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A survey for phosphoglucose isomerase with lysyl aminopeptidase activity in Vibrionaceae and non-*Vibrio* pathogens☆

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Abstract

Phosphoglucose isomerase (PGI) with a novel lysyl aminopeptidase (LysAP) activity was recently purified and characterized from *Vibrio vulnificus*. We showed that it cleaves the amino-terminal lysyl residue from des-Arg^{10}-kallidin to produce des-Arg^{9}-bradykinin, suggesting that it plays a role in virulence. A survey was conducted to determine the presence of this potential virulence-enhancing enzyme among twenty-three halotolerant human and fish pathogens from eleven species within the Vibrionaceae family, including *V. vulnificus*, *V. parahaemolyticus*, *V. cholerae*, *Aeromonas hydrophila*, and *Plesiomonas shigelloides*. In addition, fourteen species of non-Vibrionaceae pathogens were screened for LysAP activity. Cell lysates were partially purified by anion exchange chromatography and fractions were screened for LysAP and isomerase activities. PGI-LysAP activity was detected in chromatographic fractions from all the *Vibrio* species tested, but was not detected in any of the non-Vibrionaceae pathogens. Levels of isomerase and LysAP activity correlated (R^2=0.92) for nine strains of *V. vulnificus*. Since the Vibrionaceae represent an important family of human and fish pathogens, our identification of PGI-LysAP activity in a broad array of vibrios may lead to the development of improved analytical methods for their identification as well as interventions to reduce the high morbidity and mortality associated with some Vibrionaceae infections in clinical, veterinary, and aquaculture settings.
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1. Introduction

Phosphoglucose isomerase (PGI) is a multifunctional enzyme involved in glycolysis and gluconeogenesis. In mammalian cells, PGI serves as the cytokines neuroleukin [1,2] and autocrine motility factor [3] and as a growth factor known as maturation mediator [4]. In bacteria, PGI shuttles fructose into the Entner–Doudoroff pathway, while in plants, it is involved in carbohydrate biosynthesis. Recently, we identified another function of PGI as a lysyl aminopeptidase (LysAP) in *Vibrio vulnificus* [5]. Since proteases are important in bacterial pathogenesis [6–9], this LysAP function of PGI is intriguing and may help explain the pathogenicity of various *Vibrio* species. A variety of proteases have been identified in members of the Vibrionaceae family [10–15], but until recently, the LysAP activity of *Vibrio* PGI (PGI-LysAP) was unknown. We also

Abbreviations: APW, alkaline peptone water; ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; CU, calculated units; CV, column volume; FDA, United States Food and Drug Administration; L-Lys-AMC, L-lysyl-7-amino-4-methylcoumarin; LysAP, lysyl aminopeptidase; PGI, phosphoglucose isomerase; PGI-LysAP, phosphoglucose isomerase with a lysyl aminopeptidase activity; RT, room temperature; *tdh*, thermostable direct hemolysin gene; *trh*, thermostable related hemolysin gene; TSA, tryptic soy agar; TSA-N, tryptic soy agar containing 1% NaCl; TSB, tryptic soy broth; TSB-N, tryptic soy broth containing 1% NaCl; USDA, United States Department of Agriculture; USUHS, Uniformed Services University of the Health Sciences

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identified PGI-LysAP activity in *V. cholerae* and *V. parahaemolyticus* [5]. Most recently, our laboratory showed that PGI-LysAP of *V. vulnificus* hydrolyzed des-Arg^{10}-kallidin converting it to des-Arg^{9}-bradykinin [16], a known virulence-enhancing peptide. This signals a possible mechanism for vibrios to invade susceptible hosts through the formation of bradykinin metabolytes. Bradykinin is a universal trigger for bacterial invasion [17–20] and, when injected into mice, enhanced the intravascular dissemination of *V. vulnificus* [21]. Bradykinin metabolites elicit inflammatory reactions, a common symptom of *V. vulnificus* infection [16]. This paper provides the results of a survey for PGI's LysAP and isomerase activities in members of the Vibrionaceae family and in Gram positive and negative non-Vibrionaceae pathogens.

2. Materials and methods

2.1. Bacterial strains and propagation

Vibrionaceae and non-Vibrionaceae pathogens were obtained from sources listed in Tables 1–3. Non-Vibrionaceae were streaked from slants or from frozen glycerol stocks onto tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) and incubated overnight at 37 °C. The Vibrionaceae, including nine strains of *V. vulnificus*, were cultured on TSA supplemented with 0.5% NaCl (TSA-N). In preparation for enzyme assays, *V. vulnificus* colonies were picked from the TSA-N plates and subcultured in 100 ml of alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.4±0.2) and in 100 ml of tryptic soy broth (TSB, Difco) containing 1% NaCl (TSB-N). The other *Vibrio* spp. as well as two *Aeromonas* spp. and *Plesiomonas shigelloides* were picked to 100 ml of TSB-N. Non-Vibrionaceae were picked to 100 ml of TSB without added NaCl. All cultures were incubated at 37 °C with orbital shaking at 250 rpm.

Table 1
Sources of non-Vibrionaceae pathogens used in this study

Bacterium	Serotype	Source[a]	Designation
Bacillus cereus		USDA	5056
Enterobacter aerogenes		ATCC	13048
Escherichia coli	O157:H7	ATCC	43889
		ATCC	9637
Klebsiella pneumoniae		ATCC	13883
Listeria monocytogenes		USDA	Scott A
Salmonella typhimurium	DT-104	USDA	H3402
Salmonella enteritidis		USDA	92-008
Shigella flexneri	B	USUHS	2457T
Shigella boydii	C	USUHS	BS512
Shigella sonii	D	USUHS	BS513
Staphylococcus aureus		USDA	196E
Streptococcus bovis		ATCC	700338
Streptococcus pyogenes		ATCC	43202
Yersinia enterocolitica	O:8	USDA	12#83

[a] Abbreviations: ATCC, American Type Culture Collection, Manassas, VA; USDA, United States Department of Agriculture, Agricultural Research Service, Wyndmoor, PA; USUHS, Uniformed Services University of the Health Sciences, Bethesda, MD.

The length of incubation of broth cultures was evaluated in an effort to standardize bacterial propagation. Using *V. vulnificus* as a model, we evaluated the growth characteristics and the stability of LysAP activity. Three 100-ml flasks of TSB-N were inoculated with 1.0 ml of a 10-h broth culture of *V. vulnificus* strain MLT364. Flasks were incubated at 37 °C with orbital shaking at 250 rpm, and the OD_{600} and LysAP activities were directly measured. LysAP activity was determined for 1 μl of well-mixed culture each hour for 18 h. The LysAP activity remained at its highest levels throughout the stationary phase; therefore, all broth cultures in this study were incubated for 18 h.

In preparation for the measurement of PGI-LysAP and PGI isomerase activities in nine strains of *V. vulnificus*, eleven species from the Vibrionaceae family, and fourteen species of non-Vibrionaceae pathogens, isolates were streaked on TSA or TSA-N plates, as appropriate, and incubated overnight. One colony of each bacterium was picked to 100 ml of TSB or TSB-N and incubated as previously described. All cultures were diluted to an OD_{600} of 0.10 and 100 ml of each OD-adjusted culture was centrifuged at 1500×*g* for 20 min at 4 °C. Each pellet was resuspended in 250 μl of 20 mM Tris–HCl, pH 9.0. The resuspended pellets were frozen at −80 °C pending enzyme extraction.

2.2. Enzyme extraction

We evaluated the extraction of PGI-LysAP using a simple freeze/thaw and centrifugation procedure [5] where frozen cells were lysed by thawing at room temperature (RT) to release cell-associated enzyme. A modified procedure that differed only in the incorporation of an additional (sequential) freeze/thaw step was compared with the single freeze/thaw method to determine which technique would release the most enzyme from the lysed cells. Ten 100-ml cultures of *V. vulnificus* strain MLT364 were grown overnight and five were processed with a single freeze/thaw, while the remaining five were frozen and thawed twice. Freezing was for 1 h at −80 °C. After either one or two freeze/thaw cycles, the cells were centrifuged at 12,000×*g* for 5 min at RT. Supernatants, also referred to as lysates, were retained at 4 °C. One microliter of each lysate was assayed in triplicate for LysAP activity, as described in Section 2.4, below.

The extraction method used in the subsequent assays involved the single freeze/thaw procedure where cells frozen for ≥1 h at −80 °C were thawed at RT, centrifuged at 12,000×*g* for 5 min at RT, and the supernatants stored at 4 °C pending assay, usually within 3 h. This extraction procedure was used to compare: (a) the LysAP levels produced by *V. vulnificus* grown in TSB-N and APW, (b) the LysAP and isomerase activities for Vibrionaceae pathogens, and (c) the LysAP activity of non-Vibrionaceae pathogens.