Cloning, nucleotide sequence and heterologous expression of the protective outer-membrane protein P.68 pertactin from *Bordetella bronchiseptica*

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The *prn* gene encoding the 68 kDa protective outer-membrane protein of *Bordetella bronchiseptica* (P.68 pertactin) was cloned, sequenced and expressed in *Escherichia coli*. The gene was isolated by DNA:DNA hybridization experiments using a radioactively-labelled fragment of the homologous *prn* gene from *Bordetella parapertussis*. DNA sequence analysis reveals that the gene is capable of encoding a protein with a molecular mass of 93996 Da (P.94); this precursor molecule is processed to form the P.68 antigen on the surface of *B. bronchiseptica*. Heterologous expression of the full-length gene encoding P.94 in *Escherichia coli* results in similar processing, with the P.68 antigen targeted to the bacterial outer membrane. Comparison of P.94 with the P.93 and P.95 precursors, encoding homologous proteins from *Bordetella pertussis* and *B. parapertussis*, shows a high degree (>90%) of homology. The major differences between all three proteins occur in the number of repeats of the two families (Gly-Gly-Xaa-Xaa-Pro)_n and (Pro-Gln-Pro)_n of reiterated sequence motifs.

Introduction

Bordetella bronchiseptica (Pittman, 1984) is a bacterial pathogen associated with respiratory diseases in animals, particularly atrophic rhinitis in pigs (Harris & Switzer, 1968; Goodnow, 1980). The disease state is recognised by severe changes in the nasal architecture of developing piglets, pneumonia and growth retardation (Harris & Switzer, 1968). We have demonstrated that protection against *B. bronchiseptica*-mediated atrophic rhinitis correlates with the presence of an outer-membrane protein with a molecular mass of 68 kDa as determined

by SDS-PAGE (Novotny *et al.*, 1985*b*). Antibodies to this 68 kDa protein can be detected in high titre in protected piglets, while the titre is low or absent from non-protected animals (Novotny *et al.*, 1985*b*). Furthermore, a naturally occurring mutant of *B. bronchiseptica* lacking the 68 kDa protein is unable to protect piglets when used as a vaccine and is unable to induce pathological changes in infected piglets (Novotny *et al.*, 1985*b*). Administration of a vaccine consisting of a purified preparation of the 68 kDa protein to pregnant sows results in significant protection of the resulting piglets when challenged with virulent *B. bronchiseptica* (Kobisch & Novotny, 1990). In addition, passively administered monoclonal antibody BB05 (Novotny *et al.*, 1985*a*, specific for the 68 kDa protein) was able to prevent death from pneumonia and development of atrophic rhinitis in mice that had been aerosol-infected with a virulent strain of *B. bronchiseptica*.

The 68 kDa protein is a member of an immunologically cross-reactive family of *Bordetella* spp. outer-membrane proteins that are represented in *Bordetella parapertussis* as a 70 kDa moiety (P.70) and in *Bordetella pertussis* as a 69 kDa (P.69) species. The P.69 antigen has been demonstrated to be a protective antigen against *B. pertussis* infections in a mouse aerosol model (Shahin *et al.*, 1990) and in the mouse intracerebral challenge model (Novotny *et al.*, 1985*a*) and is currently under evaluation as a component of future acellular vaccines against

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Abbreviations: The designations P.68/P.94, P.69/P.93, P.70/P.95 refer to the pertactin family of outer-membrane proteins expressed in *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* respectively with molecular masses of 68, 69 and 70 kDa. The initial number (e.g. P.68) refers to the apparent molecular mass (kDa) of the processed form of the antigen that appears on the bacterial surface, while the second number (e.g. P.94) refers to the molecular mass of the unprocessed precursor as inferred from the DNA sequence.

The nucleotide sequence reported in this paper has been submitted to the EMBL Database and has been assigned the accession number X54815.

whooping cough (Novotny *et al.*, 1991; Romanos *et al.*, 1991). P.69 has also been shown to be an agglutinin (Brennan *et al.*, 1988) that can promote attachment to certain eukaryotic cells via an Arg-Gly-Asp (RGD) tripeptide sequence (Leininger *et al.*, 1991). Such sequences have been characterized for a number of eukaryotic cell-cell (Ruoslahti *et al.*, 1988) and virus-cell (Fox *et al.*, 1989) attachment processes. Because of the cell attachment behaviour of the P.69 antigen it has been renamed pertactin, the product of the *prn* gene. Nothing is known about the immunological properties of the P.70 antigen from *B. parapertussis*, although it shares high homology with the P.69 protein (Li *et al.*, 1991; Charles *et al.*, 1989) including a putative RGD binding site.

In this paper we report the cloning and characterization of the gene encoding the 68 kDa antigen from *B. bronchiseptica* (P.68 pertactin) and show it to be highly homologous to its counterparts from *B. pertussis* and *B. parapertussis*. Expression of the full-length gene, capable of encoding a protein with a calculated molecular mass of 93996 Da, in *Escherichia coli* results in the appearance of a 68 kDa form of the protein on the bacterial outer membrane. Native P.68 antigen can only be purified at low levels from cultures of *B. bronchiseptica*, but by utilizing a recombinant approach we hope to be able to produce larger samples of P.68 antigen that are completely uncontaminated by other *B. bronchiseptica* components. This will facilitate the understanding of both the molecular biological and immunological properties of this molecule.

Methods

Bacterial strains, plasmids and phage. *B. bronchiseptica* strain CN7531 was from the Wellcome culture collection (Wellcome Biotech, UK). *E. coli* K12 strains TG1 and HB101 were as previously described (Carter *et al.*, 1985; Boyer & Roulland-Dussoix, 1969). Cosmid pH79 (Hohn & Collins, 1980) was from Amersham International. Plasmid pKK233-2 (Amann & Brosius, 1985) and M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) were supplied by Pharmacia. The P.69 and P.70 expression plasmids pAYL1 and pBD845 have been described previously (Li *et al.*, 1991). Plasmid pBSSKII⁺ (Bluescript) was from Stratagene. *E. coli* strains were grown in Luria broth (LB) or LB solidified with 1.6% (w/v) agar (Miller, 1972). *B. bronchiseptica* was grown in Stainer-Scholte (Stainer & Scholte, 1962) broth or medium as previously described (Novotny *et al.*, 1985a).

DNA isolation and manipulations. *B. bronchiseptica* CN7531 chromosomal DNA was prepared as described previously (Hull *et al.*, 1981). Plasmid and bacteriophage DNA were isolated and purified by standard methods (Maniatis *et al.*, 1982). All DNA-modifying enzymes were from Gibco BRL.

Construction of a *B. bronchiseptica* genomic library. Cosmid pH79 DNA was digested with *Bam*HI and ligated with *Sau*3A-digested *B. bronchiseptica* DNA in the 40–50 kb size range as described previously (Charles *et al.*, 1990). The gene bank was plated out and 480 randomly selected colonies transferred to microtitre plates as described previously (Charles *et al.*, 1990). Colonies were transferred to Gene Screen

Plus hybridization membranes (Du Pont) and hybridized with an α^{32} P-labelled *Cla*I fragment of the *prn* gene encoding the P.70 protein from *B. parapertussis* (Li *et al.*, 1991) as described previously.

Subcloning and DNA sequencing. Recombinant phage DNA was sequenced after ligation of specific restriction endonuclease fragments of cosmid into the vectors M13mp18 and M13mp19. Sequencing was carried out using universal primer, [α^{35} S]dATP and both gradient and wedge gels (Biggin *et al.*, 1983; Sanger *et al.*, 1977). Some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, 1987) and 7-deaza-2-dGTP (Mizusawa *et al.*, 1986) using a kit supplied by Pharmacia. Gaps in the sequence were filled in using synthetic oligonucleotides made on a MilliGen 7500 DNA synthesizer (Millipore, UK) as specific primers (Charles *et al.*, 1986).

Amino acid sequencing and immunological characterization. P.68 pertactin was purified as described previously (Novotny *et al.*, 1985b) and protein sequence determined by using an Applied Biosystems 470-A gas-phase microsequencer. *E. coli* lysates harbouring recombinant P.68, P.69 and P.70 protein were prepared by harvesting 5 ml overnight cultures of *E. coli* TG1 grown in L-broth harbouring either pBD881 (P.68), pAYL1 (P.69) or pBD845 (P.70). Cell pellets were washed once in L-broth and 0.1 OD₆₅₀ units were subjected to SDS-PAGE (Maniatis *et al.*, 1982). Proteins were transferred to nitrocellulose by the method of Towbin *et al.* (1979). The monoclonal antibody BB05 (Montaraz *et al.*, 1985; Novotny *et al.*, 1985a) was used to detect P.68 protein using horse-radish-peroxidase-conjugated goat antimouse second antibody and 4-chloro-1-naphthol as substrate (Fairweather *et al.*, 1986). To test for surface expression of recombinant P.68, slide agglutination assays were carried out as described previously (Li *et al.*, 1991).

Results

Isolation of the gene encoding the P.68 antigen

The *B. bronchiseptica* genomic library was screened by hybridization with a radioactively-labelled 1.8 kb *Cla*I fragment of the *prn* gene encoding the P.70 antigen from *B. parapertussis* (Li *et al.*, 1991). Of the two cosmids returning a positive signal, one, designated pBD844, was selected for further analysis. Restriction mapping and Southern blotting experiments carried out on pBD844 demonstrated that the *Cla*I and *Sal*I hybridization pattern of the *prn* gene from *B. bronchiseptica* was identical to that described for both *B. pertussis* (Charles *et al.*, 1989) and *B. parapertussis* (Li *et al.*, 1991). These *Cla*I and *Sal*I fragments were therefore assumed to contain the entire *prn* gene encoding the P.68 pertactin and were cloned into Bluescript pBSSKII⁺ to generate pBD875 (*Cla*I) and pBD856 (*Sal*I); they were also cloned into M13mp18 and M13mp19 for DNA sequencing.

Nucleotide sequence of the *prn* gene encoding P.68: comparison with the equivalent genes from *B. pertussis* and *B. parapertussis*.

The DNA sequence was obtained initially from the *Cla*I and *Sal*I fragments cloned into M13 and using universal

Fig. 1. DNA sequence of the *prn* gene encoding the P.68 pertactin from *B. bronchiseptica*. Downward arrows indicate the sites of two possible protein cleavage regions that result in the production of the mature polypeptide. Arrows over protein sequence denotes two protein repeat motifs: (i) (GXXP)₃ encoded by nucleotides 939–983 and (ii) (PQP)₇ encoded by nucleotides 1852–1947. An RGD tripeptide sequence encoded by nucleotides 922–930 that occurs in the surface-exposed processed P.68 form of the protein is shown overlined. An additional RGD tripeptide motif occurs between residues 2245–2253 within the C-terminal fragment of P.94 that is clipped off during the processing of P.68. An inverted repeat followed by a run of T-residues, resembling a rho-independent transcription terminator, is shown between residues 2898–2909.

P. 68	MNMSLSRIVLAAPLRRTTAMALGALGAAPAYADWNNQSIKAGERQHGIHIKQSDGAGVRTATGTTIK	70aa.
P. 70	K.....Y.....I.A.....KQ..GA....T....	70aa.
P. 69	K.....H.....V.T.....QG..PG....S....	70aa.
P. 68	VSGRQAQGVLEENPAELRFQNGSVTSSGQLFDEGVRRFLGTVTVKAGKLVADHATLANVSDTRDDGIA	140aa.
P. 70	V.....R.Q.....F.E.V.....S..R.....	140aa.
P. 69	I.....Q.R.....S.D.I.....G..W.....	140aa.
P. 68	LYVAGEQAQASIA DSTLQAGGVVRVERGANVTQVSTIVDGGHIGTLQPLQPEDLPPSRVVLGDTSVTA	210aa.
P. 70	RV.....T.....T..P.....G..S...	210aa.
P. 69	QI.....A.....A..S.....R..N...	210aa.
P. 68	VPASGAPAAVSVF GANELTV DGGHITGGRAAGVAAMDGAIVHLQRATIRRGDAPAGGAVPGGAVPGG***	277aa.
P. 70	F.F..N...V.....D..I.....AVP	280aa.
P. 69	S.L..S...L.....Q..V.....AVP	280aa.
P. 68	*****FGPLLDGWYGVDSVSTVDLAQSIVEAPQLGAAIRAGRGARVTVSGGSLSAPHGNVIETGGGA	340aa.
P. 70	*****GG...L.....D.T.D.....Q.....A.....G.	345aa.
P. 69	GGFGPGG...V.....G.S.E.....E.....V.....*	349aa.
P. 68	RRFPPPASPLSITLQAGARAQGRALLYRVLPEPVKLTLAGGAQGGDIVATELPPIPGASSGPLDVALAS	410aa.
P. 70	...P.P.S.....R..R.....A...QG.....P..A.S.....	415aa.
P. 69	...A.Q.A.....H..K.....T...DA.....S..T.I.....	419aa.
P. 68	QARWTGATRAVDSLSIDNATWVMTDNSNVGALRLASDGSVDFQQPAEAGRFKCLMVDTLAGSGLFRMNVF	480aa.
P. 70	V.M.D.....	485aa.
P. 69	V.T.N.....	489aa.
P. 68	ADLGLSDKLVVMRDASGQHRLLVRNSGSEPASGNTMLLVQTPRGSAAFTTLANKDGKVDIGTYRYRLAAN	550aa.
P. 70	R.....W.....G..M.....R.....	555aa.
P. 69	Q.....W.....A..L.....L.....	559aa.
P. 68	GNGQWSLVGAKAPPAPKPAPQPGPQPG*****PQPPQPPQPPQPPQPEAPAPQPPAGRELSAAANAA	614aa.
P. 70	GPQPPQPPQPPQPPQ.....R.....	625aa.
P. 69	*****.....*	613aa.
P. 68	VNTGGVGLASTLWYAESNLSKRLGELRLNPDAGGAWGRGFAQRQQLDNRAGRRFDQKVAGFELGADHAV	684aa.
P. 70		695aa.
P. 69		683aa.
P. 68	AVAGGRWHLGGLAGYTRGDRGFTGDGGHTDSVHVGGYATYIANS GFYLDATLRASRLNDFKVAGSDGY	754aa.
P. 70	N.....	765aa.
P. 69	D.....	753aa.
P. 68	AVKGKRYRTHGVGASLEAGRRFAHADGWFLEPQAEALAVFRVGGGSYRAANGLRVRDEGGSSVLGRLGLEVG	824aa.
P. 70	V.....A.....V...A.....	835aa.
P. 69	A.....T.....A..A.....	823aa.
P. 68	KRIELAGGRQVQPYIKASVLQEPDGAGTVRTNGIAHRTELRTRAELGLGMAAALGRGHSLYASVEYSKG	894aa.
P. 70	R.....	905aa.
P. 69	H.....	893aa.
P. 68	PKLAMPWTFHACYRYSW	911aa.
P. 70		922aa.
P. 69		910aa.

Fig. 2. Protein sequence alignment of the P.68, P.70 and P.69 pertactins from *B. bronchiseptica*, *B. parapertussis* and *B. pertussis*, respectively. Downward arrows represent the position of likely protein cleavage sites. *, Missing residue within the alignment. The P.69 sequence has two corrections from the version published in Charles *et al.*, 1989; these are (i) at amino acid 264, where proline replaces leucine, and (ii) at amino acid 322 where serine replaces proline. These corrections have been reported to the sequence data bases.

primer; the overlapping sequence was compiled using a set of synthetic oligonucleotides as specific sequence primers. Computer analysis of the DNA sequence (Fig. 1) demonstrates that the ORF for the *prn* gene of P.68 pertactin is capable of encoding a protein with a molecular mass of 93996 Da (P.94). The molecular mass

of the mature protein found on the surface of *B. bronchiseptica* is, however, 68000 Da as judged by SDS-PAGE, suggesting that this molecule is a processed form of the larger precursor. By comparison with the P.69 antigen, this processing is likely to involve the removal of both N-terminal and C-terminal sequences. N-terminal

protein sequencing of purified preparations of P.68 from *B. bronchiseptica* gave the sequence Asp-Trp/Gln-Asn-Asn-Gln-Gln/Ser-Ile-Xaa-Lys-Ala. This sequence, despite having three regions of ambiguity, aligns with amino acid residues 35–44 in Fig. 2 and confirms that in common with the P.70 and P.69 sequences, a signal peptide is cleaved in the maturation process of P.68 pertactin. Cleavage of this signal peptide, of between 32 or 34 amino acid residues depending on which ATG initiation codon is used, occurs after the sequence Ala-Tyr-Ala, which is in agreement with the Ala-Xaa-Ala motif reported to be recognized by *E. coli* signal peptidases (Perlman & Halvorson, 1983). Towards the C-terminus of the P.68 protein at residues 602–603 (numbering the Asp produced after signal peptide cleavage as residue 1) is a dibasic pair of amino acids, Lys-Arg, that occur in the same position, and are flanked by identical residues, as are found in the P.69 pertactin from *B. pertussis*. This Lys-Arg sequence in *B. pertussis* was originally identified as a putative cleavage site involved in the production of the mature form of the protein (Makoff *et al.*, 1990). More recently, Capiou *et al.* (1990) have reported that purified preparations of P.69 have a C-terminal Asn residue. This Asn residue at amino acid residue position 597 is closely positioned to the Lys-Arg motif in the sequence Asn-Ala-Leu-Ser-Lys-Arg (Fig. 2). The precise processing events responsible for the generation of this C-terminal sequence are still unknown.

A comparison of the deduced protein sequences for the P.68, P.69 and P.70 proteins (Fig. 2) demonstrates the high degree of homology between the proteins. A comparison between the P.68 and P.70 proteins shows only 17 amino acid differences, while a similar comparison between P.68 and P.69 shows 80 differences and between P.69 and P.70 there are 79 differences. The majority of amino acid differences between the three deduced protein sequences occur in the number of repeat units in the two families of repeat sequences present in all three proteins. Thus, P.68 has three copies of the Gly-Gly-Xaa-Xaa-Pro repeat while P.70 has four and P.69 five. Similarly, P.68 has seven Pro-Gln-Pro repeats, P.70 has nine and P.69 has five. These findings suggest that the P.68 sequence from *B. bronchiseptica* and the P.70 sequence from *B. parapertussis* are more closely related to each other than either are to the P.69 sequence from *B. pertussis*. The finding of close sequence homology between *B. bronchiseptica* and *B. parapertussis* is in agreement with the evolutionary trees produced by Altschul (1989), based on a re-analysis of the sequence of the pertussis toxin operons from all three Bordetella species (Arico *et al.*, 1987).

Examination of the first 140 bp of the DNA sequence in Fig. 1 shows that there are no good fits with either the

consensus ribosome binding site (Shine & Dalgarno, 1975) or consensus promoter elements identified for *E. coli* genes (Rosenberg & Court, 1979). In common with the genes encoding both P.68 and P.69, there is an inverted repeat after the stop codon, and then a run of T-residues. This sequence resembles the rho-independent-like terminator that is also found at the end of the *B. pertussis* *ptx* operon (Charles *et al.*, 1989).

Heterologous expression of P.68 pertactin in E. coli

Cosmid pBD844 harbouring the *B. bronchiseptica* *prn* gene was unable to direct the expression of P.68 pertactin in *E. coli*. To express the gene in *E. coli*, the entire structural gene was cloned into the expression plasmid pKK233-2. Fig. 3 outlines the expression strategy; an *EcoRI*–*EcoRV* fragment comprising the 5' end of P.68 *prn* from plasmid pBD875 was ligated into *EcoRV*–*EcoRI* digested pBD856 (containing the 3' end of P.68 *prn*) to generate pBD876 containing the entire structural gene. Plasmid pBD876 was digested with *Afl*III–*Hind*III and ligated with *Nco*I–*Hind*III-digested pKK233-2. This ligation mixture was used to transform *E. coli* TG1 and transformants expressing P.68 pertactin identified by their ability to cross-react with the mAb BB05 in protein dot-blots.

Colonies returning a positive signal were isolated and plasmid minipreps carried out to verify that a full-length insert had been cloned. One such plasmid, pBD881, was selected for further study. Fig. 4 shows the result of a Western blot of an SDS-PAGE gel of *E. coli* TG1 harbouring (1) pBD845 (P.70/P.95); *B. parapertussis*, (2) pAYL1 (P.69/.93); *B. pertussis*, (3) pBD881 (P.68/P.94); *B. bronchiseptica* and (4) pKK233-2. The arrows represent the position of bands corresponding to proteins with molecular masses of 70 kDa (*B. parapertussis*), 69 kDa (*B. pertussis*) and 68 kDa (*B. bronchiseptica*). For both the plasmids expressing the P.70/P.95 and P.69/P.93 proteins, larger bands corresponding to the full-length 95 kDa and 93 kDa species are present in addition to a large number of lower molecular mass bands. Surprisingly, the P.68 expression construct does not produce a detectable 94 kDa higher molecular mass band, but instead produces a pair of stronger bands at around 69 kDa and 68 kDa. The pattern of bands produced for P.69 and P.70 pertactin expression differs from that reported in our earlier work (Li *et al.*, 1991). This discrepancy may be due to the different growth conditions used in preparing the lysates for Western blotting. The constructs pBD881, pBD845 and pAYL1 direct the expression of P.68, P.69 and P.70, respectively, under the control of the *tac* promoter and IPTG induction. In preliminary experiments, the use of IPTG

Fig. 3. Line drawing representing the strategy used to generate the P.68 expression plasmid pBD881. Plasmids pBD875 and pBD856 contain the 5' and 3' sections of the gene encoding *B. bronchiseptica* *prn*, respectively, and were used to generate the full-length gene encoding P.94. This full-length (non-expressing) gene harboured by pBD876 was excised by digestion with *Afl*III and *Hind*III and ligated with *Nco*I-*Hind*III-digested pKK233-2 to generate the P.68 expressing plasmid pBD881.

to induce pertactin expression led to the accumulation of unprocessed P.94, P.93 and P.95 forms of the proteins as insoluble inclusion bodies (data not shown). In our previous work (Li *et al.*, 1991) lysates were prepared from bacteria grown in M9 minimal medium to avoid this overexpression problem. The lysates used in this study and shown in Fig. 4 were prepared from bacteria grown in L-broth. The partial induction of the promoters under

these conditions leads to the accumulation of full-length forms of P.69/93 and P.70/95 that are not processed.

To test whether or not the P.68 protein was expressed on the surface of *E. coli*, slide agglutination assays were carried out using both monoclonal antibody BB05 and polyclonal anti-P.69 antibody (Li *et al.*, 1991). Agglutination occurred just as rapidly with *E. coli* harbouring pBD881 as with *vir*⁺ *B. bronchiseptica*, suggesting that the

Fig. 4. Western blot showing the expression of the pertactin family of proteins in *E. coli* TG1. Samples were prepared as described in Methods and reacted with the monoclonal antibody BB05. Lanes: 1, P.70/P.95 pertactin; 2, P.69/P.93 pertactin; 3, P.68/P.94 pertactin; 4, (negative control), pKK233-2. Arrows indicate positions of protein bands corresponding to the 70 kDa, 69 kDa and 68 kDa species. There is no signal from the negative control (track 4). In lanes 1 and 2, heavy bands can be seen just below the 97 kDa molecular mass marker. These bands probably correspond to the full-length protein precursors for P.70 and P.69. By contrast, there is no apparent band for the full-length P.68 precursor. For all three proteins, lower molecular mass bands cross-reacting with BB05 monoclonal antibody indicate that the proteins are substantially degraded in *E. coli*.

heterologous protein is surface-located. Both the positive control strains of *E. coli* expressing P.69 (pAYL1) and P.70 (pBD845) on their surface were able to demonstrate agglutination (Li *et al.*, 1991).

Discussion

Protection against *B. bronchiseptica*-mediated atrophic rhinitis in animals correlates with the presence of a 68 kDa protein (Novotny *et al.*, 1985*b*). In this paper we report the cloning, sequencing and expression of this protective antigen in *E. coli*. The *B. bronchiseptica* P.68

antigen belongs to a family of outer membrane immunologically cross-reactive proteins. In *B. pertussis*, a homologous protein has been characterized with a molecular mass of 69 kDa, as judged by PAGE, and has been the subject of much recent study as a potential candidate for inclusion in new generations of acellular whooping cough vaccines (Charles *et al.*, 1989, 1991; Shahin *et al.*, 1990; Novotny *et al.*, 1991; Romanos *et al.*, 1991; Roberts *et al.*, 1992). So far there has been little study of the equivalent P.70 protein from *B. parapertussis*, although we have recently isolated and sequenced the gene encoding the protein (Li *et al.*, 1991). A comparison between the deduced sequences for the P.68, P.69 and P.70 pertactins reveals a high degree of homology, with the *B. parapertussis* and *B. bronchiseptica* proteins being more similar to each other than to the P.69 protein from *B. pertussis*. The main regions of sequence divergence between the three proteins occur in the number of repeats within the repeated regions. The region spanning the Pro-Gln-Pro family of repeats has been demonstrated to be the immunodominant part of the P.69 molecule (Charles *et al.*, 1991). Differences in this region may have significance in determining the degree of immunological cross-reactivity between the different molecules, and may account for the observation that sera from piglets that cross-reacted in ELISA with preparations of P.68 failed to react with P.69 (Kobisch & Novotny, 1990) despite the high degree of homology between the proteins.

It has been demonstrated that the P.69 protein has the ability to bind to CHO cells in culture. This binding is mediated, at least in part, by an Arg-Gly-Asp (RGD) tripeptide sequence; a mechanism that has been characterized for a variety of adhesive proteins (Ruoslahti, 1988) including *B. pertussis* filamentous hemagglutinin (Relman *et al.*, 1990) and may suggest that P.69 has a role in bacterial adhesion. The finding of a similar RGD sequence in the P.68 pertactin also suggests that this protein may have a role in bacterial adhesion, and thus play a role in virulence. The expression of the P.68 protein on the surface of *E. coli* should enable us to produce recombinant protein that is uncontaminated by other *B. bronchiseptica* components for both immunological and cell-adhesion evaluation.

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References

- ALTSCHUL, S. F. (1989). Evolutionary trees for the genus *Bordetella*. *Journal of Bacteriology* **171**, 1211–1213.
- AMMAN, E. & BROSIUS, J. (1985). 'ATG' vectors for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* **40**, 183–190.

- ARICO, B., GROSS, R., SMIDA, J. & RAPPUOLI, R. C. (1987). Evolutionary relationships in the genus *Bordetella*. *Molecular Microbiology* **1**, 301–308.
- BIGGIN, M. D., GIBSON, T. J. & HONG, G. F. (1983). Buffer gradient gels and $\alpha^{35}\text{S}$ label as an aid to rapid DNA sequence determination. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 3963–3965.
- BOYER, H. W. & ROULLAND-DUSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology* **41**, 459–465.
- BRENNAN, M. J., ZHONG, M. L., COWELL, J. L., BISHER, M. E., STEVEN, A. C., NOVOTNY, P. & MANCLARK, C. R. (1988). Identification of a 69-kilodalton non-fimbrial protein as an agglutinin of *Bordetella pertussis*. *Infection and Immunity* **56**, 3189–3195.
- CAPIAU, C., CARR, S. A., HEMLING, M. E., PLAINCHAMP, D., CONRATH, K., HAUSER, P., SIMOEN, E., COMBERBACH, M., ROELANTS, P., DESMONS, P., PERMANNE, P. & PETRE, J. O. (1990). Purification, characterisation and immunological evaluation of the 69-kDa outer membrane protein of *Bordetella pertussis*. In *Proceedings of the Sixth International Symposium on Pertussis* [DHHS Publication no. (FDA) 90-1164], pp. 75–86. Edited by C. R. Manclark. Bethesda, Maryland: Department of Health and Human Services, United States Public Health Service.
- CARTER, P., BEDOUELL, H. & WINTER, G. (1985). Improved oligonucleotide site directed mutagenesis using M13 vectors. *Nucleic Acids Research* **13**, 443–444.
- CHARLES, I. G., KEYTE, J., BRAMMAR, W. J., SMITH, M. & HAWKINS, A. R. (1986). The isolation and nucleotide sequence of the complex AROM locus of *Aspergillus nidulans*. *Nucleic Acids Research* **14**, 2201–2213.
- CHARLES, I. G., DOUGAN, G., PICKARD, D., CHATFIELD, S., SMITH, M., NOVOTNY, P., MORRISSEY, P. & FAIRWEATHER, N. F. (1989). Molecular cloning and characterization of protective outer membrane protein P.69 from *Bordetella pertussis*. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3554–3558.
- CHARLES, I. G., LAMB, H. K., PICKARD, D., DOUGAN, G. & HAWKINS, A. R. (1990). Isolation, characterization and nucleotide sequences of the *aroC* genes encoding chorismate synthase from *Salmonella typhi* and *Escherichia coli*. *Journal of General Microbiology* **136**, 353–358.
- CHARLES, I. G., LI, J., ROBERTS, M., BEESLEY, K., ROMANOS, M., PICKARD, D. J., FRANCIS, M., CAMPBELL, D., DOUGAN, G., BRENNAN, M. J., MANCLARK, C. R., AU JENSEN, M., HERON, I., CHUBB, A., NOVOTNY, P. & FAIRWEATHER, N. F. (1991). Identification and characterisation of a protective immunodominant B-cell epitope of pertactin from *Bordetella pertussis*. *European Journal of Immunology* **21**, 1147–1153.
- FAIRWEATHER, N. F., LYNNESS, V. A., PICKARD, D., ALLEN, G. & THOMSON, R. O. (1986). Cloning, nucleotide sequencing and expression of tetanus toxin fragment C in *Escherichia coli*. *Journal of Bacteriology* **165**, 21–27.
- FOX, F., PARRY, N. R., BARNETT, P. V., MCGINN, B., ROWLANDS, D. J. & BROWN, F. (1989). The cell attachment site on Foot-and-Mouth disease virus includes the amino acid sequence RGD (Arginine–Glycine–Aspartic Acid). *Journal of General Virology* **70**, 625–637.
- GOODNOW, R. A. (1980). Biology of *Bordetella bronchiseptica*. *Microbiological Reviews* **44**, 722–738.
- HARRIS, D. L. & SWITZER, W. P. (1968). Turbinate atrophy in young pigs exposed to *Bordetella bronchiseptica*, *Pasteurella multocida* and combined inoculum. *American Journal of Veterinary Research* **29**, 777–785.
- HOHN, B. & COLLINS, J. (1980). A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**, 291–298.
- HULL, R. A., GILL, R. E., HSU, P., MINSHEW, B. H. & FALKOW, S. (1981). Construction and expression of recombinant plasmids encoding type 1 or D-mannose resistant pili from a urinary tract infection *Escherichia coli*. *Infection and Immunity* **33**, 933–938.
- KOBISCH, M. & NOVOTNY, P. (1990). Identification of a 68-kilodalton outer membrane protein as the major protective antigen of *Bordetella bronchiseptica* by using specific-pathogen-free piglets. *Infection and Immunity* **58**, 352–357.
- LEININGER, E., ROBERTS, M., KENIMER, J. G., CHARLES, I., FAIRWEATHER, N., NOVOTNY, P. & BRENNAN, M. J. (1991). Pertactin, an Arg–Gly–Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 345–349.
- LI, J. L., DOUGAN, G., NOVOTNY, P. & CHARLES, I. G. (1991). P.70 pertactin an outer membrane protein from *Bordetella pertussis*: cloning, nucleotide sequence and surface expression in *Escherichia coli*. *Molecular Microbiology* **5**, 409–417.
- MAKOFF, A. J., OXER, M. D., BALLANTINE, S. P., FAIRWEATHER, N. F. & CHARLES, I. G. (1990). Protective surface antigen P.69 of *Bordetella pertussis*: its characterization and very high level expression in *Escherichia coli*. *Bio/Technology* **8**, 1030–1033.
- MANIATIS, T., FRITSCH, E. E. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MILLER, J. M. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MONTARAZ, J. A., NOVOTNY, P. & IVANYI, J. (1985). Identification of a 68-kilodalton protective antigen from *Bordetella bronchiseptica*. *Infection and Immunity* **47**, 744–751.
- MIZUSAWA, S., NISHIMURA, S. & SEELA, F. (1986). Improvements of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. *Nucleic Acids Research* **14**, 1319–1324.
- NOVOTNY, P., CHUBB, A. P., COWNLEY, K., MONTARAZ, J. A. & BEESLEY, J. E. (1985a). *Bordetella* adenylate cyclase: a genus-specific protective antigen and virulence factor. In *Proceedings of the Fourth International Symposium on Pertussis. Development of Biological Standards* **61**, 27–41.
- NOVOTNY, P., KOBISCH, M., COWNLEY, K., CHUBB, A. P. & MONTARAZ, J. A. (1985b). Evaluation of *Bordetella bronchiseptica* vaccines in specific-pathogen-free piglets with bacterial cell surface antigens in enzyme-linked immunosorbent assay. *Infection and Immunity* **50**, 190–198.
- NOVOTNY, P., CHUBB, A. P., COWNLEY, K. & CHARLES, I. G. (1991). Biological and protective properties of the 69-kDa outer membrane protein of *Bordetella pertussis*: a novel formulation for an acellular pertussis vaccine. *Journal of Infectious Diseases* **164**, 114–122.
- PERLMAN, D. & HALVORSON, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic peptides. *Journal of Molecular Biology* **167**, 391–409.
- PITTMAN, M. (1984). Genus *Bordetella*. In *Bergey's Manual of Systematic Bacteriology*, vol. I, pp. 388–393. Edited by N. R. Kreig, J. G. Holt. Baltimore: Williams & Wilkins.
- RELMAN, D., TOUMANEN, E., FALKOW, S., GOLENBOCK, D. T., SAKKONEN, K. & WRIGHT, S. D. (1990). Recognition of a bacterial adhesin by an integrin: macrophage CR3 (MB₂, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell* **61**, 1371–1382.
- ROBERTS, M., TITE, J. P., FAIRWEATHER, N. F., DOUGAN, G. & CHARLES, I. G. (1992). Recombinant P.69/Pertactin: immunogenicity and protection of mice against *Bordetella pertussis*. *Vaccine* **10**, 43–48.
- ROMANOS, M. A., CLARE, J. J., BEELSEY, K. M., RAYMENT, F. B., BALLANTINE, S. P., MAKOFF, A. J., DOUGAN, G., FAIRWEATHER, N. F. & CHARLES, I. G. (1991). Recombinant *Bordetella pertussis* pertactin (P.69) from the yeast *Pichia pastoris*: high-level production and immunological properties. *Vaccine* **9**, 901–906.
- ROSENBERG, M. & COURT, D. (1979). Regulation sequences involved in the promotion and termination of RNA transcription. *Annual Review of Genetics* **13**, 319–353.
- RUOSLAHTI, E. (1988). Fibronectin and its receptors. *Annual Review of Biochemistry* **57**, 375–413.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 5463–5467.
- SHAHIN, R. D., BRENNAN, M. J., LI, Z. M., MEADE, B. D. & MANCLARK, C. R. (1990). Characterization of the protective capacity and immunogenicity of the 69 kD outer membrane protein of *Bordetella pertussis*. *Journal of Experimental Medicine* **171**, 63–73.

- SHINE, J. & DALGARNO, L. (1975). Determinant of cistron specificity in bacterial ribosomes. *Nature, London* **254**, 34–38.
- STAINER, D. W. & SCHOLTE, M. J. (1962). A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *Journal of General Microbiology* **63**, 211–220.
- TABOR, S. & RICHARDSON, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 polymerase. *Proceedings of the National Academy of Sciences in the United States of America* **84**, 4767–4771.
- TOWBIN, J., STAEBELIN, R. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.