The cytotoxin of *Pseudomonas aeruginosa*: Cytotoxicity requires proteolytic activation

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Abstract. The primary structure of a cytotoxin from *Pseudomonas aeruginosa* was determined by sequencing of the structural gene. The cytotoxin (31,700 Mr) lacks an N-terminal signal sequence for bacterial secretion but contains a pentapeptide consensus sequence commonly found in prokaryotic proteins which function in a TonB-dependent manner. The cytotoxin gene has a [G + C]-content of 53.8% which is considerably lower than generally observed for genes from *Pseudomonas aeruginosa*. The cytotoxin gene was exclusively detected in strain 158 but not in three other clinical isolates, as determined by Southern and Northern hybridization. The latter technique revealed that the toxin is translated from monocistronic mRNA. The promoter of the cytotoxin is inactive in *Escherichia coli*. Upon site-directed modification of the 5'-noncoding region by the polymerase chain reaction the gene was expressed under control of the trc-promoter. The gene product obtained in *Escherichia coli* was nontoxic. Toxicity was induced by subsequent treatment with trypsin. [35S]methionine-labeled cytotoxin with high specific radioactivity was obtained by in vitro transcription/translation. Like [125I] labeled material from *Pseudomonas aeruginosa* this polypeptide bound to membrane preparations from Ehrlich ascites cells, as evidenced by sedimentation through a sucrose gradient at neutral pH.

Key words: *Pseudomonas aeruginosa* — Cytotoxin structure — Proteolytic processing — Ehrlich ascites cells

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*Pseudomonas aeruginosa* is an opportunistic pathogen causing life-threatening disease in patients with weakened defense system by producing several toxic factors (Neu 1985). The cytotoxin has been characterized in autolysates of a *Pseudomonas aeruginosa* strain isolated from bovine mastitis milk. The protein accumulates in the periplasm of the bacterium (Kluftinger et al. 1989) and becomes liberated by autolysis rather than by secretion (Scharmann 1976). Isolated from bacterial autolysates, the cytotoxin has been characterized as a protein of 25,000 to 29,000 Mr which acts primarily on the plasma membranes of mammalian cells (Baltch et al. 1987; Kluftinger et al. 1989; Lutz 1979) by binding to a high affinity binding sites (Lutz 1986). A consequence, pores of about 2 nm diameter (Lutz et al. 1987) are formed resulting in a breakdown of the cellular gradient for low molecular substances. The role of the cytotoxin in the manifestation of the *Pseudomonas aeruginosa* infection, however, has not been thoroughly investigated.

In this paper, we present the sequence of the cytotoxin. We show that a posttranslational activation step involving proteolytic removal of a 3,000 Mr peptide from the carboxy-terminal end takes place during or after autolysis.

Materials and methods

Materials. Enzymes were purchased from Boehringer (Mannheim, FRG), [32P]ATP, [35S]dCTP, and L-[35S]methionine were from Amersham (Braunschweig, FRG). Rabbit reticulocyte lysate was obtained from Amersham or Promega (Heidelberg, FRG), nucleotides and ribonuclease inhibitor were from Pharmacia (Freiburg, FRG), and anti-rabbit IgG from Dako (Copenhagen, Denmark). Diethylpyrocarbonate was from Sigma (München, FRG).

Bacterial strains and plasmids. *Pseudomonas aeruginosa* strain 158 (0:6; H:a0, a2; pyocin:38,) was a clinical isolate from bovine mastitis milk. *Pseudomonas aeruginosa* strains 032 and 037 were isolated as swab samples from horse vagina or dog ear. The 054 strain was isolated from foals of a septicemic cow. All strains were propagated at 30°C on TSA/TSB (Difco, Detroit). *Escherichia coli* strains HB101 and JM101 were grown at 37°C in 2YT or M9-minimal medium. Plasmids pUC18/19 (Messing and Vieira 1982), M13mp18/19 (Norrander et al. 1983) and pRN653A,B,C (H. Niemann, A. Smid, M. Rosing, and E. Amann, unpublished) were used for establishing DNA-libraries, for DNA-sequencing, and for combined in vitro transcription/translation, respectively. For tightly regulated expression of the cytotoxin gene in *Escherichia coli* the IPTG-inducible vector pTrc99a (Amann et al. 1988) was used.
Expression of the cytotoxin in Escherichia coli. 10 ml precultures of Escherichia coli strain HB101 harboring pSN3 were prepared in LB medium and used to inoculate 90 ml of 1SB and 100 mg ampicillin per l. Cells were grown up to an optical density of OD660 nm of 1.0 and synthesis of the cytotoxin was induced by the addition of 1 ml of 0.5 mM IPTG. The incubation was continued for another 2 h at 37°C when cells were harvested by centrifugation at 16,000 × g, washed once with phosphate buffered saline (PBS), pH 7.4, and resuspended in 1 ml of PBS. The cells were incubated for 12 h at 37°C (autolysis step). Insoluble material was removed by centrifugation (30 min at 15,000 × g) and the supernatant was stored at −20°C. Toxicity assays were performed according to Gladstone and van Heyningen (1957). Proteolytic activation of the cytotoxin was achieved by the addition of 2.7% (w/w protein) 10CK-trypsin (40 U/mg, Boehringer) in lysates containing 3 mM (final concentration) of CaCl₂. After 2 h at 37°C reactions were stopped by the addition of 5-fold molar excess of trypsin inhibitor from soybean in 10 mM EDTA.

In vitro transcription/translation. The coding region of the cytotoxin gene was cloned from the EcorV site (Fig. 3) on the 3'-PstI site into Sau3AI-PstI digested pRN653C to yield pOE65EP33. The sequence of the 5'-recombination site was verified by direct sequencing using the SP6 sequencing primer. Plasmid DNA was purified by two consecutive centrifugations on CsCl-density gradients. Transcripts with SP6-polymerase and translations in rabbit reticulocyte lysate were performed as described previously (Mayer et al. 1988).

Interaction with plasma membrane preparations from Ehrlich ascites cells. Cytotoxin labeled during in vivo translation or by sodination was incubated for 2 h at 4°C and subsequently for 30 min at 30°C with plasma membrane preparations from Ehrlich mouse ascites cells (Kilbarg and Christiansen 1979). To assess membrane association, the incubation mixture was then placed on a sucrose cushion prepared in buffer that was either at pH 7.3 or at pH 11.3, as described in detail previously (Mayer et al. 1988). The pellet and the supernatant fraction were analyzed by SDS-PAGE.

gel electrophoresis and immunoprecipitation. SDS-PAGE and processing with DMSO-PPO for autoradiography were performed as described (Niemann and Klenk 1980). Rabbit antibodies against cytotoxin were purified by binding the antibodies to nitrocellulose carrying the purified toxin according to Burke et al. (1982). Protein A bearing Staphylococcus aureus, strain Cowman I, was used to bind immune complexes.

Results

Determination of the primary structure of the cytotoxin from Pseudomonas aeruginosa. To establish the primary sequence of the cytotoxin from Pseudomonas aeruginosa, we have cloned and sequenced 12 overlapping hybridization-positive chromosomal DNA-fragments, as identified with an oligonucleotide probe reflecting all the possible codons for the N-terminal amino acid sequence (Fig. 1). Figure 3 shows a continuous stretch of 1237 nucleotides containing a single open reading frame of 858 bp encoding a polypeptide of 286 amino acids with a molecular weight of 31,700. The sequence of the N-terminus was identical to the sequence determined by Edman degradation and the amino acid composition was in agreement with previous constituent analyses (Lutz 1979).

The hydrophathy plot of the cytotoxin according to Kyte and Doolittle (1982) did not indicate hydrophobic...
domains that could be involved in catalyzing membrane integration. In addition, no signal sequence for secretion and no α-helical transmembrane domains were detected using the programs of Roa and Argos (1986) or Klein et al. (1985), respectively.

**Organization and distribution of the cytotoxin gene**

A Shine-Dalgarno consensus sequence (AGGA) was found 12 nucleotides upstream from the translation start codon ATG-codon. The [G + C]-content of the coding region (53.8%) is significantly lower than that reported for chromosomally integrated genes of *Pseudomonas aeruginosa* (West and Iglewski 1988), indicating that the gene could originally stem from a different organism. This hypothesis is further supported by our finding that the cytotoxin gene is absent in three other clinical isolates of *Pseudomonas aeruginosa* as evidenced by Southern analyses (Fig. 4A, B). Even after 45 PCR-cycles (using oligonucleotides binding immediately upstream and downstream from the coding region and 20 ng of chromosomal DNA) these other strains failed to produce a signal in Southern blotting. In addition, Northern blot analyses of RNA from the individual strains also indicated the absence of cytotoxin-specific transcripts (data not shown).

The open reading frame was followed by two inverted repeat structures indicated by divergent arrows in Fig. 3. The free energy values (Tinoco et al. 1973) of these stem-loop structures, $-92.05 \text{ KJ/mol}$ and $-79.5 \text{ KJ/mol}$, suggest that they could function as transcription-termination signals. Northern blot analyses of RNA from strain 158 revealed that the cytotoxin-specific mRNA had a size of about 1100 nucleotides (data not shown). Taken together, these data support the conclusion that the cytotoxin gene is transcribed into monocistronic mRNA.
Expression of the cytotoxin gene in vitro and in Escherichia coli

To test the properties of the cloned gene product, we expressed the gene in vitro or in Escherichia coli and compared the properties of the products with those of unlabeled or [125I] labeled cytotoxin, as isolated from Pseudomonas aeruginosa. For combined in vitro transcription translations, we cloned the coding region from chromosomal DNA isolated from various Pseudomonas aeruginosa strains. Chromosomal DNA was isolated from strains 032 (lane 1), 037 (lane 2), 054 (lane 3), and 158 (lane 4), digested with PstI, and analyzed on a 0.8% agarose gel together with EcoRI/HindIII digested lambda DNA (lane M). After transfer onto a nitrocellulose sheet cytotoxin specific fragments were detected with a nick-translated EcoRV-PstI fragment from pSN3.

As shown in lanes 2 and 3 of Fig. 5B, treatment of chromosomal DNA isolated from strains 032 (lane 2), 054 (lane 3), and 158 (lane 4) with PstI and analyzed on a 0.8% agarose gel together with EcoRI/HindIII digested lambda DNA (lane M). After transfer onto a nitrocellulose sheet cytotoxin specific fragments were detected with a nick-translated EcoRV-PstI fragment from pSN3.

8 foreigns residues encoded by the polylinker region. Translation of the RNA in rabbit reticulocyte lysate produced a major polypeptide of 30,000 Mr (Fig. 5A, lane 1). This molecular species had an electrophoretic mobility that was indistinguishable from material isolated from intact Pseudomonas aeruginosa cells (compare lanes 1 and 2).

As demonstrated by Western blotting, the 30,000 intracellular form of the cytotoxin (lane 3) migrated clearly slower in SDS-PAGE than the 28,000 material that was isolated from autolysates (lane 4). Puls-chase experiments of [13S]methionine labeled sister cultures of Pseudomonas aeruginosa did not reveal a conversion of the 30,000 species into the 28,000 species (data not shown) indicating that the putative processing step had to occur during autolysis of the bacteria. Expression of the cytotoxin gene in Escherichia coli was inducible with IPTG (compare lanes 1 and 2 in Fig. 5B). Again yielding material that migrated like the non-processed form of the cytotoxin in SDS-PAGE (compare with lane 4). This material was clearly nontoxic in the granulocyte lysis assay (Fig. 6A). As shown in lanes 2 and 3 of Fig. 5B, treatment of Escherichia coli lysates with trypsin converted the 30,000 species into two smaller species of 28,000 and 26,000 (lane 3). Concomitantly a rapid increase in toxicity was observed (Fig. 6B), indicating that the removal of the
short peptide sequence led to the activation of the cytotoxin.

Binding properties of the cytotoxin to plasma membrane preparations from Ehrlich ascites cells

To see whether this proteolytic processing step altered the binding properties of the cytotoxin to cellular receptors, we performed binding studies of the in vitro synthesized cytotoxin derivative and compared it with iodinated cytotoxin as derived from Pseudomonas aeruginosa autolysates. Binding of the cytotoxin to membrane preparations was assessed by co-sedimentation of the radio-labeled cytotoxin with the membranes through a sucrose cushion of neutral pH. The results are summarized in Fig. 7. No difference was detected in the binding properties of the in vitro synthesized full-size cytotoxin and the processed cytotoxin. In both instances binding was reversible by the addition of a 100-fold excess of unlabeled cytotoxin (data not shown). However, binding apparently involved only attachment to peripheral binding sites, since a significant amount of labeled material eluted from the membranes, when the pellet fraction was resuspended and re-sedimented through the sucrose cushion (compare lanes 9 and 10 of Fig. 7). Furthermore, no co-sedimentation of the labeled material was observed when the sucrose was made up in buffer of pH 11.0, again indicating that the bound material was not converted into an intrinsic membrane protein. It is clear, however, that
such observations have to be confirmed by experiments involving binding to intact cells.

Discussion

We have established the sequence of a pore-forming cytotoxin from *Pseudomonas aeruginosa* by determining the amino-terminal amino acid sequence of the purified protein and sequencing of the structural gene, as identified by a pool of synthetic oligonucleotides. The cytotoxin sequence did not reveal a significant sequence similarity with any other known protein. It is important to note that no proteolytic processing of the N-terminus of the protein occurs during or after bacterial autolysis. As generally observed with procaryotic polypeptide carrying an asparagine residue in position 2, the methionine residue is retained in the mature toxin molecule (Ben-Bassat and Bauer 1987). In agreement with a previous report (Schermann 1976) indicating that the cytotoxin was released from the bacteria only after several days of growth, the molecule lacks a secretory signal.

Within the N-terminal domain, a remarkable homology to a pentapeptide consensus sequence (TonB-box), commonly found in outer membrane receptor proteins of the *Escherichia coli* iron transport system, was detected. As yet, the TonB-box was found exclusively in all proteins that function in a TonB-dependent manner (Braun et al. 1987). Interestingly, this group of proteins contains also some colicins known to kill closely related bacteria by pore formation. Uptake of such colicins by the target cell occurs in a receptor mediated and TonB-dependent process (Braun et al. 1987). Recent modifications of the TonB-box from the FhuA receptor by site-directed mutagenesis (Schöffler and Braun 1989) have shown that a replacement of the Val residue by aspartic acid only weakened the colicin M sensitivity of the *Escherichia coli* strain indicating that the interaction between the FhuA receptor and the TonB protein was not completely abolished. At present, we do not know whether the cytotoxin serves a colicin-like function for *Pseudomonas aeruginosa*.

The molecular weight of the cytotoxin purified from bacterial autolysates was 28,000 as determined by SDS-PAGE. This material migrated clearly faster than the 30,000 Mr species obtained by in vitro transcription/translation or by expression in *Escherichia coli*. Although the in vitro synthesized material bound specifically to membrane preparations from Ehrlich ascites cells, exhibiting properties indistinguishable from the mature [125I] labeled cytotoxin, this non-processed form was nontoxic in the granulocyte lysis assay. Cytotoxicity clearly required proteolytical processing which in autolysates was mediated by endogenous proteases. Trypsin-treatment of *Escherichia coli* lysates also restored cytotoxicity. Such processing could involve only C-terminal sequences, since identical N-termini were determined by Edman degradation and by DNA-sequencing. The mechanism by which pore formation through the cytotoxin is induced is far from being understood at the molecular level. We show here that binding to peripheral acceptor sites does not require proteolytic processing and

### Table 1
Sequence homology of the cytotoxin from *Pseudomonas aeruginosa* with various TonB-dependent proteins from *Escherichia coli*.
The TonB-specific sequences are framed.

| Protein | Number of the first residue shown | TonB sequence | Reference |
|---------|----------------------------------|---------------|-----------|
| Cytotoxin | 4 | IDITIHAW | this study |
| FhuA | 6 | IDITTVTA | Coulton et al. 1986 |
| FhuE | 5 | IDITTVTA | Sauer et al. 1987 |
| BtuB | 25 | PDDLTVTA | Heller and Kadner 1985 |
| FecA | 22 | CFITLSVDA | Pressler et al. 1988 |
| FepA | 11 | DDITTVVA | Lundrigan and Kadner 1986 |
| Car | 5 | GETMVSTA | Griggs et al. 1987 |
| IutA | 5 | DEKTVSTA | Kröck et al. 1987 |
| Colicin M | 1 | METTVSTA | Schramm et al. 1987 |
| Colicin B | 16 | GTGTVVWP | Mankovich et al. 1984 |
| Colicin 1b | 22 | HEEMAYDI | |
also does not involve the N-terminal sequences, since the in vitro synthesized cytotoxin had similar binding properties like the mature molecule. With the cytotoxin gene at hand and the development of various deletion mutants thereof further studies on the pore formation process can now be undertaken.

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