Arginine Dihydrolase Activity in Species of the Genus *Bacillus* Revealed by Thin-layer Chromatography

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INTRODUCTION

The arginine dihydrolase system catalyses the hydrolysis of arginine into ornithine, NH_3 and CO_2 with a gain of ATP:

This reaction may occur under anaerobic conditions thus providing a means for aerobic organisms to generate energy in the absence of free oxygen.

Arginine dihydrolase is detected in routine laboratory tests by the anaerobic tube method of Thornley (1960) or by one of its modifications (Taylor & Whitby, 1964; Stewart, 1971; Jagnow, 1973). Stanier, Palleroni & Doudoroff (1966) detected this enzyme activity by measuring the disappearance of arginine in phosphate buffer. However, all these procedures have been developed for Gram-negative, non-fermentative bacteria (particularly *Pseudomonas* species) but have not yet been applied to Gram-positive organisms. Preliminary studies with some *Bacillus* strains revealed unsatisfactory and misleading results with Thornley's anaerobic tube method, caused by an alkaline shift in the medium as a result of deamination and/or decarboxylation rather than arginine dihydrolase activity. Thin-layer chromatography for the detection of the arginine dihydrolase system proved suitable in species of *Pseudomonas* (Zolg & Ottow, 1973). Therefore this method was adapted to detect this system in species of *Bacillus*.

METHODS

Organisms. The 169 *Bacillus* strains and their sources are listed in Table 1. These strains represent 31 different *Bacillus* species and include strains of ten new species described after the publication of *Bergey's Manual of Determinative Bacteriology* (1957). The morphological grouping of these new species has been recorded by Ottow (1974).

Media. All strains were grown at pH 6.8 on a fortified nutrient agar (Steinigeweg & Ottow, 1974). The pH was adjusted to 6.0 for cultivation of *Bacillus pulvifaciens* CIP53139. The strains received as *B. laevolacticus* and *B. racemilacticus* were maintained on the glucose-acetate agar (pH 6.2) of Nakayama & Yanoshi (1967). The *B. fastidiosus* strains were cultivated on the nutrient agar supplemented with allantoin (20 g/l).

Incubation temperature. Unless otherwise stated, all cultures were incubated at 28 °C. Strains of *B. coagulans*, *B. circulans*, *B. laevolacticus*, *B. pantothenicus*, *B. pulvifaciens* and

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Table 1. Occurrence of arginine dehydrolase system among *Bacillus* species

Breakdown products were detected on thin-layer chromatograms after 3 h incubation of a thick cell suspension in a tube containing 0.5 ml L-arginine-hydrochloride (0.01 M, pH 6.4) solution.

Organisms	Citrulline	Ornithine	Organisms	Citrulline	Ornithine
Group I*			Group I* (cont.)		
<i>B. badius</i>			<i>B. pumilus</i>		
CCM2113	+	+	CCM340	+	+
<i>B. coagulans</i>			CCM2144	+	+
CCM102	+	+	SMG27	+	+
CCM2013	+	—	SMG296	+	+
<i>B. cereus</i>			ILM15	+	+
CCM2010	+	+	JO 36–38	+	+
ILM13, 14	+	+	<i>B. subtilis</i>		
CCM98	—	—	CCM1718	—	+
JO 48–51	+	+	CCM2216	—	—
<i>B. cereus</i> var. <i>mycoides</i>			SMG10	+	+
CCM145	+	+	ILM18	+	+
JO 13, 15, 31, 32, 36,	+	+	JO20	+	+
37, 55, 72, 75, 79, 100,			JO21	+	+
117, 120–125, 127–136,			JO22	—	—
138–40			JO23	+	+
CCM431	—	—	<i>B. thuringiensis</i>		
JO 6, 8–12, 17, 18, 25,	—	—	CCM19	+	+
52–55, 103, 132			ILM17, 18	+	+
<i>B. firmus</i>			JO 30–35	+	+
CCM2212	+	+	Group II*		
CCM37	—	—	<i>B. alvei</i>		
CCM2213	—	—	CCM1614	—	—
<i>B. insolitus</i>			CCM2051	—	—
ATCC23299	—	+	SMG29	—	—
<i>B. lentus</i>			SMG305	+	+
CCM35	—	—	ILM21	—	—
CCM2214	—	—	JO39	—	—
<i>B. licheniformis</i>			JO40	—	—
CCM864	+	+	JO41	+	+
CCM2145	+	+	JO42	+	+
SMG14	+	+	<i>B. brevis</i>		
JO24	—	—	CCM1089	—	+
JO25	—	+	CCM2050	—	+
JO26	+	+	SMG298	—	+
<i>B. macquariensis</i>			<i>B. circulans</i>		
NCTC10420	—	—	SMG11	—	+
<i>B. maroccanus</i>			SMG329	—	+
ATCC25099	+	+	CCM2048	—	—
<i>B. medusa</i>			ILM42	—	—
ATCC25621	+	+	JO43	—	—
<i>B. megaterium</i>			JO44	—	—
CCM1462	+	+	JO45	—	—
ILM19	+	+	JO46	+	+
JO 4, 5, 6, 11, 12	+	+	JO47	+	+
<i>B. pacificus</i>			<i>B. globisporus</i>		
ATCC25098	—	—	ATCC23301	—	—
<i>B. psychrophilus</i>			<i>B. laevolacticus</i>		
ATCC23304	+	+	ATCC23492	+	+
			ATCC23493	+	+

Table 1 (cont.)

Organisms	Citrulline	Ornithine	Organisms	Citrulline	Ornithine
Group II* (cont.)			Group II* (cont.)		
ATCC23494	+	+	<i>B. pulvifaciens</i>		
ATCC23495	+	+	CCM38	+	+
<i>B. laterosporus</i>			CCM39	+	+
CCM2116	—	—	CIP53139	—	—
CCM1612	—	+	<i>B. psychrosaccharolyticus</i>		
SMG25	+	+	ATCC23296	—	—
SMG335	—	+	<i>B. racemilacticus</i>		
CIP6348	—	+	ATCC23496	+	+
<i>B. macerans</i>			ATCC23497	+	+
CCM1610	+	+	ATCC23498	—	+
ATCC8244	+	—			
<i>B. pantothenicus</i>			Group III*		
CCM2049	+	+	<i>B. fastidiosus</i>		
SMG26	—	—	SMG73	—	—
<i>B. polymyxa</i>			SMG283	+	+
CCM1459	—	—	<i>B. sphaericus</i>		
SMG36	—	—	CCM1615	+	—
SMG292	—	—	CCM2120	+	+
ILM20	+	+	ILM14	+	+
JO 6-9	—	—	JO28	+	+
JO21	+	+	JO29	+	+
JO22	+	+			
JO25	—	—			

* For the demarcations of the morphological groups I, II and III, consult Wolf & Barker (1968).

ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; ccm, Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia; SMG, Sammlung für Mikroorganismen, Göttingen, Germany; CIP, Collection of Institute Pasteur, Paris, France; NCTC, National Collection of Type Cultures, London; ILM, Institut für Landwirtschaftliche Mikrobiologie collection, Giessen, Germany; JO, the author's collection.

B. racemilacticus were tested at 37 °C, those received as *B. globisporus*, *B. macquariensis*, *B. pacificus*, *B. psychrophilus* and *B. psychrosaccharolyticus* were kept at 20 °C.

Arginine dihydrolase assay. One loopful of each culture (24 to 48 h) was transferred into 0.5 ml of sterile 0.01 M L-arginine solution (pH 6.4), incubated for 3 h at 20 °C (room temperature) or 37 °C (stationary, in a waterbath), respectively, and centrifuged (15 min at 2600 g). Clear supernatant (2 µl) was applied to 0.2 mm cellulose layers ('Avicel' cellulose, Merck AG, Darmstadt, Germany) and chromatograms run with *n*-butanol-acetone-diethylamine-water (10:10:2:5) (Arx & Neher, 1963) as a solvent system. Each chromatogram included 2 µl samples of 0.01 M aqueous solutions of arginine, citrulline, ornithine and putrescine. Air-dried chromatograms were sprayed with a 1% ninhydrin solution in isopropanol. After heating the chromatograms (1 min at 90 °C) all compounds mentioned gave purple spots. This method is essentially as described by Zolg & Ottow (1973).

RESULTS AND DISCUSSION

The average R_F -values obtained were: arginine, 0.04, citrulline, 0.18, ornithine, 0.29 and putrescine, 0.77, enabling the amino acids and the amine to be clearly separated.

In Table 1 the distribution of the arginine dihydrolase system among the *Bacillus* species is listed according to the classification of the various species in one of the three morphological groups of Wolf & Barker (1968). Within the positive strains, the majority produced both

citrulline (the intermediate product) and ornithine (the end product). Only a few strains revealed ornithine or citrulline alone. These observations are ascribed respectively to (i) complete conversion of arginine to ornithine and (ii) incomplete hydrolysis, within the 3 h period. Strains that produced citrulline only were retested with the incubation time increased to 5 h. Under these conditions, all originally 'incomplete' strains (*B. coagulans* CCM2013, *B. macerans* CCM2012, *B. sphaericus* CCM1615 and *B. subtilis* ILM18) showed both citrulline and ornithine, although with different intensities. Generally speaking, the positive strains of *B. cereus*, *B. cereus* var. *mycoides* and *B. thuringiensis* revealed a weak dihydrolase activity, whereas fair to good activity was recorded with *B. brevis*, *B. licheniformis*, *B. laevolacticus*, *B. megaterium*, *B. subtilis* and *B. sphaericus*, if judged by the intensities of the spots. By far the most intense ornithine spot was observed with *B. licheniformis* JO25, suggesting a complete hydrolysis of arginine within the 3 h period.

Bacillus strains that did not metabolize arginine in the first experiments, were also re-incubated for the extended incubation time. However, neither citrulline nor ornithine could be detected.

Putrescine, which may be produced from ornithine by decarboxylation (ornithine decarboxylase, EC. 4.1.1.17), was never observed on the chromatograms of arginine dihydrolase-positive bacilli, in contrast to *Pseudomonas* species that revealed both ornithine and putrescine on chromatograms (Zolg & Ottow, 1973), suggesting the presence of both arginine dihydrolase and ornithine decarboxylase. Only in *B. cereus* var. *mycoides* JO100 and JO130, characterized by the production of brown and yellow exo-pigments respectively, purple-brown spots ($R_f = 0.56$) were observed additionally on the chromatograms. These spots may have been the unknown pigments.

From these results the following overall conclusions are drawn. Firstly, the arginine dihydrolase enzyme system is widely distributed in aerobic spore-formers; out of 169 different *Bacillus* strains, 126 (about 75 %) were capable of catabolizing arginine. Secondly, this hydrolytic enzyme is not restricted to any of the three morphological groups within the genus *Bacillus*. Thirdly, with some species, all strains tested produced arginine dihydrolase or lacked this enzyme complex.

Among the species that had four (or more) strains tested only those belonging to *B. laevolacticus*, *B. megaterium*, *B. pumilus*, *B. sphaericus* and *B. thuringiensis* all gave positive tests. Most species showed both positive and negative strains. In fact, the absence rather than the presence of the arginine dihydrolase system in certain species (*B. lentus*, *B. maquariensis*, *B. pacificus*, *B. globisporus* and *B. psychrosaccharolyticus*) may prove to be of taxonomic value. However, a more comparative study is needed for the evaluation of the importance of this arginine dihydrolase test in the classification of the genus *Bacillus*.

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