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Research article

The major surface Vsp proteins of *Brachyspira hyodysenteriae* form antigenic protein complexes

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ABSTRACT

The Vsp proteins are the major outer membrane proteins of *Brachyspira hyodysenteriae*, the causative agent of swine dysentery. Eight *vsp* genes have been identified in *B. hyodysenteriae* strain B204, arranged into two four-gene loci, and at least two of the corresponding proteins are produced in vitro. The aims of this study were to characterise the *vsp* genes of the virulent Australian *B. hyodysenteriae* strain X576 and their corresponding proteins, Genomic sequence comparison with strains B204 and WA1 demonstrated that the number of *vsp* genes varies between *B. hyodysenteriae* strains, although the chromosomal locations of the *vsp* gene loci are consistent. We identified two additional *vsp*-like genes, designated *vspI* and *vspJ*, in each of the three strains. Double SDS-PAGE was used to demonstrate that Vsp proteins of *B. hyodysenteriae* strain X576 form multimeric protein complexes in the outer membrane that are stable in 6 M urea but dissociate after boiling. The Vsp complexes primarily consisted of VspF but also contain VspE and VspI. VspD was also found in a series of complexes slightly larger than the more abundant VspF complexes. Vsp proteins are purported to be antigenic; however little direct data are available to support this claim. In this study convalescent pig sera did not bind denatured Vsp proteins by Western blotting, but did bind the Vsp complexes on Western blots, showing that conformational epitopes may be important in immune recognition of these major outer membrane proteins. This is the first definitive demonstration of the antigenicity of these proteins in swine dysentery.

1. Introduction

The enteric, anaerobic spirochete *Brachyspira hyodysenteriae* is the causative agent of swine dysentery, a severe mucohemorrhagic diarrheal disease of pigs. Relatively little is known about the mechanisms of pathogenesis of this organism or the importance of its protein antigens in immunity. To date, a relatively small number of its outer membrane proteins have been identified and characterized. The Vsp proteins are the most abundant outer membrane proteins of *B. hyodysenteriae* and are surface-exposed (Gabe et al., 1998). Studies investigating the origin of these proteins revealed a total of eight paralogous *vsp* genes organized in two clusters (*vspABCD* and *vspEFGH*) in *B. hyodysenteriae* strain B204 (McCaman et al., 1999, 2003). We have previously demonstrated that multiple *vsp* genes are transcribed simultaneously in strain B204 and that the

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protein products of at least two of these are detectable by mass spectrometry (Witchell et al., 2006). The presence of Vsp proteins has been noted for other strains of *B. hyodysenteriae* and *vsp* genes have also been annotated in the recently published genome sequence of *B. hyodysenteriae* strain WA1 (Bellgard et al., 2009). Vsp-like proteins have been found in *B. pilosicoli* (Trott et al., 2001), but homologs have not been identified in any other organism. The functions of Vsp proteins are currently unknown.

In this study, we demonstrate that multiple Vsp proteins are present in the *B. hyodysenteriae* outer membrane in high molecular mass protein complexes. We have also identified two new *vsp* genes in *B. hyodysenteriae* and found that the protein product of one of these genes is associated with these complexes. Furthermore, we provide evidence that conformational epitopes of Vsp protein complexes may be important in the generation of an antibody response against *B. hyodysenteriae*.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. hyodysenteriae strain X576 is a virulent Australian isolate which can reproducibly establish infection in inoculated and sentinel pigs (2.2); it was provided by N. Sammons, Intervet International, Bendigo, Australia. Cells were grown anaerobically (80% N_2, 10% CO_2, 10% H_2) at 37 °C in trypticase soya broth supplemented with 0.5% yeast extract, VPI salts and 5% heat-inactivated fetal calf serum (Wannemuehler et al., 1988) and harvested at mid-late log phase (between 5×10^7 and 2×10^8 cells per ml). Broth cultures were subjected to continuous agitation using magnetic stirrers. *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA) and BL21 CodonPlus strains were used for recombinant Vsp protein expression. PCR products of genes were cloned into pET151-D-TOPO (Invitrogen) for expression of recombinant proteins.

2.2. Generation of convalescent pig sera

Cultures of *B. hyodysenteriae* strain X576 used in this procedure for challenge were prepared (as above) and cell numbers enumerated microscopically. Cultures were harvested at 5×10^8 cells per mL and administered to pigs within 4 h of collection. To generate antisera, five 6-week old pigs were obtained from a herd known to be free of swine dysentery and were commingled for the period of the procedure. One animal was infected by intranasal administration of 10^9 cells in 2 mL of growth medium at 7 weeks of age and again at 9 weeks of age. The animals were observed for signs of disease; all developed clinical swine dysentery, confirmed by culture of *B. hyodysenteriae* strain X576 from feces. At 12 weeks of age all animals were medicated with oral tiamulin (200 mg/kg) for three days. At 15 weeks of age the animals were challenged with *B. hyodysenteriae* in doses of 20 mL delivered orally on three consecutive days. At 18 weeks of age, blood was collected and serum frozen at −20 °C until required. Animal experiments were approved by the Victorian Department of Primary Industries Animal Ethics Committee.

2.3. Oligonucleotides

Oligonucleotides used in this study are listed in Table 1. Forward oligonucleotides for cloning genes into pET151-D-TOPO contained a 7 bp overhang (5′-CACCATG) as per the manufacturer's instructions. Other oligonucleotides used in this study to sequence the *vsp* gene loci in *B. hyodysenteriae* strain X576 have been published previously (Witchell et al., 2006).

2.4. Molecular biology methods

Chromosomal DNA was prepared from *B. hyodysenteriae* strains using the cetyltrimethylammonium bromide (CTAB) precipitation method (Wilson, 2001). PCR amplification of DNA was performed using Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) or the Novagen KOD Hot Start DNA Polymerase (Merck KGaA, Darmstadt, Germany). PCR products required for sequencing were purified using the QIAQuick PCR Purification Kit (QIAGEN GmbH, Germany). DNA sequences were determined on an Applied Biosystems 3730S Genetic Analyzer and analyzed with Vector NTI Advance version 11 (Invitrogen, Carlsbad, CA).

Table 1
Oligonucleotides used in this study.

Primer number	Sequence[a]	Purpose
BAP3951	5′-TCG GAA ACT TAA TAG GAA GC	Forward primer for amplification of *vspEFGH* based on B204 sequence
BAP3952	5′-TGT ATC CCT TAG CCC AAT AA	Reverse primer for amplification of *vspEFGH* based on B204 sequence
BAP3953	5′-CTT GAA GGC GAG ATC ATA AC	Forward primer for amplification of *vspEFGH* based on X576 sequence
BAP3954	5′-TCG AGT CAG AAT TTC CAA AC	Reverse primer for amplification of *vspEFGH* based on X576 sequence & cloning *vspF* into pET151
BAP4112	5′-TTC CAA ATC TTT TAC CGG TGT TAT A	Sequencing *vspC* in X576
BAP5612[a]	5′-CAC CAT GGG TAT GTA TGG AGA TCA GGA C	Forward primer for cloning *vspA*/*vspC*/*vspD* into pET151
BAP5613[a]	5′-AAA TTC TGG CAA ATA CCA AGT TAT TC	Reverse primer for cloning *vspA*/*vspC*/*vspD* into pET151
BAP5614	5′-CAC CAT GAT GTA TGG TGC AGA CAA CAC	Forward primer for cloning *vspE* into pET151
BAP5615	5′-TTA TTG TAA AGC AGG TAA ATA CCA AG	Reverse primer for cloning *vspE* into pET151
BAP5616	5′-CAC CAT GGC TTT CGG TAT GTA TGG TGA CAG A	Forward primer for cloning *vspF* into pET151
BAP5631	5′-CAC CAT GTT TAG TTA TAC TGT TGT AGA AG	Forward primer for cloning *vspJ* into pET151
BAP5632	5′-TTA AAA CTT CCA AGT AAT ACC AGT AC	Reverse primer for cloning *vspJ* into pET151
BAP5633	5′-CAC CAT GGT ATT TGG TAT GTA CGG CAA C	Forward primer for cloning *vspI* into pET151
BAP5634	5′-TTA CTC TGC TGC TCC GAA AG	Reverse primer for cloning *vspI* into pET151

[a] Underlined bases represent directional cloning tag for ligation into pET151.