Pyrrolidonyl Peptidase in Bacteria: A New Colorimetric Test for Differentiation of Enterobacteriaceae

By M. MULCZYK AND A. SZEWCZUK

*Department of General Microbiology and Department of Biochemistry,
Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences, Wrocław, Poland*

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SUMMARY

A simple and rapid colorimetric test has been developed for the detection of pyrrolidonyl peptidase (PLP) activity in bacteria. Of 2354 strains belonging to various groups of enteric bacteria tested, 451 were PLP-positive. These included *Citrobacter*, 223 out of 226 strains, *Klebsiella* (178/188), *Enterobacter* (49/52), and one strain of *Serratia*.

Pyrrolidonyl peptidase was not detected in 1146 *Shigella* strains, 354 *Salmonella* strains, 333 *Escherichia* strains, 21 Arizona strains, nor in 33 strains of *Proteus*, *Providencia* and *Hafnia*.

In addition, 56 cultures of various Gram-negative and Gram-positive bacteria were tested for PLP. Enzyme activity was found in strains belonging to the genera *Bacillus*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Sarcina*, *Neisseria* and *Pseudomonas*. The possibility of employing the PLP test for differentiation within the family Enterobacteriaceae is discussed.

INTRODUCTION

The classification of some bacteria may be based upon their peptidase activity (Muftic, 1965, 1967; Muftic & Schroder, 1966; Westley *et al.* 1967). In a previous paper (Szewczuk & Mulczyk, 1969) we reported new pyrrolidonyl naphthylamides synthesized in our laboratory which could be used as chromogenic substrates for colorimetric determination of pyrrolidonyl peptidase activity. The enzyme was found in Gram-positive and Gram-negative bacteria. Pyrrolidonyl peptidase from *Bacillus subtilis* was purified and its properties and specificity were described.

This paper describes a simple colorimetric test for detection of pyrrolidonyl peptidase activity (PLP test) in bacteria and its adaptation as a new method for the differentiation of the various groups of Enterobacteriaceae.

METHODS

Reagents. (1) Substrate solution: 0.01 M-L-pyrrolidonyl- β -naphthylamide in methanol (2.5 mg./ml.). This substance was synthesized by the method previously described (Szewczuk & Mulczyk, 1969). (2) 0.5 M-tris/HCl buffer, pH 7.7. (3) 10 % (v/v) acetic acid solution in water. (4) 0.1 % tetra-azotized *o*-dianisidine (Fast Blue B) solution in water.

Strains. A total of 2354 cultures that belonged to the various groups of Enterobacteriaceae were tested for PLP. The strains had been collected for several years

Fig. I

and had been received from different sources. Recently isolated strains from diagnostic material were also included. In addition, 56 strains of different groups of Gram-positive and Gram-negative bacteria were examined.

Table 1. *The occurrence of PLP in enteric bacteria*

Group	Positive strains/tested strains	
	Group	Genera
<i>Shigella dysenteriae</i> , serotypes 1-8	0/34	0/1146 ^a
<i>S. flexneri</i> , all serotypes	0/867	
<i>S. boydii</i> , serotypes 1-15	0/45	
<i>S. sonnei</i>	0/200	
<i>Salmonella typhi</i>	0/76	0/354 ^b
<i>S. paratyphi B</i>	0/14	
<i>S. typhimurium</i>	0/67	
<i>S. enteritidis</i>	0/131	
<i>S. anatum</i>	0/19	
<i>S. newington</i>	0/20	
<i>S. heidelberg</i>	0/10	
<i>S. brandenburg</i>	0/2	
<i>S. moribificans</i>	0/15	
Escherichia,		0/333 ^c
O antigens 1-149	0/143	
H antigens 1-49	0/49	
K antigens 1-91	0/91	
From diagnostic material	0/50	
<i>Klebsiella aerogenes</i>	60/61	178/188 ^d
<i>K. pneumoniae</i>	10/10	
<i>K. edwardsii</i> var. <i>edwardsii</i>	19/20	
<i>K. edwardsii</i> var. <i>atlantae</i>	0/8	
<i>K. ozaenae</i>	28/28	
<i>K. rhinoscleromatis</i>	13/13	
<i>K. oxytoca</i>	48/48 ^e	
Citrobacter,		223/226 ^h
O antigens 1-32	87/88 ^f	
O antigens 33-42	10/10 ^g	
From diagnostic material	126/128	
Arizona, O antigens 1-21	0/21	0/21 ^h
<i>Enterobacter aerogenes</i>	34/35	49/52 ⁱ
<i>E. liquefaciens</i>	4/5	
<i>E. cloacae</i>	11/12	
Proteus species	0/20	0/20
Providencia species	0/10	0/10
Hafnia species	0/3	0/3
Serratia species	1/1	1/1
Total		451/2354

NOTES

^a Including strains from the Dysentery Reference Laboratory, London, and from Dr A. J. Weil, New York.

^b Received from Professor Z. Buczowski, Gdańsk, and Professor J. Chomiczewski, Łódź.

^c Including strains from Dr F. Ørskov, Copenhagen.

^d Including Kauffmann's strains, received from Dr I. Ørskov, Copenhagen, and eight new serotypes (Maresz-Babczyzyn, 1962; Durlakowa *et al.* 1963).

^e Received from Dr S. Kałuzewski, Warsaw.

^f Including NCDC strains.

^g Sedlak & Slajsova (1966).

^h Received from Professor J. Sedlak, Prague.

ⁱ Received from Dr L. Le Minor, Paris.

The pyrrolidonyl peptidase activity test (PLP test). This test is based on the following two reactions: pyrrolidonyl peptidase present in bacteria catalyses the hydrolysis of L-pyrrolidonyl- β -naphthylamide liberating free L-pyrrolidone carboxylic acid and β -naphthylamine (reaction I). The latter substance, after coupling with tetra-azotized *o*-dianisidine (Fast Blue B), gives the red azo substance (reaction II) (Fig. 1).

Procedure. The culture to be tested is inoculated on to an agar slant and incubated for 18 to 24 hr at 37°. The bacteria are washed off with 1.5 ml. saline and 0.4 ml. suspension is added to a test-tube. Then 0.05 ml. 0.5-M tris/HCl buffer and 0.05 ml. substrate (L-pyrrolidonyl- β -naphthylamide) are added and mixed immediately. The suspension is incubated in a water bath at 37° for 3 hr and then 0.2 ml. of acetic acid is added. The colour is developed by addition of 0.1 ml. Fast Blue solution followed by incubation at 37° in a water bath for 1 hr. A positive reaction is indicated by a strong red colour of the suspension, stable for at least one day at room temperature.

RESULTS

The PLP activities of 2354 cultures from various groups of Enterobacteriaceae are presented in Table 1. It can be seen that *Shigella*, *Salmonella*, *Escherichia*, *Arizona*, *Proteus*, *Providencia* and *Hafnia* species gave a uniformly negative result in PLP test. In contrast, *Klebsiella* species gave a positive result except for all strains of *K. edwardsii* var. *atlantae* tested and one strain each of *K. aerogenes* and *K. edwardsii* var. *edwardsii*. With few exceptions (see Table 1) *Citrobacter* and *Enterobacter* strains were also positive. The one *Serratia* strain tested gave a positive result.

In addition to the above-mentioned cultures of Enterobacteriaceae, 56 Gram-positive and Gram-negative strains were examined for PLP. The results are recorded in Table 2: except for the strains belonging to the genera *Corynebacterium* and *Saccharomyces*, strains that gave positive reactions were found in all remaining groups tested.

Table 2. *The occurrence of PLP in some Gram-positive and Gram-negative bacteria**

Genus	No. of cultures tested	PLP-positive
<i>Pseudomonas</i>	8	4
<i>Neisseria</i>	4	4
<i>Micrococcus</i>	1	1
<i>Bacillus</i>	9	8
<i>Corynebacterium</i>	3	0
<i>Streptococcus</i>	7	5
<i>Sarcina</i>	3	3
<i>Staphylococcus</i>	19	16
<i>Saccharomyces</i>	2	0

* Strains from Polish Collection of Micro-organisms, Wrocław, and from diagnostic material.

DISCUSSION

Different biochemical tests have been described that have distinct taxonomic value within the family of Enterobacteriaceae. Some tests may be readily applied in laboratory diagnosis and were found useful in the differentiation of enteric bacteria (Edwards & Ewing, 1962; Cowan & Steel, 1965). Our data indicate that the simple test for detection of pyrrolidonyl peptidase activity in bacteria described in this paper may

be considered as an additional one in the classification and differentiation of bacteria. This is supported by the fact that the cultures of some species had PLP and the others were devoid of the enzyme. Thus the PLP test may be applied in the differentiation of strains which still constitute diagnostic problems. These are strains that are closely related biochemically and serologically and that compose the Salmonella-Arizona-Citrobacter division. Since the great majority of Citrobacter strains are positive in the PLP test and those of Salmonella and Arizona are negative, the introduction of the test may be of great value in the differentiation of these organisms in addition to the tests for lysine decarboxylase (Møller, 1955) and KCN tolerance (Møller, 1954).

The lack of PLP in all eight strains of *Klebsiella edwardsii* var. *atlantae* should also be noted. However, only further studies of a larger number of Klebsiella cultures can show whether negative strains in the PLP test are limited to the subgroup of *K. edwardsii* var. *atlantae*.

Considering that the number of strains of some species that were tested was small, no attempts were made to evaluate the usefulness of the PLP test in routine diagnostic work. To do so, further studies are required on a larger number of strains of different serotypes of Enterobacteriaceae including original stock cultures and those isolated from diagnostic material. These studies are now in progress in our laboratory and the results will be the subject of a separate communication. It must be stressed here, however, that as the distribution of pyrrolidonyl peptidase is characteristic as a rule of the individual bacterial groups, the PLP test may be of great diagnostic value in differentiation within the family of Enterobacteriaceae.

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