Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis

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The gene for the osteolytic toxin of *Pasteurella multocida* has been cloned into a plasmid vector and expressed off its own promoter in *Escherichia coli*. Particular restriction endonucleases failed to cut the gene and regions flanking it, suggesting an A + T base ratio significantly greater than the remaining genome of *P. multocida*. Cloned toxin was indistinguishable from the native toxin with respect to molecular mass, antigenicity and toxicity in different tests. A single intraperitoneal injection of toxin purified from the recombinant *E. coli* reproduced in gnotobiotic pigs the pathological changes characteristic of atrophic rhinitis. The recombinant *E. coli* produced at least 10 times as much toxin as *P. multocida*.

Introduction

Atrophic rhinitis of growing pigs is characterized by atrophy of the nasal turbinate bones which, in the severest form of the disease, is complete and accompanied by the development of twisted or shortened snouts (Switzer & Farrington, 1975). The disease is also believed to cause a reduction in weight gain (Rutter, 1985). The severe form of the disease can be detected clinically whilst less severe forms may be seen only at slaughter when turbinate bones can be inspected. Only the severe condition will be referred to as atrophic rhinitis in this paper. Attempts to reproduce atrophic rhinitis were assisted by the discovery of toxigenic isolates of *Pasteurella multocida* (Il'ina & Zasukhin, 1975; De Jong *et al.*, 1980). These were identified by the toxicity of cell free extracts for mice and guinea pigs (De Jong *et al.*, 1980), and for cells grown *in vitro* (Rutter & Luther, 1984). Inoculation of pigs with cell free extracts of toxigenic *P. multocida* either intranasally (Il'ina & Zasukhin, 1975) or intraperitoneally (Rutter & MacKenzie, 1984) also reproduced the disease.

The toxin from *P. multocida*, purified by several groups (Nakai *et al.*, 1984; Chanter *et al.*, 1986b; Rimler & Brogden, 1986; Foged *et al.*, 1986; Kamp *et al.*, 1987), is a single polypeptide of molecular mass variously quoted as 112 to 160 kDa. Although purified toxin reproduced the symptoms of atrophic rhinitis (Chanter *et al.*, 1984b; Dominick & Rimler, 1986) its mode of action

is unknown. However, preliminary results suggest that it differs from other toxins (Chanter *et al.*, 1986c). It seemed likely that analysis of the toxin at the molecular level might provide important evidence about its mechanism of action. We describe here the cloning of the toxin gene in *Escherichia coli*, and the properties of its product.

Methods

Strains and growth conditions. *P. multocida* strain LFB3 is a toxigenic isolate from a pig with atrophic rhinitis (Rutter, 1983). *E. coli* HB101 harbouring plasmid pAT153 was from Dr J. G. Williams, Imperial Cancer Research Fund, London, UK. All bacteria were stored as cell suspensions at -70°C in 12% (v/v) glycerol. *P. multocida* were grown in Bacto-tryptose broth (Jones & Matthews, 1975) at 37°C with agitation. *E. coli* strains were grown on LB agar or in L broth (Maniatis *et al.*, 1982).

Chemicals and biochemicals. Restriction enzymes were from BRL or Biolabs, and were used according to the manufacturer's specifications. All other enzymes were from Boehringer. Ampicillin and tetracycline were from Sigma, and agarose from BRL. Adenosine 5'- α -[^{35}S]trithiophosphate ([^{35}S]dATP) was from Amersham. Other chemicals were from BDH.

DNA isolation and cloning techniques. *P. multocida* DNA was isolated by a modification of the method of Saito & Miura (1963). Cells were collected by centrifugation and washed in an aqueous solution of 0.15 M-NaCl, 0.1 M-EDTA, pH 8.0, and were resuspended in the same buffer at one-tenth the original volume of culture. Lysozyme (1 mg ml^{-1}) was added, and the mixture was incubated at 37°C for 30 min and was then rapidly immersed in a dry ice/acetone bath. Eight volumes of lysis solution [0.1 M-Tris/HCl, pH 9, 0.1 M-NaCl, 1% (w/v) SDS and $50\text{ }\mu\text{g ml}^{-1}$ proteinase K] was added and the mixture was incubated at 60°C for 10 min before being rapidly frozen in a dry ice/acetone bath. After thawing at 60°C for 30 min, an equal volume of

Abbreviation: EBL, embryonic bovine lung.

phenol (Maniatis *et al.*, 1982) was added and the suspension was mixed gently for 1–2 h. The suspension was centrifuged and the aqueous phase was re-extracted with phenol. After phase separation, residual phenol was removed from the aqueous phase by extensive dialysis against an aqueous solution of 50 mM-Tris/HCl, 10 mM-EDTA, 10 mM-NaCl, pH 8. The preparation was treated with ribonuclease as described by Maniatis *et al.* (1982), and the purified DNA was stored at 4 °C.

Genomic DNA from *P. multocida* was partially digested with *Sau*III A to obtain fragments of about 10 kb. The digest was fractionated on a sucrose gradient as described by Maniatis *et al.* (1982). Fractions were selected containing fragments in the size range 7–12 kb. The restricted DNA was ligated overnight at 15 °C to pAT153, previously cut with *Bam*HI and treated with phosphatase. Competent HB101 cells obtained from BRL were transformed and were plated onto L agar containing ampicillin (200 µg ml⁻¹). After incubation at 37 °C overnight, the bacterial colonies obtained (about 6500) were replica-plated onto agar containing tetracycline (12 µg ml⁻¹). About 2500 Ap^rTc^s colonies were tested for toxicity for embryonic bovine lung (EBL) cells by the overlay method (Chanter *et al.*, 1986a) and stored at -70 °C in 12% glycerol in microtitre trays.

Plasmids were isolated according to Ish-Horowitz & Burke (1981). DNA probes were labelled by nick translation, and other molecular biological techniques were as described in Maniatis *et al.* (1982).

Toxin purification. A crude extract of the toxigenic recombinants TOX1 or TOX2, grown on L agar containing ampicillin, was produced by the lysis method of Rimler & Brogden (1986). Crude extract, treated with RNAase, DNAase, benzamidine and phenylmethylsulphonyl fluoride was sequentially fractionated by DEAE-Sephacel chromatography and preparative PAGE (Chanter *et al.*, 1986b). Quantities of toxin in each fraction were measured using toxicity for EBL cells (Rutter & Luther, 1984).

Characterization of toxin purified from recombinant *E. coli*. The homogeneity and molecular mass of the polypeptide(s) in toxin purified from the recombinants were estimated in 10% polyacrylamide SDS-PAGE (Laemmli, 1970); gels were stained with silver as previously described (Chanter *et al.*, 1986b).

The antigenic similarity of the toxin purified from the *E. coli* clones with that purified from *P. multocida* was determined. Antiserum to toxin purified from *P. multocida* was produced in a gnotobiotic pig (Chanter *et al.*, 1986b). The toxins were compared in an ELISA, by immunoblotting, by a cytotoxin neutralization test (Rutter & Luther, 1984) and by crossed immunoelectrophoresis by the method of Moore & Rutter (1987).

In the ELISA, microtitre plates (Falcon – Becton Dickinson) were coated overnight with 100 µl of different concentrations of toxin in 50 mM-carbonate/bicarbonate buffer pH 9.6. Plates were washed in isotonic PBS, pH 7.0, containing 0.03% Tween 20 (PBS/Tween) and incubated at 37 °C for 1 h with serial dilutions of pig antiserum in PBS/Tween with 1% (w/v) dehydrated skimmed milk (Marvel, Cadbury). Plates were washed three times in PBS/Tween and 100 µl 1:5000 rabbit anti-swine Ig conjugated to horse radish peroxidase (Nordic Immunological Laboratories) in PBS/Tween/skimmed milk was added to each well and incubated at 37 °C for 3 h. Plates were washed and developed with 100 µl 0.04% (w/v) *o*-phenylenediamine in 0.015% (w/v) hydrogen peroxide buffered with 0.15 M-citrate/hydrogen orthophosphate, pH 5.0 for 15 min; the reaction was stopped with 10 µl 1M-sulphuric acid. Control wells, excluding either the antigen or the pig antiserum, were treated similarly. Absorbance at 492 nm was measured with a Titertec microplate reader. Control serum was from a specific-pathogen-free pig aged 8 weeks.

Proteins separated by SDS-PAGE were immunoblotted by the method of Towbin *et al.* (1979) using a Transblot apparatus (Bio-Rad). Toxicity was assayed using EBL cells (Rutter & Luther, 1984) and intraperitoneal injection of gnotobiotic pigs (Rutter & Mackenzie,

1984) and was related to protein content assayed by a Coomassie dye binding method (Bio-Rad).

Treatment of pigs with recombinant toxin. Three litters of gnotobiotic pigs were used to measure toxicity following intraperitoneal inoculation of various quantities of toxin from *P. multocida* or the *E. coli* clones. The eight pigs in the first litter were divided into four groups (A–D) when they were 7 d old. Group A was inoculated at 1 µg (kg body weight)⁻¹ with toxin purified from *P. multocida*. Group B was inoculated with a cell lysate of recombinant TOX1 with a quantity estimated to be approximately 1 µg toxin kg⁻¹, calculated from the toxicity of the lysate for EBL cells, the specific activity of purified toxin and the efficiency of purification. Group C was inoculated at 1 µg kg⁻¹ with toxin purified from recombinant TOX1 and group D was inoculated with a cell lysate of *E. coli* HB101 calculated to be equivalent to 10 times the dose of lysate received by group B.

The second litter, also of eight pigs, was divided into four equal groups (A–D) at 14 days of age. Groups A and D were treated similarly to the same groups of the first litter, but group A was given 200 ng toxin kg⁻¹. Group B received toxin at 200 ng kg⁻¹ purified from recombinant TOX2 and group C received the same preparation but at a dose of 400 ng kg⁻¹.

The third litter of six pigs was divided into two groups at 14 d old; group A was given toxin at 440 ng kg⁻¹ purified from TOX2 and group B was given a crude extract of *E. coli* HB101 containing pAT153.

Pigs were weighed at the start of the experiment and at post mortem 14 d after inoculations with toxin. All pigs in a group were fed the same amount. Damage to turbinate bones was measured by comparison of the ratio of body weight to ventral turbinate weight with that of the control group (D or B) in each case. Kidney and liver were examined for gross signs of damage. Samples of kidney, liver, bladder and ureter were fixed in neutral buffered formalin, embedded in paraffin wax, and sections cut and stained with haematoxylin/eosin. The sections were examined using a light microscope.

Results

Cloning the toxin gene

P. multocida LFB3 did not contain any plasmids; consequently, the toxin gene was isolated from a gene bank of chromosomal DNA cloned into the plasmid vector pAT153. Of the 2500 Ap^rTc^s clones with inserts, one (TOX1) was positive for toxin production. The plasmid from this clone, pAJL12, was purified and transformed into competent *E. coli* (BRL); 20 transformed bacteria were selected and all were toxigenic.

Analysis of the toxin gene

The plasmid pAJL12 contained a 10.7 kb insert. A physical map of the plasmid was constructed for 19 enzymes (Fig. 1) as follows. The sizes of the fragments generated by each enzyme were determined, and then digestions using two enzymes which each cut only once or twice were performed. It was then possible to map most of the sites by reference to the known restriction sites within the vector (Maniatis *et al.*, 1982). The other sites were mapped using further combined digestions.

Fig. 1. Restriction map of the inserts in the recombinant plasmids pAJL12 (top line) and pAJL13 (bottom line). The arrows indicate the direction of transcription from the tetracycline-resistance gene of pAT153. The insert in pAJL12 was not cut by *HpaII*, *KpnI*, *PvuI*, *PvuII*, *SalI* or *SstI*. The *BamHI* site in parenthesis was lost upon ligation of the vector *BamHI* site to the insert *SauIII*A site. Two *HindIII* sites between the *HindIII* sites at the right hand end of the insert were not mapped.

The map was unusual since the insert was not cut by *HpaII* or *MspI*, and was only cut three times by *HaeIII*. These three enzymes have a 4 bp recognition sequence which contains only G and C nucleotides. This suggested that the G + C ratio of the DNA might be quite low. The enzymes *DraI* and *SpeI*, which have a 6 bp recognition sequence containing only A and T, cut the insert at least 9 and 11 times, respectively (data not shown). Genomic DNA from *P. multocida* was prepared and cut with *HpaI* and *HpaII*, and Southern blots of the gels were probed with the *HpaI* fragment from the insert in pAJL12. Fig. 2 shows that most of the DNA was digested by *HpaII* to fragments smaller than 4 kb, but there were discrete bands of higher molecular mass. A band at approximately 15 kb hybridized to the probe. An *HpaI* fragment of approximately 6 kb reacted with the probe. Three other toxigenic *P. multocida* isolates produced similar fragments which also reacted with the probe.

The 2500 colonies in the clone bank were also probed with the *HpaI* fragment, and 12 other colonies hybridized. None produced toxin when tested with the EBL overlay test. Preliminary analysis of the 12 hybridizing colonies showed that they contained plasmids of different sizes.

Subcloning

It was not possible to excise the insert from pAJL12, since the *BamHI* cloning sites in the vector are not always regenerated after cloning *SauIII*A-generated fragments. It was therefore decided to use a *SauIII*A partial digestion to generate fragments of about 5 kb, the size of the gene predicted from the size of the toxin protein. Fragments of size 5–8 kb were selected and ligated into pAT153. Recombinants were screened for

toxicity by the EBL overlay test, and one (TOX2) contained a 5.0 kb insert and produced toxin. The recombinant plasmid (pAJL13) was mapped (Fig. 1); its insert was at one end of the insert in pAJL12. The

Fig. 2. (a) Agarose gel of chromosomal DNA from *P. multocida* LFB3: uncut (lane 1), and cut with *HpaI* (lane 2) or *HpaII* (lane 3). (b) Southern blot of the gel probed with the large *HpaII* fragment of pAJL12 (lanes 4–6 as lanes 1–3).

Fig. 3. SDS-PAGE of purified toxin from *P. multocida* LFB3 (lane 1) and *E. coli* TOX1 (lane 2).

Fig. 4. Crossed immunoelectrophoresis of purified recombinant toxin reacted against gnotobiotic pig antiserum to purified toxin from *P. multocida* LFB3.

*Bam*HI site in pAJL12, which is probably at the end of the insert, had been transferred to pAJL13, so it was possible that there was a duplication of the vector. Mapping using *Eco*RI/*Eco*RV digestion on pAJL12 and *Eco*RI/*Sal*I on pAJL13 showed that if present this was small and less than 0.1 kb (data not shown). The insert in pAJL13 was ligated to the vector in the opposite orientation to pAJL12, which implied that in at least one of the constructs the gene was transcribed from its own promoter.

Purification and properties of the toxin

The cytotoxic recombinants TOX1 and TOX2 produced a polypeptide of indistinguishable molecular mass (155 kDa) to that produced by *P. multocida*. The minimum concentration of purified toxin from TOX1 or *P. multocida* that affected EBL cells was 0.48 ng ml^{-1} and 0.5 ng ml^{-1} , respectively. The yield of both cytotoxicity and toxin protein suggested that TOX1 produced approximately five times as much toxin as TOX2 and ten times as much as *P. multocida*. Like *P. multocida* both recombinants produced a faint band above the main band. There were other lower molecular mass bands (Fig. 3), but analysis of SDS-polyacrylamide gels loaded

with tenfold dilutions of toxin purified from TOX1 or TOX2 indicated that the 155 kDa polypeptide made up 99% of the preparation.

A chequerboard titration of toxin was used to coat the microtitre plate for an ELISA and dilutions of gnotobiotic pig serum against toxin purified from *P. multocida* were made. The optimum coating concentration of antigen was $1 \mu\text{g per ml}$ coating buffer for toxin from *P. multocida* or from either toxigenic recombinant. At this concentration the serum gave an identical titre of 10^4 for all toxin preparations. Control serum did not react with any of the toxin preparations.

In a cytotoxin neutralization test the antiserum had a titre of 10^3 with 10 cytotoxic units of toxin purified from either *P. multocida* or the recombinants.

Crossed immunoelectrophoresis of toxin from the recombinants or *P. multocida* with gnotobiotic pig antiserum to toxin purified from *P. multocida* resulted in a precipitate in the basic pattern of one peak which on closer examination was seen to be composed of several closely spaced peaks (Fig. 4), although only two of these were reproducible. The same preparations in SDS-PAGE stained with silver were composed of only one major band and a fainter band with a slightly higher molecular mass.

Fig. 5. Immunoblot of gnotobiotic pig serum to the purified toxin from *P. multocida* LFB3 reacted with SDS-PAGE separations of purified toxin from strain LFB3 (lane 1), purified toxin from TOX1 (lane 2), whole-cell lysate from a non-toxicogenic recombinant containing part of the large *HpaI* fragment of pAJL12 (lane 3), and whole-cell lysate of *E. coli* HB101 containing pAT153 (lane 4). Lanes 4–8 as 1–4 but probed only with biotinylated anti-swine IgG and ^{125}I -labelled streptavidin.

In immunoblots, antiserum to the toxin purified from *P. multocida* reacted equally with toxin purified from *P. multocida* or the toxigenic recombinants, showing a major band of 155 kDa (Fig. 5); minor bands were seen when the toxin had been stored for several months. Whole-cell lysates from 3 of the 12 recombinants which cross-hybridized with the *HpaI* fragment of pAJL12, but not HB101 with the vector, contained three polypeptides which reacted with antibody to toxin (Fig. 5). These had approximate molecular masses of 76, 72 and 48 kDa. The same polypeptides were detected in crude extracts of the toxigenic recombinants TOX1 and TOX2. Other bands were seen if a crude extract of *E. coli* HB101 containing pAT153 was used as antigen in the SDS-PAGE or if blots were probed only with biotinylated anti-swine IgG and ^{125}I -labelled streptavidin, which suggested that their

Table 1. Turbinate atrophy and reduction in weight gain in pigs given recombinant toxin

Litter/ group	Inoculum	Percentage turbinate atrophy*	Percentage reduction in weight gain*
2A	200 ng kg ⁻¹ purified <i>P. multocida</i> toxin	59	25
		55	37
2B	200 ng kg ⁻¹ purified TOX2 toxin	61	0
		63	51
2C	400 ng kg ⁻¹ purified TOX2 toxin	78	31
		77	31
2D	Control	-5	-6
		5	6
3A	440 ng kg ⁻¹ purified TOX2 toxin	77	16
		43	14
		74	15
3B	Control	11	4
		5	1
		-15	-5

* Calculated with respect to the mean of controls (group D for litter 1, group B for litter 3), given a crude extract of *E. coli* HB101 containing pAT153.

detection was non-specific. The three polypeptides which reacted with antibody to toxin did not appear in either control.

Treatment of pigs with recombinant toxin

Crude or purified toxin from the recombinants was identical in its activity to that purified from *P. multocida* and was lethal for all but one of the pigs (found dead or killed *in extremis*) in the first litter. Extensive damage was caused to the liver and there was hydronephrosis of the kidneys. The surviving pig, which had been given crude toxin from the recombinant, had 59% turbinate atrophy when slaughtered 14 d after inoculation. Control pigs given an extract of *E. coli* HB101 containing pAT153 did not show signs of illness or turbinate atrophy. In litters 2 and 3, which were older and given less toxin than the first litter, all pigs survived, without signs of inappetence. There was, however, severe turbinate atrophy in pigs given toxin from *P. multocida* or the recombinant as well as a reduction in weight gain compared with control pigs (Table 1). All pigs given toxin in litter 2 showed signs of snout distortion to the left or right and all pigs given toxin purified from the recombinant had mild brachygnathia superior. The histopathological changes seen in the liver, bladder and ureters were identical to those previously found in pigs given crude toxin (Rutter & Mackenzie, 1984). The proliferation of the transitional epithelium of the bladder and ureters was most pronounced.

Discussion

The gene for the osteolytic toxin of *P. multocida* has been cloned and expressed in *E. coli*, and the protein expressed has been shown to have the same properties as the native toxin. In particular, the purified recombinant toxin reproduced severe turbinate atrophy in the pig, which is the key sign of atrophic rhinitis. The 4.9 kb insert in the subclone contains little other than the gene itself, since the predicted size of the toxin gene from the molecular mass of its product (Chanter *et al.*, 1986b) and the molar ratios of its composite amino acids (Nakai *et al.*, 1984) would be 4.8 kb. This shows that the *P. multocida* toxin is sufficient to explain the pathology observed and any mechanism directly involved must be connected to the whole toxin molecule or fragments generated from it.

Restriction data suggested that the insert encoding the toxin was very A + T rich, in contrast to the rest of the *P. multocida* genome (Carter, 1984). It was also of interest that the gene was on a *Hpa*II fragment of about 15 kb. This suggested that a simple method of cloning this gene or variants of it would be to extract the large *Hpa*II fragment and insert it into a suitable vector. The significance of the abnormal base ratio is not clear, but could perhaps reflect a difference between the toxin gene and its flanking sequences and the remaining *P. multocida* genome. The gene was expressed at a high level in *E. coli*, and produced about 15% of the total protein in one of the recombinants. The gene was not found in non-toxicogenic *P. multocida* (unpublished observations).

The significance of additional polypeptides produced by the toxigenic recombinants, and the non-toxicogenic recombinants containing part of the gene, is unknown. These polypeptides are not found in toxigenic *P. multocida* either in crude extracts or in purified toxin prepared by either affinity chromatography (Chanter *et al.*, 1986c) or immunoabsorbent chromatography using a monoclonal antibody (Foged, 1988). Since the insert in pAJL13 was only just large enough to encode the whole toxin, the additional polypeptides antigenically related to the toxin must have been encoded by the toxin gene. This suggests that there are either initiation or stop signals within the gene that are not utilized by *P. multocida* but which are recognized by the *E. coli* transcription system. Alternatively there might be post-translational breakdown of the protein in *E. coli*.

The toxin purified from the recombinant TOX2 was able to reduce weight gain compared with controls without affecting food consumption. Therefore the association of atrophic rhinitis with poor economic performance might, like turbinate atrophy, have a molecular basis ultimately dependent on the toxin of *P. multocida*. For such an effect in the natural disease the toxin would have to gain entry to the circulation from the

nasal cavity. Although in these experiments the toxin was injected intraperitoneally, there is evidence that toxin administered intranasally is able to cause liver damage in some pigs (Dominick & Rimler, 1986).

The recombinant toxin produced atrophy of the nasal turbinate bones of the pig, and induced considerable liver and kidney damage. There was also marked proliferation of the epithelium of the bladder and ureter as previously described for both unpurified and purified native toxin (Rutter & Mackenzie, 1984; Chanter *et al.*, 1986b). The possibility exists that the toxin exerts these proliferative effects directly.

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