Arylamidase Activity of *Salmonella* Species

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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- histidyl-, and isoecyl-β-naphthylamide 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 glycy1-β-naphthylamide. No relationship could be shown between virulence and ary lamidase 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 arylamidase 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. senfenberg*, *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, Altamont, 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 (LD₅₀) 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). Sonicate 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 0.0885 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
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 Goldberg 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 glycy1- and methionyl-β-naphthylamidases were hydrolyzed at approximately the same rate as leucyl-β-naphthylamide. No activity was detected for any of the test cultures against aspartyl-, cystyl-, histidyl-, and isoleucyl-β-naphthylamidases.

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-naphthylamide. 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).

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-

| Test organism                   | 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                       | 1,068       | 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. 2696                 | 1,750       | 729        | 73          |
| strain no. 2562                 | 2,065       | 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. marcescens                   | 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.
between arylamidase activity and virulence of *Salmonella*, LD<sub>50</sub> 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<sub>50</sub> values for the bacteria are reported in Table 2 along with the specific activities against alanly-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<sub>50</sub> values ranged for 2 × 10<sup>4</sup> to 4.7 × 10<sup>6</sup>, 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<sub>50</sub> values for the *S. gallinarum* and *S. thompson* strains and the wide difference noted in alanly-

### Table 2. Comparison of virulence and arylamidase activity against alanly-β-naphthylamide of three *Salmonella*

| Test organism | LD<sub>50</sub>* | Alanly-β-naphthylamide activity* |
|---------------|-----------------|----------------------------------|
| *S. typhimurium* |                |                                  |
| strain no. 1807 | 7.1 × 10<sup>6</sup> | 916                              |
| strain no. 2140 | 2.2 × 10<sup>6</sup> | 1,012                            |
| strain no. 2444 | 1.3 × 10<sup>6</sup> | 2,088                            |
| strain no. 2896 | 2.0 × 10<sup>6</sup> | 1,750                            |
| strain no. 2562 | 2.4 × 10<sup>6</sup> | 2,095                            |
| strain no. 2319 | 1.1 × 10<sup>6</sup> | 888                              |
| strain no. 2580 | 1.1 × 10<sup>6</sup> | 1,398                            |
| strain no. 2562 | 4.7 × 10<sup>6</sup> | 2,031                            |
| *S. gallinarum* | 3.0 × 10<sup>7</sup> | 270                              |
| *S. thompson* | 4.5 × 10<sup>7</sup> | 1515                             |

* Average of two trials.
* Expressed as micrograms of β-naphthylamide per hour per microgram of protein.

β-naphthylamide activity for the two organisms.

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