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BapC autotransporter protein is a virulence determinant of *Bordetella pertussis*

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ABSTRACT

A protein designated Bap-5 (GenBank accession no. AF081494) or BapC (GenBank accession no. AJ277634) has been identified as a member of the *Bordetella pertussis* autotransporter family and the present work suggests that this protein, like the previously characterised BrkA, is a Bvg-regulated serum resistance factor and virulence determinant. *B. pertussis bapC* and *brkA*, *bapC* mutants were created and, like a *brkA* mutant, showed greater sensitivity to killing by normal human serum than their parent strains but they were not as sensitive as a *bvg* mutant. Competition assays also showed an important role for BapC, like BrkA, in virulence of *B. pertussis* in mice after intranasal infection. Moreover, the *bapC* and *brkA*, *bapC* mutants, like the *brkA* mutant, were found to be more sensitive to the antimicrobial peptide cecropin P1 than the parent strains. In the genome sequence of *B. pertussis* strain Tohama, *bapC* is designated as a pseudogene due, in part, to a frameshift in a poly(C) tract near the 5′ end of the gene which creates a truncated BapC protein. Sequence analyses of the *bapC* region spanning the poly(C) tract of a number of *B. pertussis* strains showed minor nucleotide and amino acid polymorphisms but it appeared that all had an ORF that would be able to produce BapC.

1. Introduction

The autotransporters are a family of extracellular proteins, found in various Gram-negative bacteria, that have many different functions but appear to have a similar mechanism of export [1–3]. Autotransporters are composed of three main domains: a signal sequence; the passenger domain or α-domain, and a translocator unit or β-domain. The autotransporter polyprotein is exported across the inner membrane using the Sec machinery. The signal sequence is then cleaved, the β-domain inserted into the outer membrane and the passenger domain is translocated to the bacterial cell surface, where it may or may not undergo further processing [1,3].

Before publication of the *Bordetella* genome sequences [4], four autotransporters had been characterised in *Bordetella pertussis*, namely the virulence-regulated proteins pertactin, an adhesin [5]; BrkA, a serum resistance factor [6]; tracheal colonisation factor (Tcf), another adhesin [7]; and the product of virulence-activated gene-8 (Vag8) [8]. These proteins have structural homology in their β-domains (c. 30 kDa) but the passenger domains are structurally different, although they all have RGD and (except for pertactin) SGXG motifs. Another member of the *B. pertussis* autotransporter family was identified in our laboratory when a PCR amplicon with an unexpected sequence was produced using primers directed to the region encoding the β-domain of pertactin in *B. pertussis* genomic DNA. This sequence was used to identify a gene in *B. pertussis* strain Tab for what was then the fifth member of the *B. pertussis* autotransporter family, originally named Bap-5 (GenBank accession no. AF081494). An identical sequence was identified in *B. pertussis* strain Tohama and was named BapC (GenBank accession no. AJ277634). This latter designation was used subsequently in the *Bordetella* genome sequences [4]. With the publication of the genome sequences of *B. pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica*, it became apparent that the *bap5*/*bapC* sequence identified earlier was not the whole *bapC* gene. In *B. bronchiseptica* strain RB50, the *bapC* gene encoded an ORF of 993 amino acids. However, the predicted ORFs in the sequenced strains *B. pertussis* strain Tohama and *B. parapertussis* strain 12822 were shown to be truncated at 102 and 100 amino acids, respectively, due to frameshifts [1]. The genome sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* have revealed that these species have genes for 22 autotransporter proteins, although some of them, especially in

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B. pertussis and *B. parapertussis,* are pseudogenes. Expression of several of these genes, along with other, virulence-related genes, is known to be controlled by the BvgA–BvgS two component regulatory system [1]. In vitro, the Bvg system promotes virulence gene expression at 37 °C but the virulent (Bvg^+) phase can be switched off (Bvg^- phase) by certain "modulating" conditions such as lower temperature or high concentrations of sulphate ions [9,10].

Despite the above finding that *bapC* in the *B. pertussis* genome strain Tohama is a pseudogene, our earlier work had suggested that BapC was in fact expressed in the *B. pertussis* strains that we used and that BapC, like BrkA [6], could function as a serum resistance factor, by interfering with the classical pathway of complement activity ([11] and unpublished observations). In order to determine the relative contributions of these two components to serum resistance and mouse virulence of *B. pertussis*, single and double mutants were constructed in the same genetic background as the *brkA* and *bvg* mutants already available [12,13].

2. Results

2.1. Construction of bapC mutants

In initial studies, *bapC* mutants of *B. pertussis* strain Tab and our laboratory strain of Tohama were constructed by replacement of the *bapC* gene by allelic exchange with the *bapC* gene disrupted with a kanamycin-resistance (Km^r) cassette ([11] and Section 4.2). PCR was carried out on DNA extracted from selected transconjugant colonies to confirm that the wild-type *bapC* gene in the *B. pertussis* strains had been successfully replaced by the mutated *bapC* gene from the plasmid. PCR with primers BAPCF and BAPCR1 (Section 4.3) produced an amplicon of expected size (3.5 kbp) for *bapC*::Km^r and there was no evidence of a 2.2 kbp amplicon indicative of the native *bapC* gene present in the parent strains (data not shown). Southern blot analysis, using genomic DNA from the parent and *bapC* mutant strains digested with *Sac*I, showed that a *bapC*-specific probe (Section 4.3) hybridised to a fragment of *c.* 5.2 kbp in DNA preparations from the parent strains whereas it hybridised to a fragment of *c.* 6.5 kbp in both mutant strains (data not shown). The size difference (1.3 kbp) corresponded to the size of the inserted Km^r cassette.

Expression of *bapC* in the parent and mutant strains was investigated by RT-PCR with primers BAPCF and BAPCR2 (Section 4.4), expected to amplify a 505 bp fragment from the 3′ region of *bapC*. The RT-PCR result (Fig. 1) indicated that *bapC* was expressed (505 bp product) in the Tab and Tohama parent strains (lanes 3 and 5) but not in their corresponding *bapC* mutants (lanes 4 and 6), or in the *bvg* mutant strain BP338 *bvg* used as a control (lane 7). The lack of transcript with BP338 *bvg* clearly indicates that BapC expression is regulated by *bvg*. Lanes 1 and 2 show PCR products, with the same primers, obtained with genomic DNA from the Tab *bapC* mutant (505 bp + 1300bp Km^r cassette) and its parent strain (505 bp), respectively.

Next, *bapC* and *brkA*, *bapC* mutants were created in the same genetic background as a *brkA* mutant already available [12] to compare directly the role of BapC and BrkA. To do this, the *bapC* genes in *B. pertussis* strain BP338 (a Tohama derivative) and in the BP338 *brkA* mutant (BP2041) were replaced, by allelic exchange, with a *bapC* gene disrupted with a tetracycline-resistance (Tc^r) cassette (section 4.2). This was used instead of a Km^r cassette which had been used previously to create the *bapC* mutants of strains Tab and Tohama because strain BP338 *brkA* had been created by Tn5 insertion, which encodes Km^r, into the *brkA* gene [12]. After conjugation between the *Escherichia coli* mobilising strain SM10(λ_{pir}) carrying suicide plasmid pSS1129 *bapC*::Tc^r and streptomycin-resistant derivatives of BP338 and BP338 *brkA*, PCR was carried out on DNA extracted from selected transconjugant colonies to confirm that the wild-type *bapC* gene in the *B. pertussis* strains had been successfully replaced by the mutated *bapC* gene from the plasmid. PCR with primers BAPCF and BAPCR1 produced an amplicon of expected size (3.5 kbp) for *bapC*::Tc^r and there was no evidence of a 2.2 kbp amplicon indicative of the native *bapC* gene present in the parent strains (data not shown). The presence of the Tc^r gene in the amplified 3.5 kbp *bapC*::Tc^r fragment, after gel extraction, was shown by PCR amplification of the 1.3 kbp Tc^r cassette with primers TCF1 and TCR1 (Section 4.3) (data not shown). Southern blot analysis, using genomic DNA from the parent and *bapC* mutant strains digested with *Xho*I, showed that a *bapC*-specific probe hybridised to an approximately 7.7 kbp fragment in DNA preparations from both parent strains, whereas it hybridised to an approximately 9 kbp fragment in both mutant strains, BP338 *bapC* and BP338 *brk, bapC* (data not shown). The size difference (1.3 kbp) corresponds to the size of the inserted Tc^r cassette.

2.2. Serum resistance of the parent and mutant strains

A preliminary test was performed to compare the number of *B. pertussis* survivors when exposed to phosphate-buffered saline (PBS) or to a 1/40 dilution of the heat-inactivated (56 °C for 30 min) pooled human serum. There was no significant killing, and no significant difference ($P > 0.05$) between these two controls in a time-course study over 120 min (data not shown). This revealed that any agglutination by antibodies present in the human sera did not significantly affect the bacterial counts. After exposure to a 1/40 dilution of the unheated pooled human serum for up to 45 min (Fig. 2A), the *B. pertussis* BP338 parent strain survived much better than the *bapC*, *brkA* or *brkA*, *bapC* mutants. This better survival was also evident after longer exposure to normal human serum, for up to 120 min (data not shown). As shown in Fig. 2A, the *brkA, bapC*

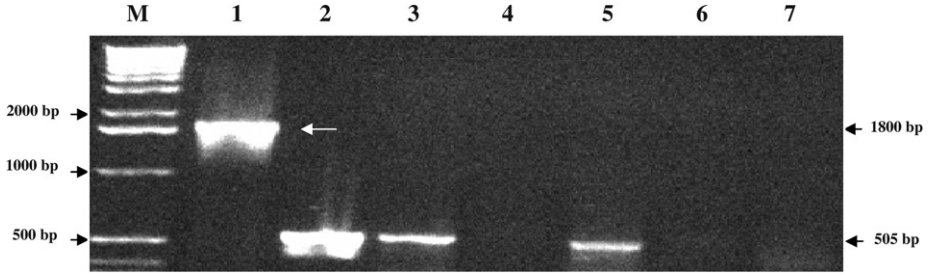

Fig. 1. RT-PCR with primers BAPCF and BAPCR2 using RNA prepared from *B. pertussis* wild-type strains, *bapC* mutants and *B. pertussis* strain BP338 *bvg*. M = markers, lane 1, PCR control with genomic DNA from *B. pertussis* Tab *bapC*; lane 2, PCR control with genomic DNA from *B. pertussis* Tab; lanes 3–7, RT-PCR with RNA from the *B. pertussis* strains: lane 3, BP Tab; lane 4, Tab *bapC*; lane 5, Tohama; lane 6, Tohama *bapC*; lane 7, BP338 *bvg*.