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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:

$$\text{arginine} \xrightarrow[+\ \text{water}\ (\text{EC } 3.5.3.6)]{\text{arginine desimidase}} \text{citrulline} + NH_3$$

$$\xrightarrow[+\ \text{water} + \text{Pi} + \text{ADP}]{\text{citrulline ureidase}} \text{ornithine} + CO_2 + NH_3 + \text{adenosine triphosphate}$$

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 μliters 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 μliters of an aqueous reference mixture consisting of arginine, citrulline, ornithine, agmatine, and putrescine (0.01 M of 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:methanol: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 *iso*-propanol) 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 R_f 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

TABLE 1. *Comparison of R_f values of arginine, citrulline, ornithine, putrescine, and agmatine obtained by TLC on cellulose sheets in different systems*

Enzyme	System					
	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

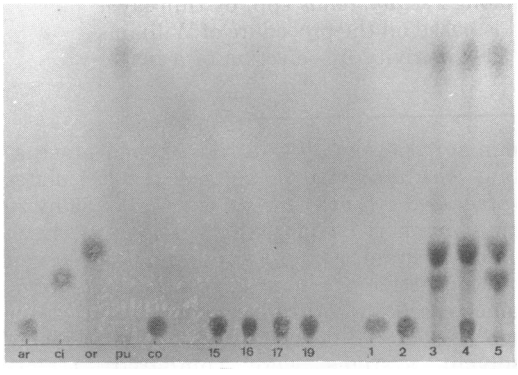

FIG. 1. *Thin-layer one-dimensional chromatogram of precoated cellulose sheets run in n-butanol: acetone: diethylamine: water (10:10:2:5) and sprayed with 1% ninhydrin (in iso-propanol). Abbreviations: ar, arginine; ci, citrulline; or, ornithine; pu, putrescine; co, control by uninoculated medium; 15,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

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

Organism	Thornley method	TLC on cellulose sheets[a]		
		Citrulline	Ornithine	Putrescine
Pseudomonas spp. 15, 16, 17, 19	−	−	−	−
P. stutzeri ATCC 17588	−	−	−	−
P. stanieri ATCC 17591	−	−	−	−
P. stanieri ATCC 17587	−	−	−	−
P. mendocina ATCC 25411	+	+	+	+
P. testosteroni ATCC 11996	−	−	+	+
P. fluorescens CCM 2115	+	+	+	+
P. aeruginosa CCM 1960	+	+	+	+
P. saccharophilia CCM 1980	−	−	−	−

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

$$\text{ornithine} \xrightarrow[\text{(EC 4.1.1.17)}]{\text{ornithine decarboxylase}} \text{putrescine} + CO_2$$

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 *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 *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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