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BACTERIAL HEMOLYSINS AS VIRULENCE FACTORS

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INTRODUCTION

Hemolysins are extracellular toxic proteins which are produced by many gram-negative (e.g. E. coli, Serratia spp., Proteus spp., Vibrio spp., Pasteurella spp., Pseudomonas aeruginosa) and gram-positive bacteria (e.g. Streptococcus spp., Staphylococcus aureus, Listeria spp., Bacillus cereus, Clostridium tetani), all of which possess a certain pathogenic potential. Hemolysins have been therefore always considered as virulence factors although direct experimental evidence for this assumption was either poor or non-existent. Most hemolysins cause lysis of erythrocytes by forming pores of varying diameters in the membrane. Many hemolysins can also attack - probably by a similar mechanism - other mammalian cells. Due to this cytolytic effect, they are also termed cytolysins. I shall discuss the following three bacterial hemolysins which we have studied in the past quite extensively with respect to their genetic, biochemical and pathogenic properties: Alpha-hemoylsin from uropathogenic Escherichia coli, Aerolysin from Aeromonas sobria, and Listeriolysin from Listeria monocytogenes.

The hemolysin-producing E. coli strains often infect extraintestinal sites of the human body and can cause cystitis, pyelonephritis or septicaemia. A. sobria frequently infects the intestinal tract but may also cause septicaemia and meningitis. In general both bacteria can be regarded as predominantly extracellular microorganisms colonizing specific tissue surfaces. L. monocytogenes, on the other hand, is a facultative intracellular bacterium which "invades" macrophages or monocytes and survives and multiplies within these professional phagocytes. Whereas high titers of antibodies against several surface antigens are found in the sera of patients suffering from uropathogenic E. coli or Aeromonas sobria (hydrophila) infections only low titers of antibodies to L. monocytogenes are normally detectable in patients with an acute listeriosis, and T cell-immunity is considered the major protective host response against a L. monocytogenes infection (1).

In the following mainly the role of the three hemolysins as virulence factors will be discussed but one should keep in mind that other bacterial components such as adhesins, serum resistance, capsules, iron transport systems and other virulence factors are connected with the pathogenicity of these bacteria (23).

RESULTS AND DISCUSSION

A.) The genetic determinants of hemolysin formation in *E. coli*, *Aeromonas sobria* and *Listeria monocytogenes* and their regulation

a) α-Hemolysin of *E. coli*

As shown by our group (2); the genetic determinant for α-hemolysin of *E. coli* consists of four genes (*hlyC*, A, B, D) which were sequenced and the amino acid composition of the expected four gene products were deduced from this DNA sequence (3,4). It was further shown (5) that the *hly*-determinant can be located either on transmissible plasmids (5) or on specific large inserts in the chromosome of *E. coli* (6). High homology exists within the four structural *hly* genes between several chromsome and plasmid-encoded *E. coli hly* determinants (7). Interestingly, high sequence homology with the *E. coli hly* determinant has been recently demonstrated also for the *hly* determinants of *Proteus mirabilis*, *P. vulgaris*, and *Morganella morganii* (8,9),

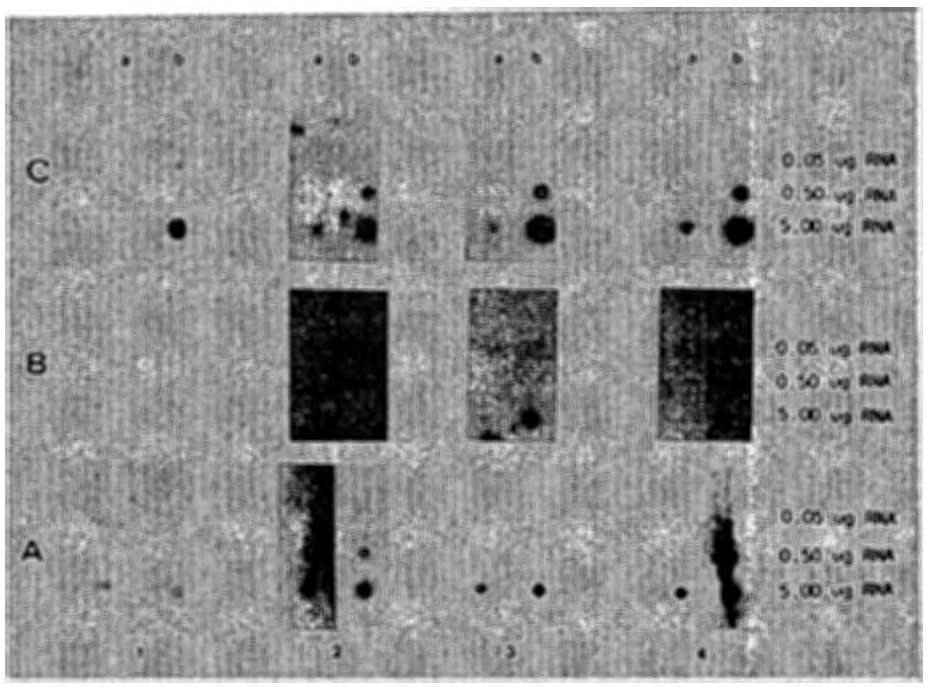

Fig. 1 The influence of *hlyR* on the transcription of *hlyC*, A, B and D. Dot blots with increasing amounts of total cellular RNA (0.05 ug, 0.5 ug and 5 ug RNA) and ^{32}P-labeled specific gene probes were performed. (A) RNA hybridized with a *hlyC*, A - specific probe, (B) RNA hybridized with a *hlyB* - specific probe and (C) RNA hybridized with a *hlyD* - specific probe. The RNAs in positions 1, 2, 3 and 4 were taken at cell densities of 2×10^8, 4×10^8, 7×10^8 and 10^9 cells/ml, respectively. (a) marks hybridization with RNA from *E. coli* 5K carrying pANN202-312 ($hlyR^-$) and (b) from *E. coli* 5K carrying pANN202-812 ($hlyR^+$).

and the genetic determinant for leukotoxin (ltx) of Pasteurella haemolytica (10). On the contrary, little sequence homology was observed between several E.coli determinants in the upstream and downstream regions flanking the four structural hly genes. A region (upstream of hlyC) of more than 400 bp was defined as a regulatory site in a chromosomal hly determinant which apparently contain two promoters from which a polycistronic m-RNA is transcribed (11). A terminator-like structure was observed in all hly determinants in the intercistronic region between hlyA and hlyB and evidence was presented that most transcripts terminate at this point (11). In a plasmid-encoded hly determinant, we have recently identified (12) a cis-acting element, which is located almost 2 kb upstream from the start of hlyC. The region between hlyC and this element, termed hlyR, includes two promoters and an IS2 element 467 bp from the start of hlyC. HlyR enhances transcription of hlyC and hlyA two to threefold, and that of hlyB and hlyD at least 50 fold (Fig. 1) and leads to 50 to 100 fold stimulation of secreted hemolysin compared to the hlyR-negative situation (12). Whether hlyR leads to an antitermination of transcription at the hlyA terminator and hence to the enhanced transcription of hlyB and hlyD or whether hlyR enhances transcription of hlyB and hlyD directly from specific promoters can not be decided at the moment, but preliminary data argue in favor of the second assumption (Hess and Gentschev, unpublished results).

b) Aerolysin of Aeromonas sobria

The aerolysin gene from a clinical isolate Aeromonas strains AB3 serotype has been cloned and its entire nucleotide sequence determined. The gene encodes a preproprotein of 492 amino acids with a molecular weight of 54.4 kD. Genetic mapping of the aerolysin gene (aerA) has shown that flanking regions both upstream (aerC) and downstream (aerB) of the gene affect its synthesis. Recently, we have determined the promoter for the aerolysin gene and have found it to be one of two non-overlapping divergent promoters present within the aerC region 5′ of the aerolysin gene. The aerC region is characterised by its extreme A+T content (68 % as compared to 41 % for the structural gene), eight copies of a core motif aATAAAa, and its absence of any extended open reading frames for 340 bp. The second promoter transcribes a hitherto unidentified 6,4 kD polypeptide whose role in toxin regulation is as yet undetermined. The downstream aerB region encodes a 82 kD protein transcribed in the same orientation as aerA from an independent promoter. The functional role of aerB in aerolysin regulation is at present unclear. However, A. sobria mutants carrying deletions within this region have altered surface properties and grow poorly in iron-restricted media.

A comparison of the aerolysin sequence determined from a A. hydrophila fish isolate to that of the A. sobria aerolysin shows an overall homology of 77 % at both nucleotide and amino acid levels. However, the corresponding aerC region for the two isolates showed only a 46 % homology at the nucleotide level, despite identity in their (A+T) content. In a study using 300 strains from diverse geographical regions the aerolysin gene was found to be present in 87 % of all hemolytic Aeromonas isolates irregardless of whether they

were from clinical or environmental sources while the aerolysin gene was present as a single copy in many strains, isolates were found that carried two copies of the aerolysin gene (Husslein and Chakraborty, unpublished results).

c) Listeriolysin of L. monocytogenes

The gene for listeriolysin (lisA) has been cloned recently from two L. monocytogenes isolates (LO28Sv1/2c and Sv1/2a EGD) and its sequence determined (15, Leimeister-Wächter and Chakraborty, unpublished data). An almost full-length lisA gene probe hybridizes in a single band with the chromosomal DNAs of Listeria monocytogenes, L. ivanovii and L. seeligeri (Leimeister-Wächter, Chakraborty, Haas, unpublished results) indicating that considerable sequence homologies exist between the genes encoding the hemolysins from these species. This finding is in contrast to our previous observation (16) and those of others (15) which failed to detect homology with the L. ivanovii and L. seeligeri genes using shorter lisA gene probes and suggests that homology

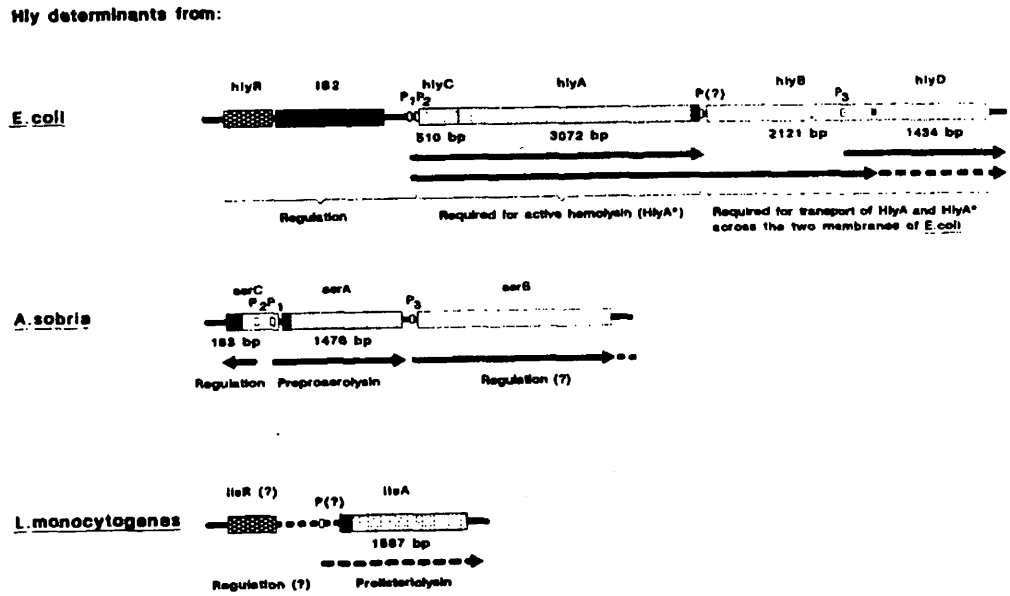

Fig. 2 The genetic determinants for α-hemolysins of E. coli, aerolysin of Aeromonas sobria and listeriolysin of Listeria monocytogenes (see text for details)

between the three listeriolysin genes may be limited to only part of the gene. In addition, the lisA gene shows homology to a C-terminal region (containing the single cys codon) of two other SH-activated cytolysins (Streptolysin O and Pneumolysin). A functional promoter for lisA has not yet been defined. Recent data (Sokolovic and Goebel, manuscript in preparation) indicate that lisA is expressed under heat shock conditions together with 12 to 14 other heat-shock proteins. Listeriolysin-negative mutants were obtained by transposon-mutagenesis with Tn916 (17, 18). Whereas one class of these Hly$^-$ mutants contains the insertion within the structural lisA gene and expresses a truncated LisA protein (17,18), the other class (17) produces very low or undetectable levels of listeriolysin, suggesting a defect in lisA gene expression. One subclass of the latter mutants appears to be impaired in the expression of most other heat shock proteins. The transposon insertion of one hly$^-$ mutant belonging to this subclass was shown to be located upstream of the start of lisA. Fig. 2 summarizes the essential features of the three hemolysin determinants and their regulation.

B.) Synthesis and Secretion of the Hemolysins from E. coli, A. sobria and L. monocytogenes

Alpha-hemolysin of E. coli is synthesized and secreted in the logarithmic growth phase. Hemolytic activity in the supernatant correlates with the secretion of a 110 kD protein, the amino acid composition of which corresponds to the unprocessed hlyA gene product. In the late log phase and in the stationary phase hemolytic activity in the supernatant drops to a low level in spite of the presence of undegraded HlyA protein in the supernatant. The hlyC gene product is required for rendering HlyA hemolytically active (5,19). The mechanism of this activation is still unknown, but active HlyA (HlyA*) can be distinguished from inactive HlyA protein by several criteria: (a) The isoelectric point of HlyA* is pH 4.0 whereas that of HlyA is pH 6.2 (as expected by the amino acid composition of HlyA), (b) HlyA* loses activity quickly upon treatment with phospholipase C and ultrasonication (20), (c) HlyA* is more stable against tryptic digestion than HlyA and (d) HlyA*, but not HlyA, forms pores of 1-2 nm in the erythrocyte membrane and in artificial lipid bilayers (21). These observations have lead us to speculate that one or more acidic phospholipids may be complexed to HlyA to yield the closed conformation of HlyA* and this "activation process" may be catalyzed by HlyC.

Using site specific mutagenesis we could identify four functional domains in HlyA, one of which appears to be connected to the activation process. There are three pronounced hydrophobic regions in HlyA between amino acids 270 to 350. Removal of the internal hydrophobic region (21 amino acids) leads to the loss of hemolytic activity and of pore formation in a lipid bilayer film. The isoelectric point of this mutant HlyA remains at pH 6.2 even in the presence of HlyC suggesting that activation by HlyC may no longer occur.

All toxins related to the α-hemolysin of E. coli (see above) have in common a repeat domain which consists in the case of HlyA of 11 repeats each of which is 9 amino acids long. In HlyA this repeat domains extends from amino acid position 739 to 849 (21). These

apparently related toxins have been therefore recently termed RTX (Repeat Toxins) (Welch, RA, personal communication). Removal of a single repeat or combinations of several repeats leads to the loss of hemolytic activity in the presence of low Ca^{2+} concentration (21). Hemolytic activity of some of these repeat deletion mutants can be restored by elevated concentrations of Ca^{2+}. The ability to form pores in artificial lipid bilayer films is unaffected in all repeat mutants. This has led us to suggest that the repeat domain of HlyA is the recognition site for a "receptor" on the erythrocyte membrane and that the proper conformation of this repeat region depends on the presence of Ca^{2+} (21).

The N-terminal end of HlyA does not carry a typical transport signal but has amphiphilic properties and may therefore interact with membranes (22).

The actual "transport signal" of HlyA for its export mediated by HlyB and HlyD, is located on the C-terminal end. Fusion of the last 37 amino acid to a (N-terminal) signal-less alkaline phosphatase gene (phoA) leads to the efficient transport of this fusion protein across both membranes of E. coli in the presence of active HlyB and HlyD

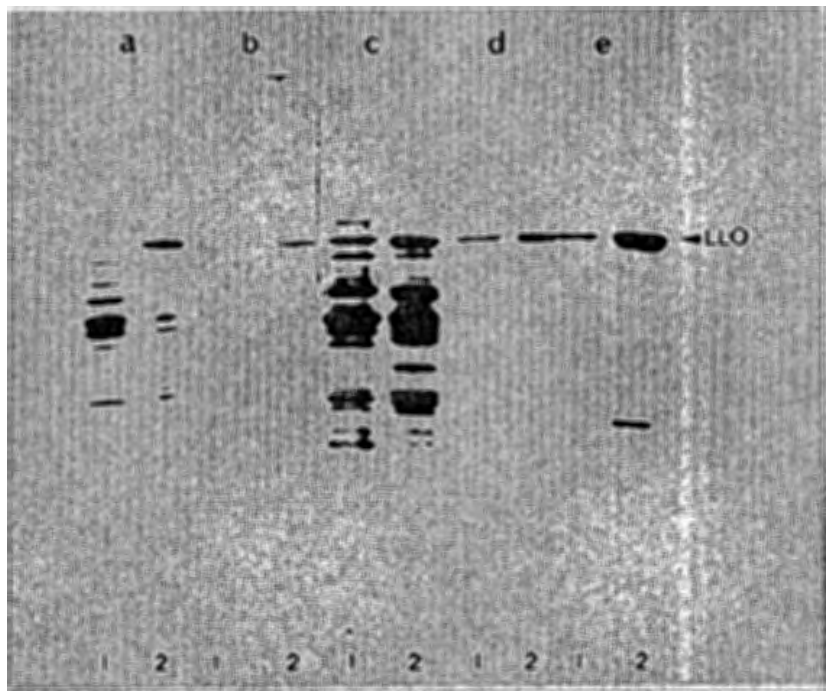

Fig. 3 Cell-associated (a-d) and cell-free listeriolysin (e) synthesized at 37°C (a,b) and 48°C (c-e) in the two L. monocytogenes strains (1) Sv1/2a EGD (termed "EGD strain" provided by S. Kaufmann, Ulm, Germany) and (2) Sv1/2a SLCC5764 (termed "Mackaness strain"). (a) and (c) show the proteins labeled at 37°C and 48°C, respectively, (b) and (d) show immunoblots of the same protein patterns developed with anti-SLO antibodies which specifically react with listeriolysin (LLO), and (e) shows the external listeriolysin synthesized and secreted at 48°C.

(Hess J, Jarchau T and Goebel W, unpublished results). The precise function of the two transport proteins for HlyA, HlyB and HlyD, both of which are incorporated in the E. coli membrane, is still unknown. Bt the N-terminal end (removal of a 23 amino acid transport leader sequence) and the C-terminal end (removal of the last 27 amino acids). Whereas cleavage of the N-terminal signal sequence occurs during transport of aerolysin across the cytoplasmic membrane, cleavage at the C-terminal end appears to occur outside the cell. This cleavage is necessary for hemolytic activity but may not be directly connected with the transport of aerolysin. However, transport of the aerolysin protein which lacks the processed Ct the N-terminal end (removal of a 23 amino acid transport leader sequence) and the C-terminal end (removal of the last 27 amino acids). Whereas cleavage of the N-terminal signal sequence occurs during transport of aerolysin across the cytoplasmic membrane, cleavage at the C-terminal end appears to occur outside the cell. This cleavage is necessary for hemolytic activity but may not be directly connected with the transport of aerolysin. However, transport of the aerolysin protein which lacks the processed C-terminal end due to a deletion in the aerA gene is considerably hampered in Aeromonas and in E. coli. This mutant aerolysin appears to quickly aggregate, suggesting that the transport competent conformation of aerolysin may require the C-terminal end. There is evidence that a short amino acid sequence of about 20 aa which immediately follows the transport signal in AerA is needed for the efficient transport of aerolysin across the outer membrane (Huhle and Chakraborty, unpublished results).

Listeriolysin seems to be produced in a precursor form containing a putative transport signal sequence of 25 amino acids (15) which is apparently cleaved off during transport across the Listerial membrane. Most of the listeriolysin protein (58 kD) is found in the supernatant but a substantial portion remains cell-associated at the normal growth temperature and under heat-shock conditions (Fig. 3). Unlike -hemolysin of E. coli and aerolysin, listeriolysin does not appear to require special activation or specific proteolytic processing to gain hemolytic activity.

C. In vitro and in vivo actions of the hemolysins

E. coli α-hemolysin. The involvement of hemolysin in virulence was demonstrated in rat, mouse and chicken embryo (23) using genetically well defined Hly$^-$ deletion mutants and reconstituted E. coli strains into which different hly determinants and genetically altered hly genes were introduced (24). It was further shown (25) that not only the amount of hemolysin correlates with the degree of toxicity, but that structural features of HlyA may also influence the virulence property. Furthermore, purified hemolysin stimulates arachidonic acid metabolism in granulocytes and causes release of the lipid mediators such as leukotrienes. Whether this effect is caused by Ca^{2+} influx into the pores formed by hemolysin or whether hemolysin triggers the intracellular event in a sublytic dose remains to be answered. Cytotoxic damage of mouse fibroblasts and human leucocytes by -hemolysin was observed, whereas human lymphocytes were relatively insensitive to this toxin (27).

Aerolysin. The aerolysin gene aerA was deleted from the chromosome of A. sobria by marker exchange mutagenesis (28). The thus obtained Hly$^-$ mutant AB3-25 exhibited a significantly reduced toxicity in the mouse (28) when the mutant strain was infected intraperitoneally. No effect on toxicity was observed with a mutant in which aerB was partially deleted. A deletion mutant AB3-5 in which parts of aerA and aerB were deleted by marker exchange exhibited the same reduced toxicity as AB3-25. Strong demonecrotic lesions with the formation of large zones of necrosis occured when the wild-type strain AB3 was injected subcutaneously into healthy mice. Surviving animals contain high titers of antibodies agains aerolysin in the blood. The mutant AB3-5, in contrast, developed only small lesions which healed within a few days. In this subcutaneous infection model the mortality rate was high when mice were infected with the wild-type strain AB3, but low when infected with the mutant strain AB3-5.

Listeriolysin. Virulent strains of L. monocytogenes are hemolytic due to the production of listeriolysin. Listeriolysin has therefore long been considered as an essential virulence factor of this facultative intracellular bacterial pathogen. On the other hand, L. ivanovii and L. seeligeri are also hemolytic and, as pointed out before, the genes for these listeriolysins exhibit homology with the lisA gene of L. monocytogenes. Yet, L. seeligeri is avirulent and L. ivanovii is only slightly virulent for humans (29). There is also a lack of correlation between the levels of cytolytic in vitro activity at normal growth temperatures and virulence of L. monocytogenes strains (30). However, the recent finding that listeriolysin is induced in L. monocytogenes strains under heat-shock conditions suggests that this toxin is synthesized more efficiently under stress conditions, which the intracellular environment, especially the phagosomal milieu, may impose on invading bacteria. In addition, the isolation of transposon-induced mutants from L. monocytogenes which fail to synthesize active hemolysin (17,18) or do so at very low levels (17) has unambiguously demonstrated the involvement of listeriolysin in the pathogenesis of L. monocytogenes. Both types of listeriolysin-negative mutants are avirulent when tested in mice. Moreover, these mutants are unable to survive in mouse peritoneal macrophages and in non-professional phagocytic cells, although the mutant bacteria can still enter these mammalian host cells. Invasion of the non-professional phagocytes is not observed by the avirulent Listeria species, even by the hemolytic ones such as L. seeligeri or L. ivanovii (31). The invasion step probably requires, among other yet unknown components of L. monocytogenes, an extracellular protein (p60), which is absent or altered in the non-invasive Listeria species (32). Evidence was presented (33) that listeriolysin may disrupt the phagosomal membrane, thereby allowing the bacterial cells to escape into the cytoplasm before lysosomes can fuse to the phagosome or afterwards. Whether this is the actual and only in vivo function of listeriolysin remains to be investigated.

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