

## Molecular Analysis of Bacterial Cytolysins

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Results of molecular and pathogenic studies of three different bacterial hemolysins (cytolysins) are presented. These exoproteins derive from the two gram-negative bacteria *Escherichia coli* and *Aeromonas hydrophila* and from the gram-positive pathogen *Listeria monocytogenes*. The hemolysin of *E. coli* is determined by an 8-kilobase (kb) region that includes four clustered genes (*hlyC*, *hlyA*, *hlyB*, and *hlyD*). This hemolysin determinant is part either of large transmissible plasmids or of the chromosome. The genes located chromosomally are found predominantly in *E. coli* strains that can cause pyelonephritis and/or other extraintestinal infections. A detailed analysis of the chromosomal *hly* determinants of one nephropathogenic *E. coli* strain revealed the existence of specific, large chromosomal insertions 75 kb and 100 kb in size that carry the *hly* genes but that also influence the expression of other virulence properties, i.e., adhesion and serum resistance. The direct involvement of *E. coli* hemolysin in virulence could be demonstrated in several model systems. The genetic determinants for hemolysin (cytolysin) formation in *A. hydrophila* (aerolysin) and *L. monocytogenes* (listeriolysin) are less complex. Both cytolysins seem to be encoded by single genes, although two loci (*aerB* and *aerC*) that affect the expression and activity of aerolysin have been identified distal and proximal to the structural gene for aerolysin (*aerA*). Cytolysin-negative mutants of both bacteria were obtained by site-specific deletion and/or transposon mutagenesis. These mutants show a drastic reduction in the virulence of the respective bacteria.

Synthesis of hemolysins has been reported throughout a broad spectrum of gram-negative and gram-positive bacterial species. The *in vivo* action of hemolysins ultimately causes physical damage to a variety of eukaryotic cells. Bacterial products possessing such a membrane-damaging action have been termed *cytolysins*, and a cytolytic action on eukaryotic cells has been demonstrated for many of the hemolysins. The biochemical basis of hemolysis (cytolysis) varies and may either be enzymatic (e.g., the phospholipase of *Pseudomonas aeruginosa*) or non-enzymatic (e.g., the thiol-activated cytolysins of several gram-positive bacteria).

The introduction of genetic techniques, especially recombinant-DNA techniques, has accelerated detailed studies of hemolysin synthesis and transport in a number of bacteria. The isolation of the genes and their gene products, in conjunction with the study of animal models, has helped to elucidate the contribution of hemolysin to the virulence of hemolytic strains. The most extensive studies of hemolysins have been performed in *Escherichia coli*,

but progress has also been made recently in the genetic analysis of other hemolysins from both gram-positive and gram-negative bacteria. Although more hemolytic extracellular proteins have been described and characterized in gram-positive than in gram-negative bacteria, less work has been reported on the genetics of these hemolysins. This lack reflects the difficulties encountered in cloning hemolysin genes from gram-positive bacteria. Cloning of such genes in *E. coli* is often accompanied by problems of limited transport, possibly incorrect processing of putative precursors, and, at times, of imperfect gene expression. Here we describe molecular studies on the hemolysins (cytolysins) of three bacterial systems: *E. coli*, *Aeromonas hydrophila*, and *Listeria monocytogenes*. It is further shown that these cytolysins represent important virulence factors.

### Results and Discussion

#### Hemolysin from *Escherichia coli*

The genetics of  $\alpha$ -hemolysin synthesis in *E. coli* was intensively studied during the past years (for review see [1, 2]). Hemolysin synthesis is observed mainly in *E. coli* strains that cause extraintestinal infections,

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and the incidence of hemolytic strains is particularly high in *E. coli* strains that cause pyelonephritis. These strains carry the *hly* determinant predominantly on the chromosome, whereas many hemolytic *E. coli* isolates from animal sources harbor plasmid-encoded *hly* determinants. The chromosomal and the extrachromosomal *hly* determinants are highly homologous and consist of four structural genes, designated as *hlyC*, *hlyA*, *hlyB*, and *hlyD* [3]. These four *hly* genes are controlled by at least two promoters. One promoter (*phly<sub>L</sub>*) is located proximal to *hlyC*. Transcription of *hlyC*, *hlyA*, and possibly *hlyB* starts from this promoter. A sequence of more than 400 base pairs (bp) proximal to *hlyC* has been shown to be necessary for full promoter activity in a chromosomal *hly* determinant [4]. The corresponding promoter sequence of most extrachromosomal *hly* determinants differs considerably in its nucleotide composition from the chromosomal sequence [5, 6] and seems to be interrupted by an IS2 insertion at position -470 bp proximal to *hlyC* (position 1 being the first nucleotide of the *hlyC* gene) since a sequence proximal to IS2 that acts in the *cis* position is necessary for the full expression of the plasmid-encoded *hly* genes. At least three different *phly* sequences have so far been identified in seven *hly* determinants analyzed [6]. A second promoter initiates the transcription of *hlyD*.

The precise location of this promoter is not yet known. The plasmid-encoded *hly* determinant is flanked in most Hly plasmids by insertion-type elements, whereas chromosomal *hly* determinants may be located on larger chromosomal inserts (70–100 kb) that carry short, directly repeated sequences at their ends [7]. Homologous recombination between the two direct repeats leads to deletions that remove these inserts precisely. The nephropathogenic *E. coli* O6 strain 536 carries two such inserts of 75 kb and 100 kb in size, both of which contain an active *hly* determinant (*hlyI* and *hlyII*). Two types of spontaneous deletion mutants, which suffered deletions of either one (*hlyI* or *hlyII*) or both inserts (*hlyI* and *hlyII*), were obtained from this strain. The latter mutant type (type I) lacks not only the hemolytic phenotype but also the expression of the mannose-resistant hemagglutination that is associated with both S-type fimbriae (*sfa*) [8] and a drastic decrease in serum resistance [9]. By analyzing type II mutants (*hlyI* or *hlyII*), it can be shown that serum resistance is associated with the *hlyII*-carrying insert. The block expression and the expression of the *sfa* genes of the



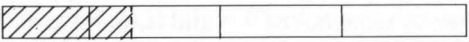
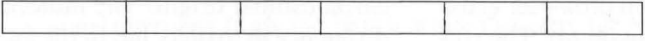
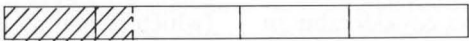

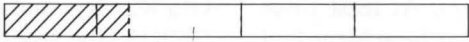

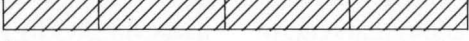
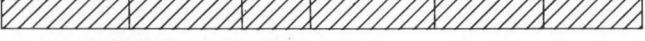

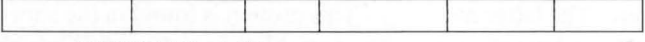
hemolytic phenotype occur at the transcriptional level [7].

Type I mutants are nonvirulent when tested in several animal models [10] and thus can be used as suitable hosts for reconstitution of virulence by reintroduction of cloned virulence genes. Hemolysin determinants from various origins were introduced with and without the *sfa* genes in such mutants. Table I shows that hemolysin alone exhibits toxic effects after injection of the manipulated strains into the mouse peritoneum or into chicken embryos. The level of toxicity depends on the *hly* determinant used and is, in general, higher for *hly* determinants that are of chromosomal origin than for those of extrachromosomal origin. The molecular cause for the difference in toxicity lies in the amount of extracellular hemolysin expressed by the *hly* determinant (which is mainly a function of sequences located proximal to *hlyC*) and in the quality of the secreted hemolysin protein, which may differ in amino acid composition between hemolysins from various hemolytic *E. coli* strains. In addition, the serotype of the bacterial host seems to influence the toxic potential of *E. coli* hemolysins, as demonstrated by O18:K5 Hly<sup>-</sup> mutants, which, in contrast with the O6:K15 Hly<sup>-</sup> strains, exhibit only minor changes in toxicity after introduction of the cloned *hly* genes (table 1).

Two *hly* determinants, one of chromosomal origin and the other of plasmid origin, were sequenced [6, 11], and the amino acid composition for the proteins was deduced from the DNA sequence. All four *hly* genes exhibit an unusually high A + T content (~60 mol% A + T) as compared with other *E. coli* genes (on the average 50 mol% A + T). The codon usage for the Hly proteins also shows unusual deviations from the average *E. coli* codon usage. Both lines of experimental data suggest that the *hly* genes found today in *E. coli* may not derive from this organism. The secreted hemolysin consists of the unprocessed *hlyA* product, a protein of 107 kilodalton (kDa).

This protein is found in the supernatant only when the two *hly* genes *B* and *D* are actively expressed. In the absence of *hlyC*, the HlyA protein is likewise secreted, albeit in lower amounts, but this product is hemolytically inactive. This finding suggests that HlyC protein activates HlyA by a yet unknown mechanism to become hemolytically active, and that HlyB and HlyD function as transport proteins for HlyA. Neither HlyA nor the other *hly* gene products that

**Table 1.** Virulence tests for *Escherichia coli*.

Bacterial strain	Plasmid-located <i>hly</i> genes*					Exported hemolysin† (%)	Virulence effects‡
	<i>hlyX</i>	<i>hlyC</i>	<i>hlyA</i>	<i>hlyB</i>	<i>hlyD</i>		
536 (Hly <sup>+</sup> , Sfa <sup>+</sup> , 06)						41.6	High
536-21 (Hly <sup>-</sup> , Sfa <sup>-</sup> , 06)						...	No
536-21 (Hly <sup>-</sup> , Sfa <sup>-</sup> , 06)						11.1	Low
536-21 (Hly <sup>-</sup> , Sfa <sup>-</sup> , 06)						71.8	High
536-21 (Hly <sup>-</sup> , Sfa <sup>-</sup> , 06)						26.1	High
536-21 (Hly <sup>-</sup> , Sfa <sup>-</sup> , 06)						0.1	No
536-21 (Hly <sup>-</sup> , Sfa <sup>-</sup> , 0.6)						33.8	Intermediate
536-31 (Hly <sup>-</sup> , Sfa <sup>+</sup> , 06)						...	Low
536-31 (Hly <sup>-</sup> , Sfa <sup>+</sup> , 06)						9.6§	Low/intermediate
536-31 (Hly <sup>-</sup> , Sfa <sup>+</sup> , 06)						50.6	High
536-31 (Hly <sup>-</sup> , Sfa <sup>+</sup> , 06)						71.6	Intermediate/high
764 (Hly <sup>+</sup> , P-Fim, 018)						61.9	High
764-2 (Hly <sup>-</sup> , P-Fim, 018)						...	Intermediate
764-2 (Hly <sup>-</sup> , P-Fim, 018)						17.6§	Intermediate
764-2 (Hly <sup>-</sup> , P-Fim, 018)						31.8	High
764-2 (Hly <sup>-</sup> , P-Fim, 018)						75.8	Intermediate
764-2 (Hly <sup>-</sup> , P-Fim, 018)						75.3	High

\* The hemolysin determinant consists of four genes, *hlyC*, *A*, *B*, and *D*. Proximal to *hlyC* an IS2 element and another sequence, *hlyX*, are located. The recombinant DNAs used parts derived from the plasmid pHly152 (filled boxes) or from the chromosomes of 06 and 018 strains, respectively (open boxes).

† The amount of released hemoglobin is given relative to complete lysis of erythrocytes by absorption at 546 nm when a constant aliquot of cell-free supernatant is incubated for 30 min at 37°C.

‡ The virulence effects were estimated in a mouse peritonitis assay (injection of  $1 \times 10^9$ - $4 \times 10^7$  bacteria into the mouse peritonium) and in a chicken embryo model (injection of  $1 \times 10^6$ - $1 \times 10^8$  bacteria into the allantoic cavity of chicken embryos).

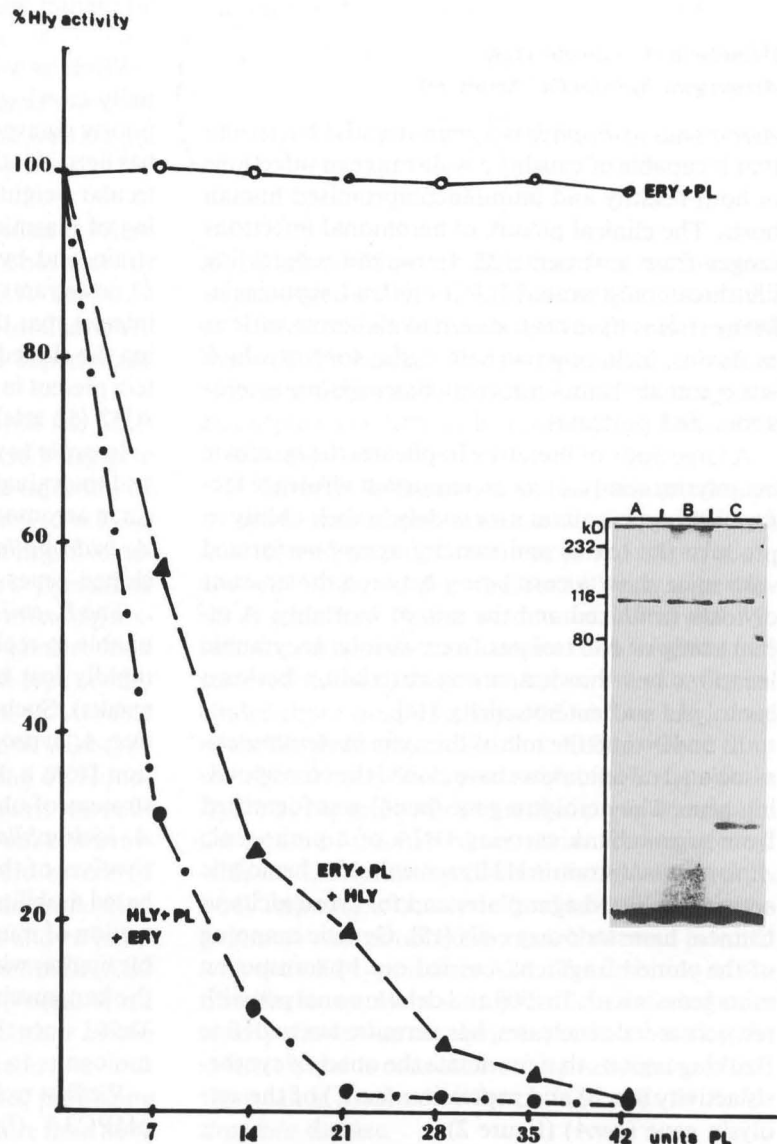
§ The amount of exported hemolysin increased by a factor of three to four in the late log phase of growth.

are required for the transport of hemolysin carry conventional N-terminal transport signal sequences. Yet, there is recent evidence that HlyA carries a sequence for its own transport across the cytoplasmic membrane, since proteins derived from fusions between *hlyA* and *phoA*, the latter being devoid of the transport signal (by  $\lambda$ Tr*phoA* [12]), are transported across the cytoplasmic membrane when <100 amino acids from the N-terminal end of HlyA are fused to PhoA. However, the transport of these fusion proteins across the outer membrane cannot be accomplished by complementation with *hlyB* and *hlyD*, a finding which suggests that possible recognition sites on HlyA for HlyB and HlyD are located closer to the

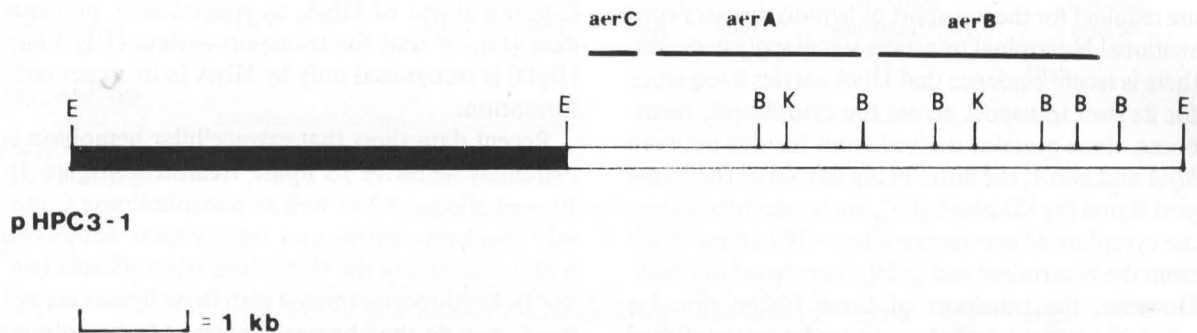
C-terminal end of HlyA, as suggested by previous data [13], or that the transport system (HlyB and HlyD) is recognized only by HlyA in its intact conformation.

Recent data show that extracellular hemolysin is extremely sensitive to lipase treatment (figure 1). Phospholipase A2 as well as phospholipase C rapidly inactivate extra- and intracellular hemolysin without affecting the size of the HlyA protein (figure 1). Erythrocytes treated with these lipases are not lysed, nor do they become resistant to hemolysin; this is evidence that association of HlyA with phospholipids may be essential for activity. How and where this association occurs remains unclear.

**Figure 1.** Sensitivity of extracellular hemolysin (HLY) from *Escherichia coli* to phospholipase C (PL). Hemolysin was prepared from the supernatant of *E. coli* 5K harboring the recombinant plasmid pANN202-812, which allows the overproduction of hemolysin determined by the plasmid pHly152 (authors' unpublished observations). Treatment of erythrocytes (ERY) with phospholipase C does not alter the sensitivity to hemolysin (upper curve, ERY + PL). Addition of phospholipase C to erythrocytes and hemolysin leads to a drop in hemolytic activity that is dependent on the concentration of phospholipase (middle curve, ERY + PL + HLY). Treatment of hemolysin with increasing amounts of phospholipase C inactivates the hemolysin and leads to a drastic reduction of lysis of added erythrocytes (lower curve, HLY + PL + ERY). Incubation with phospholipase was always for 5 min at 37°C. The insert shows the electrophoretic mobility on PAGE of the HlyA protein (107 kdal) without treatment (lane A), after treatment with phospholipase A2 (lane B), and with phospholipase C (lane C). The slower migrating bands in B and C are the respective phospholipases.







**Figure 2.** Physical map of the aerolysin-producing plasmid pHPC3-1. Open bars represent cloned *Aeromonas hydrophila* DNA; closed bars represent the vector *ptac11*. The region marked A is the structural gene for aerolysin. Regions B and C denote flanking regions that respectively modulate activity and expression of the aerolysin gene product.

### Hemolysin (Cytotoxin) from *Aeromonas hydrophila* (Aerolysin)

*Aeromonas hydrophila* is a gram-negative bacterium that is capable of causing a wide range of infections in both healthy and immunocompromised human hosts. The clinical picture of aeromonal infections ranges from gastroenteritis to rapidly progressing life-threatening wound infections and septicemia. Many strains have been shown to elaborate various exotoxins, including two hemolysins (one of which is a cytotoxic hemolysin termed *aerolysin*), enterotoxin, and proteases.

A large body of literature implicates the cytotoxic hemolysin, aerolysin, as an important virulence factor. Wild-type isolates vary widely in their ability to produce the toxin, and toxicity assays performed with mice show a correlation between the amount of toxin produced and the rate of mortality. A recent study of 686 isolates from various geographic locations has shown a strong correlation between hemolysis and enterotoxicity [14].

To understand the role of the toxin in *Aeromonas*-associated infections, we have cloned the corresponding gene. The aerolysin gene (*aerA*) was identified from a gene bank carrying DNA of a pathogenic *A. hydrophila* strain AH2 by screening for hemolytic activity on blood agar plates and for cytotoxicity to Chinese hamster ovary cells [15]. Genetic mapping of the cloned fragment, carried out by transposon mutagenesis with Tn1000 and deletion analysis with restriction endonucleases, has permitted us to define flanking regions that modulate the onset of synthesis activity (*aerB*) and expression (*aerC*) of the aerolysin gene (*aerA*) (figure 2).

While aerolysin is actively excreted in exponentially growing cultures of *A. hydrophila* AH2, it is poorly transported in *E. coli*. The cloned aerolysin has been identified as a protein with an apparent molecular weight of 54 kdal in *E. coli* by specific labeling of plasmid-encoded polypeptides in a maxicell strain and by immunoblotting of total lysates of *E. coli* strains harboring *aerA* plasmids [14]. It is of interest that the protein produced by *E. coli* carrying the cloned *aerA* (54 kdal) is larger than the protein present in culture supernatants of *A. hydrophila* AH2 (52 kdal).

In order to characterize the aerolysin gene further and to evaluate the pathogenic potential of aerolysin in aeromonal disease, we constructed mutants of *A. hydrophila* AH2 that carried deletions in the cloned genes and that failed to produce aerolysin.

The *E. coli* vector pBR322 and its derivatives are unable to replicate stably in *A. hydrophila* and are rapidly lost by segregation (authors' unpublished results). Such vectors can be efficiently introduced into *A. hydrophila* when supplied with a *mob* function from a plasmid with a broad host range. Our strategy of obtaining aerolysin-negative mutants in *A. hydrophila* was therefore to introduce deleted derivatives of the aerolysin gene cloned on a pBR322-based mobilizable vector into *A. hydrophila*. The detection of mutants required association of a selectable marker with the cloned DNA sequence. We chose the kanamycin-resistance gene from the transposon Tn903 since that gene does not carry sequences homologous to the *A. hydrophila* chromosome.

We first recloned the 5.8-kb *EcoRI* fragment from pHPC3-1 (figure 2) in the mobilizable vector

pSUP205, producing the plasmid pHPC3-700. Subsequently, 1.8 kb of aeromonal DNA sequences between the *KpnI* sites of pHPC3-700 were replaced by a 1.4-kb *KpnI* fragment carrying the kanamycin-resistance gene of Tn903. The 1.8-kb *aerA-aerB* deletion present on the resulting plasmid pHPC3-702 is a deletion-substitution, since the DNA encoding resistance to kanamycin was inserted at a position formally occupied by the deleted *aerA-aerB* DNA. *E. coli* recombinants harboring the resulting plasmid pHPC3-702 were completely devoid of both hemolytic and cytotoxic activities.

The marker-exchange procedure of Ruvkun and Ausubel [16] was used to recombine the deletion-*kan* substitution mutation of pHPC3-702 into a spontaneously derived nalidixic acid-resistant mutant of *A. hydrophila* AH2 (AB3). Subsequent in vivo recombination and segregation produced genetic recombinants that had the resident *aerA-aerB* region replaced by the substitution mutation carried by pHPC3-702. One such recombinant was used for subsequent studies.

Southern-blot hybridization was used to confirm the genetic structure of AB3 *tox-5* (data not shown). Immunoblots performed with specific antibody directed against aerolysin showed the total absence of aerolysin both in cell lysates and supernatant fluids of AB3 *tox-5*.

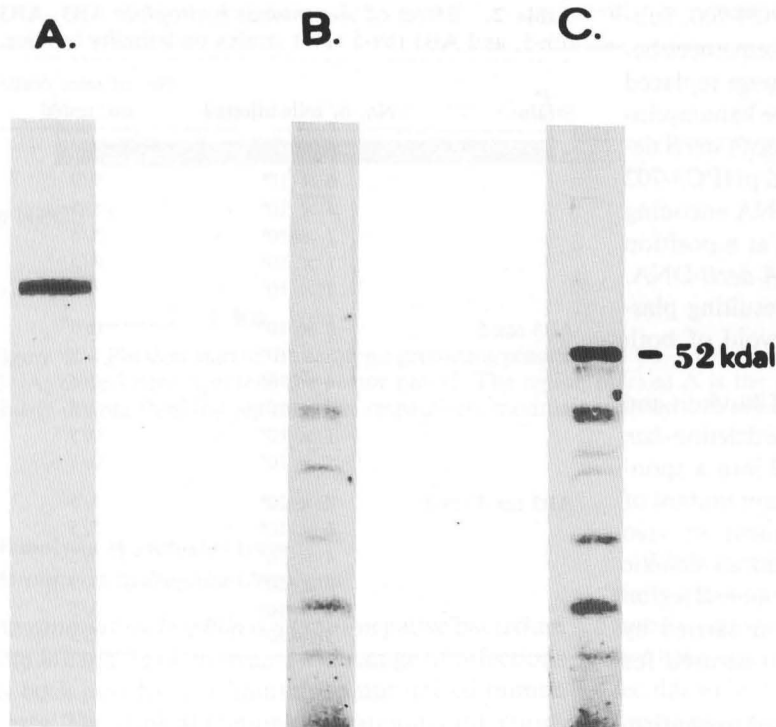
We next determined the LD<sub>50</sub> of both the wild-type strain (AB3) and its isogenic aerolysin-negative derivative (AB3 *tox-5*). Intraperitoneal injection of mice with the parental strain AB3 showed a LD<sub>50</sub> of  $\sim 5 \times 10^7$  cells, while the aerolysin-negative derivative had an LD<sub>50</sub> of  $>1 \times 10^9$  cells. To exclude the possibility that unlinked mutations affecting toxicity had been introduced during the construction of AB3 *tox-5*, we reintroduced the wild-type *aer* determinant on pHPC3-700 into AB3 *tox-5*. A double cross-over event leading to integration of wild-type *aerA-aerB* genes was detected by plating on blood-agar plates. Several stable transconjugants were obtained, and one such transconjugant (AB3 *tox-5* rev-1) was checked for its genetic structure by use of Southern hybridization and for aerolysin production in hemolysin and cytotoxin assays. As shown in table 2, full toxicity was regained with the strain (AB3 *tox-5* rev-1), which had a LD<sub>50</sub> of  $5 \times 10^7$  cells. These results clearly demonstrate the association of the toxin with lethality in mice and extend previous results obtained with wild-type isolates producing different amounts of aerolysin [14]. More than 80%

**Table 2.** Effect of *Aeromonas hydrophila* AB3, AB3 *tox-5*, and AB3 *tox-5* rev-1 strains on lethality in mice.

Strain	No. of cells injected	No. of mice dead/ no. tested
AB3	$8 \times 10^8$	5/5
	$6 \times 10^8$	9/9
	$4 \times 10^8$	5/5
	$2 \times 10^8$	5/5
	$1 \times 10^8$	3/5
	$5 \times 10^7$	1/5
AB3 <i>tox-5</i>	$8 \times 10^8$	0/5
	$6 \times 10^8$	1/9
	$4 \times 10^8$	0/5
	$2 \times 10^8$	0/5
	$1 \times 10^8$	0/5
	$5 \times 10^7$	0/5
AB3 <i>tox-5</i> rev-1	$8 \times 10^8$	5/5
	$6 \times 10^8$	5/5
	$4 \times 10^8$	5/5
	$2 \times 10^8$	5/5
	$1 \times 10^8$	3/5
	$5 \times 10^7$	2/5

of the mice died within 22 h after inoculation, and all mice died within 30 h. Moreover, pure cultures of *Aeromonas* were obtained from the spleens and livers of these mice. The murine model therefore reflects well the rapidly progressing wound infections and septicemia seen with *A. hydrophila* infections in humans.

If aerolysin is important in the pathogenesis of *A. hydrophila* disease, aerolysin should be produced in vivo, and its production could be indirectly examined by determining whether antibody to aerolysin is produced during aeromonal infections. We therefore obtained serum samples from mice that had survived an LD<sub>50</sub> dose. Control serum samples included those from mice injected with an LD<sub>50</sub> dose of the aerolysin-negative mutant AB3 *tox-5* and from uninfected mice. The results obtained are shown in figure 3. No antibody to aerolysin was detected in the serum of uninfected mice and of mice infected with the aerolysin-negative mutant. A strong antibody response to aerolysin (and several other polypeptides) was obtained in mice that were infected with the wild-type isolate AB3. The detection of antibodies to aerolysin in the serum of infected mice clearly shows that aerolysin is produced during the infection and is consistent with the hypothesis that the toxin is involved in the pathogenesis of *A. hydrophila* disease.

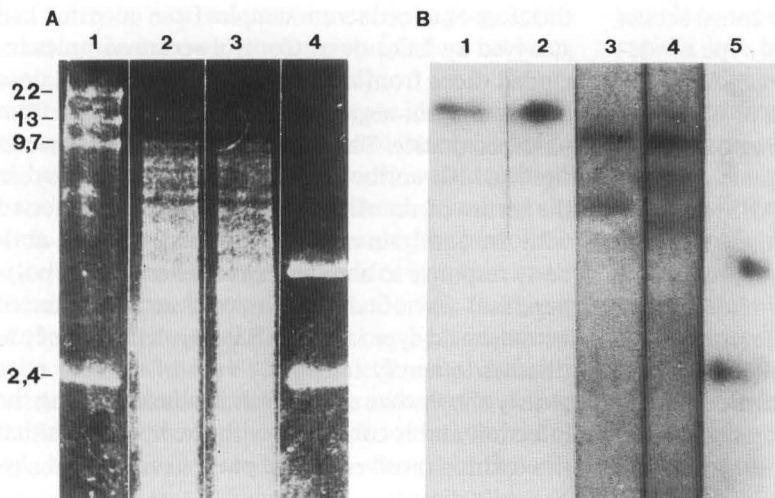


**Figure 3.** Antisera from (A) uninfected mice, (B) mice injected with strain AB3 tox-5, and (C) mice injected with AB3 were reacted with supernatant fluids from *Aeromonas hydrophila* AB3 that had been blotted after SDS-PAGE electrophoresis. The large diffuse band in A is methyl green, which has been used to mark the lane. The 52-kdal protein is aerolysin.

#### Cytolysin (Listeriolysin) from *Listeria monocytogenes*

*Listeria monocytogenes* is a gram-positive, rod-shaped bacterium that can cause severe infections in humans and animals. Most strains of *L. monocytogenes* are producers of a  $\beta$ -hemolysin, termed *listeriolysin*. This hemolysin (cytolysin) has been characterized as an extracellular protein that belongs to the group of thiol-activated, cholesterol-

inhibited cytolysins and that cross-reacts immunologically with streptolysin O (SLO) [17]. The fact that this hemolytic activity, although often relatively weak in vitro, is possessed by clinical isolates and not or rarely by strains isolated from healthy carriers or from the environment has led to the assumption that hemolysin of *L. monocytogenes* is a virulence factor. Meaningful comparisons between virulent and



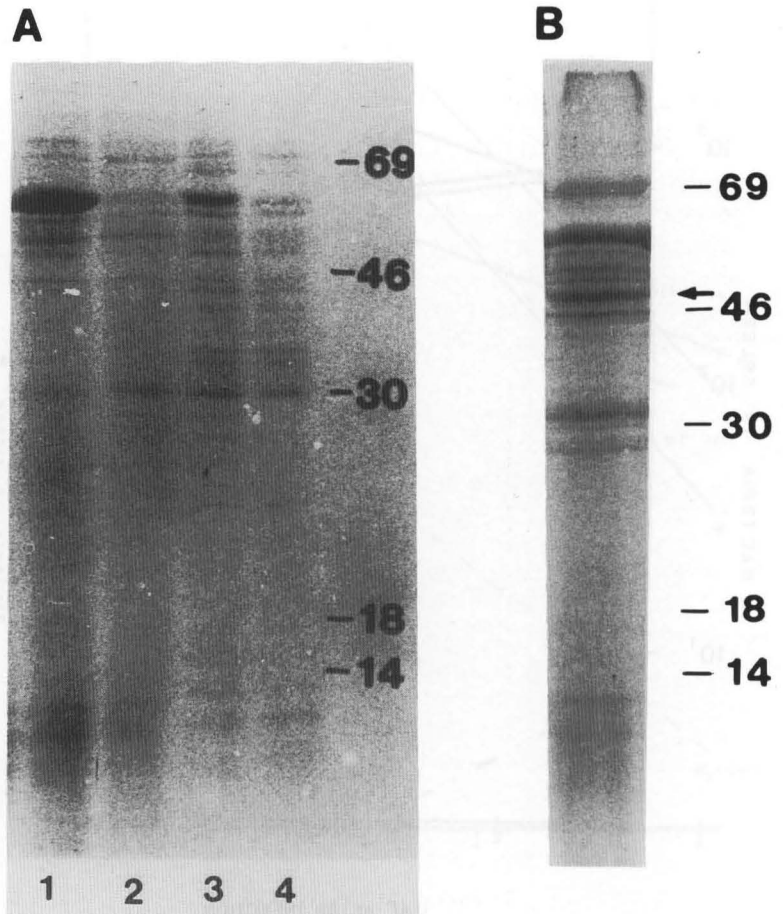
**Figure 4.** A: Agarose-gel electrophoresis of chromosomal DNA from *Listeria monocytogenes* digested with *Eco*RI. Lane 1, *Bgl*II-digested  $\lambda$  phage DNA as molecular weight standard; lane 2, Hly<sup>-</sup> mutant M3; lane 3, Hly<sup>-</sup> mutant M1; lane 4, *Eco*RI-digested DNA of plasmid pAM170 used as probe. B: Autoradiograph of Southern blots. Lanes 1 and 2, *Eco*RI-digested DNA of M3 and M1, respectively, hybridized with <sup>32</sup>P-labeled pAM170 (Southern blot prepared from the gel shown in A); lanes 3 and 4, *Hind*III-digested DNA of M3 and M1, respectively, hybridized with <sup>32</sup>P-labeled pAM170; lane 5, *Eco*RI-digested pAM170 hybridized with <sup>32</sup>P-labeled pAM170.

avirulent strains have so far been hampered by the fact that the genetic backgrounds of such wild-type strains are unclear and probably divergent with respect to several different determinants.

Moreover, there is circumstantial evidence that more than one type of listeriolysin may exist in strains of *Listeria*. A recent study described by Parrisius et al. [18] has shown that only *Listeria ivanovii* and two of 26 *L. monocytogenes* strains of various serogroups secrete a hemolysin that cross-reacts on immunoblots with SLO antibody.

The role of listeriolysin as a virulence factor was demonstrated by inactivating the genetic determinant essential for this protein in *L. monocytogenes* (MacKanness strain serotype 1/2a) by inserting the tetracycline-resistance transposon Tn916 into *L. monocytogenes* DNA (authors' unpublished observations, and P. Metz, personal communication). The transposon was transferred by means of conjugation of a streptomycin-resistant *L. monocytogenes* recipient

strain with *Streptococcus faecalis* CG110 on membrane filters [19]. Among the tetracycline-resistant transconjugants, mutants that had lost hemolytic activity were detected. Results of hybridization studies with Tn916 DNA as a probe indicate that the transposon is present on a single chromosomal *EcoRI* fragment and two *HindIII* fragments in the non-hemolytic transposon mutants (figure 4). This finding suggests that a single transposition event leads to the loss of the hemolytic phenotype, since Tn916 itself contains one *HindIII* site but no *EcoRI* site. Preliminary evidence indicates that the transposition of Tn916 in these mutants occurs directly in the structural gene for listeriolysin and not in a regulatory site influencing hemolysin synthesis and possibly other virulence properties as well. Analysis of the extracellular proteins from supernatants of wild-type *L. monocytogenes* 1/2a and of Tn916 Hly<sup>-</sup> mutants indicates the loss of a single major protein of 60 kdal, apparently the listeriolysin (figure 5). In one type of



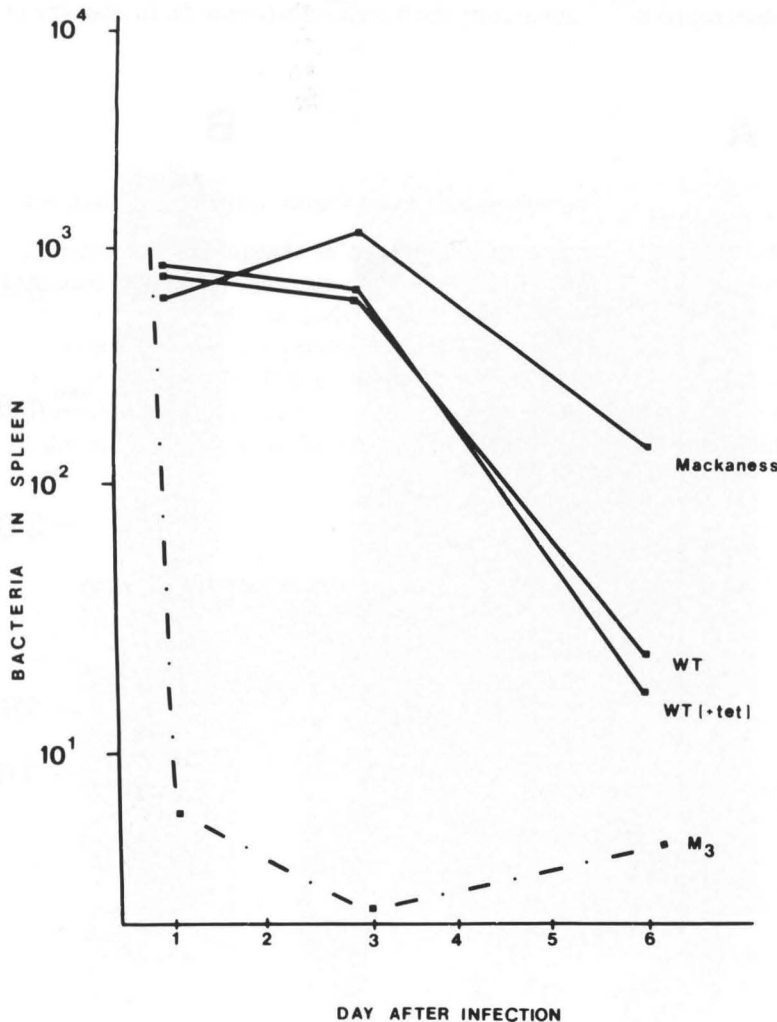
**Figure 5.** SDS-PAGE of extracellular proteins produced by transposon-bearing *Listeria monocytogenes*. **A:** Lanes 1 and 3, wild-type strain grown overnight in brain-heart infusion and tryptic soy broth, respectively; lanes 2 and 4, M3 grown overnight in brain-heart infusion and tryptic soy broth, respectively. **B:** Hly<sup>-</sup> mutant M1 grown overnight in brain-heart infusion broth. Arrow indicates the position of the presumed truncated listeriolysin.



these mutants, a smaller protein of apparent molecular size 49 kdal appears instead of the 60 kdal protein, probably representing a truncated listeriolysin protein (figure 5). The size of the *Hind*III fragments hybridizing with the Tn916 DNA probe in the two types of nonhemolytic mutants (with and without the truncated protein) suggests that Tn916 has been in each case inserted in chromosomal sites that were only about 1 kb apart. This is consistent with the idea that, in the one mutant type, Tn916 has been inserted in a site close to the 5' end of the listeriolysin gene, thus generating no, or a very small, truncated listeriolysin protein, whereas in the second mutant type, the insertion occurred in a site 1 kb more distal, generating the 49-kdal truncated protein.

After iv injection of bacteria of the wild-type (Mackness strain) into mice, the course of infection previously described by Mackness [20] for ex-

perimental infections of mice with virulent *L. monocytogenes* was observed. The number of bacteria isolated from the spleens of the animals increased after infection, reached a peak between the third and fourth day after infection, and then declined (figure 6). The number of bacteria isolated from the spleens of the animals 3 days after infection was 50 times higher than that present in the inoculum. Infection by iv injection of the Tet<sup>r</sup>, Hly<sup>+</sup> transposon-bearing wild-type strain (wild-type with respect to hemolysin production) resulted in a similar pattern of bacterial multiplication within the animals, although the extent of multiplication was somewhat lower than that observed in the case of the parental Mackness strain, and a 13-fold increase was observed. Intravenous injection of the Tet<sup>r</sup>, Hly<sup>-</sup> mutant bacteria, however, was not followed by multiplication of the bacteria in the animals; instead,



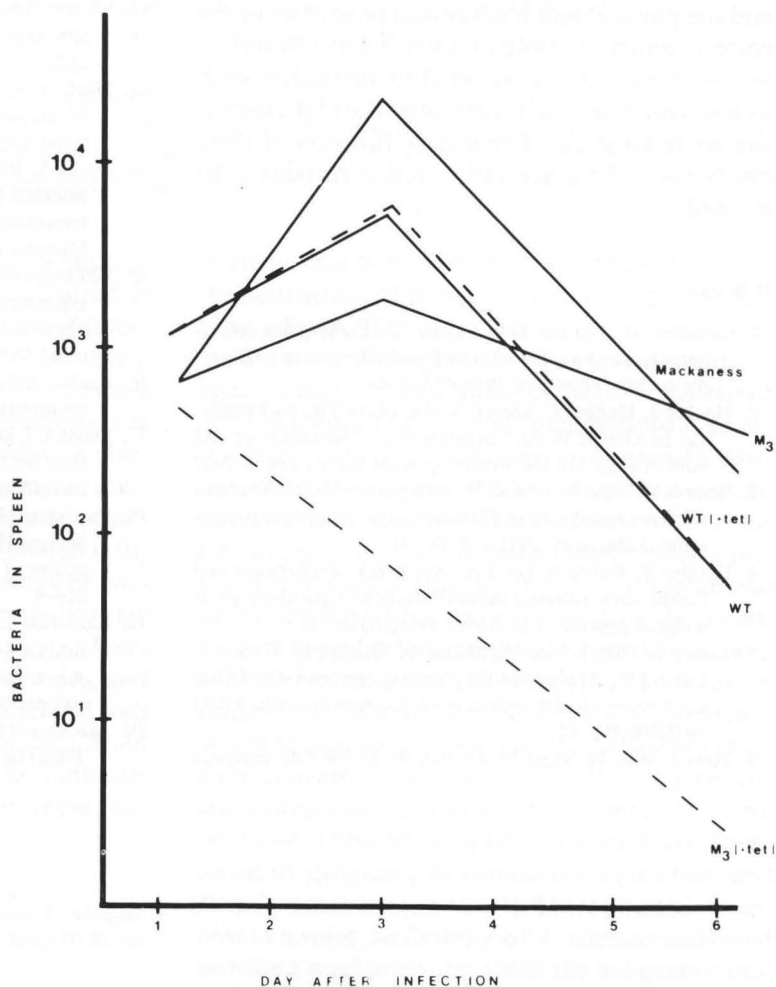
**Figure 6.** Bacterial counts per spleen 1, 3, and 6 days after intravenously induced infection. Infection dosages were  $1.4 \times 10^3$ ,  $2.7 \times 10^3$ , and  $3.2 \times 10^3$  of the Mackness, wild-type (WT), and M3 strains, respectively. The numbers are means from four or five animals.

the numbers of bacteria isolated from the spleen declined sharply after their introduction, and, as soon as 1 day after their introduction, the bacteria were virtually eliminated from the animals (figure 6). The few bacteria that could be isolated from the spleens were resistant to tetracycline and were non-hemolytic. Similar results were observed in the experimental infections of mice by other Hly<sup>-</sup> mutants and randomly selected Hly<sup>+</sup> transconjugants produced from different crosses.

The infection dose of Hly<sup>-</sup> bacteria that was used in these experiments ( $3.1 \times 10^3$  bacteria) is unlikely to contain any Hly<sup>+</sup> revertants, since the average reversion rate in vitro is  $10^{-4}$ . We expected that if such revertants were generated they would regain their ability to multiply within the animals. Moreover, on the basis of the in vitro data, we expected that most of these revertants would lose resistance to tetracy-

cline. To test these expectations, we repeated the experimental infections of mice with the three strains (Mackness strain; Tet<sup>r</sup>, Hly<sup>+</sup> strain WT; and Tet<sup>r</sup>, Hly<sup>-</sup> strain M3), using standard sublethal doses of the hemolytic strains but three orders of magnitude more bacteria of the Hly<sup>-</sup> mutant ( $1.3 \times 10^6$ ) (figure 7). The multiplication pattern of the hemolytic strains was similar to that observed earlier. The bacteria isolated from the spleens of the animals infected with WT strain were all Hly<sup>+</sup>, and similar numbers were isolated on media with or without tetracycline, an observation suggesting that the bacteria maintained their resistance to tetracycline in vivo and that loss of the transposon had not occurred. Most of the bacteria isolated from the animals that had been infected with the Hly<sup>-</sup> strain M3, however, were hemolytic and sensitive to tetracycline. Such bacteria represented 40%, 86%, and 100% of all those

**Figure 7.** Bacterial counts per spleen 1, 3, and 6 days after intravenously induced infection. Infection dosages were  $2.7 \times 10^3$ ,  $2.7 \times 10^3$ , and  $1.4 \times 10^6$  for the Mackness, wild-type (WT) and M3 strains, respectively.



that were isolated 1, 3, and 6 days after infection, respectively. The Hly<sup>+</sup>, Tet<sup>s</sup> bacteria were apparently revertants that had lost the transposon and regained the hemolytic phenotype along with the ability to multiply within the animals. Only a small fraction of the bacteria isolated from these animals were still Hly<sup>-</sup>, a finding which suggests that even when injected in high dosages the Hly<sup>-</sup> bacteria were unable to multiply within the animals and were rapidly eliminated. These data strongly suggest that the hemolysin (listeriolysin) of *L. monocytogenes* is an essential virulence factor.

### Conclusions

The three virulent bacteria described in this study, *E. coli*, *A. hydrophila*, and *L. monocytogenes*, have in common the potential for producing extracellular hemolytic toxins. The complexity of the genetic determinants encoding these hemolysins (cytolysins) and the physical and biochemical properties of the corresponding exoproteins are different. Nevertheless, each represents a potent virulence factor, since its loss reduces drastically the virulence of the respective bacterial strain. The precise function of these cytolysins in the process of infection remains to be defined.

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