

Recombinant Derivatives of *Pasteurella multocida* Toxin: Candidates for a Vaccine against Progressive Atrophic Rhinitis

S. K. PETERSEN,^{1*} N. T. FOGED,² A. BORDING,³ J. P. NIELSEN,² H. K. RIEMANN,¹ AND P. L. FRANDBSEN²

Genetic Engineering Group, The Technical University of Denmark, DK-2800 Lyngby,¹ National Veterinary Laboratory, Animal Biotechnology Research Centre, DK-1503 Copenhagen V,² and Northern Drugs and Chemicals Ltd., DK-2100 Copenhagen Ø,³ Denmark

Received 2 October 1990/Accepted 20 January 1991

Potential vaccine components for protection against atrophic rhinitis in pigs were developed. This was achieved by deletion mutagenesis of the gene encoding the *Pasteurella multocida* toxin. Four purified toxin derivatives lacking different and widely separated regions in the amino acid sequence were characterized by a lack of toxic activity. One such component was shown to induce efficient protection of vaccinated female mice and their offspring against challenge with purified *P. multocida* toxin.

Progressive atrophic rhinitis (PAR) is caused by infection of pigs with toxigenic *Pasteurella multocida* and is characterized primarily by atrophy of the nasal turbinate bones, resulting in deformation of the snout, and by reduction in rate of body weight increase (14, 19). The *P. multocida* toxin (PMT) has been shown to be the central etiological agent of this disease, and the purified toxin alone is sufficient to induce its characteristic symptoms (2, 6).

Several groups have recently described the cloning (7, 9, 16) and characterization (1, 15) of the toxin-encoding gene, *toxA*, and the production of recombinant PMT in *Escherichia coli* (7, 9, 16).

It has also been shown that vaccination with formaldehyde-inactivated PMT (fPMT) induces antibodies which protect against experimentally induced PAR in pigs (5).

In this study we have performed an analysis of the applicability of the PMT protein for vaccine purposes by using a deletion mutagenesis approach. The results of such a study can be expected to provide clues concerning regions of the protein required for efficient protection and regions required for toxic activity. This strategy has allowed us to develop deletion derivatives of PMT with impaired toxic potency, providing well-defined, putatively harmless, purified components for a future PAR vaccine. This potential was assessed by vaccination of pregnant female mice and registration of immune responses protecting vaccinated animals and their offspring against challenge with PMT.

MATERIALS AND METHODS

Construction of plasmids. Deletions in the toxin-encoding gene were made by digestion of the plasmids described in Fig. 1 with restriction enzymes, followed by treatment with the Klenow fragment of DNA polymerase I when required, and by ligation with T4 DNA ligase. The resulting deletions are schematically illustrated in Fig. 2.

Plasmid DNA preparation and DNA manipulations were carried out by conventional techniques as described previously (16). Enzymes were purchased from New England BioLabs, Beverly, Mass., and were used according to the instructions of the supplier.

To optimize the production of selected deletion proteins G, L, O, and Q (Fig. 2), the relevant deletions were

introduced into plasmid pSPE680 (Fig. 1), providing a 10-fold-higher expression of the toxin gene than pSPE312 and pSPE481 (15). For the same reason, the 3.7-kb *EcoRI* restriction fragment from pSPE680 was cloned in pOU61. This construct directs the expression of the L deletion protein. pOU61 is an R1 replicon-derived vector exhibiting a runaway replication phenotype at high temperature (8) due to control of *copB* gene expression by lambda gene *cI857* and lambda promoter *p_R*.

Bacterial strains, media, and growth conditions. *E. coli* SG21059 ($\Delta gal lon-146::\Delta Tn10 \Delta lac$), kindly provided by S. Gottesman, was used for expression of PMT and deletion derivatives. Strains were grown in L broth (10) in the presence of 50 μ g of ampicillin or 10 μ g of tetracycline per ml when appropriate. Growth was regularly measured as increase in optical density at 450 nm (OD_{450}), using a PMQ II spectrophotometer (Carl Zeiss, Germany). The L-protein-producing strain was grown at 30°C and shifted to 39°C at an OD_{450} of 0.5 to allow for massive replication of the plasmid due to its runaway replication phenotype. After two generations at this temperature, growth continued for 2 h at 30°C to minimize proteolytic degradation. The remaining cultures were allowed to grow to stationary phase at 30°C.

Protein analysis. Total protein extracts of bacterial cultures were analyzed as described previously (15) by (i) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 10% (wt/vol) acrylamide gels (acrylamide/bisacrylamide ratio of 40:1), followed by Coomassie blue staining and destaining in 5% acetic acid-50% ethanol; (ii) Western blotting (immunoblotting) with anti-PMT antiserum or a mixture of anti-PMT monoclonal antibodies (MAbs); and (iii) sandwich enzyme-linked immunosorbent assay (ELISA) involving two anti-PMT MAbs.

Purified PMT derivatives were tested for the presence of PMT epitopes by MAbs in a sandwich ELISA, and the results were compared with the results obtained with PMT. The sandwich ELISA was performed as described previously (4). In short, the wells of microtiter plates were coated with a catching MAb and incubated with 100 ng of an affinity-purified antigen (G, L, O, Q, or PMT) per ml. The antigen was detected by stepwise incubation with a biotinylated detecting MAb, peroxidase-conjugated avidin, and *o*-phenylenediamine-H₂O₂. In this study, 14 MAbs (13 anti-PMT MAbs and an unrelated control MAb) were used in all possible binary combinations of catching and detecting an-

* Corresponding author.

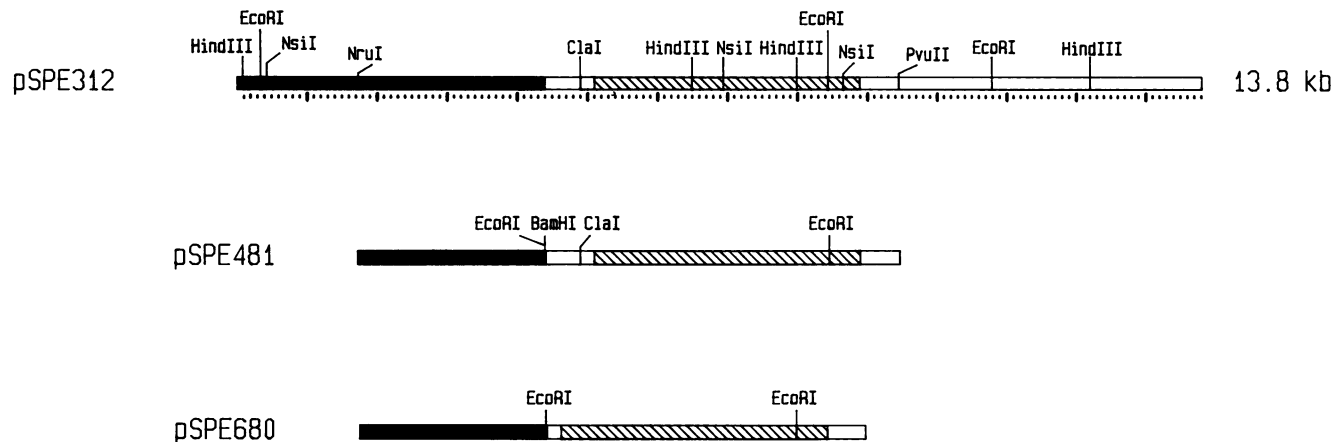


FIG. 1. Plasmids used for introduction of deletions into the PMT-encoding *toxA* gene (hatched area). Symbols: Filled area, vector DNA; open area, other *P. multocida* DNA. Restriction enzyme recognition sites used for plasmid construction and sites for enzymes with relevant recognition sequences outside the *toxA* gene are indicated. pSPE312 is a plasmid from the original *P. multocida* gene bank (16), pSPE481 was derived from pSPE312 by digestion with *PvuII* and *NruI* (15), and pSPE680 was derived from pSPE481 by digestion with *BamHI* and *ClaI* and treatment with the Klenow fragment of DNA polymerase I (15).

tibody. For each combination of MABs, the OD₄₉₀ obtained for each derivative relative to the OD₄₉₀ obtained for PMT was determined. The 13 anti-PMT MABs used were the eight used previously (4), P3F51, P3F64, P3F37, P4F58, P3F22, P4F38, P3F50, and P3F53, and five other MABs obtained by

a similar procedure but after immunization of mice with purified fPMT instead of PMT: D1F74, D1F08, D1F37, D1F24, and D1F47. Though some of these MABs bind competitively to PMT, they all show different patterns of competition and therefore bind different epitopes on PMT.

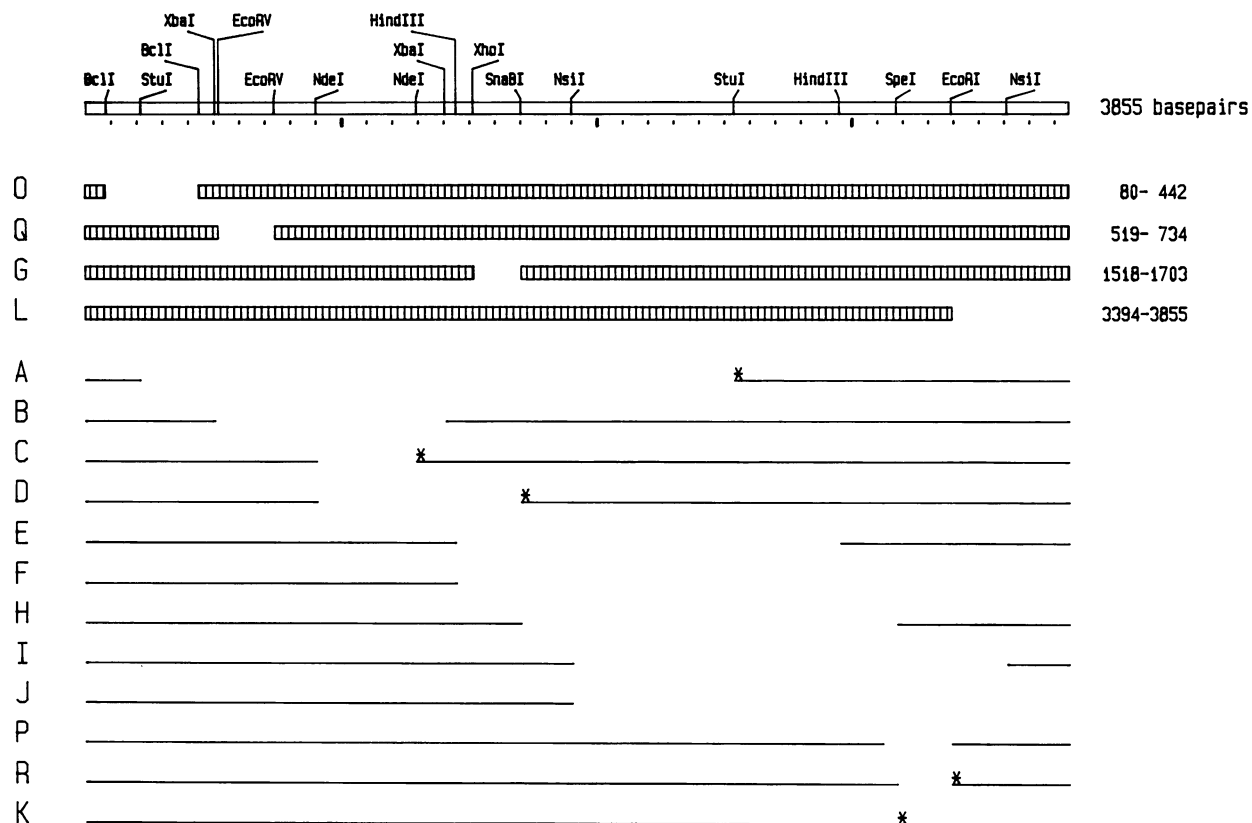


FIG. 2. Deletions introduced into the *toxA* gene. Given is a schematic representation of the structural *toxA* gene (white bar) and of the retained DNA (hatched bars) in deletions O, Q, G, and L. For deletion genes directing only poor expression of the gene, the retained DNA is indicated by a line. Recognition sites for the restriction enzymes used to introduce deletions are indicated, as are the nucleotide numbers lacking from the efficiently expressed deletion genes. Stars indicate deletions resulting in translational frameshifts.

With PMT as an antigen, this ELISA is characterized by the following properties: 128 of 196 possible combinations of catching and detecting MABs resulted in a positive reaction (data not shown). The remaining 68 combinations were negative due to competition between the antibodies (41 combinations) or lack of detection by the control MAB (27 combinations).

Protein purification. For purification purposes cells harvested from stationary-phase cultures in L broth were suspended in 10 ml of H₂O and sonicated for several 0.5-min periods at 0°C with a Branson Sonifier 250 (Branson Sonic Power Co., Danbury, Conn.). The sonic extract was diluted to 50 ml in 0.1 M Tris hydrochloride (pH 7.8) containing 0.5 ml of NaCl before application to the affinity column. Affinity chromatography was performed as described for PMT (4) by use of anti-PMT MAB P3F51 immobilized on divinyl sulfone agarose (Kem-En-Tec, Copenhagen, Denmark). The sonic extract was applied to the column, and after thorough washing, the derivative was eluted by 0.1 M glycine hydrochloride, pH 2.8. All fractions were immediately neutralized with 1 M K₂HPO₄.

Estimations of yields by the PMT-ELISA were compared with measurements of OD₂₈₀, and the purity of the eluate was estimated by SDS-PAGE visualized by silver staining (11).

Assays of biological activities. The cytopathic effects of the affinity-purified derivatives were studied by the embryonic bovine lung cell test (18), and minimal cytopathic doses (16) of these proteins were compared with those of PMT.

The dermonecrotic effects in guinea pigs after intradermal injection and the lethal effects in BALB/c mice after intraperitoneal injection were determined as described previously (16).

Mitogenicity was assayed essentially as described by Rozenfurt et al. (17) as follows: 10⁵ NIH 3T3 cells (ATCC CRL1658) were seeded onto 35-mm petri dishes in 2 ml of Dulbecco modified Eagle medium supplemented with 10% newborn calf serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 10 µg of streptomycin per ml. Fresh medium containing PMT (up to 20 ng/ml), derivative O (up to 0.5 mg/ml), or buffer (10 mM ammonium bicarbonate) was added to two equal dishes the day after plating and again 4 days later. Cells were counted 7 days after plating.

The effects of derivative O, PMT, and bovine serum albumin (BSA) after intraperitoneal injection in piglets were estimated. The piglets were obtained from an experimental farm free of *Bordetella bronchiseptica* and *P. multocida* to avoid the possibility of turbinate atrophy induced by these infective agents. One litter, consisting of 10 5-day-old piglets, was divided into three groups. The positive control group consisted of two piglets; each received an intraperitoneal injection of 0.45 µg of PMT in 4.5 ml of 0.1 M glycine hydrochloride-0.1 M K₂HPO₄ (pH 7.5). The negative control group consisted of three piglets, each of which received 225 µg of BSA in 4.5 ml of the same vehicle. The experimental group consisted of five animals which each received 189 µg of O protein in 4.5 ml of the same vehicle. At 28 days of age the piglets were sacrificed after CO₂ anesthesia. Samples from snout, tonsils, and lungs were taken for microbiological examination. These samples were tested for the presence of *B. bronchiseptica* or toxigenic *P. multocida* as described previously (5), after growth on selective media, by a hemagglutination assay and by PMT-ELISA, respectively. Samples from liver, heart, spleen, kidneys, and right concha were taken for histological examination, and the left

concha was photographed after longitudinal section of the snout.

Evaluation of immunogenicity. Two different mouse models were used. One (model A) was used to evaluate transfer of protective immunity from immunized mothers to their offspring. The other model (model B) was a direct challenge model used to establish dose-response relationships.

In both models, BALB/c mice inbred over eight generations were used. Vaccination with fPMT was used as a positive control, and vaccination with BSA was used as a negative control. All vaccine preparations were adsorbed to a 20% solution of aluminum hydroxide gel (Alhydrogel grade A; Superfos, Denmark), using the guidelines of the manufacturer. Challenge of adult and baby mice was performed with affinity-purified native PMT diluted to the desired concentrations in sterile, filtered, phosphate-buffered saline to which 0.02% normal mouse serum (Dakopatts, Denmark) was added. The 50% lethal doses of the PMT used for challenge were approximately 70 ng for grown mice and 4 to 10 ng for baby mice. Blood samples were analyzed for anti-PMT antibodies by a previously described blocking ELISA (5).

In model A, mature female mice were immunized subcutaneously twice, first at the time of mating and then 10 days before expected delivery. At 7 days of age, baby mice were challenged intraperitoneally with affinity-purified PMT and lethality was registered. After challenge of the offspring, the mothers were challenged likewise and bled. The number of dead mice within 5 days after challenge was registered. Those mice which did not survive all died within 3 days after challenge.

Model B was a simple challenge model including blood sampling immediately prior to challenge. Mice weighing 18 to 20 g were vaccinated twice subcutaneously with a 2-week interval. Two weeks after the second vaccination they received intraperitoneal injections of affinity-purified PMT, and the number of dead mice within 5 days after challenge was registered. Mice which did not survive all died within 3 days after challenge.

RESULTS

Production of deletion derivatives of PMT. A series of 16 deletions were introduced into the structural *tox*A gene in the previously described plasmids pSPE312 and pSPE481 (Fig. 1) (15). The deletions, which covered the entire toxin-encoding region except for the 28 codons in the extreme 5' end, were obtained by making use of the available restriction enzyme recognition sites as schematically outlined in Fig. 2.

E. coli SG21059 was transformed with plasmids harboring deletions A to R, and sonic extracts of stationary-phase cultures of these strains were examined for production of PMT deletion derivatives. Figure 3 shows production of PMT derivatives in strains harboring deletions A to R, visualized by SDS-PAGE. Due to the scarcity of *E. coli* protein species >100 kDa in size, this method provides a rapid survey of the production levels of the majority of deletion derivatives, which lack <30% of the PMT sequence. Only derivatives F and J could not be detected in Western blotting (data not shown). However, of the remaining 14 derivatives, only G, L, O, and Q were produced in amounts relevant for subsequent purification and further characterization as vaccine candidates. The absence of prominent bands corresponding to the full-length derivatives in the majority of strains probably could be explained by instability due to proteolytic degradation of abnormally

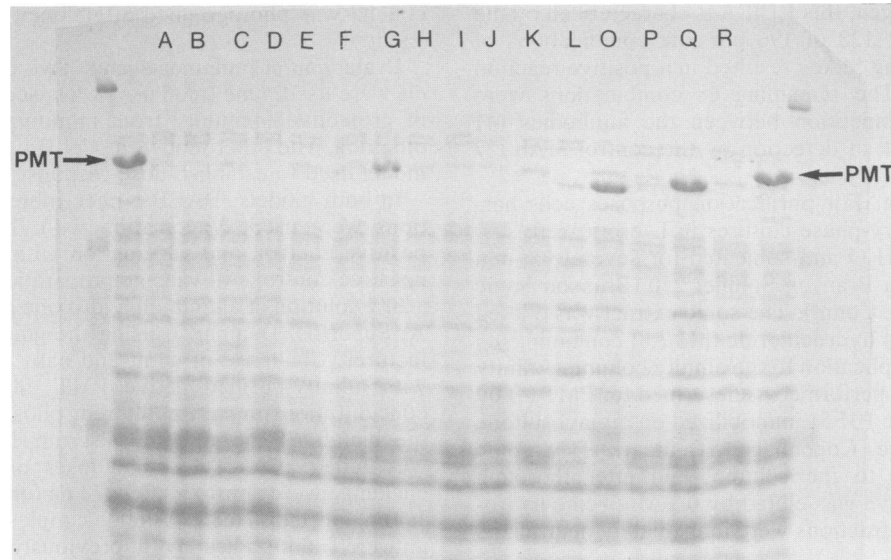


FIG. 3. Production of deletion proteins in *E. coli*. Production of toxin deletion proteins A to R (corresponding to deletion genes A to R in Fig. 2) as visualized by SDS-PAGE of sonicated *E. coli* extracts followed by Coomassie blue staining. Left- and rightmost lanes, Molecular weight markers. Only deletion protein G, L, O, and Q bands are visible.

folded mutant proteins. In accordance with this hypothesis, the four proteins present in highest amounts, and thus presumably most stable, only contained minor deletions as compared with the average deletion size (Fig. 2).

For practical reasons, the deletions were originally introduced in pSPE312 or pSPE481. Recent studies have shown that, due to a negative effect of the region upstream of the toxin-encoding gene, expression directed from these plasmids is at least 10-fold less efficient than expression directed from pSPE680 (15). This effect was eliminated for the four most stable derivatives. Enhanced production of L was achieved by directing its synthesis from a plasmid with inducibly high copy number, whereas G, O, and Q production was enhanced by introducing the corresponding deletions in pSPE680 (Fig. 1 and 2).

The G, L, O, and Q proteins were purified from sonicated bacterial extracts of strain SG21059 containing the relevant plasmids. The extracts were subjected to affinity chromatography based on immobilized anti-PMT MAbs. These derivatives were all adsorbed on the affinity column, but the resulting yields and purities varied (Table 1 and Fig. 4). A possible explanation for the low yield of Q was found by cell fractionation, which indicated that Q was primarily membrane associated (data not shown) in contrast to the native toxin which resides in the cytoplasm (12). The low yields of

the remaining derivatives was due to pH-dependent degradation observed during elution from the column with glycine hydrochloride (data not shown) and to inefficient adsorptions to the affinity column (Table 1).

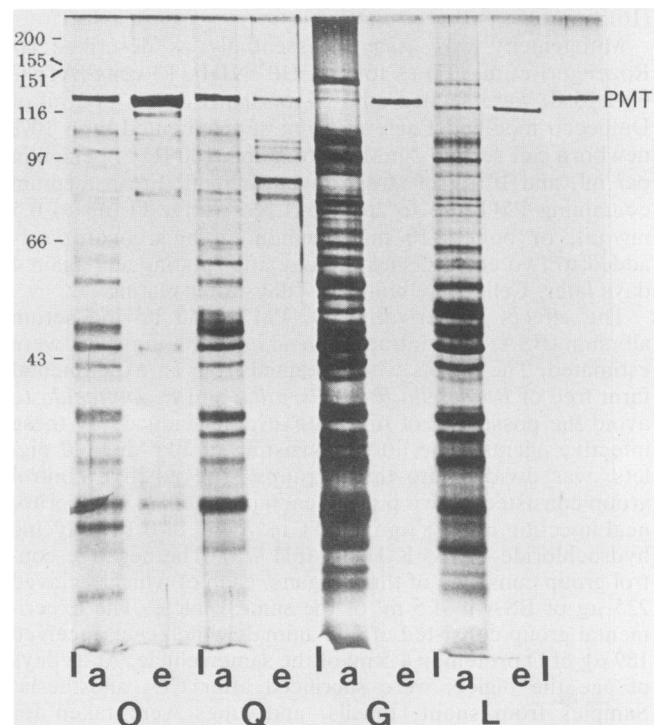


FIG. 4. Purification of toxin derivatives. The purification of derivatives O, Q, G, and L is illustrated by SDS-PAGE of sonicated *E. coli* extracts applied to the affinity column (a) and of their eluates (e). Numbers at the left indicate positions of molecular weight markers of the indicated sizes (10^3). Rightmost lane, Affinity-purified PMT. See also Table 1.

TABLE 1. Purification of deletion derivatives^a

Toxin derivative	Applied (μ g)	Outlet (μ g)	Eluate (μ g)	Yield (%)
G	430	180	80	19
L	3,100	50	640	21
O	17,500	6,900	8,200	47
Q	12,500	9,200	200	2
PMT ^b	11,100	<10	10,300	93

^a For the four sonicated *E. coli* extracts containing toxin derivatives O, Q, G, and L, the PMT-ELISA values corresponding to each step in the affinity purification procedure are shown.

^b From reference 6.

TABLE 2. Reactivities of purified deletion proteins with anti-PMT MABs

MAB	ELISA absorbance (%) ^a			
	I (G)	II		III (L)
		O	Q	
<i>n</i> = 8 ^b	102 ± 42	91 ± 35	95 ± 21	101 ± 35
D1F74	98 ± 29	5 ± 2	3 ± 2	98 ± 32
D1F08	91 ± 20	10 ± 3	6 ± 2	91 ± 25
P3F50	99 ± 8	75 ± 9	114 ± 6	18 ± 5
P3F53	93 ± 8	116 ± 18	131 ± 13	12 ± 2
D1F47	102 ± 22	86 ± 31	98 ± 18	5 ± 3

^a Results are given as relative ELISA absorbance values obtained for each deletion derivative compared with values obtained for PMT with the same MABs.

^b MABs P3F51, P3F64, P3F37, P4F58, P3F22, P4F38, D1F37, and D1F24.

Antigenicity of purified derivatives. The four purified PMT deletion derivatives lacked between 5 and 12% of the amino acid residues of PMT. The effect of introducing a deletion of this size on secondary and tertiary structures of the resulting proteins could not be anticipated. Since this effect might nevertheless be of importance for the ability to induce an immune response, the antigenicities of these proteins were assayed by sandwich ELISA.

The four deletion derivatives could be arranged in three categories, based on their reaction with MABs in this ELISA (Table 2). The category I derivative, G, shows a pattern of reaction with MABs identical to that of native toxin. The two category II derivatives, O and Q, result in absorbances below 10% of the value obtained for PMT with the two competitive MABs D1F74 and D1F08, but react like PMT with the remaining MABs. O and Q are characterized by non-overlapping deletions in the amino-terminal one-fourth of PMT. This suggests that O and Q lack the proper conformations of two closely situated structural epitopes, although it cannot be excluded that these epitopes are merely embedded in conformationally abnormal proteins. The category III derivative, L, results in absorbances below 10% of the value obtained for PMT with the competitive MABs P3F50 and P3F53 and with the unrelated MAB D1F47. Accordingly, L apparently lacks three different epitopes related to sequences in the carboxyl terminus of PMT, two of which are located close to each other in PMT. None of the PMT-negative combinations of MABs was positive when a derivative was used as antigen.

In conclusion, all four deletion proteins were recognized as efficiently as PMT by at least 10 of 13 anti-PMT MABs. Hence, no overall change in protein antigenicity of either derivative was apparent when this strategy of analysis was used.

Functional analysis of PMT derivatives. Purified L, O, and Q proteins were characterized by a total lack of toxic activity in the assays for cytopathicity, dermonecrosis, and mouse lethality (Table 3). In contrast, G had retained a considerable toxic potency which, in the same assays, amounted to approximately 1/50 to 1/500 that of PMT.

To determine whether proteins containing deletions in either end of the linear sequence were able to complement each other functionally, a combination of L and O proteins was tested in each assay, using equivalent amounts of each derivative corresponding to the highest amounts tested for L protein alone, i.e., 3, 3, and 100 µg, respectively. No complementation could be observed in any of these assays.

TABLE 3. Toxicities of purified deletion derivatives of PMT

PMT assay	Toxic activity ^a			
	G	L	O	Q
Embryonic bovine lung cell	1/500	<1/10 ⁵	<1/2 × 10 ⁷	<1/6 × 10 ⁵
Dermonecrosis	1/50	<1/100	<1/100	<1/100
Mouse lethality	1/50	<1/2,500	<1/2,500	<1/500

^a Results are given as toxic activity of a derivative compared with toxic activity of PMT.

Vaccination of mice. Unfortunately, the G derivative, which had retained all 13 PMT epitopes tested for, had also retained 1/50 to 1/500 of the toxic potency of the native protein and was therefore found unsuitable for vaccine purposes. The Q protein was omitted from the vaccination studies since it was antigenically very similar to the O protein but gave a much lower yield in the purification procedure (Table 1). O and L proteins were thus tested by means of two different mouse models. In the first set of experiments, transfer of protective immunity from mothers to offspring was registered. The results of this approach are shown in Tables 4 and 5. O and fPMT were found to be equally efficient in inducing protection in both mothers (Table 4) and their offspring (Table 5), whereas the protection induced by vaccination with L was considerably lower. The levels of anti-PMT antibodies induced were concordant with these data: L-vaccinated mice were seronegative, whereas O- and fPMT-vaccinated animals developed similar levels of anti-PMT antibodies.

A dose-response relationship was established by using a direct-challenge model. Only O and fPMT were analyzed since vaccination with L did not result in high anti-PMT serum titers. As expected, the serological effect as well as the protective effect against challenge diminished when the applied vaccination dose was decreased. However, the serological dose-response correlation for fPMT was an all-or-none effect, whereas a more clear-cut dose-response relationship was apparent for vaccination with O (Table 6).

Additional functional tests of the selected vaccine component. In a previous report on the immunogenic effect of fPMT, it was shown that two vaccination doses of 15 µg each were sufficient to protect the progeny of vaccinated gilts against experimentally induced PAR (5). Assuming that similar amounts of O would provide an equivalent protection, it would be essential to ascertain that such doses could be shown not to constitute a health risk for the sow or the piglet. PMT, O, and BSA were therefore administered to 5-day-old piglets by intraperitoneal injection to elucidate any remaining PMT-like effects of the deletion derivative. At 28 days of age, the piglets were sacrificed and examined by autopsy, chonchae atrophy was assessed, and samples were taken for microbiological examination. Neither *B. bronchi-septica* nor toxigenic *P. multocida* could be detected in any sample. The two positive control piglets, treated with 0.45 µg of PMT, had strongly degenerate dorsal and ventral chonchae of <25% of normal size (not shown). Livers, kidneys, and spleens from these animals showed the typical changes associated with exposure to PMT (3). The five piglets, which were each treated with 189 µg of O protein, all had chonchae with normal morphology. Finally, the three negative control piglets treated with 200 µg of BSA also exhibited normal chonchae. No piglets from the last two groups showed any signs of morphological changes of other

TABLE 4. Protection of vaccinated female mice against challenge with affinity-purified PMT^a

Vaccination with:	Anti-PMT titer (SD)	No. of dead mice/no. of challenged mice with given challenge dose (ng)										
		20	62.5	100	200	250	800	1,000	3,200	4,000	8,000	12,800
fPMT	6.79 (0.66)						0/2		0/4			0/4
O	6.49 (0.60)				0/2		0/4		0/4			0/2
L	Seronegative					4/4		2/4		2/2		
BSA	Seronegative	0/2	1/2	8/8	4/4	4/4		4/4				

^a Pregnant female mice were vaccinated with 1.5 µg of chemically inactivated fPMT, purified O protein, purified L protein, or bovine serum albumin (BSA) as a negative control. The mean of the natural logarithm of the anti-PMT titers induced by each vaccine is shown.

organs. Consequently, administration of O protein to piglets, in amounts well above those anticipated to be used for vaccination of sows, had no obvious damaging effects related to those observed for PMT at 400-fold lower doses.

The O protein was tested for mitogenic activity in NIH 3T3 cell cultures. It was not mitogenic in concentrations up to 25,000 times the concentration of PMT showing maximal mitogenicity. The quantitative effect of PMT was concentration dependent in a manner closely resembling the published data (data not shown) (17).

DISCUSSION

Many of the currently available vaccines for the prevention of atrophic rhinitis in pigs contain formaldehyde-treated crude extract of toxigenic *P. multocida*. A recent study showed that a vaccine containing pure formaldehyde-treated PMT can minimize the symptoms of PAR induced by a combined experimental infection with *B. bronchiseptica* and toxigenic *P. multocida* (5). Thus, a single-component toxoid vaccine is sufficient for inducing immunity against experimentally induced PAR. As the *P. multocida* toxin is highly toxic and rather difficult to produce on a large scale, it would be most advantageous if a recombinant atoxic derivative could be developed. The present study describes the development of vaccine candidates which, apart from the advantages of a subunit vaccine, exhibit several additional favorable properties, since (i) they have retained <1/100,000 of the toxic potency of PMT, whereas fPMT has retained approximately 1/100 to 1/200 (5); (ii) they are well defined and thus provide a reproducible immunogenic effect, whereas the formaldehyde treatment, especially of crude extracts, seems to result in variable levels of denaturation and cross-linking, accompanied by inconsistent levels of immunogenicity of the toxoid (data not shown); (iii) inactivation is not needed after purification of the protein, which facilitates the production procedure; (iv) production levels for derivative O are >10-fold higher than they are for native

TABLE 5. Protection of the offspring of vaccinated mice by transfer of passive immunity^a

Vaccination with:	No. of dead mice/no. of challenged mice with given challenge dose (ng) of PMT						
	4	20	30	100	150	500	750
fPMT		0/4		0/6		0/5	
O		0/4		0/7	0/10	0/7	3/8
L			0/2		7/8		8/8
BSA	0/4	2/3	3/3	3/3	7/9	4/4	4/4

^a Offspring of the female mice of Table 4, which had been vaccinated with fPMT, O, L, or bovine serum albumin (BSA), were challenged with different amounts of PMT.

PMT; and finally, (v) vaccination with deletion derivatives of PMT could allow for a distinction between infected and vaccinated animals, since these derivatives lack antigenic determinants recognized by specific anti-PMT MAbs.

In continuation of this work, we have examined the protective effects against PAR in piglets induced by vaccination of sows with deletion derivative O (13). The results of that and the present study show that the O derivative of PMT described here constitutes considerable progress and that the recombinant approach enables an efficient production of this pure, atoxic, and highly immunogenic protein. A modification of the purification procedure originally developed for purification of PMT would probably even improve the yield of O protein from 47% to a value closer to the 90 to 95% that applies to PMT.

The described close antigenic similarity between the O and Q proteins which lack different parts of the linear amino acid sequence in the N-terminal one-fourth of PMT suggests that their retained amino acid stretches in this part of the molecules are folded in a manner different from that in PMT. Since this is probably due to the large deletion sizes, smaller changes of the primary structure resulting in structurally less perturbed proteins might result in even better immunogens. Construction of such mutant proteins would, however, require a more detailed knowledge about the structure of PMT, a molecule which has no apparent amino acid sequence homology to any known proteins (15).

The deletion approach has the potential of localizing molecular regions of functional importance. Vaccination of mice with the L protein neither induced significant serum

TABLE 6. Comparison of protective effects of fPMT and O protein^a

Protein and concn (µg/ml)	No. of dead mice/no. of challenged mice	Anti-PMT titer
fPMT		
25	0/5	>6.17
5	0/5	>6.17
1	1/5	>6.17
0.2	5/5	Seronegative
0.008	4/5	Seronegative
O		
25	0/5	>6.17
5	0/5	3.44
1	0/5	1.39
0.2	3/5	Seronegative
0.008	4/5	Seronegative

^a Mature mice were vaccinated with 0.5 ml of vehicle with different concentrations of either vaccine component, as indicated, and challenged with 2.5 µg of affinity-purified toxin. The means of the natural logarithms of the induced anti-PMT titers are shown.

anti-PMT titers nor provided good protection against challenge with PMT. This could mean that an important immunogenic region is located in the C-terminal part of PMT. The completely inactive O and L proteins, each lacking an amino acid stretch close to one of either end of the primary sequence of the toxin, are both completely biologically inactive. Furthermore, they are not able to complement each other functionally. Since we have not been able to identify an antigenic epitope lacking in both proteins, this could be interpreted to mean that different functional domains are inactivated in the two proteins. However, it is remarkable that two of the three epitopes eliminated in L are differently exposed in either O or Q than in PMT. These are the epitope recognized by MAb P3F50, which results in 75% absorbance in the sandwich ELISA with O protein as compared with PMT, and the epitope recognized by MAb P3F53, which results in 131% absorbance with Q protein as compared with PMT. This could indicate an interaction between amino acid residues of the N- and C-terminal parts of PMT. Therefore, further analyses of both the structural and functional properties of PMT and the derivatives are required to ascribe specific functions, related to the toxic activity of PMT, to certain regions of the molecule. For such studies PMT derivatives will be useful tools contributing to the progress in understanding the mitogenic activity and the effect on bone metabolism of PMT.

ACKNOWLEDGMENTS

We thank Annemette Jørgensen, Henriette Lou, and Helle Grønvald for excellent technical assistance, as well as Margrethe Petersen, Ingrid Villadsen, Poul Andersson, and Claus Christiansen for critical reading of the manuscript.

This work was supported by Northern Drugs and Chemicals Ltd.

REFERENCES

1. Buys, W. E. C. M., H. E. Smith, A. M. I. E. Kamps, and M. A. Smits. 1990. Sequence of the dermonecrotic toxin of *Pasteurella multocida* ssp. *multocida*. *Nucleic Acids Res.* **18**:2815-2816.
2. Chanter, N., J. M. Rutter, and A. Mackenzie. 1986. Partial purification of an osteolytic toxin from *Pasteurella multocida*. *J. Gen. Microbiol.* **132**:1089-1097.
3. Cheville, N. F., R. B. Rimler, and J. R. Turston. 1988. A toxin from *P. multocida* type D causes acute hepatic necrosis in pigs. *Vet. Pathol.* **25**:518-520.
4. Foged, N. T. 1988. Quantification and purification of the *Pasteurella multocida* toxin by using monoclonal antibodies. *Infect. Immun.* **56**:1901-1906.
5. Foged, N. T., J. P. Nielsen, and S. E. Jorsal. 1989. Protection against progressive atrophic rhinitis by vaccination with *Pasteurella multocida* toxin purified by monoclonal antibodies. *Vet. Rec.* **125**:7-11.
6. Foged, N. T., K. B. Pedersen, and F. Elling. 1988. Characterization and biological effects of the *Pasteurella multocida* toxin. *FEMS Microbiol. Lett.* **43**:45-51.
7. Kamps, A. M. I. E., E. M. Kamp, and M. A. Smits. 1990. Cloning and expression of the dermonecrotic toxin gene of *Pasteurella multocida* ssp. *multocida* in *Escherichia coli*. *FEMS Microbiol. Lett.* **67**:187-190.
8. Larsen, J. E. L., K. Gerdes, J. Light, and S. Molin. 1984. Low-copy-number plasmid-cloning vectors amplifiable by derepression of an inserted foreign promoter. *Gene* **28**:45-54.
9. Lax, A. J., and N. Chanter. 1990. Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis. *J. Gen. Microbiol.* **136**:81-87.
10. Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117**:307-310.
12. Nakai, T., A. Sawata, and K. Kume. 1985. Intracellular locations of dermonecrotic toxins in *Pasteurella multocida* and in *Bordetella bronchiseptica*. *Am. J. Vet. Res.* **46**:870-874.
13. Nielsen, J. P., N. T. Foged, V. Sørensen, K. Barfod, A. Bording, and S. K. Petersen. *Can. J. Vet. Res.*, in press.
14. Pedersen, K. B., and F. Elling. 1984. The pathogenesis of atrophic rhinitis in pigs induced by toxigenic *Pasteurella multocida*. *J. Comp. Pathol.* **94**:203-214.
15. Petersen, S. K. 1990. The complete nucleotide sequence of the *Pasteurella multocida* toxin gene and evidence for a transcriptional repressor, TxaR. *Mol. Microbiol.* **4**:821-830.
16. Petersen, S. K., and N. T. Foged. 1989. Cloning and expression of the *Pasteurella multocida* toxin gene, *toxA*, in *Escherichia coli*. *Infect. Immun.* **57**:3907-3913.
17. Rozengurt, E., T. Higgins, N. Chanter, A. J. Lax, and J. M. Staddon. 1990. *Pasteurella multocida* toxin: potent mitogen for cultured fibroblasts. *Proc. Natl. Acad. Sci. USA* **87**:123-127.
18. Rutter, J. M., and P. D. Luther. 1984. Cell culture assay for toxigenic *Pasteurella multocida* from atrophic rhinitis in pigs. *Vet. Rec.* **114**:393-396.
19. Rutter, J. M., and A. Mackenzie. 1984. Pathogenesis of atrophic rhinitis in pigs: a new perspective. *Vet. Rec.* **114**:89-90.