Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 256-262

Identification and Expression of the *Actinobacillus actinomycetemcomitans* Leukotoxin Gene

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Received January 20, 1989

Summary: The leukotoxin produced by the oral bacterium *Actinobacillus actinomycetemcomitans* has been implicated in the pathogenesis of juvenile periodontitis. In order to elucidate the structure of the leukotoxin, molecular cloning of the leukotoxin gene was carried out. A DNA library of *A. actinomycetemcomitans*, strain JP2, was constructed by partial digestion of genomic DNA with *Sau*3AI and ligation of 0.5 to 5.0 kilobase pair fragments into the *Bam* HI site of the plasmid vector pENN-vrf. After transformation into *E. coli* RR1(λ*c*I857), the clones were screened for the production of *A. actinomycetemcomitans* leukotoxin with polyclonal antibody. Six immunoreactive clones were identified. The clones expressed proteins which ranged from 21-80 kilodaltons, and the clone designated pII-2, producing the largest protein was selected for further study. Antibodies eluted from immobilized pII-2 protein also recognized the native *A. actinomycetemcomitans* leukotoxin molecule indicating that both molecules shared at least one epitope. DNA sequence analysis demonstrated that there are regions of significant amino acid sequence homology between the cloned *A. actinomycetemcomitans* leukotoxin and two other cytolysins, *Escherichia coli* α-hemolysin and *Pasteurella haemolytica* leukotoxin, suggesting that a family of cytolysins may exist which share a common mechanism of killing but vary in their target cell specificity.

Microbial toxins play a major pathogenic role in many infections. In a series of studies, we have demonstrated that *A. actinomycetemcomitans*, a gram-negative coccobacillus shown to be associated with a form of juvenile periodontitis, produces a leukotoxin with unique biological properties. Human polymorphonuclear leukocytes (PMN), monocytes (MN) and certain human myelomonocytic leukemia cell lines (e.g., HL-60, and U937) are killed by the toxin as are PMN and MN from the great apes and Old World monkeys (1,2,3). PMN and MN of most other species, including New World monkeys, are not susceptible to the lethal effects of the toxin. Furthermore, other cells from both susceptible and nonsusceptible species, including small lymphocytes, epithelial and endothelial cells, fibroblasts, erythrocytes, and platelets are unaffected by the toxin (4,5).

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0006-291X/89 $1.50

The toxin can be extracted in relatively small amounts from freshly harvested *A. actinomycetemcomitans* by polymyxin B sulfate and subsequently purified by a combination of DEAE-cellulose, CMC-50 Sephadex and Sepharose 6B column chromatography (6). The purified toxin consists of two polypeptides of approximately 120 kDa that migrate closely after isoelectric focusing in urea gels indicating that they are similar in surface charge (7). The mechanism of interaction between the *A. actinomycetemcomitans* leukotoxin and target cells remains unknown. Killing of either HL-60 or U937 cells is inhibited in a dose-dependent manner by polyclonal (human, non-human primate or rabbit) and monoclonal (murine) antibodies against the leukotoxin. Furthermore, incubation of target cells or toxin with certain saccharides such as mannose or galactose (8), lectins such as wheat germ agglutinin and *Phaseolus vulgaris* lectin (9) and certain polar lipids (5) prevent cell destruction. These experiments suggest that the *A. actinomycetemcomitans* leukotoxin, like several other bacterial cytotoxins, binds to and alters the target cell plasma membrane.

An understanding of how the toxin associates with target cells may provide insights into the biology of granulocytes and macrophages and in addition may further delineate the role of the leukotoxin in *A. actinomycetemcomitans* infections. To address these interesting questions, it will be important to define the molecular interrelationships between the structure of leukotoxin and its biological functions. Utilization of molecular biology techniques to clone the leukotoxin gene and the employment of high level expression vectors provide an approach for delineating the structure of the leukotoxin as well as providing relatively large amounts of material for the study of its mechanism of action. In the present study, we report the molecular cloning and expression in *Escherichia coli* of a portion of the gene encoding the *A. actinomycetemcomitans* leukotoxin .

Methods

Culture conditions. *A. actinomycetemcomitans*, strain JP2 was grown in PYG medium for 24 h at 37° C in 5% CO_2 (6). The lysogenic [RR1(λcI857)], and plasmid-bearing [RR1(pRK248cIts)] derivatives of *E. coli*, were used for transformation (10). A plasmid library of the *A. actinomycetemcomitans* genome was constructed using the lysogenic host RR1(λcI857) which were grown in Luria-Bertani (LB) broth at 30° C.

Construction of *A. actinomycetemcomitans* genomic libraries and immunological screening. Genomic DNA was isolated from cultures of *A. actinomycetemcomitans* strain JP2 and partially digested with *Sau* 3A. Digests were analyzed by agarose gel electrophoresis; those containing DNA fragments of .5 to 5 kilobase pairs (kbp) were pooled and purified for library construction. pEV-vrf expression vectors (10) were modified by digestion with *Bam* HI and religated to eliminate one of the two *Bam* HI restriction sites to provide a unique cloning site (Fig 1A). The digestion also removed the *Cla* I and *Hind* III sites contained in the original vectors. These modified (designated pENN-vrf) vectors accommodate all three translational reading frames and contain a computer-generated model ribosomal binding site downstream from the λp_L promoter as well as a translational initiation codon. Genomic fragments were ligated into pENN-vrf plasmids with T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Overnight cultures of *E. coli*, RR1(λcI857) were centrifuged (5000 x g, 10 min), transformed with aliquots of the ligated DNA (11) , and plated onto nitrocellulose filters on LB agar containing 50 μg of ampicillin/ml. The plates were replicated after 24 h and were then incubated at 42° C for 2.5 h resulting in λp_L-driven transcription of *A. actinomycetemcomitans* DNA as well as partial lysis of the colonies.

The immunological screening of the library was carried out as described by Helfman *et al.* (12). Briefly, the colonies were lysed by exposure to chloroform vapor for 15 min followed by overnight treatment in lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 5 mM $MgCl_2$; 40 μg of lysozyme per ml; 1 μg of DNAse per ml). The filters were washed several times, non-specific

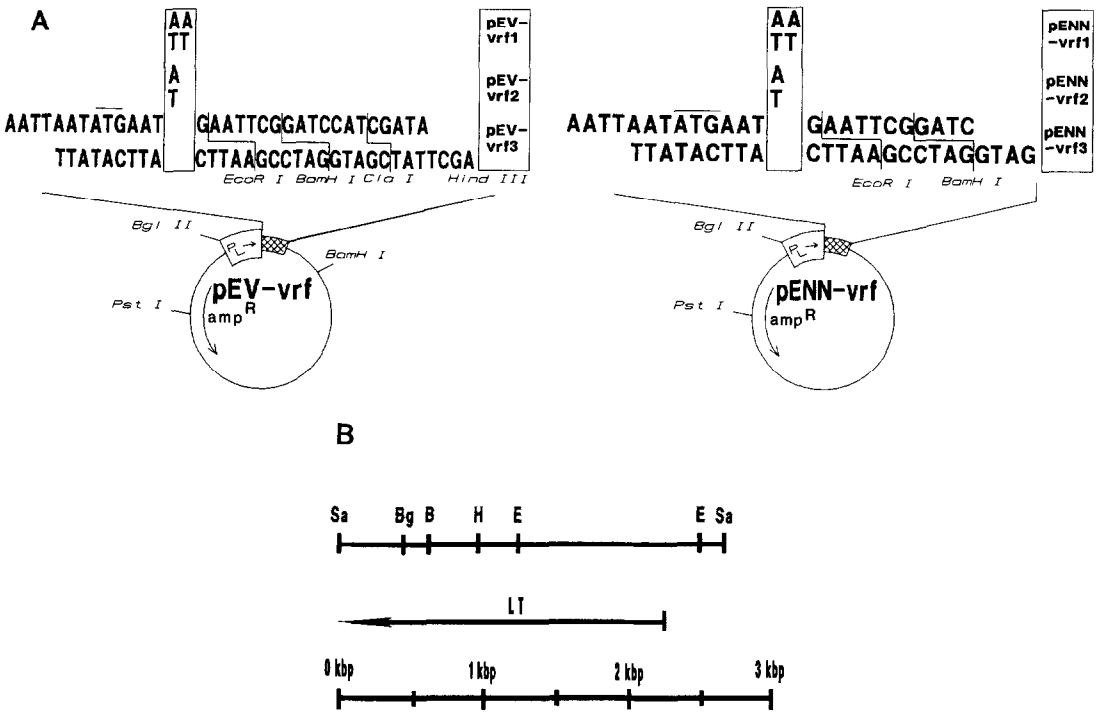


Figure 1. A. **pEV-vrf and pENN-vrf vectors.** pENN-vrf vectors were constructed from pEV-vrf vectors by digestion with *Bam* HI and religation to remove the *Bam* HI restriction fragment. The ATG start codon is overlined, and placement of additional AT bp is designated by the rectangle allowing expression in three different reading frames. The plasmids confer resistance to ampicillin (amp^R). B. **Restriction map of *A. actinomycetemcomitans* leukotoxin insert contained in pII-2.** The location and orientation of the leukotoxin gene are shown by the arrow. Restriction enzyme abbreviations are as follows: B, *Bam* HI; E, *Eco* RI; H, *Hind*III; Bg, *Bgl* II; Sa, *Sau*3AI. Only *Sau*3AI sites found at the 5' and 3' ends of the insert are indicated.

binding blocked with BLOTTO/NP-40 (5% non-fat dry milk, 0.1% NP-40, 0.01% antifoam A, 0.0001% merthiolate in PBS) (13) and reacted for 2 h with polyclonal *A. actinomycetemcomitans* leukotoxin antibody (1:1,000). Filters were washed, treated with anti-rabbit immunoglobulin G-peroxidase conjugate (Promega Biotec) for an additional 2 h, and developed with 4-chloro-1-naphthol in Tris (pH 7.5)-0.03% H_2O_2). Positive colonies were purified to homogeneity by several cycles of plating and assay.

Characterization of recombinant clones. Plasmids expressing protein immunoreactive with polyclonal antibodies to *A. actinomycetemcomitans* leukotoxin were transferred from RR1(λcI857) to RR1[pRK248cIts]. pRK248cIts is a low-copy-number plasmid that contains a gene encoding a temperature sensitive λ*cIAt2* repressor. RR1[pRK248cIts] is better suited for high level synthesis of cloned gene products than its lysogenic counterpart RR1(λcI857). Temperature-induced *E. coli* RR1[pRK248cIts] were lysed by treatment with 40 μg of lysozyme per ml followed by sonication (three 40 s bursts) on ice. Sonicated cell suspensions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-PAGE was performed on 12.5% slab gels as described previously (14) and the gels were blotted onto nitrocellulose (15).

Restriction digests were performed according to the specifications of the manufacturer, with double digests carried out sequentially. The restricted DNA was electrophoresed in 0.8% low-melting-point agarose, and when necessary, regions containing the desired fragments were excised and gel slices were melted in 0.3 M sodium acetate at 68° C and phenol extracted. The DNA was recovered by ethanol precipitation.

Affinity purification of anti-leukotoxin antibody using nitrocellulose blots. Monospecific anti-leukotoxin antibody was prepared using preparative immunoblots (16) of recombinant peptides. Immunoblots were prepared as described above. After incubation with primary antibody, the left-most and right-most lanes were excised and probed with indicator antibody to determine

the location of the recombinant peptide band. The two pieces of nitrocellulose were then used to approximate the peptide band in the remaining part of the blot. A 3 mm nitrocellulose strip, containing the immobilized peptide, was excised, cut into smaller pieces, placed into 1.5 ml of a 3M KSCN solution and agitated for 5 minutes. Control elutions were carried out on regions of the blot which contained unrelated *E. coli* proteins. The supernatants were decanted and dialyzed against 10 mM phosphate, 15 mM M NaCl, pH 7.8. All procedures were carried out at 0-4° C. The eluted antibodies were then used to probe immunoblots of native *A. actinomycetemcomitans* leukotoxin.

DNA sequencing and sequence analysis. The dideoxy chain termination method (17) in conjunction with oligonucleotides synthesized in our laboratory were used to determine the nucleotide sequence of both strands of the *A. actinomycetemcomitans* insert DNA. The DNA sequence of the leukotoxin was compared with other published sequences using GenBank Genetic Sequence Data Bank in BIONET(18) and the programs of the Genetics Computer Group of the University of Wisconsin (19) in order to identify homologies.

Results and Discussion

Polyclonal anti-leukotoxin antibodies were prepared in a manner described previously (7). The antibodies recognize two closely migrating protein bands of approximately 120 kDa which are present in a crude polymyxin B extract of *A. actinomycetemcomitans*. This preparation was used to screen expression libraries contained in pENN-vrf vectors. Initial screening of approximately 50,000 colonies in all three reading frames yielded six immunoreactive clones. Analysis of cell protein derived from lysates of each recombinant showed the clones expressed immunoreactive peptides ranging from 21 kDa to 80 kDa suggesting that only a part of the leukotoxin gene may have been cloned. Clone pII-2, expressing the largest protein, was chosen for further studies. Western blotting experiments demonstrated that this clone expressed the immunoreactive protein at higher levels when the λ promoter was uninduced than when it was induced (Fig. 2A), suggesting that the leukotoxin promoter itself may be present in the construct and that transcription initiation from the λ promoter inhibited immunoreactive protein expression. This was substantiated by experiments in which the promoter, the multiple cloning region and 400 bp of the insert were removed by digestion of the plasmid with *Bgl* II. The resulting plasmid, designated pII-2 *Bgl* II$^-$, expressed an immunoreactive protein which is approximately 10 kDa smaller than that expressed by pII-2 (Fig. 2B). Since a shortened protein was produced, this result proves that transcription was independent of the λ promoter, that the deleted sequence corresponds to the 3' portion of the transcript and suggests that the gene contained in pII-2 is oriented as shown in Fig. 1B.

To confirm the belief that the expressed pII-2 peptide was indeed the leukotoxin, a modification of the immunoblot procedure (16) was utilized. Polyclonal antibody eluted from immobilized pII-2 protein was shown to react with legitimate *A. actinomycetemcomitans* leukotoxin while eluates from control unrelated *E. coli* proteins did not (Fig. 3). This strongly suggests that the recombinant protein and the leukotoxin share at least one epitope. The molecular weight of the protein expressed by pII-2 (80 kDa) is approximately 66% of the leukotoxin molecule (120 kDa) extracted from *A. actinomycetemcomitans*. It is possible that the biologically active site site of the toxin is located in the missing carboxy-terminal region of the recombinant protein or that truncated molecules do not allow the proper folding necessary for a

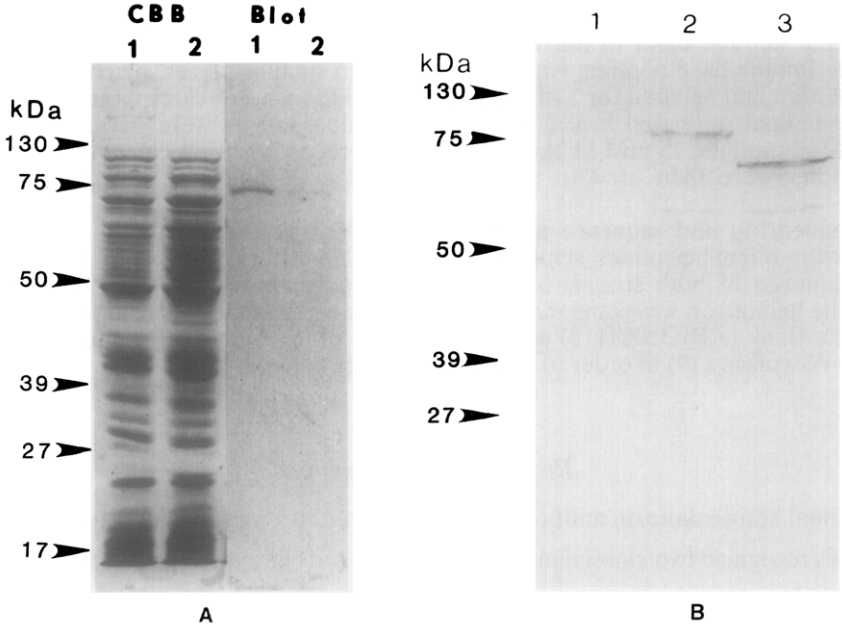
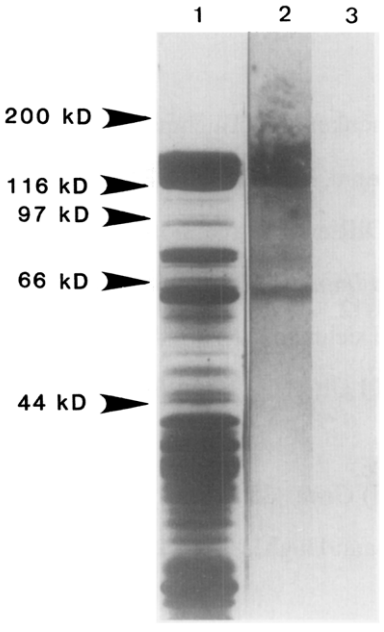


Fig. 2. A. **Analysis of protein produced in the *E. coli* containing plasmid pII-2.** Bacterial cultures of RR1[pRK248cIts] containing pII-2 were grown at 30° C and then incubated at 42° C to induce transcription from the λp_L promoter. Samples were subjected to SDS-PAGE followed by staining with Coomassie brilliant blue (CBB). lane 1, uninduced clone pII-2; lane 2 induced clone pII-2. Duplicate gels were also blotted onto nitrocellulose and developed with rabbit polyclonal anti-leukotoxin antibody (Blot). The protein M_r markers are phosphorylase B (130 kDa), bovine serum albumin (75 kDa), ovalbumin (50 kDa),carbonic anhydrase (39 kDa), soy bean trypsin inhibitor (27 kDa), and lysozyme (17 kDa). B. **Immunoblot analysis of *A. actinomycetemcomitans* proteins produced by pII-2 *Bgl* II⁻.** pII-2 was digested with *Bgl* II and religated to form pII-2 *Bgl* II⁻. Plasmid-containing bacterial cultures of RR1[pRK248cIts] were grown at 30° C. Samples were subjected to SDS-PAGE followed by immunobloting and probing with rabbit anti-leukotoxin antibody. Lane 1, pENN-vrf2 vector without insert; lane 2,clone pII-2; lane 3, clone pII-2 *Bgl* II⁻.

functional conformation. We are currently screening libraries of larger DNA fragments in λEMBL3 (20) to clone the entire gene.

Since amino acid sequence data of the leukotoxin is not yet available, the cloned insert was subjected to DNA sequence analysis in order to determine whether the deduced *A. actinomycetemcomitans* leukotoxin sequence exhibited homology to known sequences of other toxins. Screening of the GenBank data base revealed a strong homology (Fig. 4) to α-hemolysin of *E. coli* (21) and to a lesser extent, to a *P. haemolytica* leukotoxin (22). Distinct regions of highly conserved sequence exist among all three proteins, suggesting both functional and genetic homology. Results of Southern hybridization of the genomic DNA of *Proteus mirabilis, Proteus vulgaris,* and *Morganella morganii* with cloned *E. coli* α-hemolysin gene segments as probes suggest the presence of related hemolysins in these species as well (25). The *P. haemolytica* toxin appears to be specific for ruminant lymphocytes (23) and also shows sequence homology with the *E. coli* α-hemolysin (24). Taken together these analyses suggest that a family of cytotoxins may exist which share a common mechanism of killing but vary in target cell specificity.

Due to its unique tissue and species specificity, the *A. actinomycetemcomitans* leukotoxin may represent a useful reagent for studying the biology of cells of the myelomonocytic cell lineage.

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LEUTOX     WENK.YGKNY SENGYDARHS AFLEDSLKLF NELREKYKTE NILSITQQRW
ECOHYLA    WEKK.HGKNY FENGYDARHA AFLEDNFKIL SQYNKEYSVE RSVLITQQHW
PASHAEM    WEKNNHGKNY FENGYDARYL ANLQDNMKFL LNLNKELQAE RVIAITQQQW
CONSENSUS  WE.k..GKNY .ENGYDARh. AfLeD..K.. ..l...y..E ....ITQQ.W

LEUTOX     DQRIGELAGI TRNGDRIQSG KAYVDYLKRV RSLQSIAINS LNTDLDPIKG
ECOHYLA    DTLIGELAGV TRNGDKTLSG KSYIDYYEEG KRLEKKDEFQ KQV.FDPLKG
PASHAEM    DNNIGDLAGI SRLGEKVLSG KAYVDAFEEG KHI..KAD.. KLVQLDSANG
CONSENSUS  D..IGeLAGi tRnGd...sG KaYvDy.... ..l...a... ....lDp.kG

LEUTOX     NIDLSGIKGS TTLIF..... PQVRKNGKAR QSGKYEFITE LKVKGRTDWK
ECOHYLA    NIDLSDSKSS TLLKFVTPLL TPGEEIRERR QSGKYEYITE LLVKGVDKWT
PASHAEM    IIDVSNSGKA KTQHILFR.L TPGTEHRERV QTGKYEYITK LNINRVDSWK
CONSENSUS  nIDlS..k.s ttl.f..... .........r QsGKYE.ITe L.vkg...Wk

LEUTOX     VK.GVPNSNG V....YMIFY LIQHAVTRDN KVLEARLIAN LGAKDDYVFV
ECOHYLA    VK.GVQDKGA V....YDYSN LIQHASVGNN QYREIRIESH LGDGDDKVFL
PASHAEM    ITDGAASSTF DLTNVVQRIG IELDNAGNVT KTKETKIIAK LGEGDDNVFV
CONSENSUS  vk.Gv..s.. v....y.... liqha....n k..E.r.ia. LG..DD.VFv

LEUTOX     GSGSTIVNAG DGYDVVDYSK GRTGALTIDG RNATKAGQYK VERDLSG.TQ
ECOHYLA    SAGSANIYAG KGHDVVYYDK TDTGYLTIDG TKATEAGNYT VTRVLGGDVK
PASHAEM    GSGTTEIDGG EGYDRVHYSR GNYGALTIDA TKETEQGSYT VNRFVETGKA
CONSENSUS  gsGst...aG .GyDvV.Ysk g.tGaLTIDg ..aT.aG.Y. V.R.l.g...

LEUTOX     VLQETVSKQE TKRGKVT... STEYRNYKLD VVVTNKGFKA HDELNSVEEI
ECOHYLA    VLQEVVKEQE VSVGKRTEK. .TQYRSYEF. THINGKNLTE TDNLDSVEEL
PASHAEM    LHEVTSNHTA LVGNREEKIE YRHSNNQHHA GYYTKDTLKA G......EEI
CONSENSUS  vlqetv..qe ...gk.t... .t.yrny... .yyt.k..ka .d.l.svEEi

LEUTOX     IGSNLRDKFY GSKFNDVFHG HDGDDLIYGY DGDD
ECOHYLA    IGTTRADKFF GSKFTDIFHG ADGDDLIEGN DGND
PASHAEM    IGTSHNDIFK GSKFNDAFNG GDGVDTIDGN DGND
CONSENSUS  IG....DkF. GSKFnD.FhG .DGdDlI.G. DG.D
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(3) (4)

Fig. 3. **Demonstration that clone pII-2 contains epitopes present on the *A. actinomycetemcomitans* leukotoxin molecule.** Bacterial cultures of RR1[pRK248cIts] containing pII-2 were grown at 30° C. Preparative immunoblots were utilized to isolate rabbit monospecific antibodies bound to immobilized pII-2 protein. Bound antibodies were eluted with 3 M KSCN, dialyzed and used to probe immunoblots of *A. actinomycetemcomitans* leukotoxin. Lane 1, SDS-PAGE of partially purified *A. actinomycetemcomitans* leukotoxin stained with Coomassie brilliant blue. Lane 2, Immunoblot of lane 1 probed with rabbit anti-leukotoxin antisera bound to and eluted from the 80 kDa expressed protein of clone pII-2; Lane 3, Immunoblot of lane 1 probed with eluate from the 45 kDa region of the preparative immunoblot containing protein unrelated to the leukotoxin. The protein M_r markers are myosin (200 kDa), β-galactosidase (116.5 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa).

Figure 4. **Sequence comparison.** A 320 residue segment of the deduced amino acid sequence of clone pII-2 (Leutox) is compared to that of *E. coli* α-hemolysin (Ecohyla; residues 432-756) and the leukotoxin from *P. haemolytica* (Pashaem; residues 423-748). The consensus sequence indicates identical residues in all three sequences by upper case symbols. A lower case symbol was used when the Leutox sequence agreed with either of the two other sequences. Note that the homology comparison is conservative in the sense that only identities were scored and even substitutions of similar amino acids (*i.e.* leucine for isoleucine) were not counted.

The toxin selectively kills human granulocytes and the granulocytes of higher primates but granulocytes of the New World monkeys and other animals are unaffected. In addition, the fact that the toxin kills differentiated cells such as the PMN as well as HL-60 (26), a cell which has been viewed as a promyelocytic progenitor cell, indicates that susceptibility to leukotoxin-mediated killing is present from a relatively early stage of cell development. Studies are currently being carried out to determine if the human granulocyte-monocyte precursor cell (CFUgm) is toxin sensitive.

Acknowledgments. We wish to thank Dr. Robert Crowl, Department of Molecular Genetics, Roche Research Center, Nutley, N.J. for the pEV-vrf vectors and hosts and Dr. Phil Youngman,

Department of Microbiology, School of Medicine, University of Pennsylvania for helpful discussions and guidance and Joan Rosenbloom for DNA sequencing. This work was supported by National Institutes of Health grants DE08239, DE07118, RR07083 and RR01685 (BIONET).

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