Volume 277, number 1,2, 59-64 FEBS 09236 December 1990

Sequence analysis of the potent mitogenic toxin of *Pasteurella multocida*

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Received 11 October 1990

Pasteurella multocida toxin is a potent mitogen for cultured Swiss 3T3 cells where it causes an accumulation of inositol phosphates and activation of protein kinase C. The gene sequence described here coded for a 146 kDa protein. The ORF was preceded by a ribosome binding site and followed by a stem loop. There was no evidence for a signal sequence. The gene had a low G + C base ratio which differs from the rest of the *Pasteurella* genome. There was no significant homology with other known proteins, although a motif found in certain bacterial toxins which are ADP-ribosyl transferases is present. A recombinant expressing only part of the PMT gene was not mitogenic.

Mitogen; Toxin; Protein kinase C; *Pasteurella multocida*

1. INTRODUCTION

Proliferation of eukaryotic cells is controlled by complex pathways of signal transduction, which when activated lead to cell division. Some of the components of this process are known for Swiss 3T3 cells, a model system for studying signal transduction [1]. Understanding the molecular mechanisms controlling signal transduction in eukaryotic cells is of primary importance in explaining cell proliferation and has been greatly aided by the use of bacterial toxins which intervene at different stages in the signal transduction process [2,3].

A toxin produced by some strains of *Pasteurella multocida* can reproduce the pig disease atrophic rhinitis [4,5]. The main signs of disease are loss of the nasal turbinate bones, twisting of the snout and a reduction in weight gain [6]. The toxin has been purified [7-11] and the gene has been cloned [12-14]. Parenteral injection of piglets with recombinant toxin reproduces clinical atrophic rhinitis and causes damage to liver and kidney, and proliferation of cells in the ureter, bladder and turbinate mucosa [12].

We have recently shown that the *P. multocida* toxin is the most potent mitogen yet identified for cultured fibroblasts [15]. The toxin stimulates DNA synthesis and cell proliferation as effectively as serum in the absence of other synergistic factors [15]. The toxin stimulates the production of inositol phosphates and increases the phosphorylation of the 80 kDa protein, a prominent substrate for protein kinase C in 3T3 cells [16]; other proteins are phosphorylated independently of protein kinase C. The toxin does not increase the cellular content of cAMP [15].

Characterisation of the mode of action of the toxin could assist in discovering new signal transduction pathways involved in mitogenesis because the activation of protein kinase C alone is not sufficient to elicit a mitogenic response [1]. The sequence of the toxin could identify potentially important domains and motifs, and hence greatly aid molecular analysis. Whilst sequencing the mitogenic toxin gene in this laboratory the sequence of toxin genes isolated from *P. multocida* in other laboratories became available [17-19]. Consequently, it was important to ascertain whether these sequences were related to the mitogenic toxin which we have described [15]. The sequence of the mitogenic toxin gene is analysed here and compared with the other toxin gene sequences; we have also examined the mitogenic activity of one of the previously sequenced toxins [17].

2. METHODS AND MATERIALS

2.1. *Strains and growth conditions*

The *E. coli* recombinant TOX2 containing plasmid pAJL13 and the non-toxigenic recombinant containing pAJL14 [12] were grown in L broth [20]. *P. multocida* ssp *multocida* 45/78 was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, UK (Accession Number NCTC 12178), and was grown on Bacto-Tryptose broth [21] at 37°C with agitation. All bacteria were stored as cell suspensions at −70°C in 12% (v/v) glycerol [20].

2.2. *Toxin purification and assays for toxicity and mitogenicity*

We have previously described the methods used for purification of toxin [8], measurement of toxicity [22,23] and assessing DNA synthesis and cell proliferation [15].

2.3. *Chemicals and biochemicals*

Restriction and other enzymes were from BRL, Biolabs or Boehr-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

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                                                    - (2,3)
CCCTTGACCTAGAGGGGCTTTTTTATTACATCAAAAAAATAAACCCCAAACACTGCGAATGTTTGGGGTTTTATTTATAACCAAAATACATTAATATGTT     100

TATTAAGTAAGCATTATCTTACTTTAGGAATAAACTAACATAGAGGTTATGGAT ATG AAA ACA AAA CAT TTT TTT AAC TCA GAT TTT ACT  190
                                                        Met Lys Thr Lys His Phe Phe Asn Ser Asp Phe Thr

GTA AAA GGA AAA AGT GCC GAT GAA ATT TTT AGA AGA TTG TGT ACT GAT CAT CCT GAC AAG CAA TTA AAC AAT GTA
Val Lys Gly Lys Ser Ala Asp Glu Ile Phe Arg Arg Leu Cys Thr Asp His Pro Asp Lys Gln Leu Asn Asn Val

AAA TGG AAA GAA GTT TTT ATT AAT CGT TTT GGT CAG ATG ATG CTA GAT ACT CCT AAT CCG AGA AAG ATT GTA GAA      340
Lys Trp Lys Glu Val Phe Ile Asn Arg Phe Gly Gln Met Met Leu Asp Thr Pro Asn Pro Arg Lys Ile Val Glu

                                                                AAC (2)
AAA ATT ATT AAT GAA GGG CTT GAA AAA CAA GGC CTG AAA AAT ATA GAT CCT GAA ACT ACA TAT TTC AAC ATT TTT
Lys Ile Ile Asn Glu Gly Leu Glu Lys Gln Gly Leu Lys Asn Ile Asp Pro Glu Thr Thr Tyr Phe Asn Ile Phe
                                                                Asn

TCA TCT TCT GAC AGC TCC GAT GGG AAC GTT TTT CAT TAT AAC TCT TTA TCA GAA TCC TAT CGA GTT ACT GAT GCC      490
Ser Ser Ser Asp Ser Ser Asp Gly Asn Val Phe His Tyr Asn Ser Leu Ser Glu Ser Tyr Arg Val Thr Asp Ala

TGC CTA ATG AAT ATT TTT GTG GAG CGT TAT TTT GAT GAT TGG GAC TTG CTA AAT AGC TTA GCC AGT AAT GGA ATA
Cys Leu Met Asn Ile Phe Val Glu Arg Tyr Phe Asp Asp Trp Asp Leu Leu Asn Ser Leu Ala Ser Asn Gly Ile

TAT TCA GTA GGA AAA GAA GGA GCT TAT TAT CCT GAT CAT GAT TAT GGT CCA GAA TAT AAC CCT GTT TGG GGA CCA      640
Tyr Ser Val Gly Lys Glu Gly Ala Tyr Tyr Pro Asp His Asp Tyr Gly Pro Glu Tyr Asn Pro Val Trp Gly Pro

AAC GAA CAA ATT TAC CAT TCT AGA GTG ATT GCA GAT ATC CTT TAT GCT CGC TCC GTA TGG GAT GAA TTT AAA AAA
Asn Glu Gln Ile Tyr His Ser Arg Val Ile Ala Asp Ile Leu Tyr Ala Arg Ser Val Trp Asp Glu Phe Lys Lys

TAC TTC ATG GAG TAT TGG CAA AAA TAT GCT CAG CTT TAT ACC GAA ATG TTA TCT GAT ACA TTT CTT GCA ATG GCT      790
Tyr Phe Met Glu Tyr Trp Gln Lys Tyr Ala Gln Leu Tyr Thr Glu Met Leu Ser Asp Thr Phe Leu Ala Met Ala

ATT CAG CAA TAT ACA CGA CAA ACG CTT ACT GAT GAA GGC TTT CTT ATG GTT TGT AAC ACA TAT TAT GGC AAT AAG
Ile Gln Gln Tyr Thr Arg Gln Thr Leu Thr Asp Glu Gly Phe Leu Met Val Cys Asn Thr Tyr Tyr Gly Asn Lys

GAA GAA GTT CAA ATA ACT CTA CTA GAT ATC TAT GGA TAC CCT TCC ACT GAT ATA ATT TGT ATA GAG CAA AAA GGG      940
Glu Glu Val Gln Ile Thr Leu Leu Asp Ile Tyr Gly Tyr Pro Ser Thr Asp Ile Ile Cys Ile Glu Gln Lys Gly

CTT CCT ACT CCT AAA GTG ATA CTT TAC ATT CCT GGA GGA ACA CAA CCA TTT GTT GAA TTT CTT AAT ACA GAT GAT
Leu Pro Thr Pro Lys Val Ile Leu Tyr Ile Pro Gly Gly Thr Gln Pro Phe Val Glu Phe Leu Asn Thr Asp Asp

(a)                                                             CGA (2,3)
CTG AAA CAA TGG ATT GCA TGG CAT TTA AAA GAT AAC AAA CAT ATG GTC GCA TTC CGC AAA CAT TTC TCG CTA AAA     1090
Leu Lys Gln Trp Ile Ala Trp His Leu Lys Asp Asn Lys His Met Val Ala Phe Arg Lys His Phe Ser Leu Lys
                                                            Arg

CAA CGT CAG GAA GGA GAA ACG TTT ACA GGT ATA GAT AAA GCA CTT CAA TAT ATT GCA GAA GAG TCC CCT GAA TGG
Gln Arg Gln Glu Gly Glu Thr Phe Thr Gly Ile Asp Lys Ala Leu Gln Tyr Ile Ala Glu Glu Ser Pro Glu Trp

CCT GCC AAT AAA TAC ATC CTT TAT AAT CCG ACA CAT TTA GAA ACA GAA AAT TTA TTT AAC ATC ATG ATG AAG CGA     1240
Pro Ala Asn Lys Tyr Ile Leu Tyr Asn Pro Thr His Leu Glu Thr Glu Asn Leu Phe Asn Ile Met Met Lys Arg

ACA GAA CAG CGG ATG CTT GAA GAT AGT GAT GTA CAG ATT AGA TCA AAT TCA GAA GCT ACC CGT GAC TAT GCT CTT
Thr Glu Gln Arg Met Leu Glu Asp Ser Asp Val Gln Ile Arg Ser Asn Ser Glu Ala Thr Arg Asp Tyr Ala Leu

TCA TTA CTC GAA ACC TTT ATT TCA CAG TTA TCT GCA ATA GAT ATG TTA GTA CCA GCA GTA GGT ATC CCA ATT AAT     1390
Ser Leu Leu Glu Thr Phe Ile Ser Gln Leu Ser Ala Ile Asp Met Leu Val Pro Ala Val Gly Ile Pro Ile Asn

TTT GCC CTA TCA GCT ACA GCA TTA GGA CTT AGC TCG GAT ATT GTA GTT AAT GGA GAT TCA TAT GAA AAG AGA AAA
Phe Ala Leu Ser Ala Thr Ala Leu Gly Leu Ser Ser Asp Ile Val Val Asn Gly Asp Ser Tyr Glu Lys Arg Lys

TAT GGA ATT GGG TCC TTA GTG CAA TCT GCA TTA TTC ACA GGA ATT AAT CTT ATT CCA GTT ATT TCG GAA ACC GCA     1540
Tyr Gly Ile Gly Ser Leu Val Gln Ser Ala Leu Phe Thr Gly Ile Asn Leu Ile Pro Val Ile Ser Glu Thr Ala

GAA ATT TTA TCT TCT TTC TCT AGA ACA GAA GAA GAT ATT CCA GCT TTT TTC ACT GAA GAA CAA GCT TTA GCT CAA
Glu Ile Leu Ser Ser Phe Ser Arg Thr Glu Glu Asp Ile Pro Ala Phe Phe Thr Glu Glu Gln Ala Leu Ala Gln

CGC TTT GAA ATA GTA GAA GAA GAA TTA CAT TCT ATC TCA CCT GAT GAT CCT CCT CGA GAA ATT ACT GAC GAA AAT     1690
Arg Phe Glu Ile Val Glu Glu Glu Leu His Ser Ile Ser Pro Asp Asp Pro Pro Arg Glu Ile Thr Asp Glu Asn

TTA CAT AAA ATT CGT CTG GTA CGT CTT AAC AAT GAA AAT CAA CCT TTA GTT GTG TTA CGA AGA TTA GGA GGA AAT
Leu His Lys Ile Arg Leu Val Arg Leu Asn Asn Glu Asn Gln Pro Leu Val Val Leu Arg Arg Leu Gly Gly Asn

AAA TTT ATC AGA ATC GAG CCT ATA ACA TTC CAG GAA ATA AAA GGT TCT TTA GTA AGT GAA GTT ATA AAT CCA GTG     1840
Lys Phe Ile Arg Ile Glu Pro Ile Thr Phe Gln Glu Ile Lys Gly Ser Leu Val Ser Glu Val Ile Asn Pro Val

ACT AAT AAA ACG TAC TAC GTA AGC AAT GCT AAA CTA TTA GGG GGC TCT CCT TAT AGT CCT TTC CGT ATT GGA TTA
Thr Asn Lys Thr Tyr Tyr Val Ser Asn Ala Lys Leu Leu Gly Gly Ser Pro Tyr Ser Pro Phe Arg Ile Gly Leu

GAA GGT GTT TGG ACA CCA GAG GTA TTA AAA GCA AGA GCT TCC GTT ATT GGA AAG CCT ATT GGA GAA TCA TAT AAA     1990
Glu Gly Val Trp Thr Pro Glu Val Leu Lys Ala Arg Ala Ser Val Ile Gly Lys Pro Ile Gly Glu Ser Tyr Lys

AGA ATA TTA GCC AAA CTA CAA AGA ATA CAT AAC AGT AAT ATC TTA GAT GAG CGA CAA GGT TTA ATG CAT GAA CTC
Arg Ile Leu Ala Lys Leu Gln Arg Ile His Asn Ser Asn Ile Leu Asp Glu Arg Gln Gly Leu Met His Glu Leu

ATG GAG CTT ATT GAT CTT TAT GAA GAA TCG CAA CCT TCT TCA GAG CGT TTG AAT GCT TTT CGT GAA CTG CGT ACT     2140
Met Glu Leu Ile Asp Leu Tyr Glu Glu Ser Gln Pro Ser Ser Glu Arg Leu Asn Ala Phe Arg Glu Leu Arg Thr

CAA TTA GAA AAA GCG CTT TAT CTT CCT GAA ATG GAA GCA TTA AAA AAA CAA ATA CTA CAG ATT CCT AAC AAA GGT
Gln Leu Glu Lys Ala Leu Tyr Leu Pro Glu Met Glu Ala Leu Lys Lys Gln Ile Leu Gln Ile Pro Asn Lys Gly
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Fig. 1. Sequence of the toxin gene PMT1. (a) marks the end of the insert in pAJL14. Differences between PMT1 and other sequences are shown by bases above the PMT1 sequence and amino acid changes below. The sequences are: 2 [17]; 3 [18]; 4 [19].

inger, and were used according to the manufacturer's specifications. Other chemicals were from BDH.

2.4. *Sequencing strategy*

The insert in pAJL13 was isolated by digestion with *Hpa*II followed by gel electrophoresis and elution onto silica gel (Geneclean, Statech). It was digested with either *Alu*I or *Sau*IIIA and ligated into M13mp18 cut with either *Sma*I or *Bam*HI respectively (prior to ligation the cut vector was treated with phosphatase). DNA from isolated phage containing inserts was purified and sequenced using an Applied Biosystems sequencer. This strategy produced about 10 discrete short lengths of sequence. Since these sequences closely matched parts of

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TCT GGT GCC GCT CGA TTT TTA CTT CGT ACA GCC ATG AAT GAA ATG GCT GGA AAA ACC AGT GAA AGC ACG GCT GAT    2290
Ser Gly Ala Ala Arg Phe Leu Leu Arg Thr Ala Met Asn Glu Met Ala Gly Lys Thr Ser Glu Ser Thr Ala Asp

TTA ATA CGC TTT GCC TTG CAA GAT ACA GTA ATT TCA GCG CCT TTT CGC GGA TAT GCT GGT GCG ATT CCA GAG GCA
Leu Ile Arg Phe Ala Leu Gln Asp Thr Val Ile Ser Ala Pro Phe Arg Gly Tyr Ala Gly Ala Ile Pro Glu Ala

ATA GAC TTT CCT GTA AAA TAT GTA ATA GAA GAC ATA TCT GTA TTT GAT AAA ATA CAG ACA AAT TAC TGG GAA CTT    2440
Ile Asp Phe Pro Val Lys Tyr Val Ile Glu Asp Ile Ser Val Phe Asp Lys Ile Gln Thr Asn Tyr Trp Glu Leu

                                        CGA (2,3)
CCT GCT TAT GAA AGC TGG AAC GAA GGA AGT AAT AGC GCA TTA CTG CCT GGT TTG TTA CGT GAA TCG CAA AGC AAG
Pro Ala Tyr Glu Ser Trp Asn Glu Gly Ser Asn Ser Ala Leu Leu Pro Gly Leu Leu Arg Glu Ser Gln Ser Lys
                                        Arg

GGG ATG TTA AGT AAG TGT CGT ATC ATA GAA AAT AGC CTT TAT ATT GGA CAT AGC TAT GAA GAA ATG TTT TAC AGC    2590
Gly Met Leu Ser Lys Cys Arg Ile Ile Glu Asn Ser Leu Tyr Ile Gly His Ser Tyr Glu Glu Met Phe Tyr Ser

ATT TCT CCA TAT TCA AAC CAG GTT GGA GGG CCT TAT GAA TTA TAT CCT TTC ACT TTT TTC AGT ATG CTT CAA GAA
Ile Ser Pro Tyr Ser Asn Gln Val Gly Gly Pro Tyr Glu Leu Tyr Pro Phe Thr Phe Phe Ser Met Leu Gln Glu

                                              A (4)
GTA CAA GGT GAT TTA GGA TTT GAG CAG GCC TTT GCC ACA CGT AAC TTT TTC AAT ACT CTT GTT TCT GAT CGA CTA    2740
Val Gln Gly Asp Leu Gly Phe Glu Gln Ala Phe Ala Thr Arg Asn Phe Phe Asn Thr Leu Val Ser Asp Arg Leu
                                              Tyr

TCC TTA ATG GAA AAT ACG ATG TTA CTT ACA GAA AGT TTT GAT TAT ACA CCT TGG GAT GCT ATT TAT GGA GAT ATT
Ser Leu Met Glu Asn Thr Met Leu Leu Thr Glu Ser Phe Asp Tyr Thr Pro Trp Asp Ala Ile Tyr Gly Asp Ile

AAT TAT GAT GAA CAA TTT GCT GCA ATG TCT ATT AAT GAA CGC ATA GAA AAA TGT ATG AAT ACC TAT AGA GGT GTG    2890
Asn Tyr Asp Glu Gln Phe Ala Ala Met Ser Ile Asn Glu Arg Ile Glu Lys Cys Met Asn Thr Tyr Arg Gly Val

GCA TTC CAA AAC TCT TCA AAA AGT ATT GAC TTT TTC CTA AAT AAT CTA ACC ACA TTC ATT GAT AAT GGA CTA ACC
Ala Phe Gln Asn Ser Ser Lys Ser Ile Asp Phe Phe Leu Asn Asn Leu Thr Thr Phe Ile Asp Asn Gly Leu Thr

GAA ATT GCT ATA TCT GAT TTA CCG TAT GAT ATT GTG CAA CAA GAA ATC TCT CAA TTC TTA CAA GGA AGT AAT GAA    3040
Glu Ile Ala Ile Ser Asp Leu Pro Tyr Asp Ile Val Gln Gln Glu Ile Ser Gln Phe Leu Gln Gly Ser Asn Glu

TGG AAA ACA CTT GAT GCC ATG TTA TTT AAC TTA GAT AAA GGA GAT ATT AAT GGT GCT TTC AGA AAG CTT CTG CAA
Trp Lys Thr Leu Asp Ala Met Leu Phe Asn Leu Asp Lys Gly Asp Ile Asn Gly Ala Phe Arg Lys Leu Leu Gln

TCA GCA AAA GAT AAT AAT ATA AAA TTT AGA GCT ATA GGG CAT TCA GAT AAT TCT GTT CCG CCA TTT AAT AAC CCT    3190
Ser Ala Lys Asp Asn Asn Ile Lys Phe Arg Ala Ile Gly His Ser Asp Asn Ser Val Pro Pro Phe Asn Asn Pro

TAT AAG TCT TTA TAT TAT AAA GGA AAT ATA ATA GCT GAA GCA ATT GAA AAA CTA GAT CGA GAA GGT CAA AAA TTT
Tyr Lys Ser Leu Tyr Tyr Lys Gly Asn Ile Ile Ala Glu Ala Ile Glu Lys Leu Asp Arg Glu Gly Gln Lys Phe

GTT GTA TTT GCT GAT AGT TCT CTG CTC AAC AGC ACG CCT GGG ACA GGT CGT CCT ATG CCA GGA CTA GTT CAA TAT    3340
Val Val Phe Ala Asp Ser Ser Leu Leu Asn Ser Thr Pro Gly Thr Gly Arg Pro Met Pro Gly Leu Val Gln Tyr

TTA AAA ATA CCA GCA ACT GTA GTA GAT AGC GAT GGT GCA TGG CAA TTT CTT CCA GAT GTA GCT TCA AGC AGA GTT
Leu Lys Ile Pro Ala Thr Val Val Asp Ser Asp Gly Ala Trp Gln Phe Leu Pro Asp Val Ala Ser Ser Arg Val

CCT ATT GAA GTT ACA GAG TTA GAA AAT TGG CAA GTC TTA ACT CCT CCA CAA GGT AAG ATT CTT GGA TTA AAG CAA    3490
Pro Ile Glu Val Thr Glu Leu Glu Asn Trp Gln Val Leu Thr Pro Pro Gln Gly Lys Ile Leu Gly Leu Lys Gln

TTT AAG TTA ACG GCA GGT TTT CCA ACA GAA CAA AGT CGC TTA CCT CTT TTA GAG AAT TCG GTT TCT GAA GAT TTA
Phe Lys Leu Thr Ala Gly Phe Pro Thr Glu Gln Ser Arg Leu Pro Leu Leu Glu Asn Ser Val Ser Glu Asp Leu

AGG GAA GAA TTA ATG CAA AAG ATT GAT GCA ATA AAA AAT GAT GTG AAA ATG AAT AGT TTA GTG TGT ATG GAA GCT    3640
Arg Glu Glu Leu Met Gln Lys Ile Asp Ala Ile Lys Asn Asp Val Lys Met Asn Ser Leu Val Cys Met Glu Ala

GGC TCT TGT GAT TCA GTA AGC CCT AAG GTA GCT GCC CGT CTT AAA GAT ATG GGG TTA GAA GCT GGG ATG GGT GCT
Gly Ser Cys Asp Ser Val Ser Pro Lys Val Ala Ala Arg Leu Lys Asp Met Gly Leu Glu Ala Gly Met Gly Ala

TCT ATT ACC TGG TGG AGA CGT GAA GGC GGG ATG GAA TTT TCA CAT CAG ATG CAT ACT ACT GCT TCC TTT AAA TTT    3790
Ser Ile Thr Trp Trp Arg Arg Glu Gly Gly Met Glu Phe Ser His Gln Met His Thr Thr Ala Ser Phe Lys Phe

GCT GGT AAA GAG TTT GCC GTG GAT GCT TCA CAT TTA CAA TTT GTA CAC GAC CAA TTA GAT ACA ACT ATC CTG ATA
Ala Gly Lys Glu Phe Ala Val Asp Ala Ser His Leu Gln Phe Val His Asp Gln Leu Asp Thr Thr Ile Leu Ile

CTA CCT GTA GAT GAT TGG GCT TTA GAA ATA GCT CAA AGA AAT CGG GCT ATT AAT CCT TTT GTG GAA TAT GTT AGT    3940
Leu Pro Val Asp Asp Trp Ala Leu Glu Ile Ala Gln Arg Asn Arg Ala Ile Asn Pro Phe Val Glu Tyr Val Ser

AAA ACA GGA AAC ATG TTA GCA CTC TTC ATG CCT CCT CTT TTC ACA AAG CCT CGC TTA ACA AGA GCA CTA TAA CTA
Lys Thr Gly Asn Met Leu Ala Leu Phe Met Pro Pro Leu Phe Thr Lys Pro Arg Leu Thr Arg Ala Leu *

ATTAAAAACTGTATTAAAGCCTTATATTATAAGGCTTTAATTTTCTTTCAAGAATTATTAAGTAGAAGAATCAAAATCAATGAGATAGATAAAATCAAAT    4115

GTTATTACCAATACAACTTTCTTAAGTATACTTTTTGAATTTTTTGCGTTAATAAATTTATAATACCCTTAACTCAATAAAAGAAGTTATTGAGAAGTTT

AAATCTTGTGAGCAAGATGAAGATATAATTTCAGCAATCGATCTTATTAGCGCTTCATATAGAAGGGCTGTGGATGCAGTGGAACAAAGATTCGGTTCTAG    4316
```

: Shine Dalgarno ribosome binding site _____: terminator

Fig. 1 continued

the *P. multocida* toxin sequence which had become available [17], a differnt strategy was adopted to obtain the full sequence of the mitogenic toxin.

Oligonucleotides based on the available sequence [17] were synthesised on an Applied Biosystems 381A machine at intervals of about 200 bases along the gene and these were used for double-stranded sequencing using the Sequenase kit according to the manufacturer's instructions. For reasons of safety, most of the sequencing was done using a non-toxigenic derivative of the gene (pAJL14) which was created by removal of a 394 bp fragment by digestion with *Nde*I and subsequent religation. Removal of the *Nde*I fragment induces a frameshift mutation, resulting in a non-toxigenic construct (see below). Plasmid pAJL13 was used to obtain the sequence of this fragment, but in a containment laboratory. Sequence analysis was carried out using UWGCG programs.

3. RESULTS AND DISCUSSION

3.1. *Sequence of the mitogenic toxin*

The sequence (Fig. 1) contained an open reading frame (ORF) starting at base 155, which encode a pro-

tein of 1285 amino acids which would have a molecular mass of 146 kDa (Fig. 1). This is in accord with the apparent molecular mass of the mitogenic toxin as estimated by SDS-PAGE. The most likely start is the methionine shown, since the deduced amino acid sequence from this point is identical to the N-terminal amino acid sequence of PMT1 [15] except for one residue. The difference is likely to be due to experimental error in amino acid sequencing. It has been claimed that the N-terminal was blocked [17,18]. The difference may be related to the source of the toxin since one was purified from recombinant *E. coli* (unblocked) and the other from *P. multocida* (blocked). A ribosome binding site is immediately upstream of the start (Fig. 1), but no obvious *E. coli* consensus promoter sequence is present. Petersen [18] has presented convincing evidence for the location of the promoter region. The gene is expressed well in *E. coli* [12], but since little is known about *Pasteurella* gene organisation, it is not clear whether promoter sequences which function in *E. coli* do so in *P. multocida*. There were other methionine residues downstream from the presumed start. Although none had a good consensus with a ribosome binding site it is possible that some of the minor polypeptides produced by the recombinant which are antigenically related to the toxin [12] might have resulted from initiation at these residues. There was a stem loop structure typical of a terminator region starting at base 4027.

There was no evidence for a signal sequence at any of the potential start codons, which agrees with the lack of secretion from either the native *P. multocida* or the recombinant *E. coli*. A hydrophobicity plot (Fig. 2) indicated that the protein might have several domains; in particular there was a hydrophobic region between amino acids 380 and 470 which was bounded by two hydrophilic domains, and the C terminal of the protein was also hydrophobic. Experiments with the lysosomotrophic agent methylamine, which blocks entry of certain toxins [2], and neutralisation of toxin action by antibody when added early but not late to toxin treated cells both indicate that the toxin binds to the cell surface, is internalised and subjected to processing in endosomal/lysosomal compartments [15], from which it presumably exits to exert its biological effects. The hydrophobic regions revealed in Fig. 2 are likely to play a role in the interaction of the toxin with various cellular membranes.

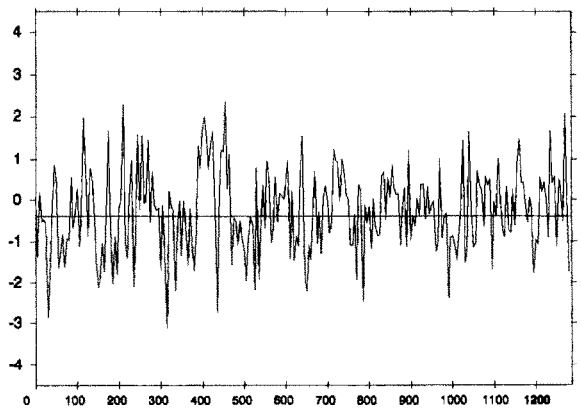

Fig. 2. Hydrophobicity plot of the PMT1 protein, calculated using a program written by Dr M.E.G. Boursnell. Positive numbers indicate hydrophobicity.

The DNA and deduced amino acid sequences were compared with the sequences on the EMBL, Genbank and Swissprot data bases, but no significant homology was found. The motif His-Glu-Trp which is common to several toxins which ADP-ribosylate their substrate [24] was found near the N-terminus of the toxin (Fig. 3). The spacing between the amino acids matched precisely the His-Glu-Trp motif found in the ADP-ribosylating toxins (Fig. 3). Amino acid motifs thought to indicate the NAD and protein substrate binding site in the ADP ribosylating toxins [25] were not present in this toxin. It should be noted that the His-Glu-Trp motif was also found in a variety of proteins that are not thought to ADP-ribosylate any substrates (unpublished).

As predicted from the restriction map [12] the gene had a low G + C content (35%). Interestingly, the G + C content of the *Pasteurella* genome differs markedly from that of the toxin gene, so it is possible that the gene has been acquired relatively recently and that there might exist a family of closely related mitogenic toxin genes in other bacteria. Indeed, recent reports indicate that the toxin gene is flanked by phage elements [17], may be carried on a prophage [26] and that some *Salmonella* isolates contain part of the *Pasteurella* toxin gene [27].

3.2. *Mitogenicity of* P. multocida *toxin from another isolate*

The sequence (hereafter called PMT1) was compared to the other *P. multocida* toxin sequences available (PMT2 [17]; PMT3 [18]; PMT4 [19]). There were 4 differences within the open reading frames of the genes, and in each case the PMT1 sequence was checked

P. multocida toxin	His	125	Glu	4	Trp
	29		155		160
Pseudomonas aeruginosa ETA	His	126	Glu	4	Trp
	426		553		558
Corynebacterium diphtheriae DT	His	126	Glu	4	Trp
	21		148		153
Bordetella pertussis S1	His	126	Glu	4	Trp
	83		210		215
Vibrio cholerae CT	His	125	Glu	3	Trp
	44		170		174
Escherichia coli LTH	His	125	Asp	3	Trp
	44		170		174

Fig. 3. Comparison of the His-Glu-Trp motif in PMT1 with other toxins. The data for the other toxins are taken from [24]. The numbers between the amino acids indicate the spacing between them; the numbers below indicate the position in each protein of the respective amino acid.

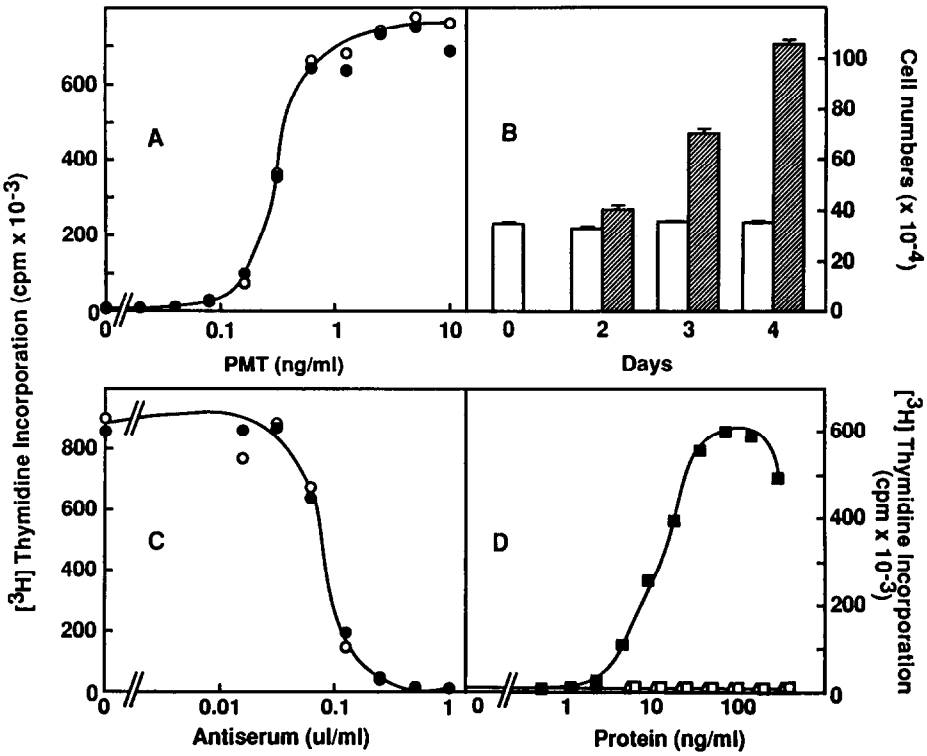

Fig. 4. Comparison of the mitogenic activity of PMT1 and PMT2. (A) Dose-response curve for the stimulation of DNA synthesis by PMT1 (○) or PMT2 (•). Cultures of Swiss 3T3 cells were incubated with PMT and DNA synthesis measured as described [15]. Each point is the mean of two determinations: 10% foetal bovine serum gave a level of incorporation of 704 × 10^3 cpm. (B) PMT2 stimulates reinitiation of growth in confluent Swiss 3T3 cells. PMT2 was added directly to the medium of Swiss 3T3 cell cultures 6 days after plating to give a final concentration of 10 ng/ml. 2, 3 and 4 days after this addition, cells from both treated (hatched bars) and untreated (open bars) cultures were trypsinised and counted using a Coulter counter. The values shown are mean ± SE (n = 5). (C) Dose-response for the inhibition by PMT antiserum (polyclonal 34B) of DNA synthesis induced by PMT1 (○) or PMT2 (•). Various concentrations of immune serum were incubated with toxin and added to confluent quiescent Swiss 3T3 cells as described [15]. Each point is the mean of two determinations: 10% foetal bovine serum gave a level of incorporation of 979 × 10^3 cpm. (D) Dose-response curve for the stimulation of DNA synthesis by extracts of *E. coli* transformed with various constructs. Swiss 3T3 cells were treated as described [15] with various concentrations of HB101 + pAJL14 (□), HB101 + pAT153 (△) or HB101 + pAJL12 (■). Each point is the mean of two determinations: 10% foetal bovine serum gave a level of incorporation of 672 × 10^3 cpm.

carefully for errors. There was a single base change between the PMT1 and the PMT4 sequences (at base position 2712) which resulted in a conservative amino acid substitution. There were 2 differences between the PMT1 gene and that of the gene for PMT3, at base positions 1064 and 2477; both resulted in a change from an alanine in PMT1 to an arginine residue in PMT3. There was one other difference between the sequences of PMT1 and PMT2 at base position 396.

These minor differences among the sequences might be due to errors in sequencing, or reflect variation among isolates. If the latter were true, it was important to investigate whether the potent mitogenicity of the protein was affected. PMT2, purified from *P. multocida* NCTC12178, and PMT1 stimulated DNA synthesis with an identical dose-response relationship in Swiss 3T3 cells (Fig. 4A); there was a similar toxin-induced increase in cell proliferation (Fig. 4B). The mitogenic activity of each toxin was completely blocked by antibody to PMT1 in a concentration dependent manner (Fig. 4C).

One of the recombinants produced in the original clone bank contained part of the gene, but did not produce toxin active on EBL cells [12]. This recombinant produced polypeptide fragments which reacted with antibody to the toxin. Restriction enzyme analysis and the DNA sequence of this plasmid showed that it contained the N-terminus of the gene, and should code for amino acids 1–287 to produce a 33.5 kDa peptide (Fig. 1). A crude preparation from this recombinant was unable to induce DNA synthesis (Fig. 4D).

4. CONCLUSIONS

We report the complete sequence of the mitogenic toxin PMT1. The deduced protein sequence did not show any significant homology to known sequences with the possible exception of a motif found in toxins which ADP-ribosylate their substrate. There appear to be two hydrophobic domains in the toxin, which could play a role in the interaction of the toxin with cellular membranes. The sequence was almost identical to other

P. multocida toxin sequences [17–19], and the toxin purified from one of these latter strains was as mitogenic as PMT1 in Swiss 3T3 cells.

It has been suggested that other bacterial toxin genes originally had a eukaryotic origin, and this might also be the case for the *P. multocida* toxin. The toxin might then be expected to have an as yet undiscovered eukaryotic homologue involved in signal transduction. It is to be hoped that analysis of the toxin action will not only explain its role in atrophic rhinitis, where it has specific effects on bone morphogenesis, but also yield important information about new signal transduction pathways.

Acknowledgements: We wish to thank Dr Ian Goldsmith and Karen Mawditt for synthesis of oligonucleotides and Fara Khurshid for automated DNA sequencing.

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