APPLIED MICROBIOLOGY, June 1975, p. 726-728
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Vol. 29, No. 6
Printed in U.S.A.

Arylamidase Activity of *Salmonella* Species

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Received for publication 19 March 1975

Arylamidase activity in cell extracts of sonically cell treated suspensions of 23 *Salmonella* strains, including 12 strains of *S. typhimurium*, was investigated. All cultures hydrolyzed five of nine different neutral and basic substrates. Activity against aspartyl-, cystyl- histidinyl-, and isoleucyl-β-naphylamide was negligible. Alanyl-β-naphthylamide was the preferred substrate for the *Salmonella* species; however, specific activities ranged widely. Of several gram-negative organisms surveyed, all except *Proteus vulgaris* hydrolyzed alanyl-β-naphthylamide at the fastest rate. The most preferred substrate for the *Proteus* culture was glycyl-β-naphthylamide. No relationship could be shown between virulence and arylamidase activity for the *Salmonella* strains.

Arylamidase are enzymes with the ability to hydrolyze amino acid-β-naphthylamide substrates. Since their discovery by Patterson et al. (10) they have been demonstrated in many biological systems (3, 5, 8, 13, 14) and have been reported to occur in various gram-negative organisms (2, 12) and to a lesser extent in some gram-positive bacteria (1). Studies by Tappel (15) and Marks et al. (8) suggested that arylamidases function at some stage in protein catabolism; however, the in vivo function of the enzyme has not been defined. Burton et al. (4) demonstrated that pathogenic strains of *Leptospira* possessed 10 to 20 times higher arylamidase activity than saprophytic strains, suggesting a possible role for the enzyme in pathogenicity.

Westley et al. (16) have proposed the use of arylamidase substrate specificity patterns as a tool to identify bacteria, and Muftic (9) suggested a similar plan to aid in differentiation of mycobacteria. Arylamidases of salmonellae have not been studied; therefore, we undertook the present study to gain an understanding of the occurrence and properties of arylamidase in salmonellae and to determine if the enzyme might play a role in the virulence of the organisms.

MATERIALS AND METHODS

Twenty-three *Salmonella* strains, including 12 strains of *S. typhimurium* and single strains of *S. anatum*, *S. new-brunswick*, *S. tennessee*, *S. london*, *S. thompson*, *S. montevideo*, *S. senftenberg*, *S. newport*, *S. worthington*, *S. derby*, and *S. gallinarum*, were obtained from the National Center for Disease Control, Salmonella Laboratory, Atlanta, Ga. Other gram-negative cultures used in this study were obtained from stock cultures of the Food Science Department, University of Georgia.

Virulence of *S. typhimurium* strains was determined by the method of Reed and Muench (11). Intraperitoneal injections (1 ml) of each serial dilution were administered to six 9- to 11-week-old white Swiss/CF mice weighing 19 to 27 g (Blue Spruce Farms, Altamount, N.Y.). The cells were grown for 24 h, collected by centrifugation (10 min at 19,000 × *g*), washed twice, and resuspended in sterile saline. Several dilutions were made, and the number of viable cells in each dilution was determined by plate count on tryptic soy agar (Difco Laboratories, Detroit, Mich.). Mean lethal dose (LD_{50}) values were based upon the deaths occurring in the six mice within a 7-day period.

Arylamidase activity of the *Salmonella* species was determined in cell extracts prepared from 24-h cultures grown with agitation at 37 C in 125 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.), pH 7.2. The cells were harvested by centrifugation (10 min at 19,000 × *g*), washed twice with 20 ml of sterile saline, resuspended in 20 ml of saline, and sonically treated for a total of 3 min (Sonifier Cell Disruptor, model W140D, Heat Systems-Ultrasonics). Sonic treatment was conducted intermittently with 15-s periods of sonic treatment followed by 20-s cooling periods. Sonic treatment under the above conditions provided maximal enzyme activity in the extract. After sonic treatment, cellular debris were removed by centrifuging at 19,000 × *g*, and the supernatant was collected. Protein concentration in the cell extracts was determined by the method of Lowry et al. (7) using bovine serum albumin as the standard.

The reaction mixture for arylamidase assay contained 0.1 ml of cell extract, 1.0 ml of 0.0685 M amino acid-β-naphthylamide (Schwartz-Mann, Orangeburg, N.Y., and Sigma Chemical Co., St. Louis, Mo.) and 0.9 ml of 0.2 M phosphate buffer, pH 7.0. The reaction was terminated after 1 h at 37 C by the

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addition of 1.0 ml of 40% trichloroacetic acid. After filtration through Whatman No. 1 filter paper, the free β-naphthylamine in the filtrate was determined by the procedure of Goldbarg and Rutenburg (6). Specific activity was expressed as micrograms of β-naphthylamine liberated per hour per milligram of protein in the cell extract. All enzyme assays were conducted in duplicate, and average results of two trials are reported.

RESULTS AND DISCUSSION

Each of the *Salmonella* cultures examined in this study possessed arylamidase activity. The specific activities of 12 *Salmonella* species against alanyl-, lysyl-, and leucyl-β-naphthylamide are given in Table 1. The most preferred substrate for each culture was alanyl-β-naphthylamide. Specific activities ranged from above 2,000 against alanyl-β-naphthylamide for some *S. typhimurium* strains to 270 for *S. gallinarum*. The highest specific activity reported for pathogenic *Leptospira* serotypes was 402 (4) against leucyl-β-naphthylamide. Pathogenic strains of *Leptospira* were shown to prefer the leucine substrate over the alanine substrate. Lysyl-β-naphthylamide was hydrolyzed by each *Salmonella* species at the next fastest rate followed by leucyl-β-naphthylamide. Although not shown in Table 1, both glycyl- and methionyl-β-naphthylamides were hydrolyzed at approximately the same rate as leucyl-β-naphthylamide. No activity was detected for any of the test cultures against aspartyl-, cystyl-, histindinyl-, and isoleucyl-β- naphthylamides.

To compare the arylamidase activity of the *Salmonella* species to that of other gram-negative bacteria, cell extracts were prepared from *Pseudomonas aeruginosa*, *P. fluorescens*, *Escherichia coli* (K-12), *Proteus vulgaris*, *Shigella flexneri*, and *Serratia marcescens*. The arylamidase activity in the cell extracts was measured against the same substrates used for the *Salmonella* cultures (Table 1). All of the gram-negative bacteria tested hydrolyzed alanyl-β-naphthylamide at the fastest rate with the exception of the *P. vulgaris* culture. Glycyl-β-naphthylamide was the first preferred substrate and alanyl-β-naphthylamide the second most preferred substrate by *P. vulgaris*. The specific activity for *P. vulgaris* was 260 for glycyl-β-naphthylamide compared to 167 for alanyl-β-napththylamide. The specificity profile of the *E. coli* culture against the five amino acid-β-naphthylamide substrates was similar to that of the salmonellae except the former possessed higher glycyl-β-naphthylamide activity than leucyl-β-naphthylamide activity (278 μg of β-naphthylamine per h per mg of protein compared to 80 μg of β-naphthylamine per h per mg of protein).

These data give some interesting insight into the possibility of using this enzyme assay as an adjunct to identification of bacteria. Westley et al. (16) working with *Bacillus* and *Escherichia* species demonstrated that there were considerable differences in the specificity profiles of different bacteria, and that a pure strain yields a reproducible profile under strictly specified conditions. In the case of *Salmonella* examined in this study, the close similarity of the specificity patterns and the wide ranges of specific activities rules out the use of the substrate profile as a tool to differentiate *Salmonella* species.

To determine if any relationship existed be-

TABLE 1. *Specific activity of Salmonella species and other gram-negative bacteria against alanyl-, lysyl-, and leucyl-β-naphthylamide*

Test organism	Sp act[a]		
	Alanyl-β-NA	Lysyl-β-NA	Leucyl-β-NA
S. senftenberg	441	193	53
S. gallinarum	270	135	58
S. anatum	290	118	59
S. montevideo	873	400	73
S. derby	946	514	95
S. tennessee	1,102	271	107
S. thompson	1,515	424	121
S. london	1,058	404	77
S. new-brunswick	1,250	414	89
S. newport	849	456	88
S. worthington	957	500	75
S. typhimurium			
strain no. 1807	916	367	66
strain no. 1976	810	345	60
strain no. 2051	594	278	52
strain no. 2140	1,012	494	53
strain no. 2444	2,088	530	103
strain no. 2896	1,750	729	73
strain no. 2562	2,095	1,087	174
strain no. 710	2,028	943	142
strain no. 2319	888	300	44
strain no. 2580	1,388	629	97
strain no. 2562	2,031	613	80
strain no. 2849	1,972	778	83
P. aeruginosa	308	144	68
P. fluorescens	283	133	67
E. coli K-12	818	307	80
P. vulgaris	167	33	59
S. flexneri	1,429	571	114
S. marcesens	333	121	76

[a] Expressed as micrograms of β-naphthylamine liberated per hour per milligram of protein. Results are average of two trials. NA, Naphthylamide.

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TABLE 2. *Comparison of virulence and arylamidase activity against alanyl-β-naphthylamide of three Salmonella*

Test organism	LD_{50}[a]	Alanyl-β-naphthylamide activity[b]
S. typhimurium		
strain no. 1807	7.1×10^5	916
strain no. 2140	2.2×10^5	1,012
strain no. 2444	1.3×10^5	2,088
strain no. 2896	2.0×10^4	1,750
strain no. 2562	2.4×10^5	2,095
strain no. 2319	1.1×10^5	888
strain no. 2580	1.1×10^6	1,388
strain no. 2562	4.7×10^6	2,031
S. gallinarum	3.0×10^7	270
S. thompson	4.5×10^7	1515

[a] Average of two trials.

[b] Expressed as micrograms of β-naphthylamide per hour per microgram of protein.

tween arylamidase activity and virulence of *Salmonella*, LD_{50} values were determined for eight *S. typhimurium* strains and for *S. gallinarum*, which had the lowest arylamidase activity of all the *Salmonella* species examined, and for *S. thompson*, which had the highest activity for the species other than *S. typhimurium* strains. LD_{50} values for the bacteria are reported in Table 2 along with the specific activities against alanyl-β-naphthylamide. All of the *S. typhimurium* strains used in this study were obtained from the Center for Disease Control, Atlanta, Ga. and had been isolated from human salmonellosis outbreaks. LD_{50} values ranged for 2×10^4 to 4.7×10^6, indicating that all were highly virulent. There appeared to be no relationship between the virulence of the organisms and the arylamidase activity. This is further supported by the similarity of LD_{50} values for the *S. gallinarum* and *S. thompson* strains and the wide difference noted in alanyl-β-naphthylamide activity for the two organisms.

ACKNOWLEDGMENTS

We thank K. M. Patel for his skillful technical assistance.

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