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***In vitro* DNA-binding properties of VirB, the *Shigella flexneri* virulence regulatory protein.**

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Key words: *Shigella flexneri*, VirB protein, transcription factor, gene regulation, leucine zipper,
helix-turn-helix motif

Abstract

The DNA binding activity of the *Shigella flexneri* VirB transcription factor was studied *in vitro*. The protein was found to bind non-specifically to DNA, but showed preferential binding to VirB-dependent promoter sequences. DNA binding was contingent on the presence of an intact helix-turn-helix motif. While high molecular mass protein-DNA complexes were formed in both specific and non-specific interactions with DNA, mutant derivatives of VirB lacking a leucine zipper domain or a carboxyl terminal-located oligomerization domain formed discrete complexes, indicating that an ability to oligomerize on DNA was responsible for the formation of high molecular mass complexes by the wild type protein.

Introduction

Shigella flexneri is a Gram-negative facultative enteroinvasive pathogen which infects the lower intestinal epithelia of humans and primates to cause bacillary dysentery. Shigellosis is a serious global health problem, causing around 600,000 deaths per annum, predominantly in the developing world [1]. Invasion of host cells by *S. flexneri* requires expression of virulence genes located on a 230 kb plasmid. The products of these genes include the Ipa invasins, which mediate the invasion of gut epithelia and macrophage apoptosis [2, 3], the Mxi and Spa proteins which form a type III secretion system [4], and the IcsA, IcsB and VirA proteins which are responsible for intercellular spreading of bacteria in the lower gut [5-7]. The virulence genes are expressed only under conditions approximating those found at the site of infection, i.e. at 37°C, a pH of 7.4 and moderate osmolarity [8-10]. A transcriptional regulatory cascade, involving both chromosomally-encoded proteins including IHF (integration host factor) and H-NS (histone-like nucleoid structuring protein) and plasmid-borne regulatory proteins, VirF and VirB, integrates environmental signals and restricts the expression of structural virulence genes to the appropriate conditions [7, 11-14].

The transcriptional cascade regulating the activation of virulence gene expression is initiated with the expression of the VirF protein, an AraC-like transcriptional regulator [10, 14]. VirF is responsible for the activation of the *virB* regulatory and *icsA* structural genes [9, 15]. The VirB protein then activates the other structural genes required for virulence [16-18]. The expression of *virB* is also regulated by H-NS, which acts as an antagonist to VirF at the promoter, and together with levels of negative DNA supercoiling, this appears to be responsible for the thermo-sensitivity of *virB* expression [19-21].

The role of the VirB protein in the regulation of virulence gene expression in *S. flexneri* was identified through transposon mutagenesis of the large virulence plasmid, when it was shown to be essential for the expression of almost all of the structural virulence genes [18]. VirB is an unusual candidate for a transcriptional activator, sharing homology with a family of plasmid partition proteins, including the ParB and SopB proteins, rather than previously described conventional transcriptional regulators [18, 22, 23]. Small and basic, VirB has been shown to form dimers, trimers and higher oligomers both *in vivo* and *in vitro* [17]. Oligomerization is independent of DNA binding [17] and occurs through two domains, a leucine zipper located between residues 193 and 228 and a region at the C-terminus predicted to form a triple coil structure (Fig. 1A) [17]. Also, VirB binds specifically to target structural gene promoters *in vivo* [17]. The ability of VirB to interact with DNA *in vivo* is dependent on the presence of the N-terminus and the integrity of a helix-turn-helix (HTH) DNA binding motif, located between residues 148-171 and essential for structural gene activation (Fig. 2A) [17]. Oligomerization of the protein contributes to DNA binding since oligomerization mutants bind to DNA with lower preference *in vivo* [17]. The interaction with virulence gene promoters implies that activation by VirB is direct, however the mechanism is unclear (Fig. 1B). It has recently been shown that HNS also binds directly to these promoters where it acts antagonistically to VirB [24].

The HTH DNA binding motif in VirB is 80% identical to the HTH in the homologous ParB protein of plasmid P1, which mediates binding of ParB to the *parS* site leading to plasmid partitioning [17, 25, 26]. Interestingly there appears to be no consensus sequence analogous to

parS for VirB DNA binding, and no homology exists between the four promoters to which VirB binds.

Although chromatin immunoprecipitation experiments indicate that VirB interacts specifically with target promoters *in vivo* [17], binding has never been demonstrated directly. This study investigates the binding of the VirB protein to target promoter DNA *in vitro* and assesses the importance of the HTH motif and the role of oligomerization in VirB binding to DNA. Evidence is presented that the VirB protein polymerizes on DNA *in vitro* echoing a phenomenon observed *in vivo* with the ParB and SopB proteins [27, 28]. This raises interesting questions about the nature of DNA binding *in vivo* and the mechanisms by which VirB may activate structural gene expression.

Materials and Methods

Bacterial strains plasmids and growth conditions

Plasmids were derivatives of the pET19b Novagen vector containing VirB, or VirB mutants K152E, K164E, L224R, Δ LZ, or Δ 244-309 [17]. Proteins were expressed in *Escherichia coli* K-12 strain BL21DE3 containing the LacI-expressing plasmid pDIA17. Cells were grown in Luria-Bertani broth (LB) containing chloramphenicol (Cm; 20 μ g/ml) and carbenicillin (Ca; 50 μ g/ml) at 30°C.

Purification of VirB

Expression of N-terminally His-tagged VirB was induced with 0.1 mM IPTG in exponentially growing 500 ml cultures. After 3 hours the cells were harvested and lysed in a French pressure cell. Lysates (~15 ml) were applied to a His-bind® Quick column (Novagen) pre-equilibrated with binding buffer [17]. The column was then washed with binding buffer (50 ml) and wash buffer (25 ml) [17] and the protein eluted in 1 ml fractions of 2 x 15 ml of elution buffer (10% glycerol, 50 mM Tris HCl pH 7.9, 0.5 M NaCl, 0.1 mM PMSF) containing 100 mM imidazole and 500 mM imidazole. Fractions were analyzed by SDS-PAGE and those containing VirB were pooled and dialyzed three times against 1 L 50 mM Tris HCl pH 7.5, 1 mM EDTA pH 8, 300 mM NaCl, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT). VirB was estimated to be approximately 95% pure. Preparation and use of anti-VirB rabbit polyclonal antibodies was described previously [17]. The His-tagged wild type protein was found to be fully functional *in vivo* (data not shown).

Small scale protein purification

VirB and VirB mutants for use in electrophoretic mobility shift assays were purified from crude extracts by metal affinity chromatography using the His-Bind Magnetic Beads (Novagen). Induction of protein expression and cell lysis were performed as described above. 1.5 ml of the soluble extract was applied to 500 μ l of pre-equilibrated beads in a 2 ml microfuge tube and incubated for 5 min. The magnetic beads were pelleted using a magnetic rack (Novagen) and the supernatant removed. The remaining soluble extract was applied to the beads as before and the beads were then washed with three times with 2 ml wash buffer (as before) to remove contaminating proteins. Bound protein was eluted with 500 mM imidazole and analysed by SDS-PAGE. Proteins purified in this manner were deemed to be 85-90% pure by SDS-PAGE followed by Coomassie staining, and were dialysed in storage buffer (as above) and stored at -20°C.

Electrophoretic mobility shift analysis of protein-DNA interactions

A 340-bp DNA fragment encompassing the *icsB-ipgD* promoter region was amplified by the polymerase chain reaction using oligonucleotide primers IcsB5' 5'-TATAGGTCCTGTATTGCTTGCCCTGAAT-3' and IcsB3' 5'-AAATGAGGATCCATGCAATCCCAAATTAGT-3'. A 350-bp DNA fragment internal to the *icsP* gene was amplified with primers IcsP5' 5'-ACTCTCGACTTTAAAAGGATGGGA-3' and 5'-CTACTCACCTTCTTGTTCTCTCGC-3'. Radiolabelled probe DNA was incubated with increasing concentrations of His-tag purified wild-type VirB or mutant VirB for 20 min at room temperature in a 20 μ l reaction cocktail containing 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 20 mM Tris HCl pH8, 50 mM KNa and 2 mM spermidine. Similar

results were obtained when these mixtures were incubated at 30°C or 37°C. Each reaction mixture contained 1.45 μM DNA (5 ng) and VirB protein concentrations in the range 0 to 500 μM . Protein-DNA complexes were resolved by electrophoresis through an 8% polyacrylamide gel, for 4 h at 4°C. The gel was dried under vacuum onto Whatmann 3 MM paper and examined by autoradiography.

Results and Discussion

Ability of purified VirB protein to bind to target promoter DNA

The *icsB-ipgD* intergenic region was selected as the target DNA in electrophoretic mobility shift assays, as this region contains the promoters for two divergently transcribed VirB-dependent operons (Fig. 1). A DNA fragment internal to the open reading frame of the *icsP* gene was used as a negative control. Binding reactions with increasing concentrations of (His)₁₀-VirB protein were carried out in an optimised buffer at room temperature.

Purified VirB showed a higher preference for binding to *icsB-ipgD* DNA than to *icsP* DNA (Fig. 2). At a protein:DNA molar ratio of 4:1, the *icsB-ipgD* probe is beginning to be shifted to the wells in the presence of 6.25 μ M VirB (Fig. 2A). In contrast, the *icsP* probe requires a protein:DNA molar ratio of 80:1 (125 μ M VirB in the reaction mixture) to achieve a comparable shift (Fig. 2B). VirB was observed to display a similar preference for binding to the *spa* and *virA* structural gene promoters *in vitro* as it does for *icsB-ipgD* (data not shown). These results indicate that VirB probably binds directly to the target promoters *in vivo* to effect the activation of structural gene expression, which is consistent with previous findings from *in vivo* analyses [17].

Interestingly, no discrete protein-DNA complexes, which normally indicate the attachment of an active protein to its binding site on DNA via an HTH motif [29], were observed in the binding of VirB to either probe. One possible explanation for this pattern is that VirB binds to DNA as a large complex. Another possibility is that on binding, VirB polymerizes rapidly along the DNA, forming a large complex that traps the probes in the gel wells. With the exception of differing binding preferences, the pattern of binding appears similar for both the target promoter DNA and the negative control DNA. Indeed, in further bandshift experiments

VirB was capable of shifting many unrelated DNA probes in a similar manner (data not shown), but it always showed a strong preference for binding to target DNAs that contained VirB-dependent promoters.

Purification of VirB mutants

To test the effect of mutations in the HTH putative DNA binding motif of VirB on the ability of the protein to bind DNA, the K152E and K164E mutants were purified for use in electrophoretic mobility shift assays. Both of these proteins contain mutations at key residues in the motif, proposed to disrupt protein-DNA interactions [17]. These changes were previously shown to abolish the ability of VirB to activate structural gene transcription, without affecting the oligomeric state of the protein [17]. To test the requirement for oligomerization in the DNA binding activity of VirB, several representative mutants that had been shown to be defective in oligomerization, L224R, Δ LZ, and Δ 244-309 [17], were also purified. An attempt was made to purify a truncate lacking the N-terminus of the protein, to examine the role of that region in DNA binding, but this protein proved unstable when over-expressed. The proteins were purified as described in Materials and Methods and were judged to be >90% pure by SDS PAGE and densitometric analysis (Fig. 3).

Ability of VirB HTH mutants to bind DNA in vitro

The observation that mutagenesis of key charged residues in the HTH DNA binding motif of VirB abolished the ability of the protein to activate structural gene expression and reduced *in vivo* binding of VirB to promoters of the virulence structural genes [17], indicated that this motif was functional and contributed to DNA binding by VirB in *S. flexneri*. To confirm directly the

disruption of protein-DNA interactions by these mutations, purified K152E and K164E were used in electrophoretic mobility shift assays. Fig. 4 shows the results of binding reactions of the HTH mutants with an *icsB-ipgD* promoter probe and an *icsP* control probe. Clearly both of the HTH mutants were unable to bind to either promoter DNA or to the negative control *icsP* DNA, in contrast to the wild type VirB protein binding to the *icsB-ipgD* probe (Fig. 4). In some cases, small quantities of probe were trapped in the wells spontaneously, even in the absence of any protein (for example, Fig. 4B; *icsP* probe). The reasons for this are not known. These results are consistent with the HTH motif in VirB being functional and contributing to the DNA binding ability of the protein. By implication, HTH mutations would block gene activation by VirB in *S. flexneri* because they render the protein unable to bind to DNA. In previous work we have shown that these HTH mutants remain fully functional for oligomerization *in vitro* [17] so the effects reported here are unlikely to involve deficiencies in protein-protein interaction. VirB differs from other DNA binding proteins having an HTH motif in apparently not binding to a consensus binding site [29, 30] and may, like its antagonist H-NS, bind to a consensus structure in DNA [31]. If so, other aspects of VirB may contribute to the specificity of its binding activity.

Ability of oligomerization deficient mutants of VirB to bind DNA in vitro

To test the role of oligomerization in the ability of VirB to bind DNA three mutants, L224R, Δ LZ, and Δ 244-309 were purified for use in bandshift assays (Fig. 3.B). These mutants had previously been shown to have a reduced capacity to oligomerize *in vivo* [17]. All of the oligomerization deficient mutants showed a reduced preference for promoter *icsB-ipgD* DNA and the *icsP* internal DNA compared with wild type VirB *in vitro* (Fig. 5). Unlike the native protein, none of the mutants was capable of fully shifting the DNA probes at any protein:DNA molar

ratio tested, indicating that VirB probably interacts with DNA most efficiently in its oligomeric form. This is in agreement with *in vivo* experiments showing a reduced DNA binding capacity for VirB mutants impaired in oligomerization [17].

Interestingly the VirB mutants with an inability to oligomerize showed a different pattern of binding to the *icsB-ipgD* promoter DNA to the wild type protein (Fig. 5). Wild type VirB shifted the promoter DNA probe directly into the wells, whereas each of the oligomerization deficient mutants produced a single intermediate complex of the protein with promoter DNA before a shift into the wells (Fig. 5). Thus it appears that the L224R, Δ LZ, and Δ 244-309 mutants form an initial complex with DNA, followed by polymerization of the protein on the DNA forming high molecular weight complexes that remain in the wells of the gel. The reduced ability of these proteins to interact with each other appears to slow this reaction sufficiently to separate initial complex formation from oligomerization. The L224R and the Δ LZ mutants bound to and shifted promoter DNA at a protein:DNA molar ratio of 40:1, a ratio at which the wild type shifted the probe completely to the wells. The Δ 244-309 mutant formed the intermediate complex only at a protein:DNA molar ratio of 218:1, possibly indicative of the severity of the carboxyl-terminal deletion that removed a major oligomerization domain.

Thus it appears that oligomerization of the VirB protein plays a key role in the binding of the protein to DNA *in vitro*. Proteins defective for oligomerization showed a reduced preference for promoter DNA and a different binding pattern compared with native VirB. The results presented here demonstrate direct binding of the VirB protein to target promoters *in vitro* for the first time. In addition they are consistent with an essential role for the HTH motif in this DNA binding activity. Finally it appears that oligomerization of VirB is also required for effective DNA binding and there is an indication that VirB possesses the ability to polymerise along DNA

in vitro, possibly echoing a similar phenomenon described *in vivo* for the homologous ParB and SopB plasmid partitioning proteins [26-28]. Perhaps VirB has evolved from a plasmid partition function that was diverted to a role as a regulator of virulence genes. The *S. flexneri* plasmids possess dedicated partition functions related in amino acid sequence to VirB [32, 33] and this redundancy might have permitted such an event to occur.

Acknowledgements

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Figure legends

Fig. 1 Model of structural domains and activity of the VirB protein.

The organization of structural domains within the VirB protein is shown in schematic form in A. Structural features, their positions and their functions are highlighted on the diagram. A model for the activation of structural gene expression is shown in B. VirB binds directly at these promoters to activate gene expression, probably acting as an antagonist to the H-NS repressor [24].

Fig.2. Binding of purified recombinant VirB to DNA in vitro.

Gel mobility shift analysis of VirB interactions with the target promoter *icsB-ipgD* DNA and control *icsP* DNA. Increasing amounts of purified recombinant (His)₁₀-VirB (indicated below each gel) were incubated with 1.45 μM ³²P-labelled *icsB-ipgD* (A) and *icsP* (B) DNA fragments for 20 minutes at room temperature. Bound and free probes were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. The migration of free and bound probes is indicated with arrows.

Fig. 3. Purification of recombinant VirB mutants.

SDS PAGE analysis of overexpression and purification of the VirB mutant K164E is shown in A. Strain BL21 (DE3)/pDIA17 harbouring the overexpression plasmid pET19K164E was grown to mid-exponential phase, and *virB* expression induced by the addition of 0.1 mM IPTG. Protein samples of induced (+) and uninduced (-) culture, the insoluble and soluble fraction prior to passage over a Ni²⁺ charged column, and the elution fractions are indicated on the gel. The band corresponding to K164E is indicated with a horizontal arrow. B shows SDS PAGE analysis of 1 μg of each of the VirB mutants purified to show concentration, purity and integrity of the proteins.

Fig. 4. Ability of VirB HTH mutants to bind DNA in vitro.

Gel mobility shift analysis of interactions of the K152E and K164E HTH mutants of VirB with the target promoter *icsB-ipgD* DNA and control *icsP* DNA. Increasing amounts (indicated below each gel) of purified recombinant (His)₁₀-K152E (A) and (His)₁₀-K164E (B) were incubated with 1.45 μM ³²P-labelled *icsB-ipgD* or *icsP* DNA fragments for 20 minutes at room temperature. Bound and free probes were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. The migration of free probes is indicated with arrows. In each case, the mobility shift achieved with 62.5 μM wild type VirB protein binding to the *icsB-ipgD* probe in a 40:1 protein:DNA molar ratio is shown for comparison in the lane on the extreme right.

Fig. 5. Ability of oligomerization deficient VirB mutants to bind DNA in vitro.

Gel mobility shift analysis of interactions of the L224R, ΔLZ and Δ244-309 oligomerization deficient mutants of VirB with the target promoter *icsB-ipgD* DNA and control *icsP* DNA. Increasing amounts (indicated below each gel) of purified recombinant (His)₁₀-L224R(A), (His)₁₀-ΔLZ (B) and (His)₁₀-Δ244-309 (C) were incubated with 1.45 μM ³²P-labelled *icsB-ipgD* and *icsP* DNA fragments for 20 minutes at room temperature. Bound and free probes were separated by polyacrylamide gel electrophoresis and visualised by autoradiography. The migration of free and bound probes is indicated with arrows. In each case, the mobility shift achieved with 62.5 μM wild type VirB protein binding to the *icsB-ipgD* probe in a 40:1 protein:DNA molar ratio is shown for comparison in the lane on the extreme right.

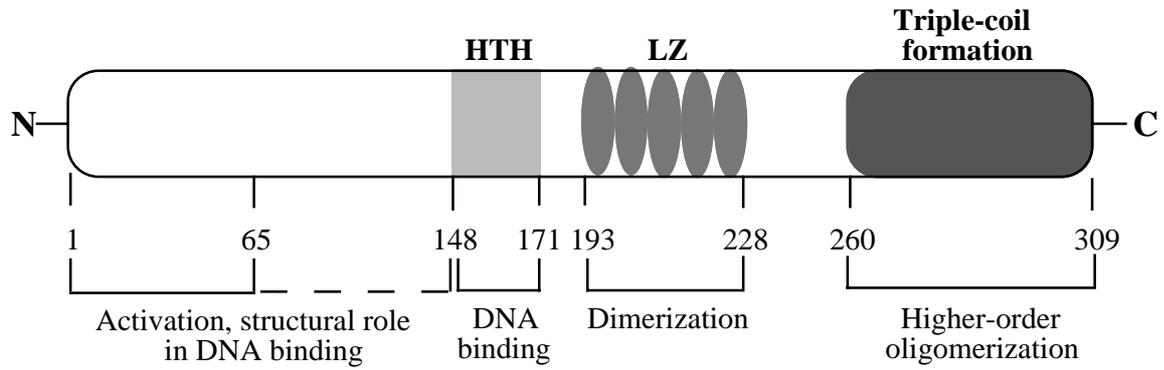
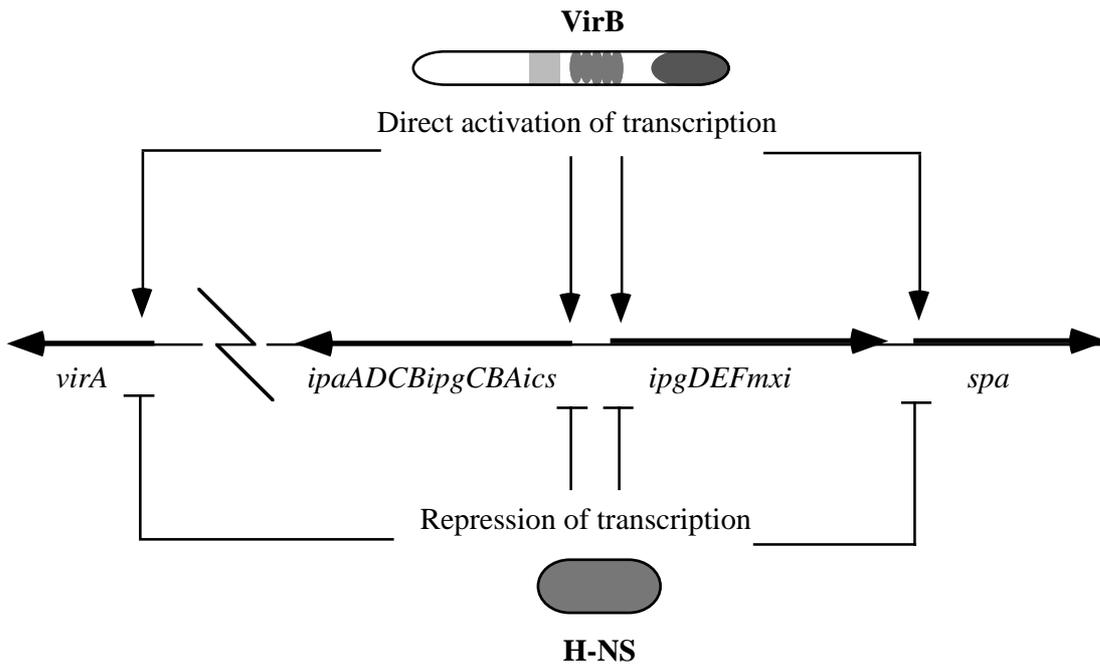
A**B**

Fig 1

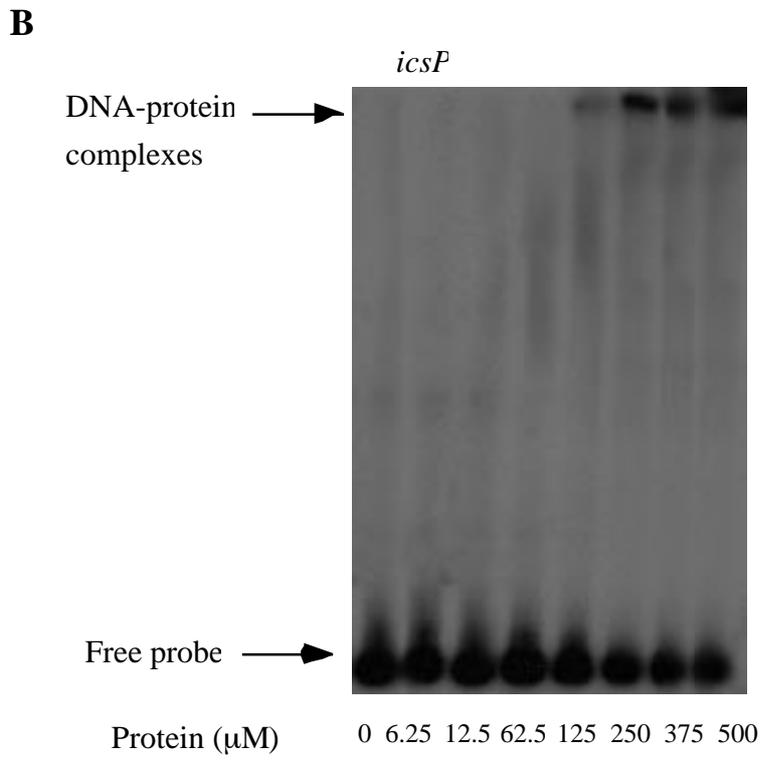
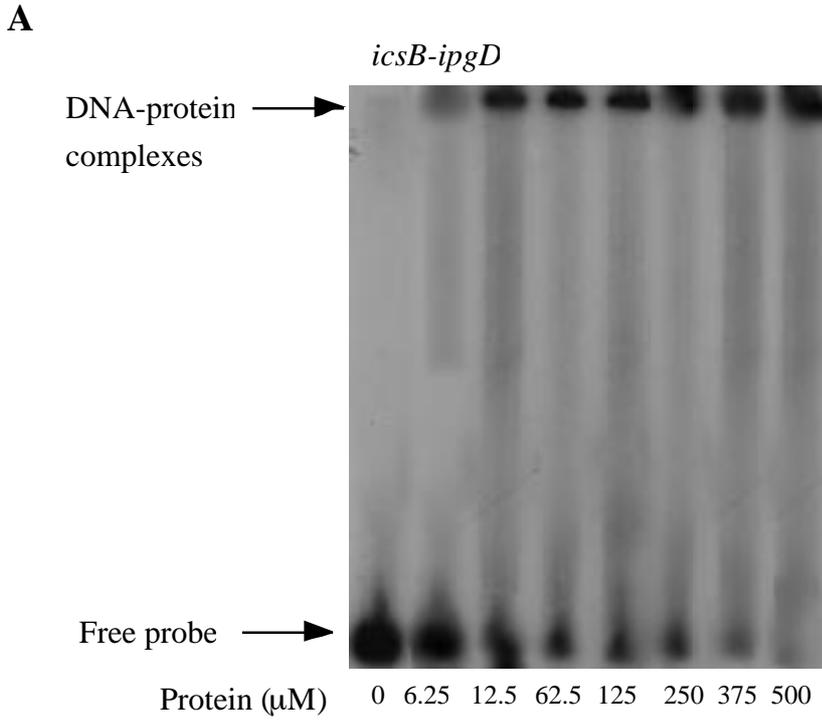


Fig. 2.

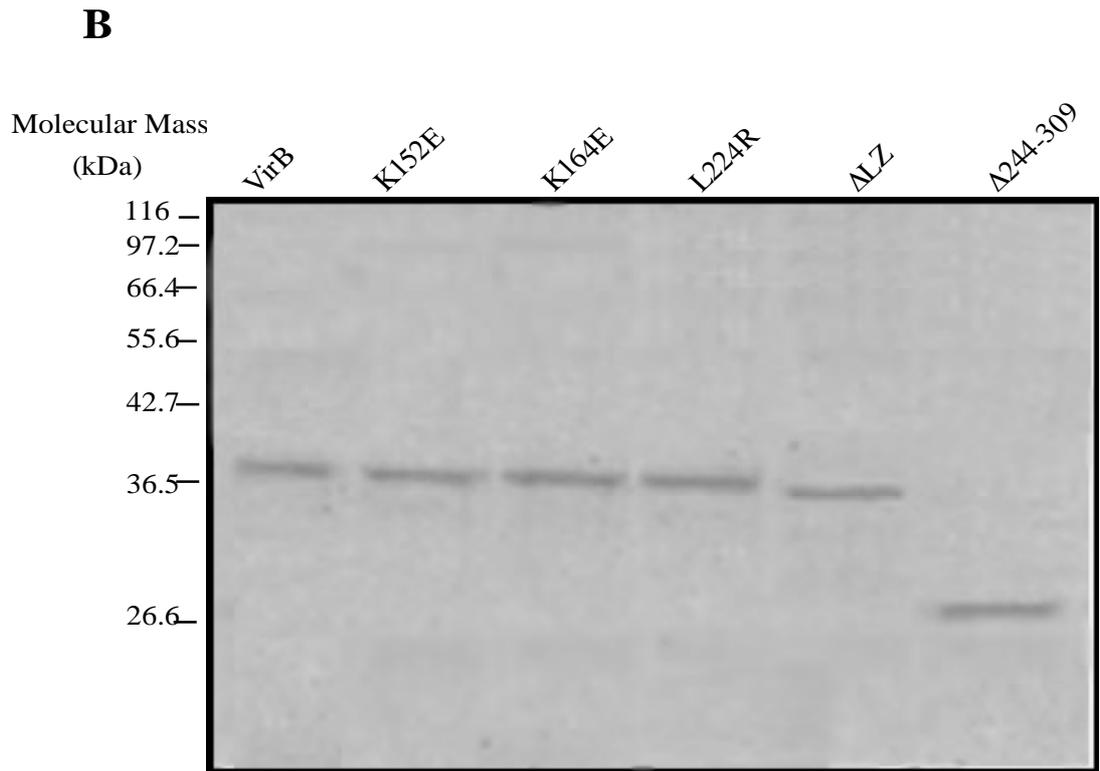
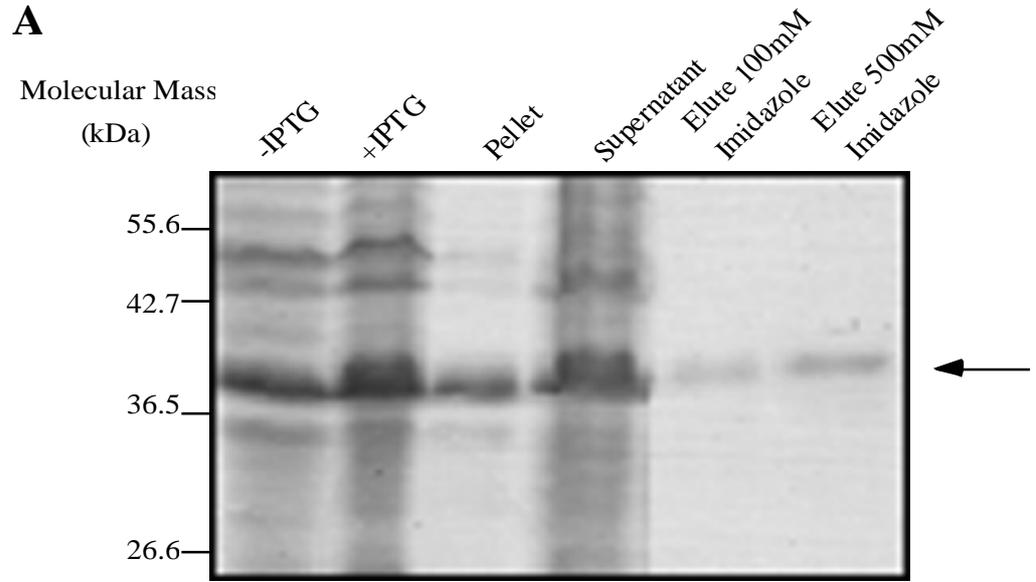
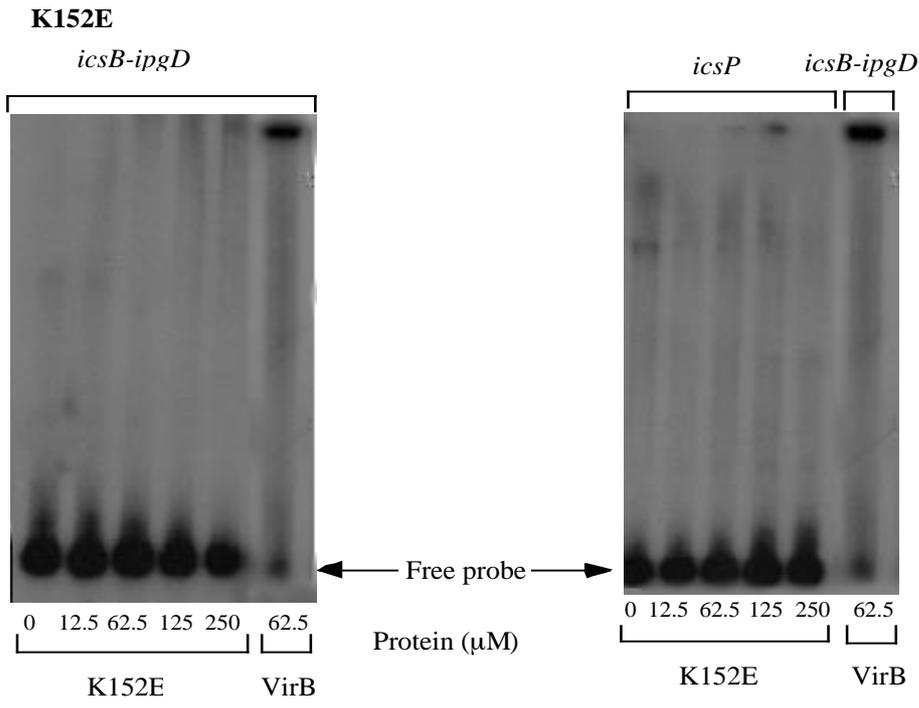


Fig. 3.

A



B

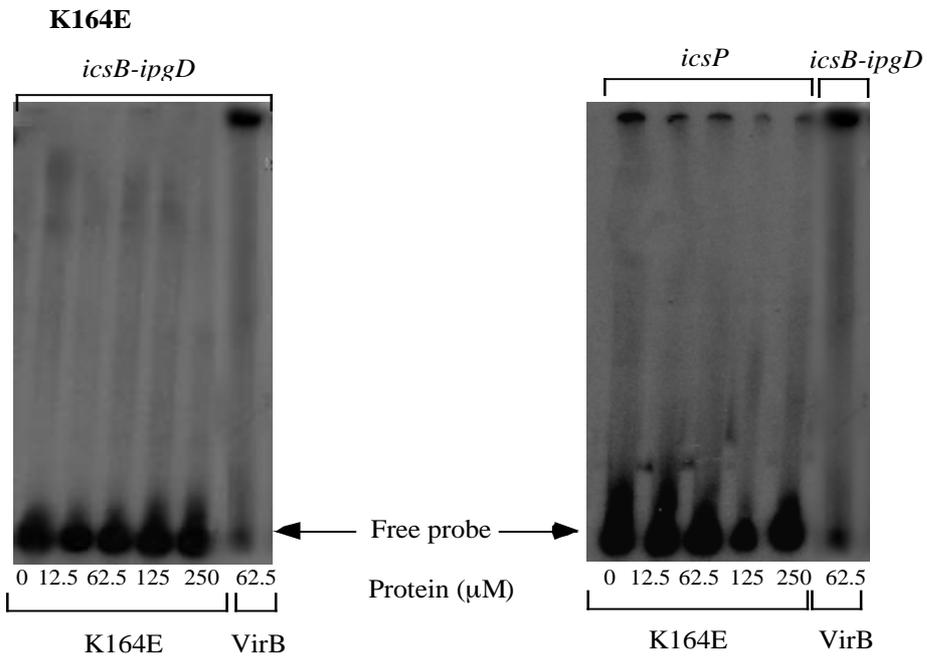
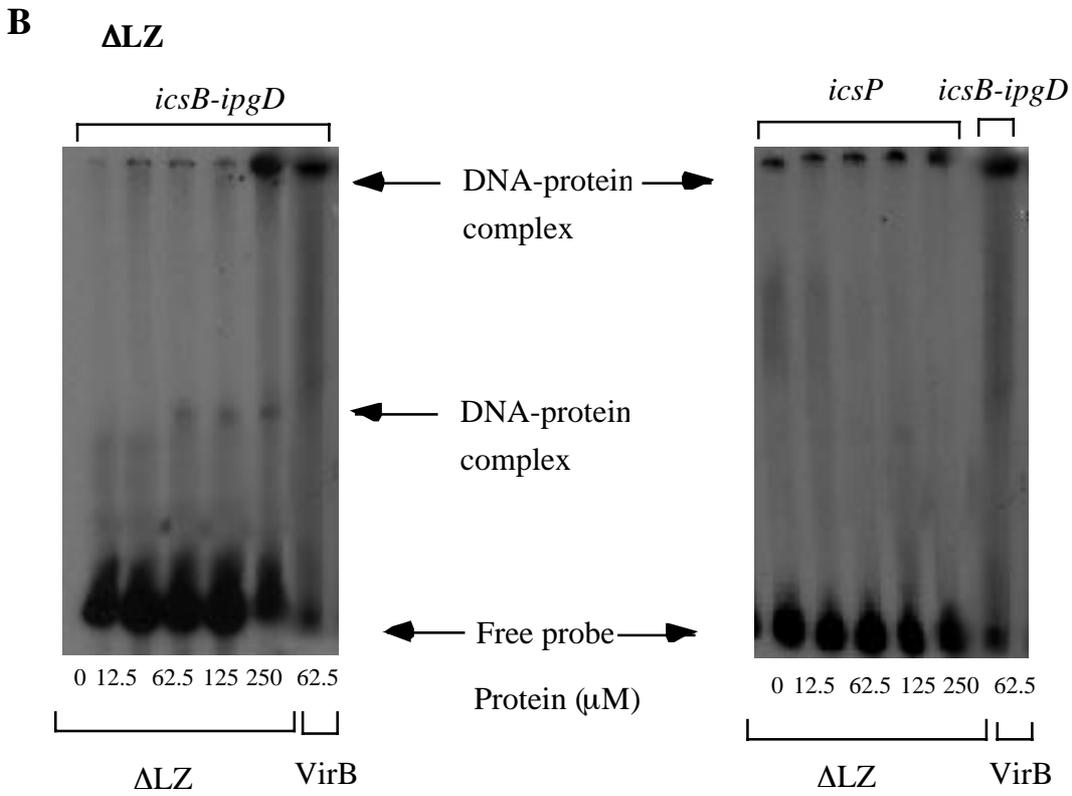
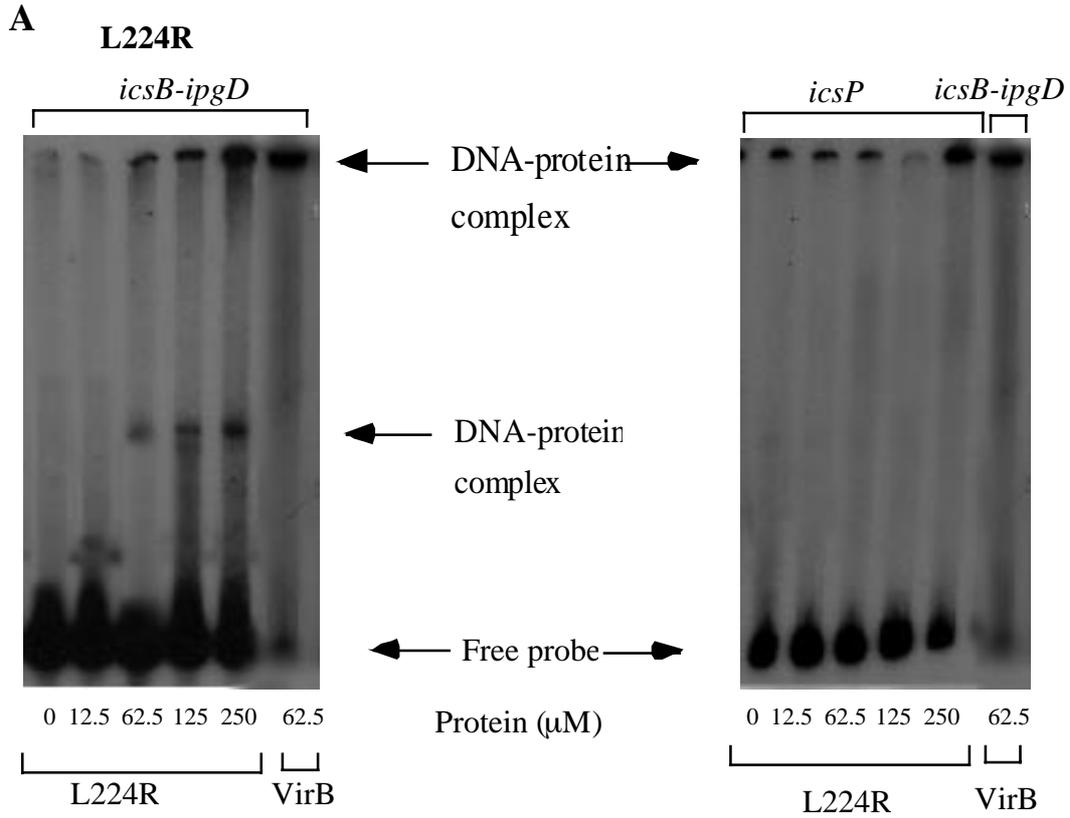


Fig 4.



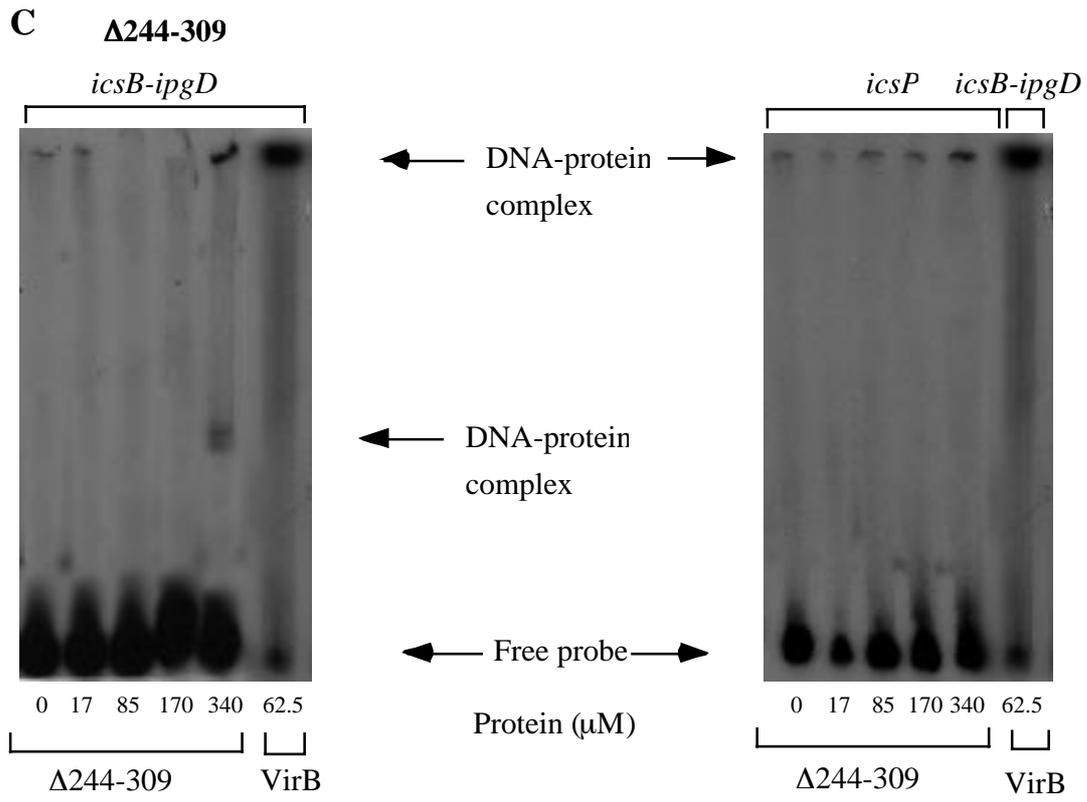


Fig. 5.