Dual inhibition of DNA polymerase PolC and protein tyrosine phosphatase CpsB uncovers a novel antibiotic target

Alistair J. Standish[1*], Angela A. Salim[2], Robert J. Capon[2] & Renato Morona[1]

[1]School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA, 5005, Australia

[2]Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, St Lucia, Queensland, Australia

*Corresponding author. Address: University of Adelaide, North Terrace, Adelaide, SA, 5005, Australia. Ph: +61883130232. Fax +61 8 8313 7532. Email address : alistair.standish@adelaide.edu.au

Abstract

Increasing antibiotic resistance is making the identification of novel antimicrobial targets critical. Recently, we discovered an inhibitor of protein tyrosine phosphatase CpsB, fascioquinol E (FQE), which unexpectedly inhibited the growth of Gram-positive pathogens. CpsB is a member of the polymerase and histidinol phosphate phosphatase (PHP) domain family. Another member of this family found in a variety of Gram-positive pathogens is DNA polymerase PolC. We purified the PHP domain from PolC ($PolC_{PHP}$), and showed that this competes away FQE inhibition of CpsB phosphatase activity. Furthermore, we showed that this domain hydrolyses the 5'-*p*-nitrophenyl ester of thymidine-5'-monophosphate (*p*NP-TMP), which has been used as a measure of exonuclease activity. Finally, we showed that FQE not only inhibits the phosphatase activity of CpsB, but also ability of $PolC_{PHP}$ to catalyse the hydrolysis of pNP-TMP. This suggests that PolC may be the essential target of FQE, and that the PHP domain may represent an as yet untapped target for the development of novel antibiotics.

Keywords

Bacteria, Antibiotic, DNA polymerase, exonuclease, polymerase and histidinol phosphate domain

Introduction

The search for novel antimicrobials is of the utmost importance with ever-increasing rates of resistance to the current breed of antibiotics, along with the lack of drugs in the development pipeline. With the polysaccharide capsule the major virulence factor

of *Streptococcus pneumoniae*, our work has concentrated on identifying inhibitors of capsule as a novel anti-virulence target by focusing on a phosphoregulatory system, highly conserved amongst all but two pneumococcal serotypes, as well as across bacterial species [1]. During a recent search for inhibitors of CpsB, a protein tyrosine phosphatase from *S. pneumoniae*, we discovered an inhibitor, Fascioquinol E (FQE), which inhibited CpsB both *in vitro* and *in vivo* [2]. Unexpectedly, FQE also inhibited the growth of a number of Gram-positive pathogens, including *Streptococcus pneumoniae* and *Staphylococcus aureus*, while not affecting Gram-negative bacteria [3]. This was surprising as the gene encoding for CpsB is not essential [4].

CpsB belongs to the polymerase and histidinol phosphatase (PHP) family of proteins (PFAM: PF02811) [4,5]. Over 95% of these proteins are found in prokaryotes and along with the protein tyrosine phosphatases involved in regulation of capsular polysaccharides, also include histidinol phosphate phosphatases from bacteria and yeasts, DNA polymerases from a range of Gram-positive and Gram-negative bacteria, as well as a number of as yet un-characterised proteins [5]. The presence of DNA polymerases piqued our interest; as such enzymes are essential and are thus critical for the ability of organisms to survive.

Prokaryotic replicative polymerases belong to the C family of DNA polymerases and show little similarity with polymerases found in eukaryotes. For this reason, DNA polymerase have been the target for the development of antimicrobials, with these being primarily nucleotide analogues [6,7]. Low-GC bacteria, such as the pneumococcus, possess two DNA polymerases, DnaE and PolC [8]. Recently, the *in vitro* reconstitution of the Gram-positive replication machinery from *Bacillus subtillus*

showed that PolC is responsible for rapid, processive chromosomal replication, while DnaE is responsible for the extension of RNA primers [9]. This differs from the protypical system in *E. coli* where both functions are maintained by the DnaE polymerase.

While both PolC and DnaE possess a PHP domain, the PHP domain of PolC is bisected by an exonuclease domain (Figure 1A) [10,11]. A number of point mutations isolated in the PolC PHP domain in *B. subtillis* and *S. aureus* have illustrated that this domain is essential, and of importance for the exonuclease activity of the enzyme [10,12]. One such residue that has been mutated in *S. aureus* PolC (H339) is highly conserved throughout PHP domains, and additionally is important for CpsB phosphatase activity [2,13]. Interestingly, the PHP domains present in DnaE polymerases contain mutations in a number of conserved metal binding sites, questioning the importance of the domain in this polymerase [5]. Thus, we hypothesised that inhibition of the PHP domain from PolC may be responsible for the Gram-positive specific growth inhibition seen with FQE.

In this study, we have investigated whether the PHP domain of PolC is a target for the development of antibiotics, using a previously characterised inhbitor of CpsB, FQE. We have shown that addition of purified $PolC_{PHP}$ can reverse the inhibition of CpsB phosphatase activity. Furthermore, we have developed a novel assay to screen for PolC inhibitors by showing that the enzyme catalyses the hydrolysis of 5'-*p*-nitrophenyl ester of thymidine-5'-monophosphate (*p*NP-TMP), an activity associated with a variety of exonucleases. Using this assay, we have shown that PolC activity is inhibited by FQE, suggesting that the PHP domain may be a novel target for the

development of antibiotics that target a broad range of PHP domain containing proteins.

Materials and Methods

Growth Media and Growth Conditions

Escherichia coli strains were grown in Luria-Bertani broth (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl) broth or agar, with transformation carried out using $CaCl_2$-treated cells. *E. coli* K12 strain DH5α was used for routine cloning, while strain M15 (Qiagen) was used for induction of the 6xHis-$PolC_{PHP}$ protein.

CpsB purification and phosphatase assay

CpsB purification, as well as hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) was undertaken as described previously [2].

Computational analysis

Protein structure comparison was undertaken using Phyre2 [14], with structures of CpsB from *S. pneumoniae* (PDBID: 2WJD) and PolC from *Geobacillus kaustophilus* (PDBID: 3F2B). Sequence analysis was undertaken using DNAMAN.

Cloning and overproduction of PolC PHP.

The PHP domain from D39 PolC (Gene ID as from KEGG database: SPD_0254) was amplified from *S. pneumoniae* D39 chromosomal DNA (AS65 cgcGGATCCgttgagtttcatgctcatactaac and AS66 tgcGGTACCttattaaataaccagtttacgagccagttc) (nt1017-2024). The PHP domain was identified by comparison with the known structure and sequence of *Geobacillus kaustophilus* (Gene ID as from KEGG database: GK1258)[11] and CpsB (Gene ID as from KEGG database: SPD_0316) [15] from *S. pneumoniae*. This DNA was subsequently cloned into the *Bam*HI and *Hin*dIII sites of pQE30 and the construct

confirmed by DNA sequencing. pQE30:PolC$_{PHP}$ was then transformed into M15. For induction, an overnight culture was subscultured 1/20 for 2 hrs (OD600 ≈ 0.4) at 37°C, and then transferred to 20 °C where it was induced for 16 hrs with 1 mM IPTG. 6 L were then pelleted (8000*g* for 20 min) and resuspended in phosphate buffer pH 7.4, 500 mM NaCl and 20 mM imidazole. Bacteria were lysed in a Constant Systems Cell Disruptor constant pressure cell, and the soluble recombinant protein was purified using an AKTA prime plus (GE Life Sciences) with a HiTrap column as described by the manufacturer. Protein was eluted using gradient elution performed up to 500 mM imidazole. SDS-PAGE analysis was used to identify the fractions containing purified PolC$_{PHP}$ and then these were dialysed into 20mM Tris pH 8, with 50% glycerol and subsequently stored at -20 °C in aliquots. Final concentration was 0.04 mg/mL, with purity estimated at > 90%. Protein concentration was estimated by comparing to known concentration of BSA.

_p_NP-TMP assay

The hydrolysis of *p*NP-TMP by PolC$_{PHP}$ was investigated using assays as described previously [16]. Assays were routinely carried out in duplicate in 100 µl volumes in a 96 well microtitre tray, with change in OD_{420} monitored every minute on a Powerwave XS (Biotek) at 25 °C. Assay buffer consisted of 50 mM Tris pH 8, 150 mM NaCl and 1 mM DTT. *p*NP-TMP (Sigma, cat no. T4150) was diluted with assay buffer (50mM Tris pH 8, 150 mM NaCl), and stored in single use aliquots. Initial rates were estimated by reading A_{420} at t = 10 min versus t = 0 min. The rate of *p*NP-TMP hydrolysis was calculated using a value of 12 950 $M^{-1}cm^{-1}$ for the e420 of *p*-nitrophenyl at pH 8.0.

The effect of enzyme concentration was measured using 2 to 32 nM of $PolC_{PHP}$, using standard assay conditions, with $[Mn^{2+}]$ = 1mM. In order to estimate Michelis-Menten kinetic paramters, data was measured with *p*NP-TMP concentrations from 0.15 to 10 mM where $[Mn^{2+}]$ = 1 mM. Km and Kcat were determined by non-linear regression using GraphPad Prism. The effect of metal ions on the rate of reaction was studied using $[Mg^{2+}]$ = 1 mM and $[Mn^{2+}]$ = 1 mM under standard assay conditions. The IC_{50} of FQE was determined using $[PolC_{PHP}]$ = 12.5 nM, and [ρNP-TMP] = 2 mM. The data was analysed with GraphPad Prism using a non-linear fit of $\log_{10}$ [FQE] vs normalized response. Ki values were determined using the Cheng-Prusoff equation [17]. Data reported here are the means of three independent assays ± standard error of the mean. The mode of inhibition of FQE was investigated by varying the concentrations of FQE alongside varying the concentrations of *p*NP-TMP. The data was plotted as double reciprocal plots and assessed using Lineweaver-Burk analysis.

Results

Alignment of CpsB and PolC from *S. pneumoniae*

The recent structure of *Geobacillus kaustophilus* PolC (PDB: 3F2B) has illustrated that it contains numerous domains, including a PHP domain which is bisected by the region necessary for the exonuclease activity of the protein (**Figure 1A**) [11]. As we postulated that an inhibitor of CpsB phosphatase activity may also inhibit the function of fellow PHP domain member PolC, we aligned published structures of the two proteins as well as their four conserved motifs as determined by Aravind & Koonin [5] (**Figures 1B & 1C**). While there is a published structure of CpsB from *S. pneumoniae*, we used the PolC structure from *Geobacillus kaustophilus*, which Phyre2 predictions mapped with 100% accuracy to *S. pneumoniae* PolC, as well as

other Gram-positive PolC proteins, such as that from *S. aureus* [14]. Alignment of the protein structures of CpsB and PolC via RCSB showed that they are indeed highly similar (*P*-value of 1.37e-04 by jFATCAT_rigid algorithm) [18] (**Figure 1B**). Additionally, comparison of the protein sequence of the conserved motifs found that the PHP domains from PolC and CpsB share conservation at the majority of these sites (**Figure 1C**). Interestingly, one essential amino acid in *S. aureus* PolC (H339) [12] constitutes a hypothetical conserved metal binding site found across PHP domains, and indeed has been shown to be important for CpsB phosphatase activity [2,13]. This prompted us to investigate the possibility that CpsB phosphatase inhibitor FQE also inhibits the PolC activity.

Cloning and purification of *S. pneumoniae* PolC$_{PHP}$

Initially, we attempted to clone and express full length *S. pneumoniae* PolC protein, however, while a clone was successfully produced, we were not able to induce expression in a range of *E. coli* strains (data not shown). As we were interested specifically in the PHP domain, we cloned and expressed the PHP domain from *S. pneumoniae* D39 PolC (aa 339-808) as a His6-tagged protein. While the majority of PolC$_{PHP}$ was insoluble after induction (approximately 90%), we were able to purify a significant quantity from the soluble fraction with > 90% purity as judged by Coomassie blue stained PAGE (**Figure 2A**).

Purified His6-PolC$_{PHP}$ suppresses FQE inhibition of CpsB phosphatase activity

We required an assay to test our hypothesis that FQE was able to inhibit PolC$_{PHP}$ function. Unlike CpsB, PolC$_{PHP}$ did not catalyse the hydrolysis of *p*-nitrophenyl

phosphate (data not shown). Thus, we investigated whether adding increasing concentrations of $PolC_{PHP}$ into a CpsB phosphatase assay could decrease the efficacy of the inhibitor FQE. Increased levels of $PolC_{PHP}$ resulted in significantly increased CpsB phosphatase activity and counteracted the inhibitory effect of FQE (**Figure 2B**). This suggested that $PolC_{PHP}$ was able to bind or sequester FQE. Addition of BSA to the assay did not produce such effects (data not shown).

Hydrolysis of ρNP-TMP by $PolC_{PHP}$

Having shown that $PolC_{PHP}$ was likely to bind FQE, we next developed an assay to directly test whether FQE can inhibit $PolC_{PHP}$ function. $PolC_{PHP}$ contains the PHP domain bisected by an additional domain important for the enzyme's intrinsic 3'-5' exonuclease activity (**Figure 1A**) [10]. While the PHP domain does not possess exonuclease activity itself [11], mutational alteration of various amino acids of the PHP domain do impact exonuclease as well as polymerase activity [10]. In recent times, the *E. coli* proof reading exonuclease (ε) subunit of the DNA polymerase machinery has been shown to hydrolyse the 5'-ρ-nitrophenyl ester of TMP (*p*NP-TMP)[16]. *p*NP-TMP is a phosphodiester analog of a natural nucleic acid substrate. Numerous other exonucleases have also been shown to hydrolyse *p*NP-TMP [19]. Thus, we hypothesized that $PolC_{PHP}$ would hydrolyse *p*NP-TMP.

Incubation of $PolC_{PHP}$ with *p*NP-TMP resulted in the release of *p*-nitrophenyl from *p*NP-TMP. Preliminary results showed that a pH of 8 was optimal (data not shown). Values of *v0* were proportional to the concentration of $PolC_{PHP}$ over a 20 fold range (**Figure 3A**). Additionally, varying the substrate concentration illustrated the kinetics of the reaction fitted well with Michelis-Menten Kinetics. The kinetic parameters for

the hydrolysis of *p*NP-TMP were as follows; Km = 1.9 ± 0.1 mM and Kcat = 13 ± 0.22 s^{-1} (**Figure 3B**).

As with other PHP proteins, as well as the other exonucleases investigated for the ability to hydrolyse *p*NP-TMP, there was a requirement for a metal ion in order for the reaction to proceed. Mn^{2+} was by far the preferred ion, with activity significantly higher than when Mg^{2+} was used (**Figure 2C**). This also correlates with the phosphatase activity of CpsB, with Mn^{2+} being the metal ion of preference for optimal phosphatase activity [15].

FQE inhibits $PolC_{PHP}$ activity

Having developed a suitable assay to measure $PolC_{PHP}$ activity, we investigated the ability of FQE to inhibit this activity. FQE was able to inhibit the ability of $PolC_{PHP}$ to catalyse the hydrolysis of *p*NP-TMP. FQE was confirmed to be a competitive inhibitor using Lineweaver-Burk analysis (Supplementary Figure 1). Although it was unlikely that FQE was acting as a chelator as we used [Mn] = 1mM, varying [Mn] also did not effect inhibition, providing further evidence FQE was not a simple divalent metal chelator (data not shown). The inhibition constant (Ki) was calculated to be 2.1 ±0.04 µM for FQE inhibition of $PolC_{PHP}$ (Figure 4). This result provided direct evidence that the enzymatic activity of the PHP domain of PolC could be inhibited by FQE, suggesting that this may be the essential target of FQE in *S. pneumoniae*, and possibly other Gram-positive pathogens such as *S. aureus* [3].

Discussion

The polymerase and histidinol phosphate phosphatase domain was first described nearly 15 years ago [5], and since has been further characterised via the production of structures for a great number of its members [11,15,20] as well as functional analyses [4,13,15]. Its relative abundance in prokaryotes, along with its scarcity in eukaroytes suggests that is makes an attractive target for the development of a new breed of anti-microbials that target a variety of proteins, resulting in broad range specificity. The discovery of novel targets for the development of antimicrobials is becoming increasingly desperate, with the advent of so called superbugs such as methicillin resistant *S. aureus* (MRSA) [21]. This study has shown that the PHP domain is a drugable target, for which one inhibitor may inhibit many critical functions of a bacterial pathogen, targeting both essential cellular processes as well as activities important for virulence.

In a recent study describing a high throughput screen for inhibitors of protein tyrosine phosphatase CpsB from *Streptococcus pneumoniae*, we discovered an inhibitor, FQE, which resulted in *in vitro* inhibition of CpsB phosphatase activity, as well as subsequent reduction of capsular polysaccharide production in whole *S. pneumoniae* [2]. Interestingly, FQE also inhibited the growth of *S. pneumoniae*, as well as a number of other Gram-positive but not Gram-negative pathogens [3]. Thus, we were interested if FQE was also targeting an essential component of Gram-positive bacterium. As PolC, and in particular the PHP domain of PolC, is known to be essential, we hypothesised that this domain was another target of FQE. Indeed, the PHP domains from these proteins are highly similar, both structurally as well as

through sequence conservation (**Figure 1B & 1C**). While we were unsuccessful in purifying whole PolC from *S. pneumoniae*, we were able to purify sufficient quantities of a truncated form of PolC containing the PHP domain. While $PolC_{PHP}$ did not show any activity against *p*NPP, the addition of $PolC_{PHP}$ to FQE-CpsB phosphatase inhibitor assays significantly increased phosphatase activity (**Figure 2B**), suggesting $PolC_{PHP}$ was able to sequester FQE and decrease inhibition of CpsB. This provided the first evidence that FQE was able to bind the DNA polymerase PolC.

In order to demonstrate the true effect of FQE on PolC, we required a functional assay. Hamdan et al. (2002) [16] have shown that the proof reading exonuclease (ε) subunit of *Escherichia coli* DNA polymerase III was able to hydrolyse *p*NP TMP. Indeed, oligoribonucleases from a number of species have also been shown to have such activity [19]. While traditional studies of exonuclease activity have involved the use of radiolabelled ssDNA or primer template substrates that have mismatched termini, this method provides a simple continuous method that can be monitored on a spectrophotometer. We found that $PolC_{PHP}$ was able to hydrolyse *p*NP TMP. The recorded Km of 1.94 was not dissimilar to that reported for other enzymes previously shown to hydrolyse *p*NP TMP [16,19]. There was also other similarities with these enzymes, with $PolC_{PHP}$'s ability to hydrolyse *p*NP TMP showing an absolute dependence on the presence of a metal ion. While the addition of Mg^{2+} promoted some activity, the best ion by far was Mn^{2+}, as for other assayed enzymes. Additionally, the optimum pH was pH 8. Interestingly, as well as showing similar characterisitics to other exonucleases assayed with this substrate, all these characteristics are the same as that seen for PHP domain family member CpsB, when we are assaying the PTP's ability to catalyse the hydrolysis of *p*NPP.

When we investigated if FQE inhibits the ability of $PolC_{PHP}$ to hydrolyse *p*NP TMP, we found that it did so with a similar IC_{50} to that of CpsB phosphatase activity. Ki for FQE ($Ki_{PolCPHP}$ = 2.1 µM and Ki_{CpsB} = 4 µM) were also similar. This suggests that FQE is a general PHP domain inhibitor, as it is able to inhibit two different activities of two different enzymes at similar concentrations. Furthermore, this also provides further evidence that while the PHP domain of PolC is not sufficient for exonuclease activity [11], the PHP domain is essential, perhaps due to its ability to recruit metal ions required for exonuclease activity. Interestingly, the PHP domain of DnaE, another bacterial DNA polymerase present in Gram-positive and also Gram-negative pathogens, has been shown to possess exonuclease activity in thermophiles [22], while in *E. coli* the PHP domain has been shown to bind the ε subunit, which itself possess exonuclease activity [23]. Thus, it would be of interest to see if FQE would also inhibit the function of DnaE, both from Gram-positive and Gram-negative bacterial pathogens.

The fact that FQE inhibits two different functions of two different PHP domain containing proteins, suggests that it is a general PHP domain inhibitor. Thus, it is interesting to consider other proteins FQE could potentially inhibit. These would include histidinol phosphate phosphatase (HPP). PHP domain containing Hpps are present in a number of bacteria [24] and also in yeast. In some bacteria, the histidine biosynthesis pathway has been shown to be a antibiotic target [25], while in yeast such Hpps have been postulated as ideal targets for the development of novel anti-fungals [26]. Other possible targets include the family X DNA polymerases involved in DNA repair [27], the TatD proteins encoding a DNase [28], as well as proteins with

as yet undefined roles such as YcdX [29]. We are currently investigating whether FQE can also inhibit the function of other PHP proteins.

In a time of increasing antibiotic resistance, and few new drugs in development, the identification of a novel targets for the development of antimicrobial agents is critical. Furthermore, the identification of one which targets multiple proteins, is critical not only for increasing its spectrum of action, but also as a method of limiting the development of antibiotic resistance. The PHP domain, with its significant prevalence in bacteria but not eukaryotes, is one such target.

Abreviations

PHP, polymerase and histidinol phosphatase; Hpp, histidinol phosphate phosphatase; FQE, fascioquinol E; *p*NP-TMP, 5'-*p*-nitrophenyl ester of thymidine-5'-monophosphate ; *p*NPP, *p*-nitrophenyl phosphate;PTP, protein tyrosine phosphatase.

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Figure Legends

Figure 1. **Alignment of CpsB and PolC**. **A**. Cartoon schematic of PolC structure illustrating the PHP domain with the exonuclease domain present in the middle. **B**. Structure comparison between Cps4B (Pdb: 2WJE) (Black Shading) and PolC (Pdb:3F2b) (White shading) shows similar structures (Aligned using RCSB). **B**. Sequence conservation of conserved motifs of PHP between PolC (top) and CpsB (bottom) from *S. pneumoniae* with putative active site residues highlighted in inverse black (Generated using DNAMAN).

Figure 2. **$PolC_{PHP}$ supresses FQE inhibition of CpsB phosphatase activity**.**A**. 1 µg purified $PolC_{PHP}$ separated on 12 % SDS-PAGE and stained with Coomassie Blue shows > 90% purity. **B**. Increasing concentrations of $PolC_{PHP}$ (33 nM, 16.5 nM and 8.25 nM) were added to CpsB phosphatase assay where CpsB concentration was 2.5 nM. Addition of $PolC_{PHP}$ significantly increased the activity of CpsB phosphatase activity in the presence of inhibitor FQE (10 µM) (*** - $P < 0.001$; student's t-tailed test).

Figure 3. **$PolC_{PHP}$ <u>hydrolyses</u> ρNP-TMP**. **A**. The rate of hydrolysis of *p*NP-TMP as a function of $PolC_{PHP}$ concentration. The concentration of *p*NP-TMP was 2 mM and the concentration of $MnCl_2$ was 1 mM. **B**. Varying concentration of *p*NP-TMP produces results that fit well with Michelis Menten kinetics. **C**. Rate of hydrolysis of *p*NP-TMP by $PolC_{PHP}$ with either 1mM $MnCl_2$ or 1 mM $MgCl_2$. Concentration of $PolC_{PHP}$ was 12.5 nM and concentration of *p*NP-TMP was 2 mM .

Figure 4. **FQE inhibits PolC$_{PHP}$ hydrolysis of ρNP-TMP.** PolC$_{PHP}$ hydrolysis of ρNP-TMP in the presence of FQE. IC_{50} of FQE was 4.2 µM. [Mn^{2+}] was 1 mM and [*p*NP-TMP] was 2 mM.

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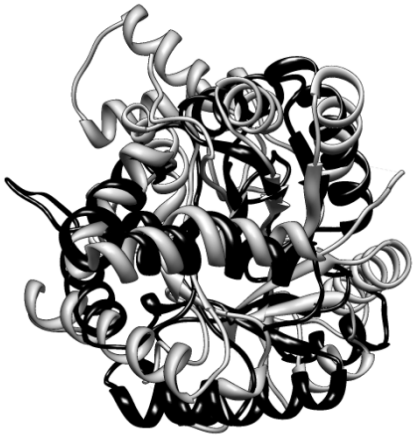
A

B



C

```
Motif 1
339   VEFHAHTNMSTMDA
      ::.|.|. ... |:
2     IDVHSHIVFDVDDG

Motif 2
358   EIVATAAKWGHKAVAITDH
      .::|.. : |.:.:. |.|
   24 ALLAESYRQGVRTIVSTSH

Motif 3
609   SYKKARIKHATIY
      |  .: | | . |
128   SGITPVIAHIERY

Motif 4
736   LATGNVHYIE
      : .:::| ::
195   VIASDMHNLD
```

Figure 1

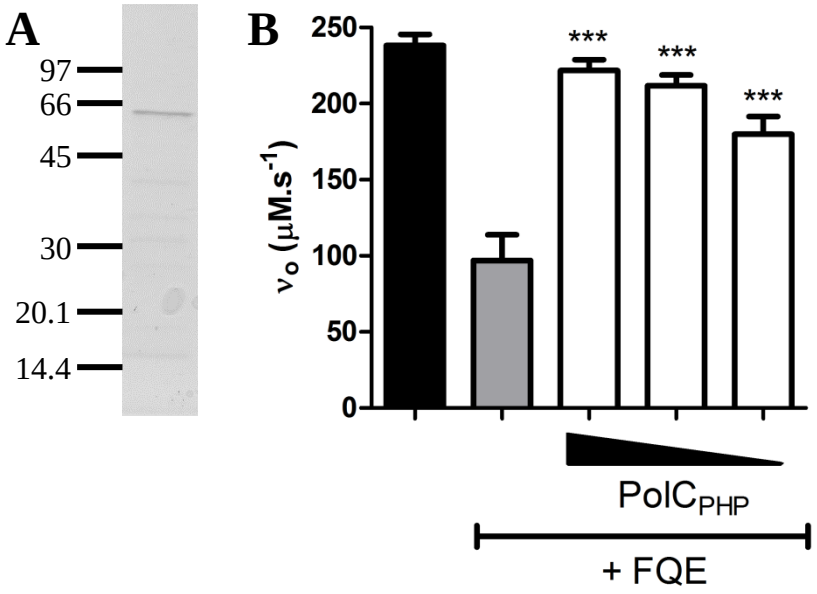
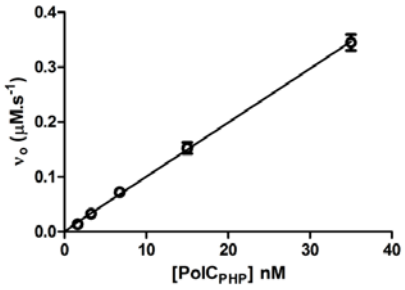
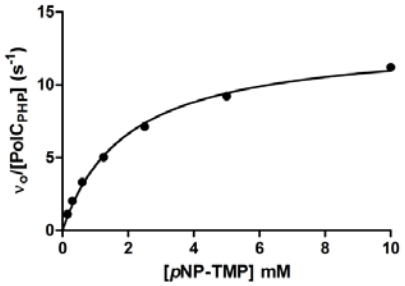


Figure 2

A


0.4
0.3
0.2
0.1
0.0
v_o (μM.s^{-1})
0
10
20
30
40
[PolC$_{PHP}$] nM

B


15
10
5
0
v_o/[PolC$_{PHP}$] (s^{-1})
0
2
4
6
8
10
[*p*NP-TMP] mM

C

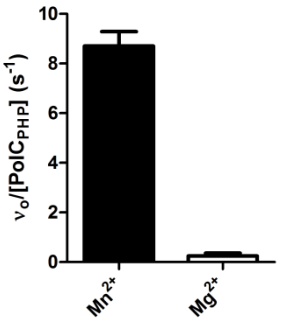

10
8
6
4
2
0
v_o/[PolC$_{PHP}$] (s^{-1})
Mn^{2+}
Mg^{2+}

Figure 3

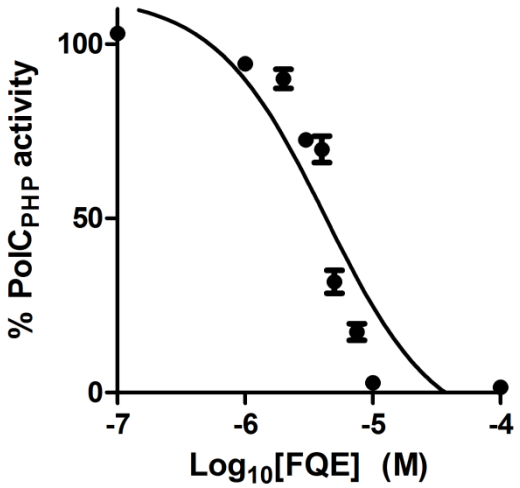


Figure 4