NOTES

Improved Thin-Layer Technique for Detection of Arginine Dihydrolase Among the Pseudomonas Species

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The detection of arginine dihydrolase among Pseudomonas spp. by thin-layer chromatography is described. The method is based on the procedure of Williams and co-workers (1971) but was improved in sensitivity by selection of a better solvent system.

The arginine dihydrolase system is one of the key enzyme systems in the differentiation of aerobic Pseudomonas spp. from other nonfermentative gram-negative bacilli (3, 10) as well as within its own species (7–10). This hydrolytic system catalyzes the following anaerobic reaction with a gain in energy:

\[
\text{arginine desimidase} \xrightarrow{+ \text{water}} \text{citrulline} + \text{NH}_4^+ \\
\text{citrulline ureidase} \xrightarrow{+ \text{water} + \text{Pi} + \text{ADP}} \text{ornithine} + \text{CO}_2 + \text{NH}_4^+ + \text{adenosine triphosphate}
\]

The ability to produce these two hydrolytic enzymes is detected in routine tests according to the anaerobic agar-tube method of Thornley (11) by measuring the disappearance of arginine in phosphate buffer (10) or by thin-layer chromatography (TLC) of its end product (12).

In an attempt to classify four Pseudomonas strains isolated from soil, we improved the TLC method of Williams and co-workers (12) both by a better solvent system and in sensitivity.

The organisms used in these studies were four unidentified Pseudomonas strains and eight physiologically related Pseudomonas cultures (see Table 2).

A loopful of each culture (24 h, 28 C) grown on triple sugar-iron agar was inoculated into Thornley test medium (11) as well as into a tube of arginine-hydrochloride (0.01 M, pH 6.4) as reported by Williams et al. (12). The arginine broth was sterilized by filtration, incubated at 30 C for 3 h (water bath) and centrifuged (15 min at 6,000 rpm). Two μl of each supernatant was applied to a number of TLC plates (precoated cellulose sheets, 0.1 mm by 20 by 20 cm, Merck AG, Darmstadt, Germany). Each chromatogram also received 2 μl of an aqueous reference mixture consisting of arginine, citrulline, ornithine, agmatine, and putrescine (0.01 M each, pH 6.4). The following solvent systems were compared: (i) 1-butanol:acetic acid:water (4:1:5, organic phase) (13); (ii) 1-butanol:acetone:acetic acid:water (35:35:10:20) (12); (iii) tert-butanol:ethylmethylketone:acetone:me- thanol:ammonia d = 0, 88:water (40:20:20:1:5:14) (5); (iv) 1-butanol:acetone:diethylamine:water (10:10:2:5) (1); (v) phenol:water:ammonia d = 0, 91 (100:20:0.3) (3); and (vi) phenol:acetic acid:water (6:1:6) (4).

Developed, air-dried chromatograms were sprayed with either ninhydrin solution (1% in isopropanol) or cadmium acetate-isatine reagent (6) and heated (90 C) for 1 or 10 min, respectively. With ninhydrin, all of the compounds mentioned above gave purple spots, whereas the cadmium acetate-isatine reagent colored arginine and citrulline red, ornithine purple, and agmatine as well as putrescine blue.

The average Rf values for arginine, citrulline, ornithine, agmatine, and putrescine obtained with the six different solvent systems are compared in Table 1. From this scheme it is evident that the arginine dihydrolase products (citrulline, ornithine) as well as the arginine decarboxylase metabolites (agmatine, putrescine) are best separated by n-butanol:acetone:diethylamine:water (10:10:2:5). This system remained stable throughout the chromatographic...
fractionation. An example of the results obtained is illustrated by Fig. 1. It shows the clear-cut differentiation between negative Pseudomonas cultures and those producing citrulline, ornithine, and putrescine. Second best in the separation of the amino acids and amines in question was the system phenol: water: ammonia (2). The solvent system used by Williams et al. (12) was less successful for the intended purpose in study.

The distribution of arginine dihydrolase among the Pseudomonas cultures tested is given in Table 2. The following conclusions are drawn from it. First, except with the P. testosteroni strain, a complete correlation between the results obtained with Thornley's method and the TLC method is found. Second, both ornithine (the arginine dihydrolase end product) and citrulline (the intermediate stage) are detected on the chromatograms spotted with P. aeruginosa, P. fluorescens, and P. mendocina. On the other hand, the intermediate citrulline was never found with P. testosteroni ATCC 11996, although ornithine was detected with all of the six solvent systems used. Third, all cultures that produced ornithine from arginine simultaneously showed spots corresponding to putrescine (see also Fig. 1). Putrescine, however, can be produced from ornithine only by decarboxylation, under the experimental conditions that were described.

Table 1. Comparison of $R_v$ values of arginine, citrulline, ornithine, putrescine, and agmatine obtained by TLC on cellulose sheets in different systems

| System | Enzyme | 1-Butanol: acetic acid: water (4:1:5) (ref. 13) | 1-Butanol: acetone: acetate acid: water (35:35:10:20) (ref. 12) | tert-Butanol: butanone: acetone: methanol: ammonia (0, 88): water (40:20:20:1:5:14) (ref. 5) | 1-Butanol: acetone: diethylamine: water (10:10:2:5) (ref. 1) | Phenol: water: ammonia (0, 91) (100:20:0,3) (ref. 2) | Phenol: acetic acid: water (6:1:6) (ref. 4) |
|--------|--------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Arginine | 0,10   | 0,13                            | 0,03                            | 0,04                            | 0,67                            | 0,75                            |
| Citrulline | 0,13   | 0,15                            | 0,04                            | 0,17                            | 0,50                            | 0,72                            |
| Ornithine | 0,07   | 0,08                            | 0,07                            | 0,25                            | 0,42                            | 0,58                            |
| Putrescine | 0,08   | 0,17                            | 0,75                            | 0,75                            | 0,69                            | 0,77                            |
| Agmatine | 0,13   | 0,19                            | 0,29                            | 0,27                            | 0,83                            | 0,80                            |

Fig. 1. Thin-layer one-dimensional chromatogram of precoated cellulose sheets run in n-butanol: acetone: diethylamine: water (10:10:2:5) and sprayed with 1% ninhydrin (in isopropanol). Abbreviations: ar, arginine; ci, citrulline; or, ornithine; pu, putrescine; co, control by uninoculated medium; 15, 16, 17, and 19, isolated Pseudomonas spp.; 1, P. stutzeri ATCC 17588; 2, P. staniieri ATCC 17591; 3, P. mendocina ATCC 25411; 4, P. testosteroni ATCC 11996; 5, P. fluorescens CCM 2115.

Table 2. Comparison of Thornley and TLC methods for Pseudomonas arginine dihydrolase determinations

| Organism       | Thornley method | TLC on cellulose sheets* |
|----------------|-----------------|--------------------------|
| Pseudomonas spp. |                 |                          |
| 15, 16, 17, 19 |                 |                          |
| P. stutzeri    |                 |                          |
| ATCC 17588     |                 |                          |
| P. staniieri   |                 |                          |
| ATCC 17591     |                 |                          |
| P. staniieri   |                 |                          |
| ATCC 17587     |                 |                          |
| P. mendocina   |                 |                          |
| ATCC 25411     |                 |                          |
| P. testosteroni|                 |                          |
| ATCC 11996     |                 |                          |
| P. fluorescens |                 |                          |
| CCM 2115       |                 |                          |
| P. aeruginosa  |                 |                          |
| CCM 1960       |                 |                          |
| P. saccharophilia |               |                          |
| CCM 1980       |                 |                          |

* Run for 90 min in the system n-butanol: acetone: diethylamine: water (10:10:2:5) and sprayed with either 1% ninhydrin (in isopropanol) or cadmium acetate-isatine reagent.
ornithine decarboxylase

\[ \text{ornithine} \xrightarrow{(EC 4.1.1.17)} \text{putrescine} + \text{CO}_2 \]

Apparently, under the experimental conditions given, arginine dihydrolase is subsequently followed by ornithine decarboxylase activity.

The procedure followed by us is more sensitive than the one recommended by Williams and co-workers (12), since the latter neither detected the intermediate citrulline nor was putrescine observed on their chromatograms.

One puzzling aspect of this study is that the intermediate citrulline could never be found with \textit{P. testosteroni}, although the experiment was repeated several times using both prolonged (5 h) and reduced (1.5 h) incubation periods. However, if it is assumed that \textit{P. testosteroni} produces an arginase (arginine amidohydrolase EC 3.5.3.1) which hydrolyzes arginine into ornithine and urea directly rather than via citrulline, the absence of the intermediate citrulline may be explained in an acceptable manner.

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