Glutamate residues in the putative transmembrane region are required for the function of the VirS sensor histidine kinase from *Clostridium perfringens*

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The causative agent of gas gangrene, *Clostridium perfringens*, is a Gram-positive anaerobe which produces a number of extracellular toxins and enzymes. The production of several of these toxins is regulated by the VirS/VirR two-component signal transduction system. The sensor histidine kinase, VirS, contains motifs that are conserved amongst sensor histidine kinases, although not in the same relative positions. In this study, the conserved histidine residue (H255), the GXGL and DXGXG motifs, and two glutamate residues located in putative transmembrane domains were altered by site-directed mutagenesis to examine their significance for VirS function. Introduction of the mutated *virS* genes into the *virS*::Tn916 mutant, JIR4000, showed that the altered *virS* genes were not able to complement the host mutation. These results demonstrate that the conserved motifs, including the cytoplasmic DXGXG motif which is located between the putative transmembrane domains 4 and 5, are functional. Furthermore, it is concluded that charged residues located within two of these transmembrane domains are also required for the structural or functional integrity of the VirS sensor kinase.

Keywords: *Clostridium perfringens*, two-component, signal transduction, sensor histidine kinase, transmembrane domain

INTRODUCTION

Bacteria live in an ever-changing environment, and to survive they must constantly monitor their surroundings and respond quickly and efficiently to various environmental cues. The most common adaptive response involves two-component signal transduction systems. These regulatory networks have been identified in both Gram-positive and Gram-negative bacteria and have been found to control a variety of environmental responses, including chemotaxis, nitrogen regulation, phosphate regulation, osmoregulation, antibiotic resistance and virulence (Stock *et al.*, 1989, 1995). Two-component signal transduction systems usually consist of two proteins, a membrane-associated sensor histidine kinase and its cognate cytoplasmic response regulator.

In general, sensor histidine kinases can be divided into two domains. The more conserved domain extends over

approximately 250 amino acids and is usually located at the cytoplasmic C-terminus of the protein (Albright *et al.*, 1989). Sequence alignments of sensor kinases reveal that there are five regions that show stronger conservation. These motifs include an invariant histidine residue that acts as the site of autophosphorylation and GXGL, DXGXG, N and F motifs, which have been proposed to form a nucleotide-binding surface (Parkinson & Kofoed, 1992; Stock *et al.*, 1995). The N-terminal domain is less well conserved than the C-terminus and most sensor kinases contain transmembrane regions which have been postulated to function as membrane receptors (Stock *et al.*, 1989). In general, these proteins contain two N-terminal membrane-spanning segments (Parkinson & Kofoed, 1992), with the extracytoplasmic loop between the transmembrane segments proposed to be the site of interaction with the environmental stimulus (Stock *et al.*, 1995).

In the Gram-positive anaerobe *Clostridium perfringens*,

Abbreviation: RT, reverse transcriptase.

Fig. 1. Transmembrane model of the VirS sensor histidine kinase. Grey bars represent the putative membrane-spanning regions. Extracytoplasmic and cytoplasmic loops are shown as loop structures, while the positions of the amino acid at the loop/transmembrane domain interface are indicated adjacent to each loop structure. Negatively and positively charged amino acids in the potential transmembrane regions are represented by the open ovals and solid triangles, respectively. The positions of the H255 (●), NA (■), GXGL (●) and DXGXG (◆) motifs are indicated as shown.

which is the causative agent of gas gangrene (Rood & Cole, 1991), toxin production is regulated by the VirS/VirR two-component signal transduction system (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994). The *virR* and *virS* genes form an operon (Ba-Thein *et al.*, 1996), with *virR* encoding the cytoplasmic response regulator and *virS* encoding the sensor histidine kinase (Lyristis *et al.*, 1994). Both proteins contain motifs and residues that are conserved amongst their two-component homologues. However, the VirS sensor histidine kinase differs in two ways. First, it contains six putative transmembrane regions, whereas most sensor kinases have two putative membrane-spanning regions. Secondly, the DXGXG motif is postulated (Lyristis *et al.*, 1994) to be in a cytoplasmic loop between the fourth and fifth putative transmembrane segments (Fig. 1); this motif is generally situated near the GXGL motif in the cytoplasmic C-terminal catalytic domain in most sensor kinases (Parkinson & Kofoid, 1992).

The VirS/VirR system differentially regulates the production of several of the toxins potentially involved in pathogenesis and virulence, namely α -toxin (phospholipase C), perfringolysin O (θ -toxin), collagenase (κ -toxin), sialidase and protease (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994). It is proposed that upon the detection of an as yet unknown environmental or growth phase stimulus by the N-terminal transmembrane regions, the VirS protein undergoes a conformational change. This change initiates the autophosphorylation process, whereby a phosphoryl group is transferred from ATP to the conserved H255 residue. The phosphorylated VirS protein then acts as a phosphodonor, leading to the phosphorylation of the D57 residue of VirR. The resultant activated VirR molecule subsequently regulates the transcription of the various target genes, leading to increased toxin production (Lyristis *et al.*, 1994; Rood & Lyristis, 1995).

In this study, the conserved histidine residue H255, the two glycine-rich motifs GXGL and DXGXG, and two

glutamate residues in the putative transmembrane region were changed by site-directed mutagenesis. To assess the effect of these mutations and therefore the functional significance of the original amino acids, complementation assays were carried out by introducing the mutated genes into the *virS*::Tn916 mutant JIR4000 (Lyristis *et al.*, 1994).

METHODS

Bacterial strains, plasmids and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37 °C on/in 2 × YT agar or broth or in SOC broth (Sambrook *et al.*, 1989), supplemented with 100 µg ampicillin ml⁻¹ or 150 µg erythromycin ml⁻¹. *C. perfringens* strains were grown at 37 °C in trypticase-peptone-glucose (TPG) broth (Rood *et al.*, 1978), Brain Heart Infusion (BHI) broth (Oxoid), fluid thioglycolate medium (FTG) (Difco) or nutrient agar (Rood, 1983) supplemented with 50 µg erythromycin ml⁻¹ or 10 µg tetracycline ml⁻¹. When screening for perfringolysin O production, *C. perfringens* transformants were grown on horse blood agar (HBA) (Lyristis *et al.*, 1994). All agar cultures of *C. perfringens* were incubated in an atmosphere of 10% (v/v) H₂, 10% (v/v) CO₂ in N₂.

Molecular techniques. Plasmid DNA from *E. coli* cells was routinely isolated by an alkaline lysis method (Morelle, 1989). When used for sequencing, DNA was obtained using the modified mini alkaline-lysis/PEG precipitation procedure outlined in the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). *C. perfringens* plasmid DNA was isolated using a modified Magic Mini Prep kit method (Promega) (Lyristis *et al.*, 1994). Rubidium-chloride-competent *E. coli* cells were prepared and transformed as described previously (Hanahan, 1985). Transformation of electrocompetent *E. coli* cells (Smith *et al.*, 1990) was carried out with a Bio-Rad Gene Pulser in 0.1 ml cuvettes (Bio-Rad) under conditions outlined by the manufacturer. Electrocompetent *C. perfringens* cells were prepared and transformed as before (Scott & Rood, 1989).

Nucleotide sequence analysis. Nucleotide sequences were determined by use of a PRISM Big Dye Terminator Cycle

Table 1. Relevant strains and plasmids

Strain/plasmid	Characteristics	Reference/origin
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ 80d <i>lacZ</i> ΔM15Δ(<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17</i> (<i>r_k⁻m_k⁺</i>) <i>deoR thi-1 supE44 gyrA96 relA1</i>	Bethesda Research Laboratories
<i>C. perfringens</i>		
JIR325	Strain 13 NaI ^R Rif ^R	Lyrstis <i>et al.</i> (1994)
JIR4025	JIR325 Rif ^S	Lyrstis <i>et al.</i> (1994)
JIR4000	JIR4025 <i>virS</i> ::Tn916	Lyrstis <i>et al.</i> (1994)
Plasmids		
pBluescript II SK(+)	Cloning vector, Ap ^R	Stratagene
pJIR751	<i>C. perfringens</i> – <i>E. coli</i> shuttle vector, Em ^R	Bannam & Rood (1993)
pJIR888	pJIR872Δ(<i>HpaI</i> – <i>EcoRV</i> , 2·7 kb)(<i>virS virS</i> ⁺)	Lyrstis <i>et al.</i> (1994)
pJIR1364	pBluescript II SK(+)Ω(<i>SalI</i> / <i>PstI</i> :pJIR888, 1·6 kb)	Recombinant
pJIR1216	pJIR751Ω(<i>SalI</i> / <i>PstI</i> :pJIR1446, 1·6 kb)(<i>virS-H255I</i>)	Lyrstis (1996)
pJIR1481	pJIR751Ω(<i>SalI</i> / <i>PstI</i> :pJIR1446, 1·6 kb)(<i>virS-G400A</i>)	Cloned site-directed mutant
pJIR1483	pJIR751Ω(<i>SalI</i> / <i>PstI</i> :pJIR1460, 1·6 kb)(<i>virS-G156A</i>)	Cloned site-directed mutant
pJIR1506	pJIR751Ω(<i>Asp718/BamHI</i> :pJIR1501, 1·6 kb)(<i>virS-E19A</i>)	Cloned site-directed mutant
pJIR1712	pJIR751Ω(<i>Asp718/BamHI</i> :pJIR1643, 1·6 kb)(<i>virS-E19D</i>)	Cloned site-directed mutant
pJIR1715	pJIR751Ω(<i>Asp718/BamHI</i> :pJIR1639, 1·6 kb)(<i>virS-E19Q</i>)	Cloned site-directed mutant
pJIR1507	pJIR751Ω(<i>Asp718/BamHI</i> :pJIR1502, 1·6 kb)(<i>virS-E102A</i>)	Cloned site-directed mutant
pJIR1713	pJIR751Ω(<i>Asp718/BamHI</i> :pJIR1640, 1·6 kb)(<i>virS-E102D</i>)	Cloned site-directed mutant
pJIR1714	pJIR751Ω(<i>Asp718/BamHI</i> :pJIR1641, 1·6 kb)(<i>virS-E102Q</i>)	Cloned site-directed mutant

Sequencing Ready Reaction kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems) according to the manufacturer's instructions. Sequencing samples were resolved and analysed on a 373 DNA STRETCH Sequencer (Applied Biosystems). Sequence analysis was performed using the Sequencher 3.0 software (GeneCodes). Transmembrane protein structure was predicted by use of TopPred II software (Claros & von Heijne, 1994).

Construction of recombinant plasmids. Plasmid DNA was digested in the presence of the appropriate restriction enzyme(s) and digestion buffer (Boehringer Mannheim; New England Biolabs) under the conditions specified by the manufacturers. Insert and vector DNA were isolated using the BRESA-CLEAN Nucleic Acid Purification kit (Bresatec). Vector and insert DNA were ligated with T4 DNA ligase (3 units μl⁻¹; Promega) at 16 °C overnight. The target plasmid for site-directed mutagenesis, pJIR1364, was obtained by cloning the 1·6 kb *SalI*–*PstI* fragment of pJIR888, which contained the *virS* gene, into pBluescript II SK(+) (Stratagene).

Site-directed mutagenesis. Site-directed mutagenesis of the *virS* gene was carried out by the unique site replacement method (Deng & Nickoloff, 1992) with a U. S. E. Mutagenesis kit (Pharmacia Biotech) by a modification of the manufacturer's instructions. After annealing the selection and mutagenic primers to the plasmid template, synthesis of a mutant DNA strand was carried out at 42 °C for 1 h. The second round of restriction enzyme selection was performed in a total volume of 20 μl. The digested DNA was then used to transform rubidium-chloride-treated competent *E. coli* cells; the cells were heat-shocked at 37 °C for 2 min before inoculation into SOC broth. Plasmids were screened using restriction analysis and the desired mutations were verified by nucleotide sequence analysis. The oligonucleotide primers

used for mutagenesis (Table 2) were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer. All mutated *virS* genes were completely sequenced to confirm that no additional mutations had been introduced.

Perfringolysin O assays. Perfringolysin O activity was determined by measuring the haemolysis of horse erythrocytes. *C. perfringens* cultures and supernatants were obtained as described previously (Awad *et al.*, 1995). Haemolysin assays were performed by a doubling-dilution assay as before (Stevens *et al.*, 1987), except that 5 mM DTT was used. The titre was defined as the reciprocal of the last well that showed complete haemolysis.

Isolation of RNA. To obtain cells for RNA extraction, 3·0 ml of each overnight FTG culture of the relevant *C. perfringens* strain was subcultured into 20 ml BHI broth and grown at 37 °C until late exponential phase. Cells were harvested by centrifugation at 16300 g and the supernatant was discarded. The cells were resuspended and incubated in 1 ml lysis solution (10 mM Tris/HCl, pH 7·5; 0·4 M sucrose; 5 mg lysozyme ml⁻¹) for 15 min at 37 °C, harvested by centrifugation, and the supernatant was removed. Total RNA was then isolated using TRIzol Reagent (Gibco-BRL/Life Technologies) as described previously (La Fontaine & Rood, 1996), with a few modifications. To remove contaminating DNA, RNA samples were treated with 1 unit RQ1 RNase-free DNase I (Promega) in the presence of 0·1 vol. 10 × DNase I buffer (40 mM Tris/HCl, pH 7·9; 10 mM NaCl; 6 mM MgCl₂; 10 mM CaCl₂) and 40 units RNasin RNase Inhibitor (Promega) for 1 h at 37 °C. Samples were then re-extracted with TRIzol according to the manufacturer's instructions, but using only half the original reagent volumes.

RT-PCR. Reverse transcription was carried out using 2 μg RNA as the template, following the manufacturer's instructions

Table 2. Oligonucleotide primers used in this study

Primer no.	Sequence (5'–3')	Purpose
2281	GGCGGCCGCTCCAGAACTAGTG	<i>Xba</i> I selection primer
3537	CTTATATTTTTTAAGGCCAATAGCATGTAAAAAC	G400A mutation
3908	TAAAGTCTCCCTTTGCAATATCTACACG	G156A mutation
4622	CTTACCCATGCTATAATC	E19A mutation
5323	CTTACCCAATCTATAATC	E19D mutation
5322	CTTACCCATTGTATAATC	E19Q mutation
4623	GAAATAGATAGAGCTGCTACTG	E102A mutation
5324	GATAGAGCGTCTACTGTAAGC	E102D mutation
5325	GAAATAGATAGAGCTTGTACTG	E102Q mutation
1001	GATCTCATTAATATTTTGGT	RT-PCR
1495	TACATATATCAAAGTACTCAAT	RT-PCR

(Promega), with minor modifications. The reaction was performed at 42 °C for 50 min, followed by heat inactivation of the AMV reverse transcriptase at 70 °C for 15 min.

RT and PCR reactions were carried out consecutively. PCR was performed in a total volume of 25 µl; the reaction mixture consisted of 0.2 vol. of the RT reaction, 0.5 µM of each internal *virS* primer (Table 2), 0.1 vol. 10×PCR buffer (Boehringer Mannheim), 2.5 units *Taq* DNA polymerase (Boehringer Mannheim) and sterile distilled water. Reactions were carried out in a GeneAmp PCR System 2400 (Perkin Elmer), where the 94 °C denaturation (1 min), 50 °C annealing (2 min) and 72 °C extension (3 min) steps were carried out for 30 cycles. The final cycle consisted of 2 min annealing and 5 min extension at the temperatures indicated. RT-PCR products were resolved and visualized by agarose gel electrophoresis.

RESULTS

Functional analysis of the conserved H255 residue and GXGL motif

To confirm that the VirS protein was a functional sensor histidine kinase we used site-directed mutagenesis to examine the functional significance of the conserved H255 residue and GXGL motif. The proposed site of autophosphorylation, H255, was changed to isoleucine to observe the effect of a change from a basic hydrophilic amino acid to a hydrophobic amino acid that could not act as a phosphoacceptor. The VirS GXGL motif has been postulated to be the conserved nucleotide binding site (Lyristis *et al.*, 1994). To determine whether this motif was functional in VirS, the first glycine residue of the putative GXGL motif, G400, was changed to alanine. The target plasmid, pJIR1364, was subjected to mutagenesis as described in Methods and potential H255I and G400A mutants were identified by restriction endonuclease digestion and preliminary DNA sequencing. The *virS* gene regions of potential mutants were then completely sequenced and no additional mutations were found.

To examine the effect of the H255I and G400A mutations on VirS function in the native *C. perfringens* host, the mutated *virS* genes were subcloned into an *E. coli*–*C. perfringens* shuttle vector, pJIR751 (Bannam & Rood, 1993). The resultant constructs, pJIR1216 and pJIR1481, respectively, were then introduced into the *C. perfringens* strain, JIR4000. This strain carries a chromosomal *virS* mutation which alters its toxin production profile to the extent that α -toxin and collagenase production are reduced and perfringolysin O production is completely eliminated (Lyristis *et al.*, 1994). Complementation of the mutation with a plasmid carrying a wild-type *virS* gene results in restoration of the ability to produce perfringolysin O, depending upon the level of expression of the wild-type gene. This complementation can be readily observed as a zone of haemolysis on HBA.

Unlike the *virS*⁺ positive control, pJIR888, the mutated *virS* plasmids, pJIR1216 and pJIR1481, did not complement the chromosomal *virS* mutation. No zone of haemolysis was observed on HBA. This observation was confirmed by carrying out quantitative perfringolysin O assays using culture supernatants (Table 3). As expected, the wild-type strain JIR325 produced high levels of perfringolysin O, while the mutant strain JIR4000 and the mutant strain carrying the shuttle plasmid, JIR4000(pJIR751), did not produce detectable levels of haemolysis. Note that lower levels of perfringolysin O activity were observed in supernatants from JIR4000(pJIR888), presumably because this plasmid does not have the native *virRS* promoter. These results were in agreement with the phenotypes observed on HBA.

Although these results indicated that the H255I and G400A mutations had altered the function of VirS, it was necessary to confirm that the mutated gene was still transcribed. Total RNA was isolated from *C. perfringens* strains carrying either the control plasmid or the mutated *virS* genes. Following DNase I treatment, the RNA samples were used as templates in RT-PCR experiments. A positive DNA control, which used the

Table 3. Effect of site-directed mutagenesis on perfringolysin O production

Strain	Plasmid <i>VirS</i> mutation	Nucleotide change	PfoA titre*
JIR325	Wild-type		8.2 ± 0.4
JIR4000	Chromosomal <i>virS</i> mutant		<1.0
JIR4000(pJIR751)	Negative control		<1.0
JIR4000(pJIR888)	Positive control		2.8 ± 0.3
JIR4000(pJIR1216)	H255I	CAT → ATC	<1.0
JIR4000(pJIR1481)	G400A	GGT → GCT	<1.0
JIR4000(pJIR1483)	G156A	GGA → GCA	<1.0
JIR4000(pJIR1506)	E19A	GAA → GCA	<1.0
JIR4000(pJIR1712)	E19D	GAA → GAT	<1.0
JIR4000(pJIR1715)	E19Q	GAA → CAA	<1.0
JIR4000(pJIR1507)	E102A	GAA → GCA	<1.0
JIR4000(pJIR1713)	E102D	GAA → GAC	<1.0
JIR4000(pJIR1714)	E102Q	GAA → CAA	<1.0

* PfoA titre refers to the mean perfringolysin O titre obtained from duplicate assays carried out using supernatants from three independent cultures of each strain.

same oligonucleotide primers and pJIR888 as the template, was included at the PCR stage to amplify a product of the same size as that expected from RT-PCR. As anticipated, RNA samples isolated from JIR4000 derivatives carrying pJIR888, pJIR1216 or pJIR1481 all yielded products of the same size (739 bp) as the positive DNA control (Fig. 2). No PCR product was observed from RNA samples isolated from JIR4000 carrying the shuttle vector pJIR751. The absence of a *virS* transcript in this background strain has previously been demonstrated and was attributed to the insertion of Tn916 in the chromosomal *virS* gene (Ba-Thein *et al.*, 1996), the position of which has been mapped previously (Lyristis *et al.*, 1994). The absence of PCR products in the reactions in which RT was omitted confirmed that the RNA samples were DNA-free and that the products observed were the result of the amplification of cDNA molecules derived from the RT reaction. These products were isolated, sequenced and confirmed to be the internal *virS* region, thereby demonstrating that the mutated *virS* genes were expressed and that the observed effects on VirS function could be attributed to the mutations. Based on these results it was concluded that VirS was a functional sensor histidine kinase and that the H255 and G400 residues represented conserved components of the respective motifs.

Functional significance of the DXGXXG motif

The cytoplasmic DXGXXG motif of sensor histidine kinases is also involved in nucleotide binding (Parkinson & Kofoid, 1992). However, this motif appears to be located in the cytoplasmic loop between the fourth and fifth putative transmembrane domains of VirS (Fig. 1). Due to its unusual position in comparison to other sensor histidine kinases, the putative DXGXXG motif of VirS was mutated to ensure that it was functional. Site-directed mutagenesis was used to change the first glycine residue (G156) of the motif to alanine. Sequence analysis

of the resultant plasmid confirmed that no additional mutations had been introduced. The mutated *virS* gene was cloned into the shuttle plasmid to construct pJIR1483, which was then introduced into strain JIR4000 and analysed as before.

The results showed that the mutated *virS* gene was being expressed, as indicated by RT-PCR (Fig. 2a), and that the G156 residue in the DXGXXG motif was required for VirS function, since pJIR1483 was not able to complement the *virS* mutation of JIR4000, i.e. there was no detectable haemolytic activity on HBA and no demonstrable perfringolysin O activity in culture supernatants (Table 3). These results implied that despite its unusual position, the DXGXXG motif had a functional role in VirS activity.

Role of glutamate residues in the putative transmembrane domains

Topological analysis of the VirS protein was carried out by use of the TopPred II algorithm (Claros & von Heijne, 1994). The optimal theoretical conformation, i.e. the highest K + R difference, was obtained with a window of 21 amino acid residues and predicted the presence of six transmembrane domains (Fig. 1). Examination of each proposed membrane-spanning region revealed the presence of several charged residues (Fig. 1). Of these amino acids, E19 and E102 were the only negatively charged acidic residues predicted to be well within these domains. Other glutamate residues, E12 and E130, were also found in the putative first and fourth transmembrane domains, respectively. However, these amino acids were situated adjacent or very close to putative cytoplasmic or external regions of the protein.

Since the presence of acidic residues located deeply within putative transmembrane domains is thermodynamically undesirable in a stable membrane protein

Fig. 2. Agarose gel electrophoresis of RT-PCR products. Lanes: 1, λ cl857 DNA digested with *Hind*III; 2 and 3, the positive [JIR4000(pJIR888)] and negative [JIR4000(pJIR751)] plasmid DNA controls, respectively; 4 and 6, the positive and negative RNA controls, respectively; 5 and 7, the respective no RT controls. The last lane contains PCR markers (Promega). (a) Lanes 8, 10 and 12 contain RT-PCR products derived from total RNA isolated from JIR4000 cells harbouring plasmids containing the H255I, G400A and G156A mutations, respectively. Lanes 9, 11 and 13 contain the respective no RT controls. (b) Lanes 8, 10, 12, 14, 16 and 18 contain RT-PCR products derived from total RNA isolated from JIR4000 cells harbouring plasmids carrying the E19A, E19D, E19Q, E102A, E102D and E102Q mutations, respectively. Lanes 9, 11, 13, 15, 17 and 19 contain the respective no RT controls.

our analysis suggested that the E19 and E102 residues may be of functional importance. To examine their significance these residues were each changed to alanine, aspartate and glutamine by site-directed mutagenesis with the oligonucleotides listed in Table 2. The six mutated *virS* genes (Table 3) were then analysed in the same way as the previous mutations. Again sequence analysis confirmed that no other mutations had been introduced and RT-PCR studies (Fig. 2b) showed that the mutated genes were expressed in *C. perfringens*. Analysis on HBA and perfringolysin O assays (Table 3) showed that none of the mutated *virS* genes complemented the chromosomal *virS* mutation of JIR4000. These results imply that the glutamate residues in the putative transmembrane domains have functional or structural significance in the VirS sensor histidine kinase.

DISCUSSION

In this study, site-directed mutagenesis of various residues in the kinase and putative transmembrane regions of the sensor histidine kinase, VirS, was carried out to determine if these residues were required for VirS function. Previously, sequence alignment of VirS with other sensor histidine kinases predicted the site of autophosphorylation to be at residue H255 (Lyristis *et al.*, 1994). Studies with well-characterized sensor histidine kinases, such as EnvZ from *E. coli* (Kanamaru *et al.*, 1990; Yang & Inouye, 1991, 1993), VirA from *Agrobacterium tumefaciens* (Jin *et al.*, 1990) and BvgS from *Bordetella pertussis* (Uhl & Miller, 1994), have

shown that mutation of the conserved histidine residue abolishes the function of the protein. In this study, complementation experiments have shown that a *virS* gene carrying the H255I mutation could not complement a chromosomal *C. perfringens virS* mutation. This result confirmed that H255 is required for VirS function.

Comparative sequence analysis of VirS also revealed the presence of other conserved regions, namely the N, DXGXXG and GXGL motifs (Lyristis *et al.*, 1994). The glycine-rich DXGXXG and GXGL motifs have been proposed to be involved in nucleotide binding due to their similarity to the glycine loops that are characteristic of many ATP-binding sites (Parkinson & Kofoed, 1992). Mutagenesis studies of other sensors have shown that all three motifs are essential for kinase activity (Hsing *et al.*, 1998; Yang & Inouye, 1993). In this study, *virS* genes encoding glycine to alanine substitutions in the first glycine-encoding codons of both the DXGXXG and GXGL motifs could not complement the *virS* mutation of the host strain. These results indicate that these glycine residues, and by implication the respective motifs, are of functional or structural importance. By analogy with other sensors it is concluded that the respective VirS motifs are likely to have the same function as the equivalent residues in other histidine kinases.

Recently, the structures of the histidine kinase domains of EnvZ (Tanaka *et al.*, 1998) and the CheA chemotaxis sensor from *Thermotoga maritima* (Bilwes *et al.*, 1999) were determined. In these kinases, the N, DXGXXG and

GXGL motifs lined the catalytic core that was predicted to be involved in ATP binding (Bilwes *et al.*, 1999; Tanaka *et al.*, 1998). Based on our results it is likely that the DXGXXG and GXGL motifs also form part of the catalytic domain of VirS. In almost all sensor histidine kinases, the DXGXXG motif is found in close proximity to the GXGL motif and is located in the more conserved C-terminal domain (Parkinson & Kofoed, 1992; Stock *et al.*, 1989, 1995). However, the VirS DXGXXG motif, which we have now shown to be functional, is predicted to be in one of the cytoplasmic loops of the putative transmembrane domains in the variable N-terminal region (Fig. 1). Although not common, the conserved kinase motifs of other sensors have been found in different positions or found to be absent altogether. For example, the histidine residue that acts as the site of autophosphorylation of CheA is located in a region distinct from the conserved kinase domain (Hess *et al.*, 1988), while the N motif and DXGXXG motif are absent in FrzE and AgrORF2, respectively. Similarly, the GXGL motifs are absent in NarX and NarQ (Parkinson & Kofoed, 1992). We postulate that the conformation of VirS is such that the DXGXXG motif, despite its unusual position, can still form the catalytic domain and subsequently bind ATP. Specifically, we propose that the cytoplasmic loop 4–5 must be located in close proximity in the cytoplasm to the conserved C-terminal domain of VirS. Recent studies on EnvZ have indicated that its ATP-binding ability was not affected when the G box (GXGL of VirS) or D/F box (DXGXXG of VirS) was altered (Hsing *et al.*, 1998). In contrast, ATP binding was abolished in the N347D mutant of EnvZ (Tanaka *et al.*, 1998). The G box and D/F box are presumably important in positioning the phosphate moieties of ATP (Hsing *et al.*, 1998). Therefore, it is possible that mutation of the DXGXXG and GXGL motifs of VirS interferes with the positioning of the bound ATP, rather than with actual ATP binding.

The N-terminal domains of sensor histidine kinases are more variable than their C-terminal domains (Parkinson & Kofoed, 1992). The majority of sensor histidine kinases are membrane-associated and have two transmembrane segments located on either side of a periplasmic domain that presumably interacts with the external stimulus (Parkinson & Kofoed, 1992; Stock *et al.*, 1989). However, there are several histidine kinases, such as ComP from *Bacillus subtilis* (Weinrauch *et al.*, 1990), AgrC from *Staphylococcus aureus* (Lina *et al.*, 1998) and UhpB from *E. coli* (Island & Kadner, 1993; Island *et al.*, 1992), which have from six to ten potential transmembrane domains. Similarly, our topological model of VirS predicted the presence of six putative transmembrane domains, with the N- and C-termini on the cytoplasmic side of the membrane (Fig. 1). Amino acid sequence analysis of the membrane-spanning segments revealed the presence of two negatively charged glutamate residues that were clearly located within two putative transmembrane domains. These residues were targeted in our mutagenesis experiments because the presence of an acidic amino acid in a

hydrophobic membrane region is highly unfavourable for thermodynamic reasons (Lee *et al.*, 1992).

We postulated that the presence of the E19 and E102 residues in putative transmembrane regions implied that they had a role in VirS activity. Evidence in support of this hypothesis was obtained from the mutagenesis experiments. These results showed that when these glutamate residues were changed to alanine, aspartate or glutamine, the mutant VirS proteins were not functional. Glutamate was specifically required at these positions since replacement with an amino acid of similar charge, aspartate, or of similar size, glutamine, did not result in complementation. However, the function of these residues is as yet unknown, and we do not know if these glutamates are of structural or functional significance. It is possible that these mutations interfered with the ability of VirS to undergo a conformational change in the transmembrane domain that is required for signal transfer between the external receptor domain of VirS and the C-terminal kinase domain. Unfortunately, transmembrane signalling in bacteria is poorly understood and what little is known is gleaned from work on the methyl-accepting chemotaxis proteins (MCPs) which act as chemoreceptors in *E. coli* (Parkinson & Kofoed, 1992). The similarity in structure and function of MCPs to sensor kinases allows some parallels to be drawn. The MCPs have been found to exist as stable homodimers that signal via an intradimer conformational change (Chervitz & Falke, 1996; Falke *et al.*, 1997). Similarly, work on EnvZ suggests that interaction between the two membrane-spanning regions is crucial for transmembrane signalling (Tokishita & Mizuno, 1994). Alternatively, the glutamate residues may be involved in the transport of a charged molecule across the cell membrane, as previously suggested (Lyristis *et al.*, 1994).

Although there are still many details about the VirS/VirR system that are yet to be determined, the results obtained in this study provide important information about the sensor component. In particular, the significance of the conserved motifs and glutamate residues in the putative membrane-spanning regions has now been established experimentally. Further insights into the structural and functional significance of the N-terminal input domain of VirS will require the experimental verification or modification of the transmembrane model.

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