explain the production of ornithine without the ammonia which is necessary for the pH shift in Thornley's medium. Further studies are being done to ascertain which enzymes are responsible for these results. Whatever the explanation, the TLC method has demonstrated that P. maltophilia can produce ornithine and agmatine from arginine. This would be useful in distinguishing P. maltophilia from other members of the genus Pseudomonas.

Other workers have reported P. maltophilia as being negative for arginine dihydrolase. However, they used different methods for determining this, Pickett and Pedersen (4) and Gilardi (1) relying on pH change and Stanier et al. (6) measuring the amount of residual arginine left after incubation. Our results have shown that TLC is more accurate and more sensitive for arginine dihydrolase detection, since it reveals definitive products. The TLC method is also simple and rapid and could be used easily in the clinical laboratory for the detection of arginine breakdown.

**TABLE 1. Comparison of Thornley and TLC methods for arginine dihydrolase**

| Organism                | No. tested | No. positive Thornley's | No. positive TLC |
|-------------------------|------------|-------------------------|-----------------|
| *Pseudomonas maltophilia* | 23         | 0                       | 23              |
| *P. aeruginosa*         | 31         | 31                      | 31              |
| *P. fluorescens*        | 5          | 5                       | 5               |
| *P. putida*             | 1          | 1                       | 1               |
| *P. stutzeri*           | 1          | 1                       | 1               |
| *P. odorans*            | 1          | 1                       | 1               |
| *P. denitrificans*      | 1          | 0                       | 0               |
| *P. alcaligenes*        | 1          | 0                       | 0               |
| *Herellea vaginicola*   | 12         | 0                       | 0               |
| *Mima polymorpha*       | 9          | 0                       | 0               |
| *Mima polymorpha* var. oxidans* | 1        | 0                       | 0               |
| *E. coli*               | 1          | 1                       | 1               |
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