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Author(s)	Hosoda, Naohiko; Kurokawa, Yoichi; Sako, Yoshihiko; Nagasaki, Keizo; Yoshida, Takashi; Hiroishi, Shingo
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Title

The functional effect of Gly209 and Ile213 substitutions on lysozyme activity of family 19 chitinase encoded by cyanophage Ma-LMM01

Authors

Naohiko Hosoda・Yoichi Kurokawa・Yoshihiko Sako・Keizo Nagasaki・Takashi Yoshida・and Shingo Hiroishi

N. Hosoda
Departmennt of Marine Bioscience, Fukui Prefectural University, 1-1 Gakuen-cho, Obama, Fukui 917-0003, Japan
E-mail: s0894004@s.fpu.ac.jp
Y. Kurokawa
Department of Bioscience, Fukui Prefectural University, 4-1-1 Matsuoka-Kenjojima, Eiheiji-cho, Yoshida-gun, Fukui 910-1195, Japan
E-mail: kurokawa@fpu.ac.jp
Y. Sako
Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
E-mail: sako@kais.kyoto-u.ac.jp
K. Nagasaki
National Research Institute of Inland Sea, Fisheries Research Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan
E-mail: nagasaki@affrc.go.jp
S. Hiroishi
Departmennt of Marine Bioscience, Fukui Prefectural University, 1-1 Gakuen-cho, Obama, Fukui 917-0003, Japan
E-mail: hiroishi@fpu.ac.jp

Corresponding author
T. Yoshida
Mailing address: Laboratory of Marine Microbiology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan.
Tel: +81-75-753-6218; Fax: +81-75-753-6226
E-mail: yoshiten@kais.kyoto-u.ac.jp

Abstract

ORF69 in the cyanophage infecting *Microcystis aeruginosa*, Ma-LMM01, shows homology to the family 19 chitinases where the catalytic domain has structural similarity to lysozyme. Chitinases hydrolyze chitin, a β-1, 4-linked monopolymer of *N*-acetylglucosamine (GlcNAc); whereas lysozymes hydrolyzes peptidoglycan, alternating β-1, 4-linked copolymers of *N*- acetylmuramic acid (MurNAc) and GlcNAc. Using amino acid sequence comparison to ORF69, the putative sugar binding residues, Gln162 and Lys165, from the barley chitinase (the model enzyme for the family 19 chitinases) corresponding to subsites -4 and -3 were found to be replaced with Gly209 and Ile213, respectively, in ORF69. To analyze their contribution to substrate binding affinity, ORF69 was cloned into *Escherichia coli*; and two mutant proteins G209Q and I213K were prepared using site-directed mutagenesis. The wild-type gene product (gp69) showed both lysozyme and chitinase activities. In contrast, the I213K mutant showed a decrease (70%) in lysozyme activity and a significant increase (50%) in chitinase activity; whereas, the G209Q mutant almost completely abolished both enzyme activities. The data suggest the Ile213 residue is involved in recognizing the substrate MurNAc; and Gly209 has significant contribution in chitinase and lysozyme activities for the wild-type gp69.

Keywords
Microcystis, cyanophage, family 19 chitinase, site-directed mutagenesis

Introduction

Throughout the world, *Microcystis aeruginosa* is one of the common bloom-forming species in eutrophic freshwaters. Some strains produce cyclic peptide toxins called microcystins that cause serious health problems in water management [1]. Previously we isolated a cyanophage, Ma-LMM01, infecting the toxic *M. aeruginosa* strain NIES298 [2]. The genome of Ma-LMM01 contains 184 ORFs [3]. The majority of the predicted genes have no detectable homologues in present databases including other *Myoviridae*; and thus Ma-LMM01 was assigned as a member of a new lineage of the *Myoviridae* family [3, 4]. Of the ORFs, ORF69 is predicted to encode for a member of the family 19 chitinases whose catalytic domain has structural similarity to lysozyme [5].

Chitinase (EC 3.2.1.14) is a glycoside hydrolase that hydrolyzes chitin, a linear β-1, 4-linked monopolymer of *N*-acetylglucosamine (GlcNAc). Based on structures and catalytic mechanisms, the chitinases are classified into two families, 18 and 19 [6, 7]. Family 18 chitinases are widely distributed in a variety of organisms such as bacteria, fungi, bacteriophages, animals and higher plants (classes III and V); whereas family 19 chitinases are found only in higher plants (classes I, II and IV). Recently, however, some members of the family 19 chitinases have been found in genomes of

actinobacteria, proteobacteria, nematodes and bacteriophages [8]. Based on amino acid sequence comparisons, phylogenetic analysis shows the family 19 chitinases are separated into five clusters (clusters I to V) [8]. Of these, cluster III of the family 19 chitinases are most distantly related to the other clusters [8]. The cluster III family 19 of chitinases consists only of those from proteobacteria and bacteriophages and the genes in proteobacteria are often found within the phage-related regions. The cluster III family 19 chitinase genes in PA0629 from *Pseudomonas aeruginosa* and PFL_1227 from *P. fluorescens* Pf-5 are located within a region of one of the variants of a defective phage (pyocin) and prophage, respectively. Their recombinant proteins have lysozyme activity that hydrolyses peptidoglycans, alternating β-1, 4-linked residues of *N*- acetylmuramic acid (MurNAc) and GlcNAc [9, 10].

The family 19 chitinases are shown to have highly conserved catalytic residues and substrate-binding residues using crystal structure analyses, e.g. those from barley (cluster I) [11], Jack bean (cluster I) [12], ChiC of *Streptomyces griceus* HUT6037 (cluster II) [13] and ChiG of *S. coelicolor* A3(2) (cluster II) [14]. We found two residues were replaced in the putative substrate-binding residues of ORF69 when compared to the other family 19 chitinases. Here, we determined the lysozyme and chitinase activities of ORF69 gene products from cyanophage Ma-LMM01; and

examined the function of the two residues in recognition of the substrates, chitin and peptidoglycan, using site-directed mutagenesis.

Materials and methods

Cloning of ORF69 and derivatives

The genomic DNA of Ma-LMM01 was purified as described previously [2]. To isolate the full sequence of the ORF69 gene, a PCR reaction was performed with a forward primer MaPOrf69InF and a reverse primer MaPOrf69InR1 containing *Eco*RI site (Table 1). The PCR was performed in a 50 μl containing 200 ng Ma-LMM01 DNA, 10 μM primers, 250 μM each dNTPs, 1X PCR buffer for KOD-plus- and 1U KOD-Plus- (TOYOBO, Osaka, Japan). The reaction conditions were: 2 min initial denaturing at 94 °C followed by 35 cycles: 94 °C for 15 s, 45 °C for 30 s and 68 °C for 90 s. The reaction mixture was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The pTrc-OmpA vector was fused with the OmpA signal sequences upstream of a multiple cloning site; thus the resulting expressed protein is transported to the periplasm in the transformants [15]. The purified DNA fragments were digested with *Eco*RI (TOYOBO) and cloned into expression vector pTrc-OmpA. The coding region of the ORF69 was inserted downstream of the OmpA

signal sequence yielding pTrc-OmpA-ORF69. *E. coli* JM109 (TOYOBO) was transformed with the pTrc-OmpA-ORF69; and the transformant was selected on LB plates containing 100 μg/ml carbenicillin disodium salt (Nacalai Tesque, Kyoto, Japan) and 0.5% glucose. The DNA sequence of the resultant plasmids was verified using the primers Trc-F and Trc-R2 (Table 1).

Site-directed mutagenesis

Three mutant proteins (G209Q, I213K and E122A) were constructed using PCR-based site-directed mutagenesis. The mutagenesis primers are shown in Table 1. The PCR reaction mixtures contained 100 ng plasmid DNA template, 10 pmol each of the forward and reverse primers containing the desired mutation, 2 mM dNTPs, reaction buffer and PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA). The reaction mixture was subjected to 20 cycles of PCR (95 °C for 30 s; 55 °C for 1min; and 68 °C for 12 min); then, the resultant plasmids were digested with *Dpn* I. Finally, the mutated plasmids were transformed into *E. coli* JM109 and expressed.

Preparation of culture supernatant from transformants.

E. coli JM109 cells containing the plasmid pTrc-OmpA-ORF69 or mutated plasmids were independently grown overnight at 30 °C in LB liquid medium containing 100 μg ml^{-1} carbenicillin disodium salt and 0.5% glucose. Two-ml of the culture was

diluted to 100 ml of fresh LB medium and grown with shaking at 30 °C until the OD_{660nm} = 0.5. Protein expression was induced adding isopropyl-*β*-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growth for another 6 h, the supernatant of two consecutive centrifugations (15,000g, 5 min, at 4 °C) was stored as the culture supernatant at -20 °C until used. The pelleted cells were resuspended in 50 mM sodium phosphate buffer (pH 6.2) and disrupted using sonication. After centrifugation, the crude extracts were also stored at -20 °C. Amount of total protein was measured by method of Bradford with BSA as a standard [16].

Enzymatic activities of culture supernatant from *E. coli* cells expressing the wild-type ORF69 and its mutants.

(i) Lysozyme activity

Lyophilized cells of *Micrococcus lysodeikticus* ATCC4698 (Nacalai) were re-suspended at a concentration of 0.25 mg/ml in 10 ml of 50 mM sodium phosphate buffer (pH 6.2); then, 100 μg of a culture supernatant (see above) was added to 2 ml of the *M. lysodeikticus* cell suspension. Cell lysis was measured continuously by monitoring the decrease in turbidity (OD_{660nm}) using a Ultraspec 2100 *pro* (GE Healthcare, Buckinghamshire, UK) for 15 minutes at 37 °C. Lysozyme activity was calculated from the linear portion of the digestion graph representing absorbance versus

time. One unit of enzyme activity was defined as the amount of enzyme causing an absorbance decrease of 0.01 OD_{660nm} per min at 37 °C [17].

(ii) Chitinase activity

The chitinase assay was performed using *p*-nitrophenyl chitooligosaccharide *p*NP-$(GlcNAc)_n$ (n= 2 to 5) (Yaizu Suisan Chemical Co. Ltd., Shizuoka, Japan) as the substrate [18]. The reaction mixture (200μl) contained 2.5 mM of substrate in 50 mM sodium phosphate buffer (pH 6.2). The reaction was initiated adding 100 μg of a culture supernatant to the above reaction mixture pre-incubated for 5 min; and incubated for 15 min after addition at 37 ˚C. The reaction was stopped by adding 250 μl 0.2 M Na_2CO_3 and the released *p*-nitrophenol was measured at OD_{420nm}. One unit of chitinase activity was defined as the amount of enzyme causing 1μmol releasing of *p*-nitrophenol per min at 37 ˚C.

Results

The amino acid sequence of ORF69

A phylogenetic analysis showed the amino acid sequence of ORF69 was clustered within the cluster III family 19 chitinases (data not shown). Comparison of the conserved domain database analysis [19] showed the C-terminal region of ORF69 (residues 110-251) contains a domain similar to a glycoside hydrolase family 19

chitinase (cd00325). There are two catalytic residues and seven putative sugar binding residues in the family 19 chitinases.

The position of the 161-166 residues (the 161-166 loop) in the cluster I barley family 19 chitinase contains two polar amino acids (Gln162 and Lys165) (Fig. 1) [20]. Gln162 and Lys165 are thought to form the substrate-binding site, namely subsite -4 and -3, respectively (subsites are numbered according to the standard nomenclature; cleavage occurs between the sugar units bound in subsites -1 and +1 [21]). These residues in ORF69 and other cluster III family 19 chitinases are replaced with the non-polar amino acids, Gly209 and Ile213. Therefore, we predicted mutations in residues Gly209 and Ile213 of ORF69 would affect the recognition of substrates. To confirm this hypothesis, site-directed mutagenesis was performed.

Lysozyme activity of gp69 and its mutants

Based on the vector pTrc-OmpA-ORF69 encoding the wild-type ORF69 enzyme (gp69), two vectors encoding mutants in the putative sugar biding site (G209Q, I213K) and one in the catalytic site (E122A) were constructed.

The growth of transformants was monitored measuring the change in OD_{660nm}. After induction with IPTG, the OD value of both transformants containing pTrc-OmpA-ORF69 and pTrc-OmpA-ORF69-I213K declined about 30% from 4 to 8 h

182 (data not shown). In contrast, normal growths were observed in transformants
183 containing pTrc-OmpA-ORF69-G209Q and pTrc-OmpA-ORF69-E122A.
184 In transformants containing either pTrc-OmpA-ORF69 or
185 pTrc-OmpA-ORF69-I213K, induction of protein expression caused cell lysis and most
186 of the lysozyme activities were observed in the culture supernatant fraction (data not
187 shown). In the transformants containing pTrc-OmpA-ORF69-G209Q, no lysozyme
188 activity was observed in both culture supernatant fractions and crude protein from
189 pelleted cells (data not shown). In addition, a 10-fold concentration of the crude extracts
190 had no impact on lysozyme activity. The activities of wild-type gp69, G209Q and
191 I213K for *M. lysodeikticus* were 61.3 ± 17.2, 1.1 ± 1.2 and 18.1 ± 12.9 U/mg,
192 respectively (Table 2) suggesting mutations in these residues have effects on the
193 lysozyme activity. Lysozyme activity from the mutant protein, E122A (having a
194 mutation in the catalytic site) was not detected.
195 **Chitinase activity of gp69 and its mutants digesting chitooligosaccharides.**
196 To determine the chitinase activity of wild-type gp69 and its mutants, we used
197 the culture supernatant fraction to measure the release of *p*-nitrophenol, using several
198 chitooligosaccharides [*p*NP-$(GlcNAc)_n$ (n= 2 to 5)] as substrates. The highest
199 hydrolyzing activity of wild-type gp69 was observed when *p*NP-$(GlcNAc)_3$ was used as

200 the substrate (Fig. 2). Whereas, the hydrolytic activity to *p*NP-$(GlcNAc)_5$ was
201 approximately one-half compared to *p*NP-$(GlcNAc)_3$; and the hydrolysis activity for
202 *p*NP-$(GlcNAc)_4$ was not detected (Fig. 2). Compared to the gp69, the hydrolytic activity
203 of mutant protein I213K had approximately a 1.3-fold increase using *p*NP-$(GlcNAc)_3$
204 and the hydrolytic activity to *p*NP-$(GlcNAc)_4$ was increased. Thus, the I213K mutation
205 increased the hydrolyzing activity towards *p*NP-$(GlcNAc)_3$ and *p*NP-$(GlcNAc)_4$ even
206 though the lysozyme activity of I213K was the 30% of that of wild-type (Table 2). In
207 contrast, the mutant protein, G209Q in both fractions, showed no detectable activity
208 towards any substrate. In addition, a 10-fold concentration of the crude extracts of
209 G209Q did not show chitinase activities (data not shown).

210 **Discussion**

211 We tried to construct various vectors for the expression of ORF69 where
212 spontaneous mutations were observed in the cloned sequences. This was possibly due to
213 the toxicity of the gene products for *E. coli* [22]. We obtained only one clone with the
214 correct sequence of ORF69 using the pTrc-OmpA vector that allows expression of
215 OmpA signal-fused protein that was guided to the periplasmic space of the *E. coli* cell.
216 For this reason, crude extracts from the transformants were used in the enzymatic
217 experiments.

In general, the family 19 chitinases are endo-type chitinases that generate various sizes of chitooligomers [23]. However, the release of *p*-nitrophenol from the gp69 using the chitooligosaccharides as substrate suggests the wild-type gp69 is not typically an endo-like enzyme. Chi19 from *Vibrio proteolyticus* hydrolyzes colloidal chitin to release small oligosaccharides at the early stage of the reaction; and it is thought to be an exo-like family 19 chitinase [18]. Hen egg white lysozyme (HEWL) has six subsites (from -4 to +2) [24]. Also, the subsite structures of higher plant family 19 chitinases (cluster I) are assumed to be represented by (-4)(-3)(-2)(-1)(+1)(+2) [20] or (-3)(-2)(-1)(+1)(+2)(+3) [25] except for those from *Carica papaya* [26] and *Picea abies* (Norway spruce) [27]. In HEWL, the corresponding subsite -3 is known to be responsible for interaction with MurNAc [24]. Combined, our data suggests the Ile213 residue corresponding to subsite -3 in ORF69 contributes to the interaction with MurNAc and the replacement of Ile213 with Lys may emphasize the affinity for *p*NP-$(GlcNAc)_3$ and *p*NP-$(GlcNAc)_4$ than for *M. lysodeikticus.* Whereas, the I213K mutation decreased the hydrolyzing activity to *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_5$ where one possible explanation is interference of substrate access to the catalytic site due to the small oligosaccharides but we do not have any useful data concerning this hypothesis. The substitution of Gly209 to Gln caused a significant decrease in both

lysozyme and chitinase activities. There is a possibility the single amino acid replacement altered the conformation of recombinant protein G209Q [28]. Further study is required to determine the effect of the Gly209 residue in conformational changes.

Nineteen cluster III family 19 chitinases (17 genes in bacterial genomes and 2 genes in bacteriophages) were in the current database [8, 29]. Two residues, Asn124 and Lys165 (according to the barley family 19 chitinase numbering), are presumed to be responsible for the subsite -3 activity. These residues are highly conserved among family 19 chitinases [20]. In ORF69 of Ma-LMM01, the amino acids corresponding to the Lys165 residue in 15 genes of the 19 cluster III family 19 chitinases are replaced with non-polar amino acids. In the remaining four genes, the amino acids corresponding to Lys165 is replaced with a Tyr residue. Further research focusing on the role of the Tyr residue is necessary to determine its contribution in sugar binding.

Family 19 chitinases are hypothesized to be horizontally transferred from higher plants to bacteria [29, 30]. In the evolutionary history of the family 19 chitinases, mutation in key residues (ex. corresponding to subsite -3) may lead to alternation of affinity for substrates; and may have spread to bacteria and phages as a lytic enzyme.

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Tables

Table 1. Plasmids and primers used in this study

Plasmids or primers	Characteristics or sequences (5' to 3')	Sources or references
Plasmids		
pTrc-OmpA	Amp^R, $lacI^q$, ori (pBR322), trcP, rrmB T1T2 terminator	Kurokawa, *et al.*, [15]
pTrc-OmpA-ORF69	pTrc-OmpA with an insert of nucleotides	
	52314 to 53069 from cyanophage Ma-LMM01	This study
pTrc-OmpA-ORF69-G209Q	Substitute of ORF69 Gly209 to Gln209	This study
pTrc-OmpA-ORF69-I213K	Substitute of ORF69 Ile213 to Lys213	This study
pTrc-OmpA-ORF69-E122A	Substitute of ORF69 Glu122 to Ala122	This study
Primers		
MaPOrf69InF	CTA AGA AAC ATT GAT AGA GC	This study
MaPOrf69InR1[a)]	CGG AAT TCT CAT GTC AGC ACC GCC TGT A (*Eco*RI)	This study
ORF69-E122A-F[b)]	CAG CTG ATG CAC GCG TCA *GGG* AAC CTA CG	This study
ORF69-E122A-R[b)]	CGT AGG TT*C CC*T GAC GCG TGC ATC AGC TG	This study
ORF69-G209Q-F[b)]	AGC GCA CTA CTG GAG AAC A*CC A*GG GCT AAA TGA AAT AGC AGA C	This study
ORF69-G209Q-R[b)]	GTC TGC TAT TTC ATT TAG CCC *TGG* TGT TCT CCA GTA GTG CGC T	This study
ORF69-I213K-F[b)]	GAA CAC GGG GGC TAA ATG AAA *AGG* CAG ACA AGA ATG ATA TAA A	This study
ORF69-I213K-R[b)]	TTT ATA TCA TTC TTG TCT G*CC T*TT TCA TTT AGC CCC CGT GTT C	This study
Trc-F	ACA TCA TAA CGG TTC TGGC	Kurokawa, *et al.*, [15]
Trc-R2	CAA ATTC TGT TTT ATC AGA CC	Kurokawa, *et al.*, [15]

a): The restriction site is underlined.

b): The mutation sites are in italics.

Table 2. Lysozyme activity of the culture supernatant of *E. coli* cells expressing the wild-type gp69 and its mutants.

Protein	Activity (U/ mg)	Relative activity (%)
Gp69	61.3 ± 17.2	100
G209Q	1.1 ± 1.2	1.8
I213K	18.1 ± 12.9	29.5
E122A	ND	-
Vector	ND	-
HEWL*	1.6 ± 0.01	2.6

The activity was measured by the decrease in absorbance at OD_{660nm}. The reaction mixtures were incubated in 50 mM sodium phosphate buffer, pH6.2 at 37 °C. The data are the means of three independent assays ± SD. -, Activity not detected. *, Activities of hen egg white lysozyme (HEWL; 0.6μg) was used as positive control. One unit of enzyme activity was defined as the amount of enzyme that causes an absorbance decrease of 0.01.

Figure legends

Figure 1. Alignment of amino acid sequences for the catalytic domains of family 19 chitinases. Sequence alignment was performed using MEGA version 4 software [31]. Residues conserved in the sequences are indicated by black backgrounds, whereas residues conserved in >80 and >60% of the proteins examined are indicated by white type on a dark gray background and by black type on a light gray back ground, respectively. The two catalytic amino acid residues from the family 19 chitinases are indicated by closed triangles. Residues predicted to interact with saccharide molecules in theoretical models of the barley chitinase/ $(GlcNAc)_6$ complex [19] are indicated by closed circles. The 161-166 residues (using the numbering of the barley enzyme; see text) are indicated by dots above the sequence. Based on higher plant and *Streptomyces* chitnase, the putative secondary structure assignments are indicated with "h" for α-helix and "s" in the β-strand. ORF69, Ma-LMM01 ORF69 (accession no. YP_851083); PA0629, lytic enzyme of *P. aeruginosa* PAO1 (NP_249320); putative lytic enzyme of ORF_PML14, *P. aeruginosa* (YP_788803); PFL_1227, lytic enzyme of *P. fluorescens* Pf-5 (YP_258358); ChiG, ChiG of *Streptomyces coelicolor* A3(2) (BAA75648); ChiC, ChiC of *S. griseus* HUT 6037 (BAA23739); Jackbean, class II chitinase of *Canavalia ensiformis* (CAA07413); and Barley, class II chitinase of *Hordeum vulgare* (P23951).

375 Figure 2. Hydrolytic activity for various chitooligosaccharides using the culture

376 supernatant from *E. coli* cells expressing the wild-type ORF69 and its mutants. The

377 reaction mixture was 2.5 mM substrate, 50 mM sodium phosphate buffer (pH6.2) and

378 mixed with 100 μg of the culture supernatant. pNP-$(GlcNAc)_2$, white bars;

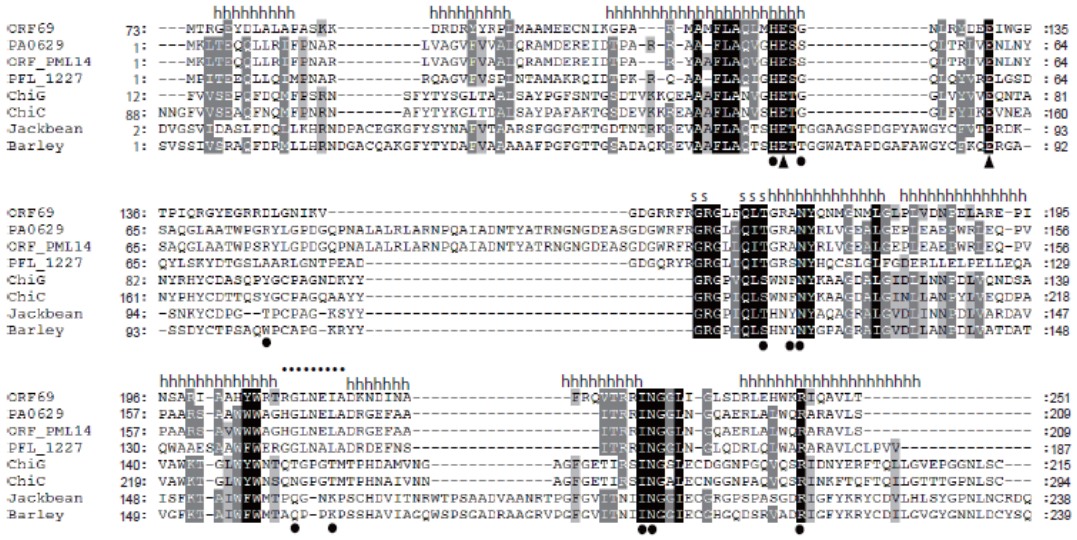
379 pNP-$(GlcNAc)_3$, pale-grey bars; pNP-$(GlcNAc)_4$, dark-grey bars; and pNP-$(GlcNAc)_5$,

380 black bars. The reaction mixtures were incubated for 15 min in 50 mM sodium

381 phosphate buffer (pH6.2) at 37 °C. The bars are an average of three independent

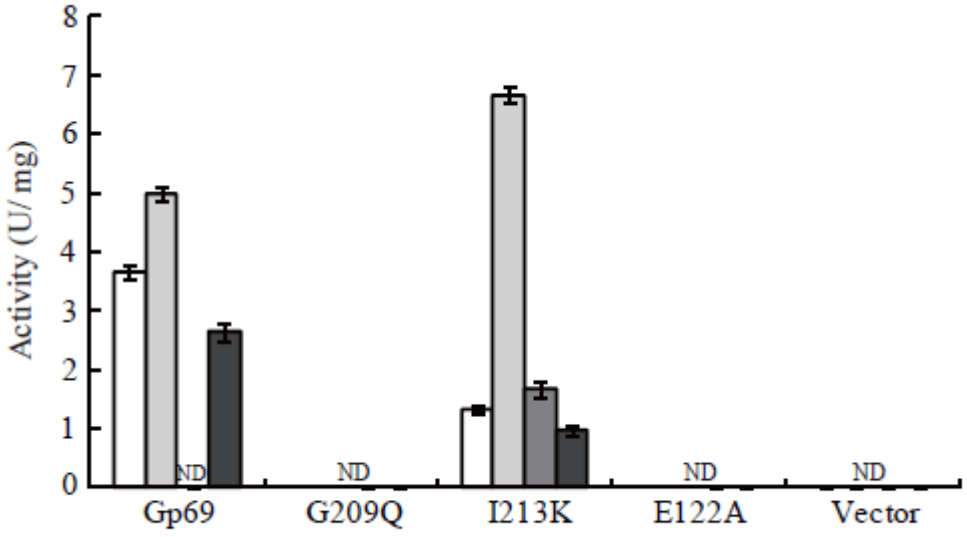
382 measurements. N.D: Not detected.

383 Fig.1



384

385 Fig. 2


Activity (U/ mg)
8
7
6
5
4
3
2
1
0
ND
ND
ND
ND
Gp69
G209Q
I213K
E122A
Vector

386