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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
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Citation	Fisheries Science (2011), 77(4): 665-670
Issue Date	2011-07
URL	http://hdl.handle.net/2433/143681
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Type	Journal Article
Textversion	author

Kyoto University

1 **Title**

2 The functional effect of Gly209 and Ile213 substitutions on lysozyme activity of family
3 19 chitinase encoded by cyanophage Ma-LMM01

4

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35

36 **Abstract**

37 ORF69 in the cyanophage infecting *Microcystis aeruginosa*, Ma-LMM01,

38 shows homology to the family 19 chitinases where the catalytic domain has structural

39 similarity to lysozyme. Chitinases hydrolyze chitin, a β-1, 4-linked monopolymer of

40 *N*-acetylglucosamine (GlcNAc); whereas lysozymes hydrolyzes peptidoglycan,

41 alternating β-1, 4-linked copolymers of *N*- acetylmuramic acid (MurNAc) and GlcNAc.

42 Using amino acid sequence comparison to ORF69, the putative sugar binding

43 residues, Gln162 and Lys165, from the barley chitinase (the model enzyme for the

44 family 19 chitinases) corresponding to subsites -4 and -3 were found to be replaced with

45 Gly209 and Ile213, respectively, in ORF69. To analyze their contribution to substrate

46 binding affinity, ORF69 was cloned into *Escherichia coli*; and two mutant proteins

47 G209Q and I213K were prepared using site-directed mutagenesis. The wild-type gene

48 product (gp69) showed both lysozyme and chitinase activities. In contrast, the I213K

49 mutant showed a decrease (70%) in lysozyme activity and a significant increase (50%)

50 in chitinase activity; whereas, the G209Q mutant almost completely abolished both

51 enzyme activities. The data suggest the Ile213 residue is involved in recognizing the

52 substrate MurNAc; and Gly209 has significant contribution in chitinase and lysozyme

53 activities for the wild-type gp69.

54 **Keywords**
55 *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

74 actinobacteria, proteobacteria, nematodes and bacteriophages [8]. Based on amino acid
75 sequence comparisons, phylogenetic analysis shows the family 19 chitinases are
76 separated into five clusters (clusters I to V) [8]. Of these, cluster III of the family 19
77 chitinases are most distantly related to the other clusters [8]. The cluster III family 19 of
78 chitinases consists only of those from proteobacteria and bacteriophages and the genes
79 in proteobacteria are often found within the phage-related regions. The cluster III family
80 19 chitinase genes in PA0629 from *Pseudomonas aeruginosa* and PFL_1227 from *P.*
81 *fluorescens* Pf-5 are located within a region of one of the variants of a defective phage
82 (pyocin) and prophage, respectively. Their recombinant proteins have lysozyme activity
83 that hydrolyses peptidoglycans, alternating β-1, 4-linked residues of *N*- acetylmuramic
84 acid (MurNAc) and GlcNAc [9, 10].
85 The family 19 chitinases are shown to have highly conserved catalytic residues
86 and substrate-binding residues using crystal structure analyses, e.g. those from barley
87 (cluster I) [11], Jack bean (cluster I) [12], ChiC of *Streptomyces griceus* HUT6037
88 (cluster II) [13] and ChiG of *S. coelicolor* A3(2) (cluster II) [14]. We found two
89 residues were replaced in the putative substrate-binding residues of ORF69 when
90 compared to the other family 19 chitinases. Here, we determined the lysozyme and
91 chitinase activities of ORF69 gene products from cyanophage Ma-LMM01; and

92 examined the function of the two residues in recognition of the substrates, chitin and

93 peptidoglycan, using site-directed mutagenesis.

94

95 **Materials and methods**

96 **Cloning of ORF69 and derivatives**

97 The genomic DNA of Ma-LMM01 was purified as described previously [2]. To

98 isolate the full sequence of the ORF69 gene, a PCR reaction was performed with a

99 forward primer MaPOrf69InF and a reverse primer MaPOrf69InR1 containing *Eco*RI

100 site (Table 1). The PCR was performed in a 50 μl containing 200 ng Ma-LMM01 DNA,

101 10 μM primers, 250 μM each dNTPs, 1X PCR buffer for KOD-plus- and 1U

102 KOD-Plus- (TOYOBO, Osaka, Japan). The reaction conditions were: 2 min initial

103 denaturing at 94 °C followed by 35 cycles: 94 °C for 15 s, 45 °C for 30 s and 68 °C for

104 90 s. The reaction mixture was purified using a Wizard® SV Gel and PCR Clean-Up

105 System (Promega, Madison, WI). The pTrc-OmpA vector was fused with the OmpA

106 signal sequences upstream of a multiple cloning site; thus the resulting expressed

107 protein is transported to the periplasm in the transformants [15]. The purified DNA

108 fragments were digested with *Eco*RI (TOYOBO) and cloned into expression vector

109 pTrc-OmpA. The coding region of the ORF69 was inserted downstream of the OmpA

110 signal sequence yielding pTrc-OmpA-ORF69. *E. coli* JM109 (TOYOBO) was

111 transformed with the pTrc-OmpA-ORF69; and the transformant was selected on LB

112 plates containing 100 μg/ml carbenicillin disodium salt (Nacalai Tesque, Kyoto, Japan)

113 and 0.5% glucose. The DNA sequence of the resultant plasmids was verified using the

114 primers Trc-F and Trc-R2 (Table 1).

115 **Site-directed mutagenesis**

116 Three mutant proteins (G209Q, I213K and E122A) were constructed using

117 PCR-based site-directed mutagenesis. The mutagenesis primers are shown in Table 1.

118 The PCR reaction mixtures contained 100 ng plasmid DNA template, 10 pmol each of

119 the forward and reverse primers containing the desired mutation, 2 mM dNTPs, reaction

120 buffer and PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA). The

121 reaction mixture was subjected to 20 cycles of PCR (95 °C for 30 s; 55 °C for 1min; and

122 68 °C for 12 min); then, the resultant plasmids were digested with *Dpn* I. Finally, the

123 mutated plasmids were transformed into *E. coli* JM109 and expressed.

124 **Preparation of culture supernatant from transformants.**

125 *E. coli* JM109 cells containing the plasmid pTrc-OmpA-ORF69 or mutated

126 plasmids were independently grown overnight at 30 °C in LB liquid medium containing

127 100 μg ml^{-1} carbenicillin disodium salt and 0.5% glucose. Two-ml of the culture was

128 diluted to 100 ml of fresh LB medium and grown with shaking at 30 °C until the

129 OD_{660nm} = 0.5. Protein expression was induced adding

130 isopropyl-*β*-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After

131 growth for another 6 h, the supernatant of two consecutive centrifugations (15,000g, 5

132 min, at 4 °C) was stored as the culture supernatant at -20 °C until used. The pelleted

133 cells were resuspended in 50 mM sodium phosphate buffer (pH 6.2) and disrupted using

134 sonication. After centrifugation, the crude extracts were also stored at -20 °C. Amount

135 of total protein was measured by method of Bradford with BSA as a standard [16].

136 **Enzymatic activities of culture supernatant from *E. coli* cells expressing the**

137 **wild-type ORF69 and its mutants.**

138 **(i) Lysozyme activity**

139 Lyophilized cells of *Micrococcus lysodeikticus* ATCC4698 (Nacalai) were

140 re-suspended at a concentration of 0.25 mg/ml in 10 ml of 50 mM sodium phosphate

141 buffer (pH 6.2); then, 100 μg of a culture supernatant (see above) was added to 2 ml of

142 the *M. lysodeikticus* cell suspension. Cell lysis was measured continuously by

143 monitoring the decrease in turbidity (OD_{660nm}) using a Ultraspec 2100 *pro* (GE

144 Healthcare, Buckinghamshire, UK) for 15 minutes at 37 °C. Lysozyme activity was

145 calculated from the linear portion of the digestion graph representing absorbance versus

146 time. One unit of enzyme activity was defined as the amount of enzyme causing an

147 absorbance decrease of 0.01 OD_{660nm} per min at 37 °C [17].

148 **(ii) Chitinase activity**

149 The chitinase assay was performed using *p*-nitrophenyl chitooligosaccharide

150 *p*NP-$(GlcNAc)_n$ (n= 2 to 5) (Yaizu Suisan Chemical Co. Ltd., Shizuoka, Japan) as the

151 substrate [18]. The reaction mixture (200µl) contained 2.5 mM of substrate in 50 mM

152 sodium phosphate buffer (pH 6.2). The reaction was initiated adding 100 µg of a culture

153 supernatant to the above reaction mixture pre-incubated for 5 min; and incubated for 15

154 min after addition at 37 °C. The reaction was stopped by adding 250 µl 0.2 M Na_2CO_3

155 and the released *p*-nitrophenol was measured at OD_{420nm}. One unit of chitinase activity

156 was defined as the amount of enzyme causing 1µmol releasing of *p*-nitrophenol per min

157 at 37 °C.

158 **Results**

159 **The amino acid sequence of ORF69**

160 A phylogenetic analysis showed the amino acid sequence of ORF69 was

161 clustered within the cluster III family 19 chitinases (data not shown). Comparison of the

162 conserved domain database analysis [19] showed the C-terminal region of ORF69

163 (residues 110-251) contains a domain similar to a glycoside hydrolase family 19

164 chitinase (cd00325). There are two catalytic residues and seven putative sugar binding

165 residues in the family 19 chitinases.

166 The position of the 161-166 residues (the 161-166 loop) in the cluster I barley

167 family 19 chitinase contains two polar amino acids (Gln162 and Lys165) (Fig. 1) [20].

168 Gln162 and Lys165 are thought to form the substrate-binding site, namely subsite -4

169 and -3, respectively (subsites are numbered according to the standard nomenclature;

170 cleavage occurs between the sugar units bound in subsites -1 and +1 [21]). These

171 residues in ORF69 and other cluster III family 19 chitinases are replaced with the

172 non-polar amino acids, Gly209 and Ile213. Therefore, we predicted mutations in

173 residues Gly209 and Ile213 of ORF69 would affect the recognition of substrates. To

174 confirm this hypothesis, site-directed mutagenesis was performed.

175 **Lysozyme activity of gp69 and its mutants**

176 Based on the vector pTrc-OmpA-ORF69 encoding the wild-type ORF69

177 enzyme (gp69), two vectors encoding mutants in the putative sugar biding site (G209Q,

178 I213K) and one in the catalytic site (E122A) were constructed.

179 The growth of transformants was monitored measuring the change in OD_{660nm}.

180 After induction with IPTG, the OD value of both transformants containing

181 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.

218 In general, the family 19 chitinases are endo-type chitinases that generate
219 various sizes of chitooligomers [23]. However, the release of *p*-nitrophenol from the
220 gp69 using the chitooligosaccharides as substrate suggests the wild-type gp69 is not
221 typically an endo-like enzyme. Chi19 from *Vibrio proteolyticus* hydrolyzes colloidal
222 chitin to release small oligosaccharides at the early stage of the reaction; and it is
223 thought to be an exo-like family 19 chitinase [18]. Hen egg white lysozyme (HEWL)
224 has six subsites (from -4 to +2) [24]. Also, the subsite structures of higher plant family
225 19 chitinases (cluster I) are assumed to be represented by (-4)(-3)(-2)(-1)(+1)(+2) [20]
226 or (-3)(-2)(-1)(+1)(+2)(+3) [25] except for those from *Carica papaya* [26] and *Picea*
227 *abies* (Norway spruce) [27]. In HEWL, the corresponding subsite -3 is known to be
228 responsible for interaction with MurNAc [24]. Combined, our data suggests the Ile213
229 residue corresponding to subsite -3 in ORF69 contributes to the interaction with
230 MurNAc and the replacement of Ile213 with Lys may emphasize the affinity for
231 *p*NP-$(GlcNAc)_3$ and *p*NP-$(GlcNAc)_4$ than for *M. lysodeikticus.* Whereas, the I213K
232 mutation decreased the hydrolyzing activity to *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_5$
233 where one possible explanation is interference of substrate access to the catalytic site
234 due to the small oligosaccharides but we do not have any useful data concerning this
235 hypothesis. The substitution of Gly209 to Gln caused a significant decrease in both

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

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

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

252 **Acknowledgement**

253 This study was supported by a Grant-in-Aid for Science Research (No. 20310045). NH

254 is a research fellow supported by the Japan Society for the Promotion of Science for

255 Young Scientists (No. 218877).

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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.

340 Table 2. Lysozyme activity of the culture supernatant of *E. coli* cells expressing the
341 wild-type gp69 and its mutants.
342

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

350
351 The activity was measured by the decrease in absorbance at OD_{660nm}. The reaction
352 mixtures were incubated in 50 mM sodium phosphate buffer, pH6.2 at 37 °C. The data
353 are the means of three independent assays ± SD. -, Activity not detected. *, Activities of
354 hen egg white lysozyme (HEWL; 0.6μg) was used as positive control. One unit of
355 enzyme activity was defined as the amount of enzyme that causes an absorbance
356 decrease of 0.01.

357 **Figure legends**

358 Figure 1. Alignment of amino acid sequences for the catalytic domains of family 19
359 chitinases. Sequence alignment was performed using MEGA version 4 software [31].
360 Residues conserved in the sequences are indicated by black backgrounds, whereas
361 residues conserved in >80 and >60% of the proteins examined are indicated by white
362 type on a dark gray background and by black type on a light gray back ground,
363 respectively. The two catalytic amino acid residues from the family 19 chitinases are
364 indicated by closed triangles. Residues predicted to interact with saccharide molecules
365 in theoretical models of the barley chitinase/ (GlcNAc)$_6$ complex [19] are indicated by
366 closed circles. The 161-166 residues (using the numbering of the barley enzyme; see
367 text) are indicated by dots above the sequence. Based on higher plant and *Streptomyces*
368 chitnase, the putative secondary structure assignments are indicated with "h" for α-helix
369 and "s" in the β-strand. ORF69, Ma-LMM01 ORF69 (accession no. YP_851083);
370 PA0629, lytic enzyme of *P. aeruginosa* PAO1 (NP_249320); putative lytic enzyme of
371 ORF_PML14, *P. aeruginosa* (YP_788803); PFL_1227, lytic enzyme of *P. fluorescens*
372 Pf-5 (YP_258358); ChiG, ChiG of *Streptomyces coelicolor* A3(2) (BAA75648); ChiC,
373 ChiC of *S. griseus* HUT 6037 (BAA23739); Jackbean, class II chitinase of *Canavalia*
374 *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

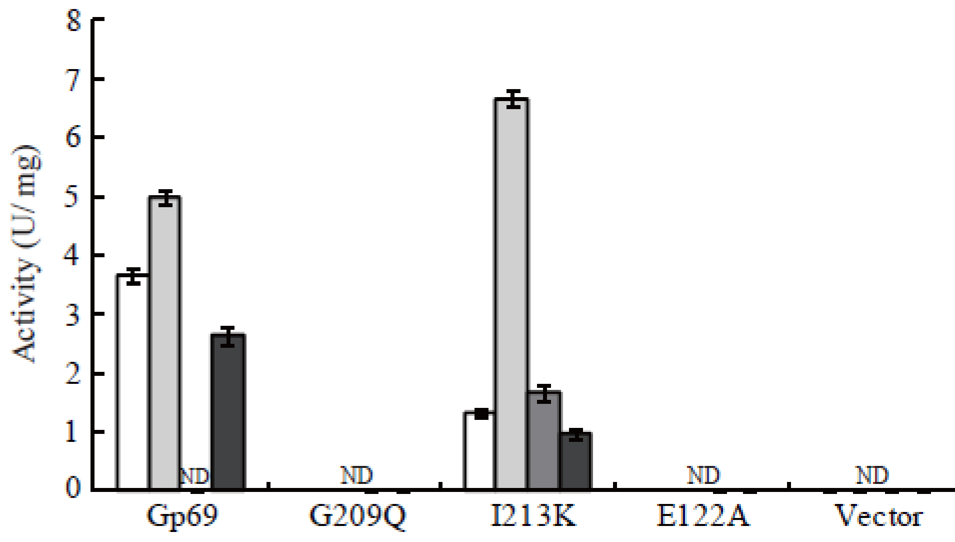
381 phosphate buffer (pH6.2) at 37 °C. The bars are an average of three independent

382 measurements. N.D: Not detected.

383 Fig.1

384

385 Fig. 2



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