EXPRESSION OF *PASTEURELLA HAEMOLYTICA* ANTIGENS IN *ESCHERICHIA COLI* USING LAMBDA GT11

By

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PREFACE

This thesis contains three chapters plus an appendix. The materials and methods presented in Chapter II contain brief descriptions of the individual experiments. Detailed protocols of each experiment are listed in the appendix.

I wish to express heartfelt thanks to my major advisor, Rebecca Craven, for her wisdom, guidance and most of all, her understanding. Without her patience and humor, giving up would have been all to easy.

Special thanks to T. C. Confer for spending more time than he could truly spare with this project. Long live Louisiana Cajun French and Conifer and the Pines.

I also wish to thank the other members of my committee, Dr. John W. Wills and Dr. Mark R. Sanborn for their instruction, encouragement and most of all, their humor.

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CHAPTER I

LITERATURE REVIEW

Introduction

Pasteurella is a genus of aerobic, gram negative, encapsulated bacteria (68). Two of the species in this group, *P. haemolytica* and *P. multocida*, are of medical importance. Although *P. multocida* can be an opportunistic pathogen of humans, these two species are important veterinary pathogens (17). Both *P. multocida* and *P. haemolytica* can cause shipping fever in cattle. However, *P. haemolytica* biotype A serotype 1 is most frequently cultured from pneumonic lungs and from calves with respiratory disease (35,49). *P. haemolytica* also has been implicated in pneumonia and septicemia in sheep (80). Although *P. haemolytica* is considered to be part of the normal flora of the upper respiratory tract in healthy cattle, it is not generally considered a normal resident of the bovine lung (16,39). Despite its importance as a veterinary pathogen, relatively little is known about *P. haemolytica* virulence mechanisms.

Virulence Mechanisms

The Capsule

One structure implicated as a virulence factor is the polysaccharide capsule (95). While production of the capsular material is not affected by repetitive transfer (33), its presence has been shown to be age-dependent (23). Substantial amounts of polysaccharide material are present on cells in logarithmic phase, yet it is nearly undetectable using light microscopy on cells in stationary phase (23). The age-dependency and the anti-phagocytic properties of the capsule were substantiated by the work of Walker et al. (95). They reported the failure of pulmonary macrophages to phagocytize cells from log-phase cultures of P. haemolytica, whereas phagocytosis of cells from stationary-phase cultures occurred readily. In addition to providing resistance to phagocytosis, possession of a capsule may protect Pasteurella from the antibacterial activities of pulmonary surfactants and the opsonizing effect of specific antibody (24). The protection from host defense mechanisms conferred by the capsule could aid in the initial bacterial colonization of the bovine lung (68,95).

Fimbriae

Colonization may be enhanced by fimbriae acting as primary attachment points to the epithelial cells of the nasopharyngeal mucosa and tonsil (68). Little information

is known about these structures in Pasteurella species. A recent report by Morck et al. (68) described the existence of at least two different types of fimbriae in P. haemolytica. One type is described as fairly rigid and approximately 12nm in length. The other is more flexible and approximately 5nm in length.

Endotoxin

Endotoxin is another component of Pasteurella that may contribute to pathogenesis. Keiss et al. (53) reported 12 to 25% of the dry weight of P. haemolytica to be comprised of endotoxin with a potency similar to that for Salmonella species in the induction of hemorrhagic necrosis of skin. Although P. haemolytica endotoxin may not aid in the colonization of the lung, it apparently plays an important role in lung injury. During the middle to later stages of the pneumonic process, the effects of endotoxin are evident. The effects include edema, thrombosis of capillaries, veins and lymphatics, activation of the alternate complement pathway (causing an increase in capillary permeability), and polymorphonuclear leukocyte (PMN) chemotaxis (74,92). These changes, combined with degranulation of PMNs (during the course of phagocytosis or upon cell death) result in extensive damage to the pulmonary tissue (54,74).

Leukotoxin

Although the importance of the aforementioned constituents of P. haemolytica as virulence factors is in question, there is accumulating evidence that leukotoxin is a major virulence determinant (3,12,13,41,51,63,65,76, 80,82,91). All pathogenic isolates of P. haemolytica produce a leukotoxin under optimal growth conditions (81). The toxin, like typical exotoxins, is a product of metabolically-active cells (5,7). Toxic activity is observed with live P. haemolytica and sterile P. haemolytica culture supernatants but not with heat-killed or x-irradiated cells (5,51).

Physicochemical characterization of leukotoxin has shown that it is heat labile, oxygen stable, pH stable, nondialyzable, nonhemolytic, water soluble, and trypsin sensitive (11). Although the molecular weight of the toxin has been debated, two independent groups have recently cloned the leukotoxin gene and reported the molecular weight to be 105 kDa (14,60). P. haemolytica leukotoxin has a marked specificity for bovine leukocytes but is not hemolytic for bovine, ovine or porcine erythrocytes (80). The reasons for the species and cell type specificity are unknown. Membrane receptors or membrane composition and structure could play a role (80).

The mechanism of action of leukotoxin also is unknown, and attempts to purify it have resulted in loss of toxicity (3,41,69).

Effects of Leukotoxin

The pulmonary alveolar macrophage is considered to be the first line of defense against inhaled bacteria (3,52,66). Other components contributing to the overall pulmonary defense against bacteria include secretory antibody, bactericidal substances, and the mucociliary apparatus (52). Efficient elimination of pathogens from the lung is accomplished by pulmonary alveolar macrophages and neutrophils in conjunction with locally-produced or systemically-derived opsonins (6).

Phagocytosis of P. haemolytica by pulmonary alveolar macrophages occurs with no indication of intracellular replication or resistance to degradation (5,63). Phagocytosis of P. haemolytica, in the absence of opsonizing antibody, has been reported to be poor (63). Maheswaran et al. (63) observed normal phagocytosis of opsonized P. haemolytica by pulmonary macrophages when the ratio of bacteria to macrophage was 10:1. When the ratio was increased to 20:1, as many as 10% of the bacterial cells were not phagocytized and prolonged incubation resulted in cytotoxic changes in the macrophages. In an earlier study, Benson et al. (5) showed that heat-killed P. haemolytica was readily phagocytized by alveolar macrophages, whereas exposure of macrophages to live bacteria resulted in a decreased rate of phagocytosis and severe cytotoxic effect to the cells. Phagocytosis is not a requirement for toxicity because lymphocytes are killed in the presence of

an excess of P. haemolytica (51).

The toxic effects of P. haemolytica on the target cell include aggregation of cytoplasmic matrices, rupturing of the cell membrane, the presence of numerous cytoplasmic vesicles, pyknotic nuclei, and evidence of foamy granular coagulative lysosomolysis (3). The effects of leukotoxin on phagocytic cells are dose dependent and can range from minor inhibition of luminol-dependent chemiluminescence to lysis of the cell (12,80). The activity responsible for inhibition of chemiluminescence in PMNs and for lysis of ruminant phagocytes and lymphocytes have not been rigorously proven to be the result of a single toxic principle (11). The specificity of leukotoxin for phagocytic cells is the basis for considering the toxin to be a major virulence factor (3).

Neuraminidase and Protease Activity

Cell-bound neuraminidase activity has been observed in typeable strains and in culture supernatants of P. haemolytica (29). Because neuraminidase is produced by several genera of pathogenic and nonpathogenic bacteria, its role in pathogenesis is most likely limited. Neuraminidase activity varied in the different strains of P. haemolytica, and there was no direct association between cytotoxicity of culture supernatants and neuraminidase activity (72). Chang et al. (11) have proposed a mode of action for P. haemolytica neuraminidase similar to that of

Vibrio cholera, whereby receptor sites on target cells are unmasked in order to get full activity of the secreted leukotoxin.

A neutral, metal ion-dependent protease activity is associated with some but not all P. haemolytica strains (72). Proteases from bacteria such as Legionella pneumophila and Pseudomonas aeruginosa have been implicated in the pathogenesis of the pneumonia caused by those organisms (43,57), and both organisms are toxic for leukocytes. Unlike P. haemolytica, however, their toxicity is not limited to leukocytes nor is their activity host-species specific (11). The in vitro effects of P. aeruginosa proteases on rabbit alveolar macrophages include agglutination, intense vacuolization, and ultrastructural damage to the cell membrane (57). Cell death is not induced by exposure to this enzyme (57). Chang et al. (11) hypothesized that the protease of P. haemolytica could act on leukocytes by either rendering it susceptible to the cytolytic activity or unmasking a receptor required for leukotoxin activity. An alternate proposal suggested that leukotoxin is released as a prototoxin, like Clostridium botulinum exotoxin A or Escherichia coli heat-labile enterotoxin, and for toxin activation, requires cleavage in a limited and highly specific manner by a protease (11).

Other Membrane-Damaging Toxins

The diverse group of membrane-damaging toxins can be

defined by their mode of action. While the mode of action of the majority of toxins is still unknown, several different mechanisms have been determined. The alpha toxin of Clostridium perfringens (phospholipase C), the beta toxin of Staphylococcus aureus (sphingomyelinase) and the lethal toxin of Corynebacterium ovis (phospholipase D) cause membrane damage by enzymatic degradation of the membrane phospholipids (1,88). Lysis of the target cell does not necessarily follow exposure to these toxins (88). C. perfringens alpha toxin and S. aureas beta toxin are classified as hot-cold hemolysins (88). That is, lysis of the affected target cell occurs only after cooling below the initial incubation temperature (88). Therefore, lysis is a secondary effect. The lethal toxin of C. ovis does not lyse erythrocytes despite the cell membrane damage caused by removal of choline from sphingomyelin by the toxin (88). Thiol-activated cytolysins, which include streptolysin O, Streptococcus pneumoniae pneumolysin and Clostridium perfringens perfringolysin among others, exert their toxic effects through the binding and sequestration of membrane-associated cholesterol (1,88). Thiol-activated toxins only act on cells containing cholesterol in the cell membrane; therefore, most mammalian cells are susceptible (88).

'Surface-active' toxins such as Staphylococcus delta toxin and Bacillus subtilis subtilysin act like a detergent by dissolving the cell membrane (1,88). The generalized action of these toxins confers a broad target range.

The toxins previously described are active on a number of cell types. There are other bacterial toxins that exhibit target cell specificity. Staphylococcus leukocidin, pseudomonal leukocidin, Fusobacterium necrophorum leukocidin and Actinobacillus actinomycetemcomitans leukotoxin are toxic for leukocytes but not erythrocytes (1). Although Staphylococcus leukocidin is probably the best characterized, the basis for its specificity and its mode of action are unknown (88). Staphylococcus leukocidin alters the permeability of the target cell membrane resulting in an efflux of K^+ and influx of Ca^{2+} (88). Degranulation and release of degradative enzymes occurs; however, phosphate, nucleotide sugars, and cytoplasmic proteins are retained by the cell (88). Degranulation can be prevented by removal of Ca^{2+} from the medium (88).

Many toxins, including C. perfringens alpha toxin and Staphylococcus leukocidin are Ca^{2+} dependent (88). Calcium ions may have an effect on the target of the toxin rather than on the toxin itself; perhaps conferring a positive charge which enables or enhances the binding of the negatively charged toxin molecule (4,100). P. haemolytica leukotoxin also requires Ca^{2+} for activity as demonstrated by reduced leukotoxic activity in the absence of Ca^{2+} in the medium and recovery of that activity upon addition of Ca^{2+} (34). Recovery of leukotoxic activity does not occur upon addition of Mg^{2+} (34). The exact nature of the relationship between Ca^{2+} and P. haemolytica leukotoxin is not known.

Viral-Bacterial Synergism

Shipping fever pneumonia of cattle (bovine pneumonic pasteurellosis) is thought to be the result of the interaction of many different host and pathogen-related factors (59,94). Physical and mental stress, extremes of heat and cold, and etiologic agents acting independently or in combination enhance the calf's susceptibility to Pasteurella-induced pneumonia (63,94). P. haemolytica normally present in the upper respiratory tract may be inhaled into the lung in higher numbers as a result of deeper breathing brought on by cold stress (86). Physical and mental stress can immunologically compromise the calf for extended periods of time through elevation of cortisol levels and suppression of the immune response (77,88).

Etiologic agents such as viruses enhance bacterial colonization of the lung in several ways. Viral infections inhibit the bactericidal mechanisms of the lung (37). Reduced neutrophil function has been observed and could result in decreased clearance of bacteria (44,45). Alterations in the mucus layer covering the epithelial cells due to prior viral infection may occur also, increasing the likelihood of bacterial adherence (26,75,78). Infection with bovine herpesvirus I induces a general suppression of the host's immune response (71). Each of these viral-induced changes severely impairs the ability of the lung to dispose of bacterial pathogens. Thus, Pasteurellae is given a replicative advantage (63,66).

Babiuk (2) estimated that 90% of all bacterial pneumonias occur after viral infection. Therefore, viral predisposing factors contribute to the establishment of shipping fever pneumonia. However Slocombe et al. (84) experimentally produced Pasteurella pneumonia in young calves without prior viral infection or stress. Therefore, the possibility that Pasteurellae can act as a primary bovine pathogens cannot discounted.

Disease Manifestations

Bovine pneumonic pasteurellosis is characterized by inflammation of the airways, severe congestion, massive fibrin deposition, and lobular necrosis (46). Pasteurellosis can affect any age group, but is most often seen in calves when stressed due to weaning, handling and transportation (25,38,48,79). The morbidity rate associated with the disease is variable, and the mortality rate is low (35).

Jensen et al. (46) reported that Pasteurella species colonize bronchiolar epithelium, bronchioles, alveoli, and alveolar ducts. The heaviest concentrations were observed at the peripheries of affected lobules (46). Many factors could contribute to the colonization of the lung by P. haemolytica. Damage to the mucociliary mechanism and pulmonary tissues may allow Pasteurellae to colonize the bovine lung (90). Loss of cell-surface fibronectin, induced by stress, has been associated with colonization of human buccal cells by other gram-negative bacteria and could be an

important factor in Pasteurellae infection (98). Prior viral-induced damage to the respiratory mucosa and to neutrophils could seriously impair lung resistance to bacterial pathogens and aid in colonization (36,61). The antiphagocytic and antibactericidal properties attributed to the bacterial polysaccharide capsule and possibly fimbriae could be vital in the establishment and replication of P.haemolytica in the lung (33,68,95).

Release of leukotoxin by P. haemolytica results in dysfunction and/or lysis of pulmonary macrophages and PMNs (6). The consequential release of leukocytic lysosomal enzymes may be responsible for the severe tissue damage, inflammation and fibrin deposition characteristic of this disease (47). Slocombe et al. (86) found that neutrophil-depleted calves did not have as severe clinical signs of experimental pneumonic pasteurellosis as did normal calves. Gross examination of lungs from the normal group revealed lesions characteristic of pneumonic pasteurellosis, whereas the lungs from the neutrophil-depleted group appeared to be normal. This data indicates that release of PMN and macrophage lysosomal contents is responsible for severe pulmonary tissue damage.

Lopez et al. (62) observed in cattle that inhalation of P. haemolytica caused the normal neutrophil to macrophage ratio in bronchoalveolar washings to be reversed. They also reported that the neutrophilic influx preceded the exudation of fibrin. Because earlier studies showed P. haemolytica to

be a poor chemotactic agent and because fibrin apparently was not responsible for neutrophil recruitment (10), Lopez et al. (62) hypothesized that endotoxin acted as a neutrophil chemotactic factor. Endotoxin also has been implicated as the cause of edema in the lungs (85). Tickoff et al. (93) reported that increased pulmonary vascular constriction resulting in pulmonary edema occurred in calves injected with endotoxin. The pathology of shipping fever pneumonia is clearly a complex, multifactorial interaction of Pasteurella virulence factors, viral-bacterial synergism and stress-induced sequelae.

Immunology

Efficient removal of bacteria from the bovine lung is accomplished by pulmonary alveolar macrophages in conjunction with opsonizing antibody (6). Adult bovine serum, nasal secretions, and lung washings contain antibodies against Pasteurellae (15,63). In addition, intrapulmonic challenge following aerosol vaccination with live P. haemolytica demonstrated the existence of a resistance state (94). These findings substantiate that an immune response occurs following natural or experimental exposure to P. haemolytica (18,27,31,73,96). Vaccination of cattle with commercially-prepared bacterins did not afford protection against experimental pneumonic pasteurellosis (20,31). In fact, bacterins may enhance pasteurellosis by eliciting opsonizing antibody (97). Markham and Wilkie (66)

suggested that opsonization of P. haemolytica may not be desirable because increased uptake of the organism may enhance toxicity to macrophages. Vaccination of calves with P. haemolytica bacterins containing water-in-oil adjuvants enhanced resistance to experimental pneumonic pasteurellosis (21).

Vaccination of cattle with live P. haemolytica protected against experimental challenge (73). Therefore, bacterial replication may be required to stimulate protective immunity (20). The age of the bacterial cultures used to make live vaccines influenced the degree of protection, possibly the larger quantities of capsular material and leukotoxin produced in younger cultures was responsible for the increased protection (19). Baluyut et al. (3) demonstrated leukotoxin-neutralizing activity in serum from both immunized and adult cattle. The neutralizing activity was due to specific antibodies (15). Gentry et al. (32) demonstrated a direct correlation between leukotoxin-neutralizing antibody titers and protection against experimental challenge.

Other types of immunizing agents (i.e., potassium thiocyanate extracts, sodium salicylate extracts and chemically-altered P. haemolytica) have been found to enhance resistance to the disease (30,101).

Specific Aims

As described above, little is known about P.

haemolytica virulence mechanisms. The pathogenesis of shipping fever pneumonia is multifactorial, involving environmental factors such as stress and etiologic agents, as well as the Pasteurellae virulence factors. P. haemolytica leukotoxin may be an important virulence factor because of its specificity for bovine phagocytic cells (3). In addition, Gentry et al. (32) demonstrated that leukotoxin-neutralizing antibody correlates with protection against experimental challenge. Lo et al. (60) and Chang et al. (14) have reportedly cloned and sequenced the leukotoxin gene. However, the existence of a single toxin species has not been rigorously proven. Baluyut et al. (3) reported retention of leukotoxin on an XM300 membrane and reported the molecular weight to be 300,000 daltons or more. Himmel et al. (41) reported isolation of a 150 kDa leukotoxic antigen from P. haemolytica A1 culture supernatants which dissociated to 20 kDa and 50 kDa components when subjected to SDS-polyacrylamide gel electrophoresis. The investigators also demonstrated serotype specificity with the antigen and suggested it to be of capsular origin. Chang et al. (12) found no evidence that the activity responsible for inhibition of the chemiluminescence in PMNs and the activity causing lysis of ruminant phagocytes and leukocytes were the same.

The inability to purify leukotoxin from P. haemolytica culture supernatants has frustrated efforts to assess its role in pathogenesis and to assess the potential of antibody

to purified leukotoxin to protect against the disease. The use of recombinant DNA technology to clone the leukotoxin gene would facilitate such assessments. Cloning of the gene would allow production of large amounts of purified toxin for use in vaccination studies. Vaccination with a purified antigen has two advantages. First, the ability of antibody specific for leukotoxin to protect against pneumonic pasteurellosis could be determined. Second, the use of a purified component of an organism, rather than the whole, would avoid the side effects associated with injection of live organisms and might avoid the induction of opsonizing antibodies. In addition, recombinant DNA technology could be used to construct a mutant P. haemolytica strain unable to produce leukotoxin. Studies using the mutant strain would provide a better understanding of the pathogenesis of the organism. This technique could also be applied to other P. haemolytica antigens. Homology studies involving the toxin genes of other bacteria could be undertaken and might provide insights into the mechanism of action of the P. haemolytica leukotoxin.

The goals of this study were two-fold. The first was to construct a P. haemolytica-containing recombinant library in lambda gt11. This would allow identification of clones expressing P. haemolytica antigens, leukotoxin in particular. Identification would be facilitated using specific antisera. The second goal involved the limited characterization of the antibody-positive clones as to

their biological and physical properties.

Although the leukotoxin gene has been cloned, my studies should either substantiate or dispute the previous findings. As mentioned above, the possibility exists that there is more than one leukotoxin.

General Cloning and Screening Strategy

Chromosomal DNA from P. haemolytica A1 will be isolated and sheared to produce random DNA fragments. Ligation of the DNA fragments to lambda gt11 DNA will result in a recombinant bacteriophage. Packaging to form fully infectious particles will be done in vitro using commercial packaging extracts. Induction of the lacZ gene carried on the vector could result in the production of a fusion protein between beta-galactosidase and the peptide specified by the P. haemolytica DNA. Bacteriophage expressing P. haemolytica antigens will be identified with antisera.

Initial screening of the library will be done with a serum that recognizes many P. haemolytica antigens. Rescreen of recombinant bacteriophage identified by the serum will be done using a serum specific for leukotoxin.

The Lambda Gt11 Vector

Lambda gt11 is a 43.7 kilobase pair (kbp) cloning and expression vector designed to accept foreign DNA inserts up to 7 kbp (figure 1). It carries the lacZ gene of Escherichia coli which contains a unique EcoRI site 53 base

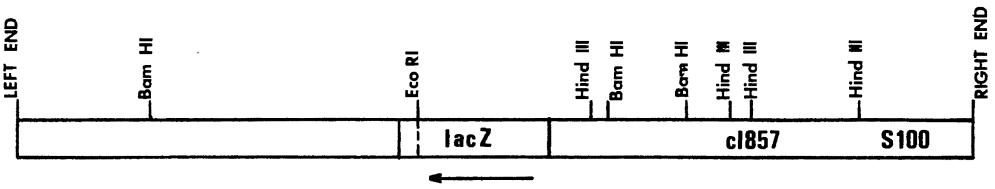
pairs upstream from the termination codon. This vector is ideal for the proposed cloning strategy for several reasons. First, insertion of P. haemolytica DNA into the lacZ gene in the correct orientation and translational reading frame results in lac promotor-directed synthesis of a fusion protein in which 16 amino acids at the carboxyl terminus of the beta-galactosidase protein is replaced with a portion of a P. haemolytica protein. Translation of the P. haemolytica antigen, therefore, is not dependent upon a functioning P. haemolytica promotor and can be regulated by addition of a synthetic inducer, isopropyl-B-D-thiogalactopyranoside (IPTG), to the medium. Because beta-galactosidase activity is destroyed via insertional inactivation, recombinant bacteriophage are easily identified by the inability to form blue plaques on E. coli Y1090 host cells in the presence of the chromogenic indicator 5-bromo-4-chloroindolyl-B-D-galactopyranoside (X-gal).

Translational control of a potentially toxic product such as leukotoxin is an important aspect of the proposed strategy. Leukotoxin is a membrane-damaging protein. Therefore, recombinant bacteriophage coding for leukotoxin may be toxic to the E. coli host. By cloning only a portion of the leukotoxin gene this complication may be avoided.

Stability of the foreign peptide in E. coli may be enhanced because of its fusion with beta-galactosidase and by propagation of recombinant bacteriophage on the lon host. This system is superior to plasmid cloning

because 10^4 to 10^6 pfu/plate can be screened as opposed to 10^3 to 10^4 colonies (maximum) per plate for a typical plasmid vector.

Figure 1. The lambda gt11 cloning and expression vector.


LEFT END
Bam HI
Eco RI
Hind III
Bam HI
Bam HI
Hind III
Hind III
Hind III
RIGHT END
lacZ
cI857
S100

CHAPTER II

Materials and Methods

Bacterial and bacteriophage strains and plasmids. All strains used in this study are listed in Table 1.

Media. All strains of E. coli were grown on Luria-Bertani (LB) medium. Each liter of medium contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl, and was adjusted to pH 7.5 with NaOH (64). Where appropriate, ampicillin was added to a final concentration of 50 μg/ml. Bacterial cultures used as bacteriophage hosts were grown in LB broth supplemented with maltose at 0.2%. Bacteriophage strains assayed for beta-galactosidase activity were combined with the appropriate host strain and grown in 0.7% LB top agar containing 160 μg/ml isopropyl-B-D-thiogalactopyranoside (IPTG), 400 μg/ml 5-bromo-4-chloroindolyl-B-D-galactopyranoside (X-gal), 2 mM $CaCl_2$, 10 mM $MgCl_2$ and 0.7% Bacto-agar on LB bottom agar containing 1.5% Bacto-agar.

Chemicals, immunochemicals, and DNA. Restriction enzymes, DNA polymerase 1, T4 DNA ligase, EcoRI methylase, T4 polynucleotide kinase, and DNA size markers were purchased from International Biotechnologies, Inc., New

TABLE 1

BACTERIA, BACTERIOPHAGE, AND PLASMID STRAINS

Strain	Genotype	Source or Reference
E. coli Y1090	Δ*lac* U169(lacIPOZYA) *proA*$^+$Δ*lon* *ara*D139 *str*A *sup*F [trp C22::Tn10] *hsd*R$^-$ *hsd*M$^+$ (pMC9) pMC9=pBR322-*lac*I$^+$	Promega Biotec
E. coli Y1089	Δ*lac* U169 *proA*$^+$ Δ *lon* *ara*D139 *str*A *hfl*A [chr::Tn10] *hsd*R$^-$ *hsd*M$^+$ (pMC9)	Promega Biotec
E. coli JM103	Δ*lac* *pro* *sup*E *thi* *str*A *end*A *sbc*B15 *hsd*R4 F' *tra*D36 *pro*AB *lac*Iq z Δ M15	Bethesda Research Laboratories
E. coli TB-1	Δ*lac*-*pro* *str*A *ara* *thi* Ø80dlacZ ΔM15 *hsd*R	Bethesda Research Laboratories
P. haemolytica	biotype A, serotype 1 OSU strain, amp^r	Corstvet et al. 1973. (22)
pUC18	2.7 kbp cloning vector carrying Ap^r	Norrander et al. 1983. (71)
pRC13	4a insert fragment carried in pUC18	This study
pRC14	12a insert fragment carried in pUC18	This study
pRC15	20C insert fragment carried in pUC18	This study
Lambda gt11	*lac*5 cI857 nin5 s100	Promega Biotec
Lambda h80 Δ 9	lytic bacteriophage	Gift of R.C. Essenberg

TABLE 1 (CONTINUED)

Lambda b221CI26	lytic bacteriophage	Gift of R. C. Essenberg
Lambda SW4a	4a insert fragment carried in lambda gt11	This study
Lambda SW12a	12a insert fragment carried in lambda gt11	This study
Lambda SW20c	20c insert fragment carried in lambda gt11	This study

Haven, CT., or New England Biolabs, Beverly, MA. Bacterial alkaline phosphatase was purchased from New England Nuclear, Boston, MA. Biotinylated Protein A and Streptavidin-biotinylated Horseradish Peroxidase complex were purchased from Amersham Corporation, Arlington Heights, IL. Protoclone GT, Packagene systems and lacZ immunoaffinity columns were purchased from Promega Biotec, Madison, WI. EcoRI linkers were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. [α^{32}P]dATP was purchased from ICN Radiochemicals, Irvine CA.

Antisera. 476 antiserum is a hyperimmune anti-P. haemolytica serum prepared by multiple parenteral injections of a steer with live organisms (58). This serum has a leukotoxin neutralizing titer of > 1:256. For all procedures, the final dilution of this serum was 1/25. The anti-leukotoxin serum was prepared in a rabbit by the method of Lo et al. (60). The final dilution of this serum, unless otherwise indicated, was 1/50. Both sera were adsorbed with sonicates and whole cells of E. coli DH1 (pUC19) prior to use. One liter of an overnight culture of E. coli DH1 cells in LB broth was centrifuged at 13,500 x g for 30 minutes. The cell pellet was resuspended in 20 ml of distilled water in a disposable 50 ml centrifuge tube. The cell suspension was then boiled for 5-10 minutes. The portion of the lysate to be sonicated was then divided into 2 ml aliquots. Each aliquot was subjected to sonication at 70% duty cycle for 80 seconds. The aliquots were then combined and sonicated once

more using the same parameters. One milliliter of lysate was added for every 100 mls of diluted serum. After an overnight incubation at 4° C on a rocker, cellular debris was removed by centrifugation 13,500 x g for 30 minutes. The supernatant was then filtered through a 0.2 micron filter. All sera were adsorbed three times by this procedure unless otherwise indicated.

The guinea pig sera were prepared by subcutaneous injection of purified fusion proteins 12a (9.4 μg), 20c (2.7 μg), purified beta-galactosidase derived from E. coli after infection with lambda gt11 that did not contain insert DNA (118.3 μg) and leukotoxin. The leukotoxin (kindly supplied by Dr. Confer) was purified from supernatants of P. haemolytica A1 grown in RPMI 1640 medium supplemented with 3.5% bovine serum albumin (BSA) and precipitated with ammonium sulfate at 50% saturation. The antigens were mixed with equal volumes of Freund's complete adjuvant. The amount of antigen administered to each animal was dependent on the relative concentration of the antigen in each preparation and the amount of antigen available. The animals were boosted with the same amount of antigen 29 days after inoculation.

Isolation and preparation of insert DNA. Chromosomal DNA from the virulent P. haemolytica A1 (OSU strain) was isolated by the method of Marmur (67)(see Appendix). The DNA was mechanically sheared using a French pressure cell

and fractionated on a 10-40% continuous sucrose gradient to yield fragments 0.5 to 2.0 kbp in size. Preparation of the DNA for ligation into the lambda gt11 vector was performed as described by Huynh et al. (42)(see Appendix). Briefly, fragments were treated with EcoRI methylase and the sheared ends filled in using DNA polymerase I. Kinase-treated EcoRI linkers were ligated to the fragments, and the excess linkers were removed by EcoRI digestion.

Construction and amplification of the lambda gt11 library. Insert fragments were ligated into the bacteriophage vector and packaged in vitro to form fully infectious particles. The recombinant bacteriophage were then titered on Y1090 host cells according to the manufacturer's specifications (see Appendix). The protocol used for amplification in Y1090 and storage of the recombinant library was as described by Maniatis et al. (64)(see Appendix).

Antibody screening. Bacteriophage of the recombinant library were plated on Y1090 host cells. Plaque lifts from these plates were performed by the method of Huynh et al. (42)(see Appendix). Following an initial three hour incubation at 42° C, the plates were overlayed with nitrocellulose filters (Schleicher and Schuell, Keene, NH) and saturated with 10 mM IPTG. Incubation was continued for three more hours at 37° C. A modification of the method of Helfman et al. (40)(see Appendix) was used for the antibody

screening procedure. Deviations from the original protocol included omission of the initial lysing step, substitution of 5% nonfat dry milk in TBS buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) for the blocking agent and adaptation to a biotinylated detection system rather than autoradiography. The detection system employed biotinylated Protein A as a second antibody, and Stretpavidin-Horseradish Peroxidase (HRP) complex and HRP color development reagent for visualization of antigen-antibody complexes on the filter. Recombinants identified with the 476 serum were picked and plaque purified once using standard methods (64). One blue and three white plaques from the resulting plates were picked into 1 ml of SM buffer containing 5.8 g NaCl, 2 g $MgSO_4$-$7H_2O$, 50 mls 1 M Tris Chloride (pH 7.5), 5 mls 2% gelatin per liter plus one drop of chloroform. Five microliters of each plaque suspension was spotted in rows on a lawn of Y1090 bacteria and allowed to dry. The plates were then carried through the plaque lift procedure described previously. Rescreening was performed in duplicate using both 476 and anti-leukotoxin sera.

Preparation of lysogens and crude lysates. Lysogens of the lambda gt11 clones were made in y1089 host cells. Lytic lambda bacteriophage were used in this procedure to aid in the identification of the lysogens. Bacterial cells not already infected with the lambda gt11 would be lysed as a consequence of infection with the lytic lambda bacteriophage strains (See Appendix A). One hundred microliters of a 1:1

mixture of the lambda h80Δ9 and lambda b221 cI26 were spread onto LB plates. An aliquot of a Y1089 overnight culture was diluted in 10 mM $MgSO_4$ to 1 x 10^5 cells/ml and 100 ul combined with 5 ul of a 1 x 10^5 pfu/ml stock of recombinant bacteriophage. The bacteriophage were allowed to adsorb to the bacteria for twenty minutes at 37° C, and the mixture was subsequently spread on top of the lytic bacteriophage mixture. The plates were incubated at 32° C overnight. Colonies were tested for temperature sensitivity at 42° C. Lysis of the host cells at 42° C and not at 37° C confirmed the presence of a lambda gt11 prophage.

Crude lysates of induced lysogens were prepared from the lambda gt11 recombinant lysogens by the method of Huynh et al. (42)(see Appendix). Briefly, a 100 ml culture of each lysogen was grown at 31° C in LB broth in flasks until the absorbance at 600 nm (A_{600}) reached 0.5 (measured with a 1 cm cuvette). The cultures were transferred to a 42° C shaking water bath for 20 minutes before adding IPTG to 10 mM. The flasks were immediately moved to 37° C and allowed to incubate 45 minutes. The cells were harvested at room temperature by centrifugation, 4100 x g for 5 minutes. The pellet was then resuspended to 1/50 of the culture volume in 50 mM Tris-HCL (pH 6.8), containing 1 mM phenyl-methylsulfonyl fluoride, and stored at -70° C.

Electrophoresis and Western blot analysis. Small polyacrylamide slab gels (8.2 cm x 8.2 cm x 1.5 mm) for immunoblotting were prepared using a 10% resolving gel with

a 6% stacking gel. Recipes were provided by Hoeffer Scientific Instruments, San Francisco, CA. Large polyacrylamide slab gels (14 cm x 15 cm x 1.5 mm) for molecular weight determination were prepared using a 7% resolving gel with a 3% stacking gel. All gels were run in the SDS buffer system of Laemmli (55)(see Appendix). Electrophoretic transfer of the proteins to nitrocellulose was done in a Hoeffer Mini Transphor (TE-22) unit according to the manufacturer's instructions. The electroblots were probed with 476 serum, anti-leukotoxin serum, mouse monoclonal anti-beta-galactosidase antibody (Promega Biotec, Madison, WI) (diluted 1/2500), or the guinea pig sera diluted 1:10 as the primary antibody. The detection system used was biotinylated protein A for rabbit and guinea pig source sera or biotinylated rabbit anti-goat IgG for bovine source sera. Streptavidin-horseradish peroxidase complex and 4-chloro-1-naphthol (BioRad, Richmond, CA) were used for color development.

Molecular weight estimations. Crude induced lysates were run in duplicate on an SDS polyacrylamide gel. One half of the gel was stained with Coomassie blue stain, whereas the other one half of the gel was subjected to electrophoretic transfer to nitrocellulose. The nitrocellulose was subsequently blotted with mouse monoclonal anti-beta-galactosidase antibody. The mobility of the bands in the stained gel that corresponded to the beta-galactosidase fusion product on the Western blot were

compared to that of the BioRad high molecular weight standards.

Purification of the fusion proteins. Frozen lysates of lambda gt11 lysogens were thawed and subjected to brief sonication (10 seconds, continuous power) to insure that the bacterial cells were lysed. Protein content of the lysates was determined by the method of Bradford (9)(see Appendix). The fusion proteins were purified from crude lysates by affinity chromatography using Protosorb lacZ immunoaffinity columns (1 ml bed volume). The purification procedure was done according to the manufacturer's specifications (see Appendix). The fusion proteins in the collected fractions were detected by Western blotting.

Cytotoxicity assays. Approximately 1 ml of the purified fusion product was dialyzed against three changes of PBS (pH 7.2). The protein content of the dialyzed fusion preparations was estimated with A260/280 readings using the formula (1.45 x A280) - (0.74 x A260) (56). Toxicity of the preparations was determined by trypan blue exclusion using a suspension cell line of bovine lymphocytic leukemia cells (BL3) as the target cells (obtained from Dr. G.H. Theilen, University of California, Davis). Varying dilutions of the purified fusion proteins were combined with the BL3 cells and allowed to incubate at 37° C for one hour. The percentage of viable cells, defined by exclusion of the trypan blue dye and calculated as (#live-#dead) x $(\#live)^{-1}$,

was determined for zero time and 1 hour. Lyophilized crude leukotoxin (derived from P. haemolytica serotype 1 culture supernatants and supplied by Dr. Confer) resuspended in 1 ml of RPMI 1640 medium served as the positive control. The endpoint dilution of toxicity for the crude leukotoxin is 1:128. Beta-galactosidase (1000 units reconstituted with 25 µl of 0.1 M sodium phosphate buffer, pH 7.3) (Sigma Chemical Co., St. Louis, MO.) was diluted 1/4 in PBS for use as a negative control.

Neutralization assays involved a 2 hour to overnight incubation step of the fusion protein with heat-inactivated anti-leukotoxin serum before combination with the target cells.

Isolation of recombinant bacteriophage DNA. Plate lysates of the recombinant bacteriophages SW12a and SW20c were prepared by infecting E. coli Y1090 lawns according to standard methods (64)(see Appendix). Bacteriophage DNA was isolated and purified from these lysates using the method of Xu (100). To remove the chromosomal DNA from the lysates, a Whatman DE52 slurry was added, mixed for three minutes and centrifuged twice for 5 minutes, 7700 x g at 4° C. The supernatant was extracted with phenol-chloroform and the resulting bacteriophage DNA was purified by ion-exchange chromatography on Elutip-D columns (Schleicher and Schuell, Keene, NH). The DNA was extracted again with phenol-chloroform and precipitated with ethanol. The insert fragments contained within the lambda gt11 vector were

excised by digestion with EcoRI and isolated by preparative electroelution for ligation into a plasmid vector.

Subcloning. pUC18 was isolated from a one liter culture of *E. coli* JM103 by chloramphenicol amplification in rich medium and SDS lysis (64). EcoRI-digested pUC18 was treated with alkaline phosphatase and ligated with the insert DNA. Recombinant plasmids were introduced into *E. coli* TB-1 cells by transformation and the transformants were recovered on LB plates containing ampicillin, X-gal and IPTG. pUC18 contains a unique EcoRI site located near the amino terminus of the *lacZ* gene. Cloning into that site results in insertional inactivation of the beta-galactosidase gene. Therefore, colonies containing the recombinant plasmid should appear white. White transformants were picked and the recombinant plasmids reisolated by the SDS lysis method (64). Purified plasmid DNA was digested with EcoRI and the insert sizes compared with those of the original recombinant bacteriophage. pRC14 carries the insert from SW12a and pRC15 carries the insert from SW20c.

Southern blotting. pUC18 and the recombinant plasmids pRC14 and pRC15 were labeled with [α^{32}P]dATP by nick translation for use as hybridization probes (64)(see Appendix). Chromosomal DNA from SW12a and SW20c lysogens and from *P. haemolytica* A1 was isolated by a small scale SDS lysis procedure (see Appendix). These DNAs, along with the

original recombinant bacteriophage DNA and the recombinant plasmids pRC14 and pRC15, were digested with EcoRI, separated by agarose gel electrophoresis on three identical gels, and transferred to nitrocellulose paper by the method of Southern (88) as described by Maniatis et al.(61). The filters were then hybridized with the three radioactive probes, washed, dried, and exposed to Kodak X-OMAT AR film.

CHAPTER III

RESULTS

Library construction. A library was constructed in lambda gt11 incorporating 0.5 to 2.0 kbp fragments of P. haemolytica DNA. Recombinant bacteriophage DNA was packaged in vitro to form fully infectious particles. Multiple packaging extracts were combined to give a total of 6.3 x 10^4 bacteriophage that contained DNA fragments. Because the antibody screening procedure demands expression of proteins from the P. haemolytica DNA, insertion into the vector must be in the proper orientation and maintain the correct translational reading frame. Therefore, 1/6 of the recombinant bacteriophage would be expected to produce a peptide of interest. The number of recombinant bacteriophage required for representation and expression of any P. haemolytica DNA (assuming the genome is 2.7 x 10^6 base pairs in size [72]) sequence is 7.4 x 10^4 (61). Our library contains 1.04 x 10^4 bacteriophage expressing P. haemolytica DNA. The titer of the library after amplification was 5.6 x 10^{11} pfu/ml.

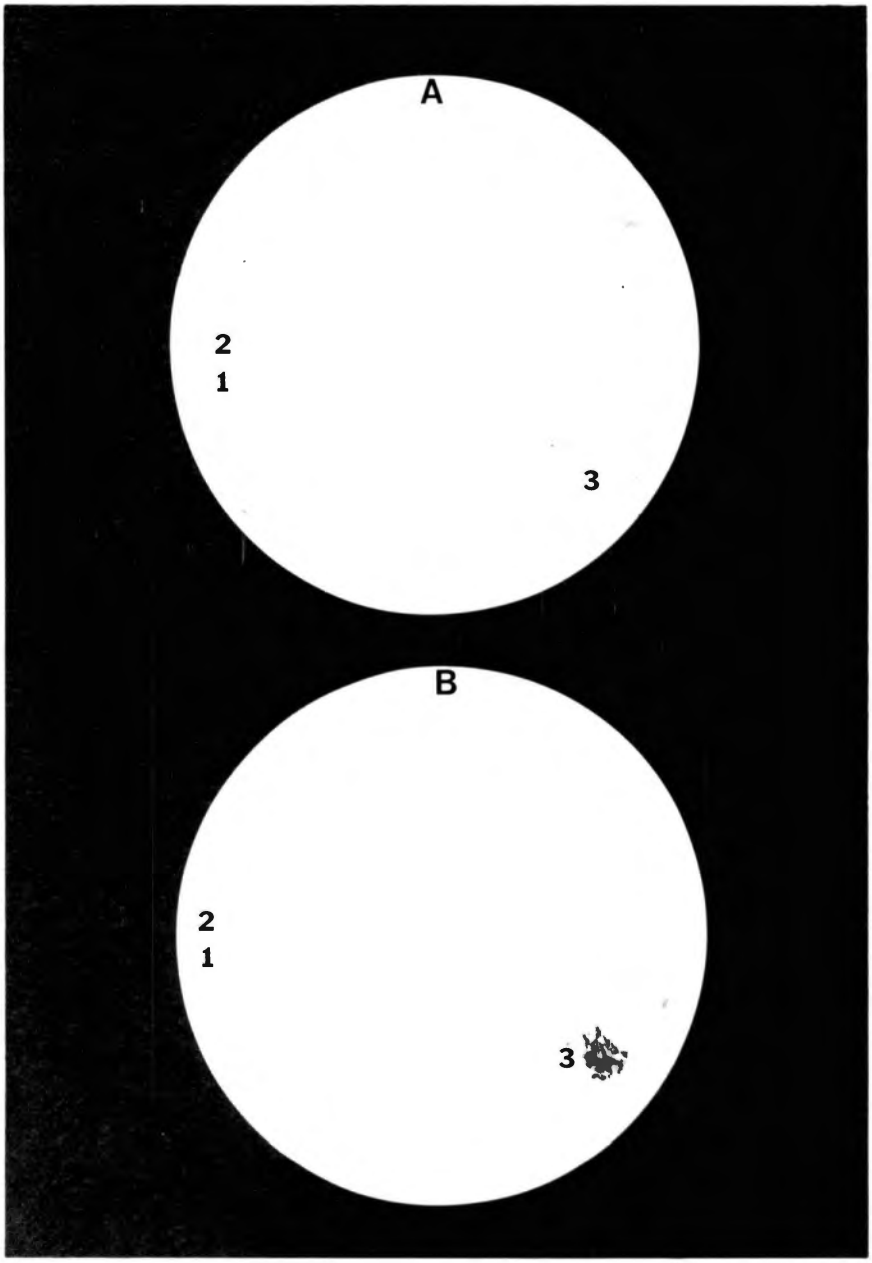
Antibody screening. Initial screening of the library was done with 476 serum. Because it was prepared by multiple injections of the live organism, it should detect

all antigens encountered in a P. haemolytica infection (including leukotoxin). Of the 2.5 million plaques screened, 40 clones were identified by their reaction with the 476 serum. These 40 were plaque purified and rescreened with rabbit anti-leukotoxin serum. Six of the 40 clones reacted with anti-leukotoxin serum. Three of these (SW4a, SW12a, and SW20c) were chosen for further characterization based on the intensity of the color reaction (figure 2).

Preparation of lysogens and crude induced lysates. Further analysis of SW4a, SW12a, and SW20c required large quantities of the foreign gene product. To achieve this, E. coli Y1089 were infected with recombinant bacteriophage. The cI857 gene carried on the vector encodes a temperature sensitive repressor that governs all genes required for the lytic pathway, thus favoring the establishment of the lysogenic state with the infected bacteria at low temperature (30° C). These lysogens can be induced to form bacteriophage products by a temperature shift to 42° C. Concomitant induction of lacZ with IPTG allows efficient translation of the foreign gene. Because of the S100 mutation in the vector, the bacteriophage are unable to lyse the host cell, therefore, accumulation of the bacteriophage products, including products of the foreign genes, occurs.

Crude lysates were prepared by freeze-thaw and sonication of lysogens. Fifteen microliters of each lysate was analyzed by SDS-PAGE and Western blotting for evidence of beta-galactosidase fusion products (antigenic protein

Figure 2. Dot immunoblot screening of duplicate nitrocellulose filters. Filter A was incubated with 476 serum; filter B was incubated with anti-leukotoxin serum. 1, positive control; 2, negative control; 3, SW12a.


A
2
1
3
B
2
1
3

bands greater than 116 kDa) or for the appearance of small antigenic bands indicating an autonomously functioning P. haemolytica promoter. In both SW12a and SW20c, discreet protein bands greater than the beta-galactosidase molecular weight marker were identified by the 476 serum and the rabbit anti-leukotoxin serum (figure 3A). SW4a did not consistently react with the anti-leukotoxin serum and was not further characterized. Fusion of P. haemolytica antigen with beta-galactosidase was confirmed by reactions with mouse monoclonal anti-beta-galactosidase antibody (figure 3B). A molecular weight for each band was determined from a standard curve. 12a was estimated to be approximately 169 kDa, and 20c was estimated to be 170 kDa (figure 4).

Purification of fusion proteins. Because the results of preliminary cytotoxicity assays using induced crude lysates showed no evidence of toxic activity, the fusion protein was purified from contaminating E. coli constituents using the lacZ immunoaffinity columns and following the manufacturer's protocol. The purified fusion protein following elution from the column was verified by Western blot using anti-leukotoxin serum (figure 5). A bacteriophage that did not contain insert DNA (gt11) was subjected to the same procedures as the recombinant bacteriophage, identified on Western blots and was used as a negative control in subsequent experiments.

Cytotoxicity assays. The pH and osmolality of the

Figure 3. Western blot of crude induced lysates. Filter A was incubated with anti-leukotoxin serum; filter B was incubated with anti-beta-galactosidase serum.

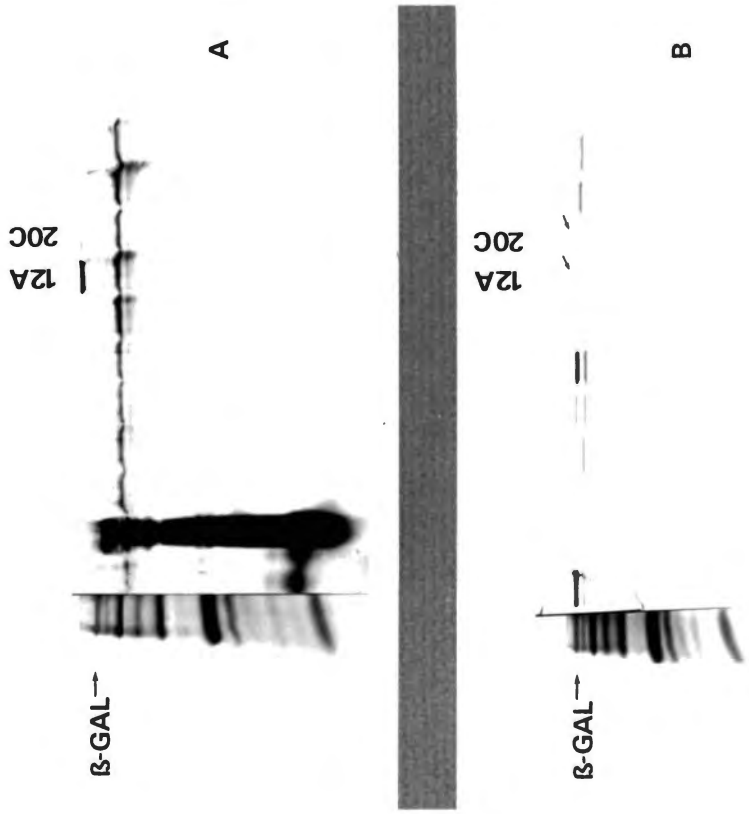

A
12A
20C
ß-GAL
B
12A
20C
ß-GAL

Figure 4. SDS-polyacrylamide gel of crude induced 12a and 20c lysates stained with Coomassie blue.

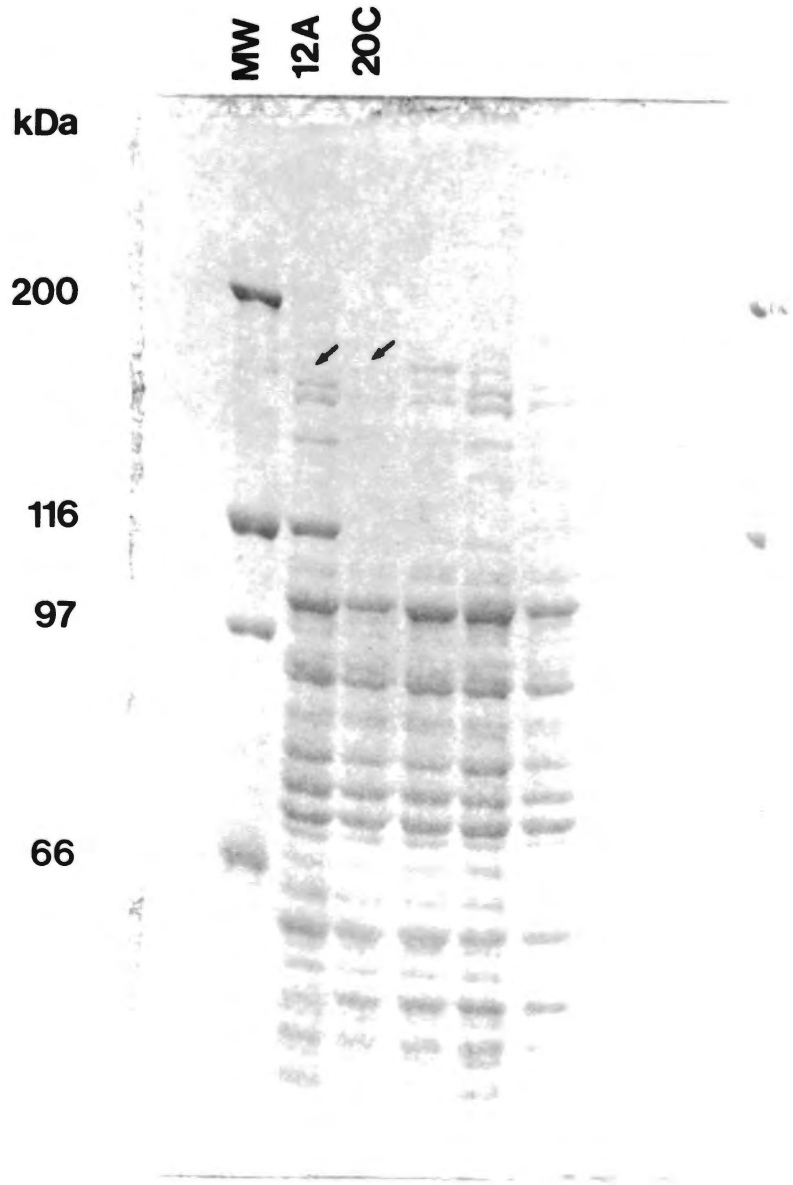
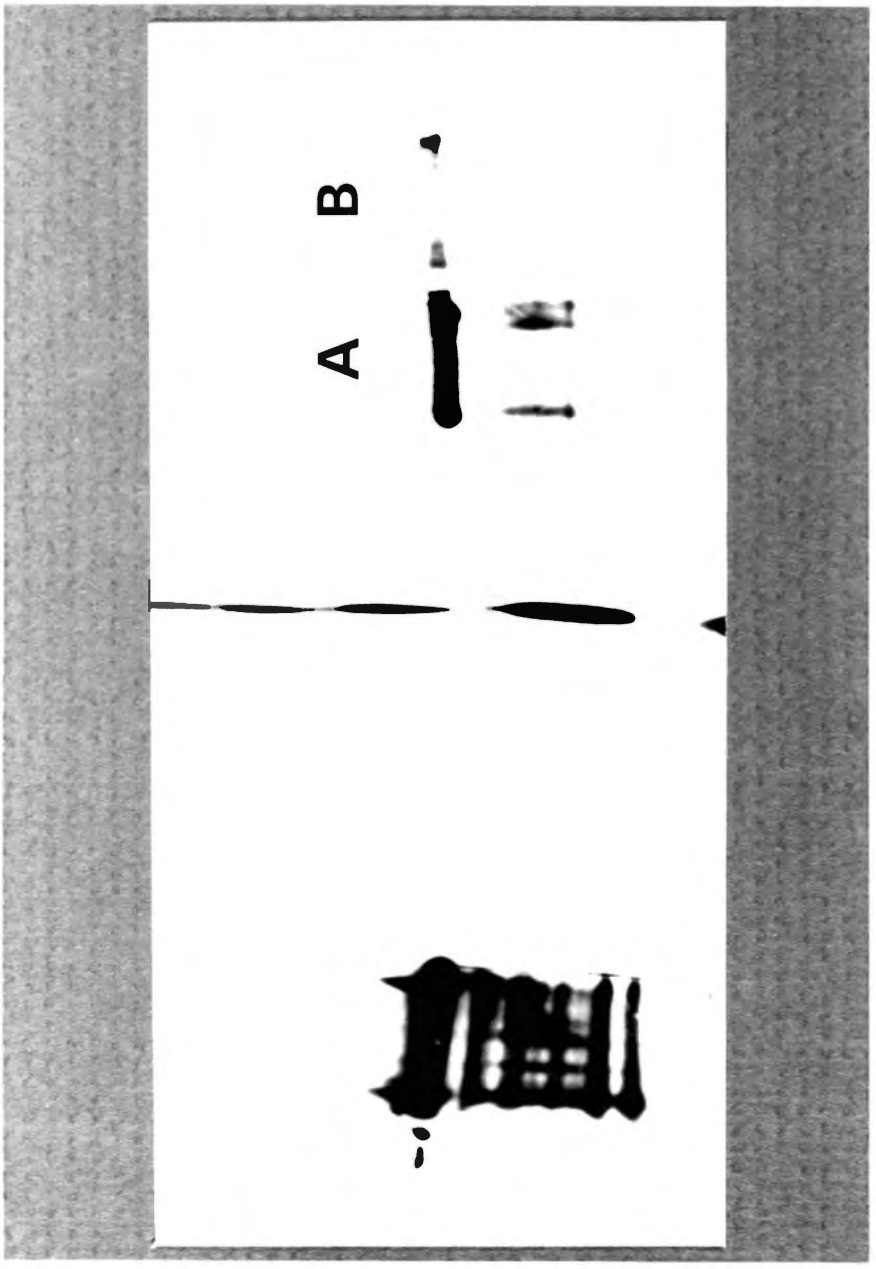

MW
12A
20C
kDa
200
116
97
66

Figure 5. Western blot using anti-leukotoxin serum. A, 20c crude induced lysate; B, purified fusion protein from 20c lysate.


A
B

recovered fractions were adjusted by dialysis against PBS (pH 7.2). The leukotoxin control caused 100% of the BL3 cells to take up trypan blue after sixty minutes of incubation (figure 6A). Twenty micrograms of the 12a and 20c fusion proteins were immediately toxic for the target cells. One hundred percent of the cells took up the trypan blue at 0 minutes for 20c and 30 minutes for 12a (figure 6B,C). The purified beta-galactosidase from the lambda gt11 lysogen and commercially obtained beta-galactosidase were not toxic for BL3 cells (Table 2).

Time course photography of a cytotoxicity assay illustrates the toxic effects of the leukotoxin control and the 12a and 20c fusion proteins (figures 7 and 8). The first noticeable morphologic change was the swelling of the cells to twice their normal size and loss of refractility (figure 7, 20 minutes, figure 8, 1 minute). After this event, uptake of trypan blue would occur, followed by rupture of the cellular membrane and finally aggregation of the cells (figure 7, 60 minutes, figure 8, 30 minutes).

The toxic effects of the leukotoxin control were neutralized by the anti-leukotoxin serum but not the normal rabbit serum (figure 6A). The toxic effects of 12a and 20c were also neutralized by anti-leukotoxin and not by the normal rabbit serum (figure 6B,C).

Subcloning and Southern blot analysis. To better characterize the inserts contained within SW12a and SW20c and to facilitate their use as probes for Southern

Figure 6. Time course of cytotoxicity and the effects of preincubation with anti-leukotoxin serum and negative rabbit control serum. Panel A, Crude leukotoxin preincubated with: ○,by itself; △ , anti-leukotoxin serum diluted 1:8; ▲ , anti-leukotoxin serum diluted 1:16; ■ , negative rabbit control serum diluted 1:8; □ , negative rabbit control serum diluted 1:16. Panel B, 20c fusion protein preincubated with: ○ , by itself; △ , anti-leukotoxin serum diluted 1:8; ▲ , anti-leukotoxin serum diluted 1:16; □ , negative rabbit control serum diluted 1:8; ■ , negative rabbit control serum diluted 1:16. Panel C, 12a fusion protein preincubated with: ○ , by itself; △ , anti-leukotoxin serum diluted 1:8; □ , negative rabbit control serum diluted 1:8.

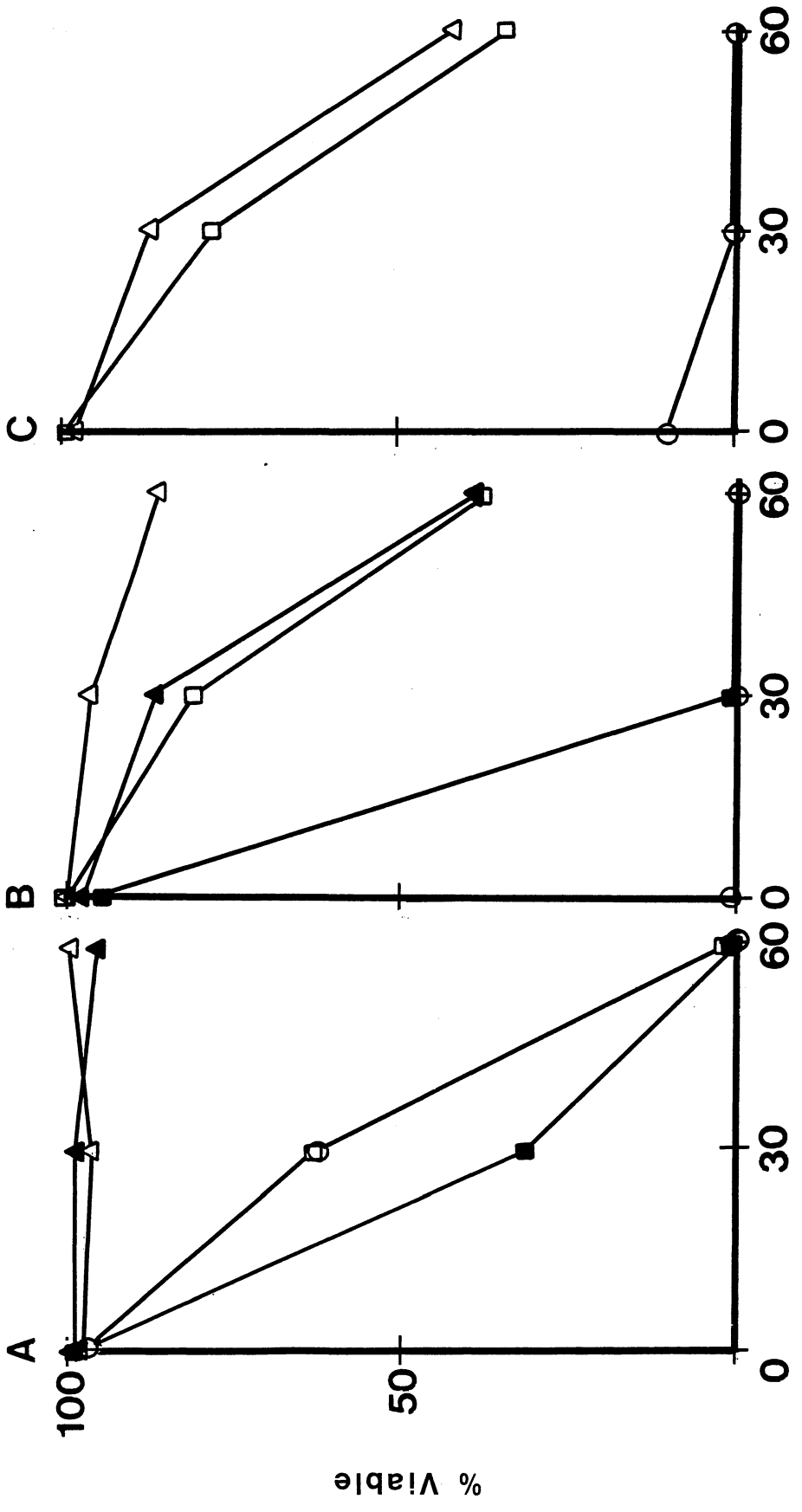

% Viable
100
50
0
A
B
C
0
30
60
Time (min.)

TABLE 2

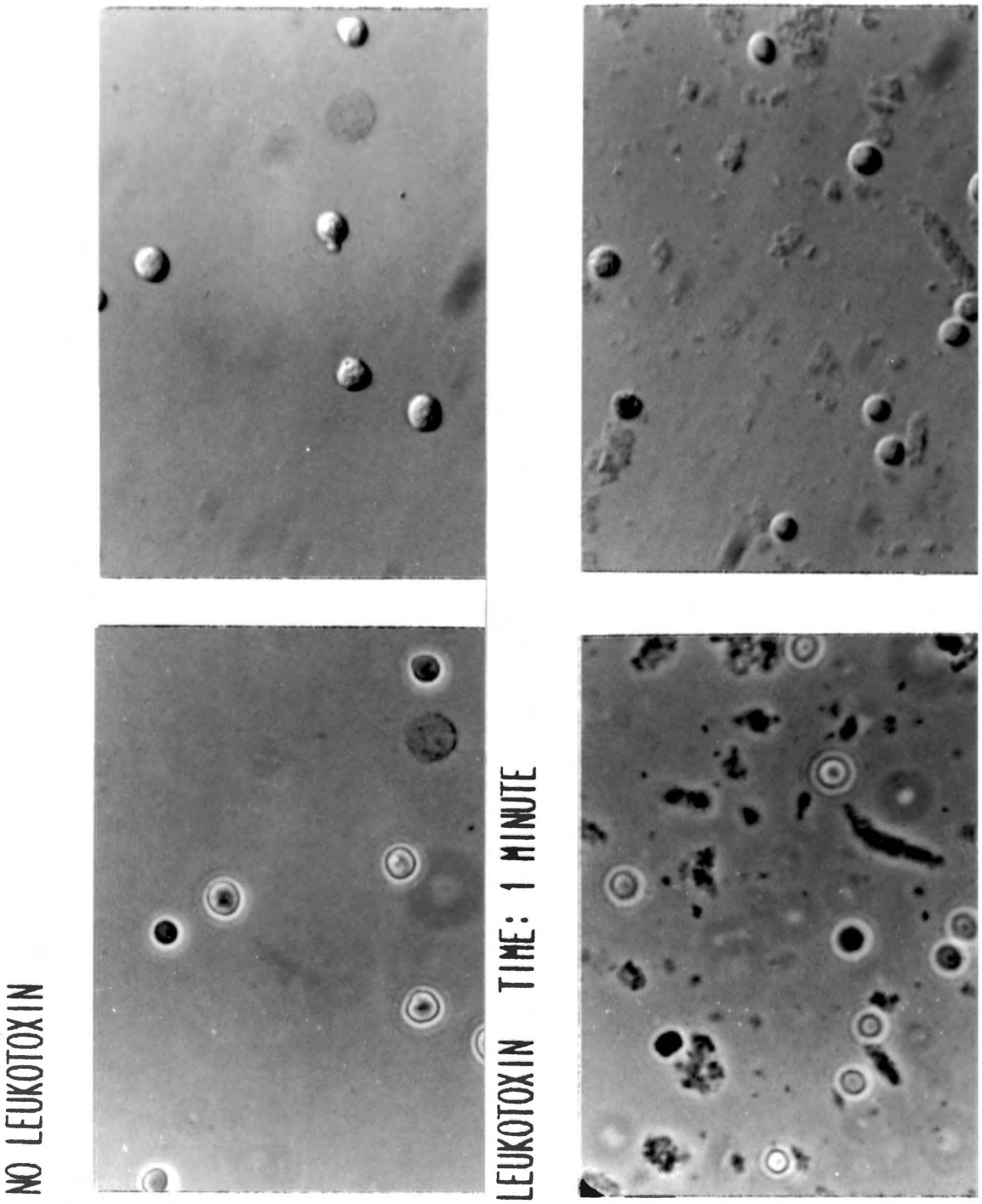
CYTOTOXICITY OF PURIFIED FUSION PROTEINS

Treatment[a]	% Viable Cells[b]	
	Exp. 1	Exp. 2
Cells alone	80	85
Cells + 2.0 µg 20c	37	<1
Cells + 0.5 µg 20c	--	82
Cells + 0.125 µg 20c	--	68
Cells + 2.0 µg 12a	--	0
Cells + 0.5 µg 12a	--	82
Cells + 0.125 µg 12a	--	78
Cells + 2.0 µg beta-galactosidase	--	86
Cells + 2.0 µg gt11	--	85

[a] The assay mixtures were incubated at 37° C.

[b] The % viable cells was determined after 30 minutes of incubation.

Figure 7. Time course photography of cytotoxicity assay using lyophilized leukotoxin. Pictures on the left were photographed using phase contrast light microscopy. Corresponding pictures on the right were photographed using light microscopy with Hoffman modulation. Magnification is 100X.


NO LEUKOTOXIN
LEUKOTOXIN TIME: 1 MINUTE

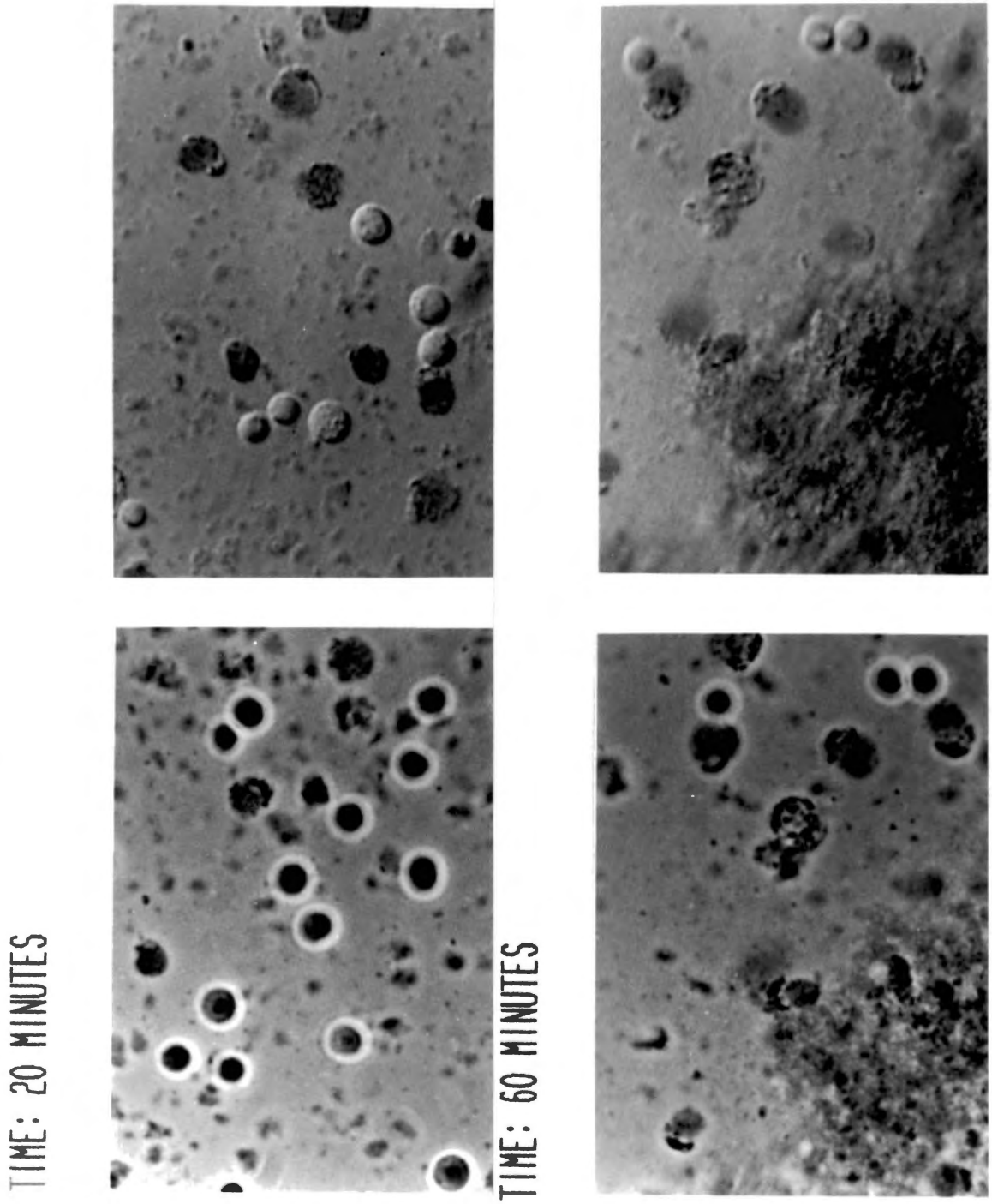
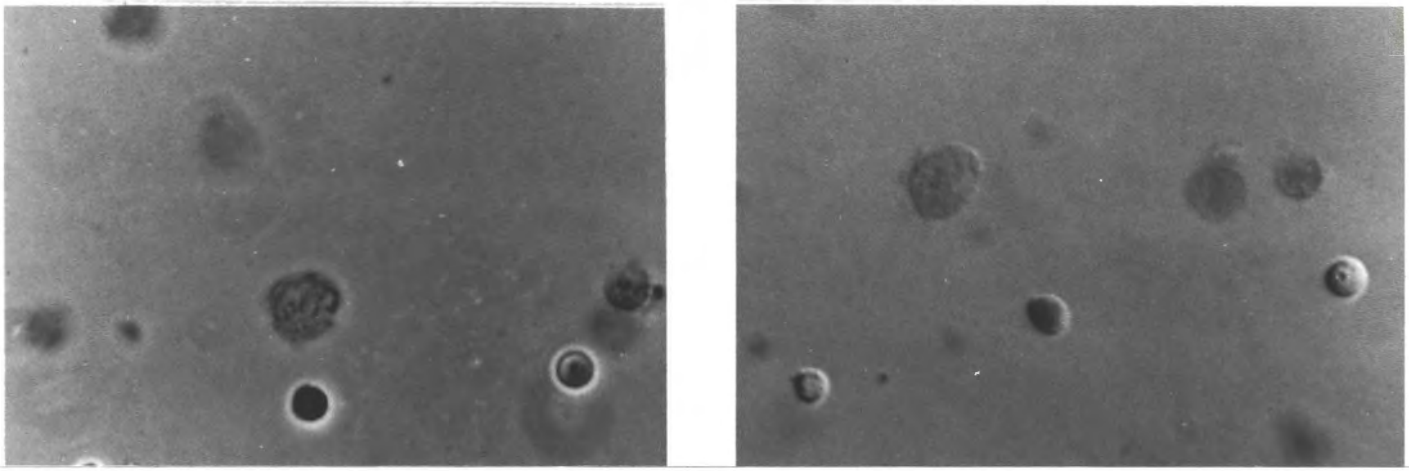
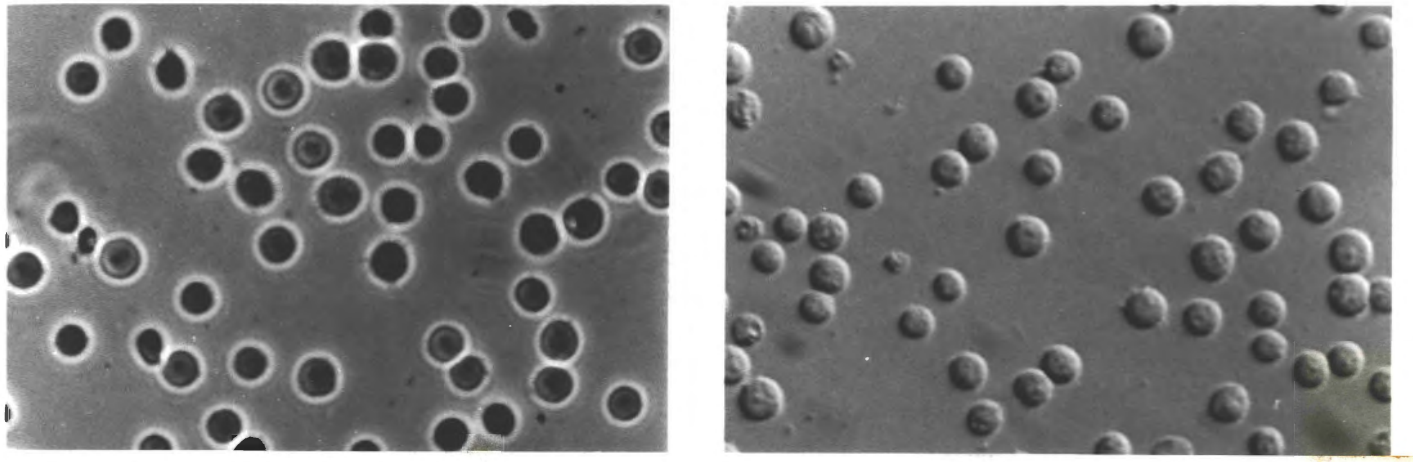

TIME: 20 MINUTES
TIME: 60 MINUTES

Figure 8. Time course photography of cytotoxicity assay using the 20c fusion protein diluted 1:27. Pictures on the left were photographed using phase contrast light microscopy. Corresponding pictures on the right were photographed using light microscopy with Hoffman modulation. Magnification is 100X.

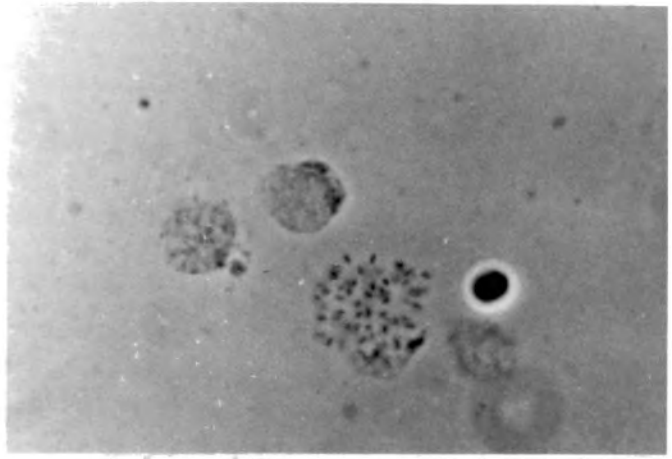
20C FUSION PROTEIN (DILUTED 1/27) TIME: 1 MINUTE

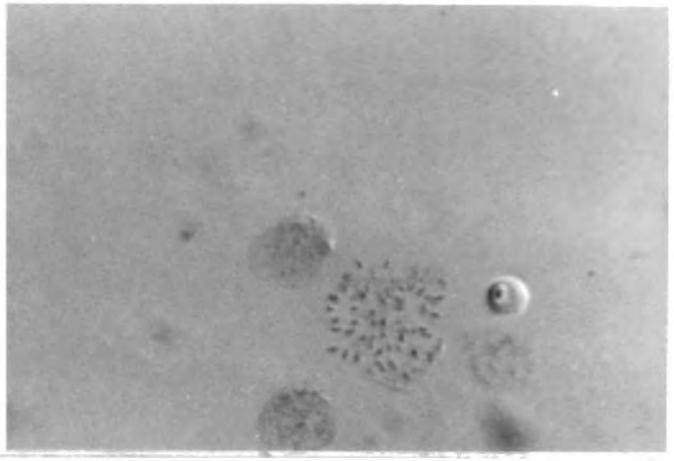


NO 20C FUSION PROTEIN

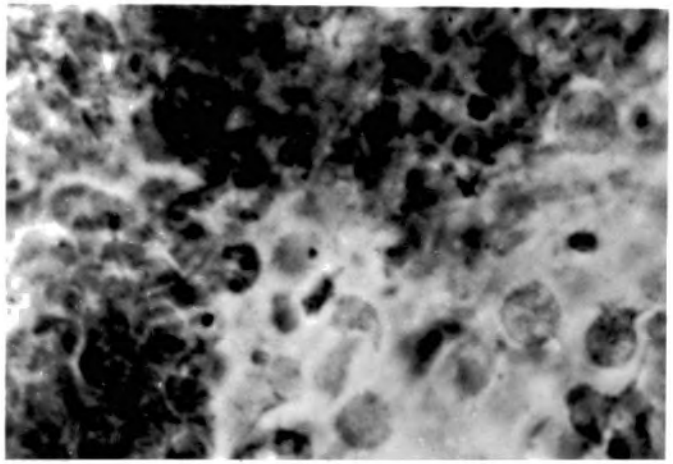


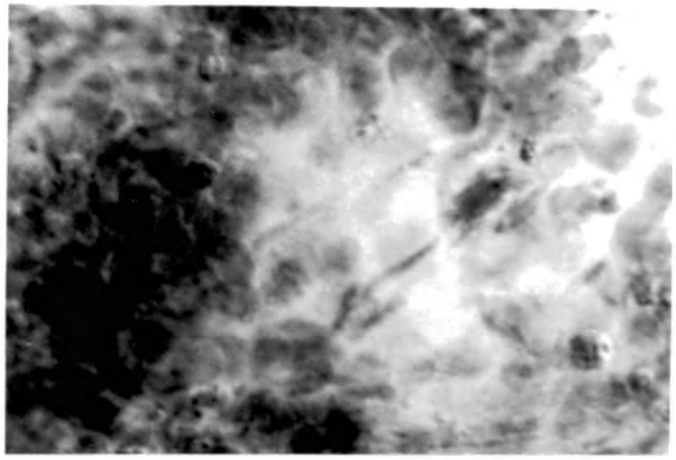
TIME: 10 MINUTES





TIME: 30 MINUTES





hybridizations, the insert DNA from the original recombinant bacteriophage was subcloned in the polylinker region of the plasmid vector. The desired recombinants were identified as white transformants on medium containing X-gal. Plasmid DNA was isolated from the recombinants, digested with EcoRI and the fragments separated by agarose gel electrophoresis (figure 9). The size of the DNA insert fragments in pRC14 (12a) and pRC15 (20c) were estimated to be 0.5 kbp and 1.2 kbp, respectively. IPTG-induced cells containing pRC14 and pRC15 were solubilized in sample buffer and were analyzed by SDS-PAGE and Western blotting with anti-leukotoxin sera. Antigen positive bands were not recognized indicating that the inserts were not being expressed in the plasmids (data not shown).

pRC14, pRC15, and pUC18 labeled with [α^{32}P]dATP were used as probes in Southern blots (figure 10). Homology of the inserts with the original bacteriophage DNA was confirmed (figure 10, lanes 8 and 9). pRC14 and pRC15 EcoRI-digested DNA hybridized with the homologous and heterologous probes demonstrating that the 12a and 20c sequences share homology (lanes 1 and 2). EcoRI-digested Pasteurella DNA probed with pRC15 had a single band confirming the origin of the insert DNA (figure 10, lane 7). A band was not detected with pRC14.

Western blots using guinea pig sera. Duplicate samples of SW12a and SW20c crude induced lysates and whole P. haemolytica cell lysates were subjected to SDS

Figure 9. Agarose gel electrophoresis of EcoRI restriction digestions of recombinant plasmids pRC13, pRC14, and pRC15. Lanes 1 and 10, HindIII-digested lambda DNA size markers; lane 2, undigested pUC18 DNA; lane 3, EcoRI-digested pUC18 DNA; lane 4, undigested pRC13 DNA; lane 5, EcoRI-digested pRC13 DNA; lane 6, undigested pRC14 DNA; lane 7, EcoRI-digested pRC14 DNA; lane 8, digested pRC15 DNA; lane 9, EcoRI-digested pRC15 DNA.

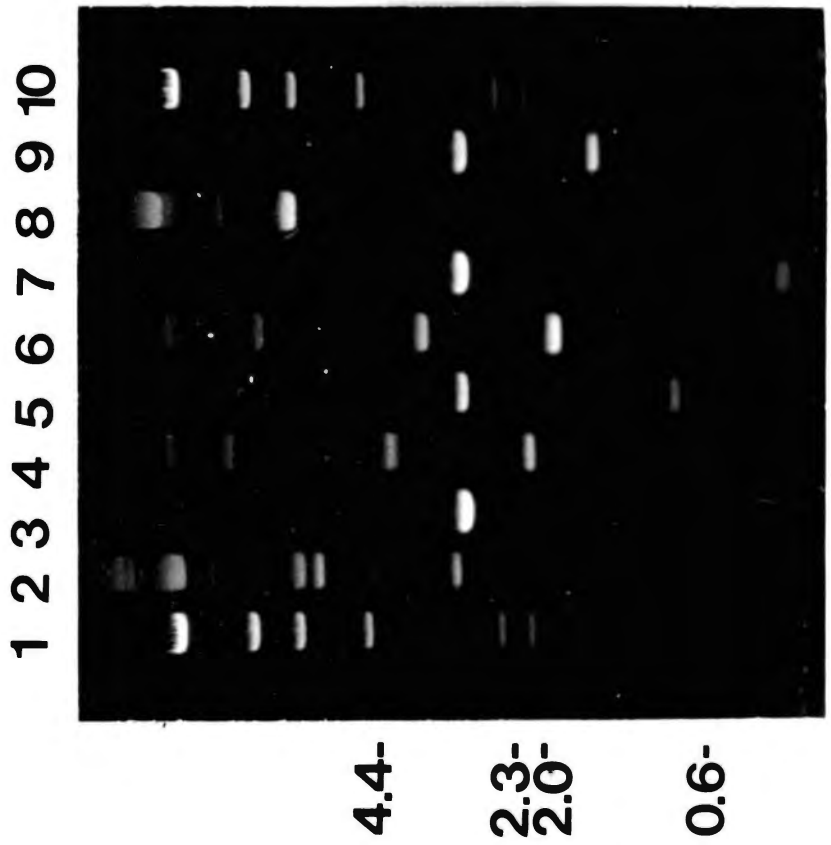
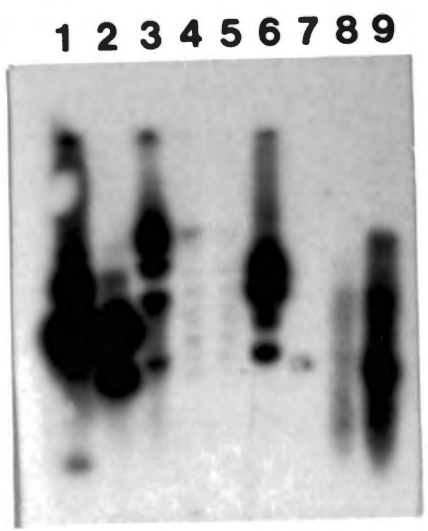
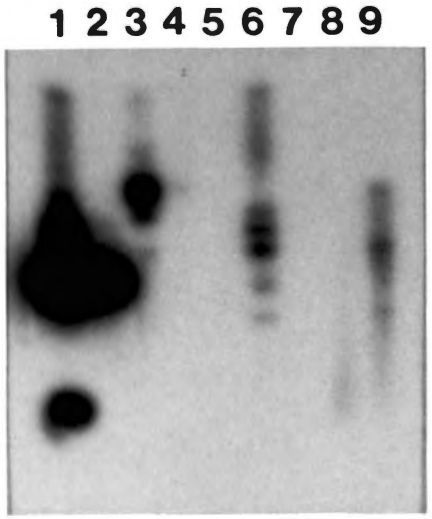

1 2 3 4 5 6 7 8 9 10
4.4-
2.3-
2.0-
0.6-

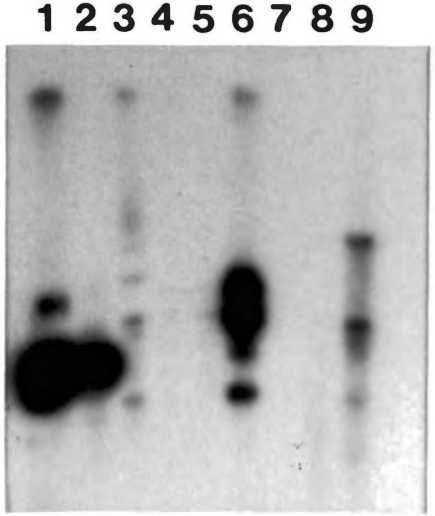
Figure 10. Autoradiogram of Southern hybridizations using [α^{32}P]dATP-labeled pRC14 (pUC18/12a), pRC15 (pUC18/20c) or pUC18. Lane 1, EcoRI-digested pRC14 DNA; lane 2, EcoRI-digested pRC15 DNA; lane 3, EcoRI-digested SW12a lysogenized Y1089 DNA; lane 4, EcoRI-digested SW20c lysogenized Y1089 DNA; lane 5, EcoRI-digested gt11 lysogenized Y1089 DNA; lane 6, EcoRI-digested pMC9 DNA; lane 7, EcoRI-digested P. haemolytica DNA; lane 8, EcoRI-digested SW12a DNA; lane 9, EcoRI-digested SW20c DNA.



pUC18/20C



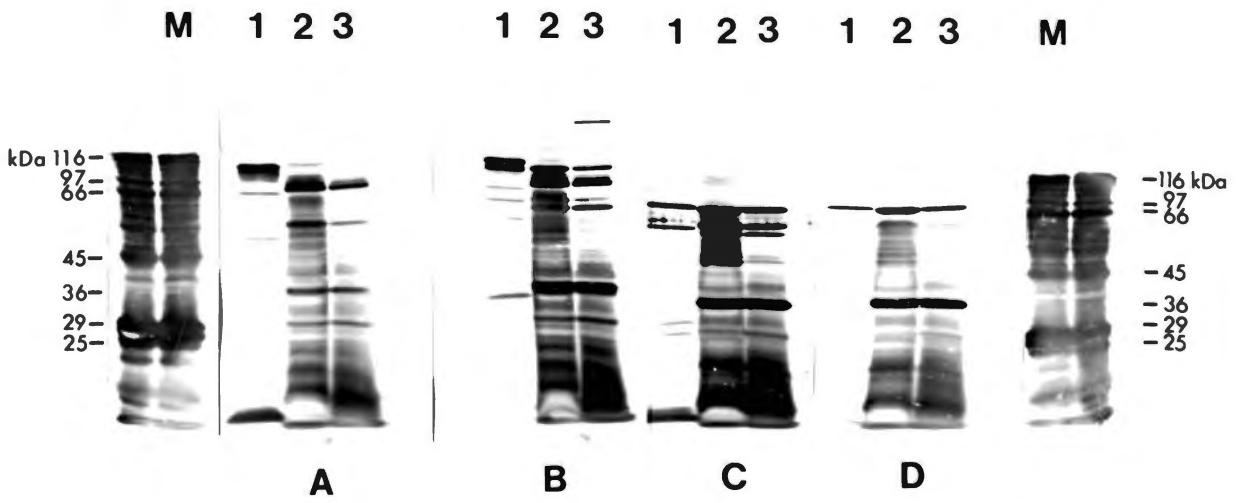
pUC18/12A



pUC 18

polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose filters. The nitrocellulose filters were incubated with anti-12a, anti-20c, anti-beta-galactosidase or preinoculation guinea pig sera (figure 11). The anti-20c serum recognized both the 12a and 20c fusion proteins. The anti-12a serum recognized the 12a fusion protein. Both sera identified a band of approximately 100 kDa in the P. haemolytica lysate. The 100 kDa band was not identified by the anti-gt11 or the preinoculated guinea pig sera.

Figure 11. Western blots using four different guinea pig sera. A, anti-12a; B, anti-20c; C, anti-beta-galactosidase; D, preinoculated control sera. Lane 1, P. haemolytica A1; lane 2, 12a crude induced lysate; lane 3, 20c crude induced lysate.


M 1 2 3
1 2 3
1 2 3
1 2 3
M
kDa 116
97
66
45
36
29
25
116 kDa
97
66
45
36
29
25
A
B
C
D

CHAPTER IV

DISCUSSION

Library Construction

The cloning strategy used not only required representation of any P. haemolytica DNA sequence, but also demanded its expression. The effects of the orientation of the insert fragment with respect to the vector and reading frame within the insert DNA that is being expressed from the lambda promotor must be taken into account. There are two possible ways to orient the insert DNA sequence and three possible reading frames. Therefore, approximately 1/6 of the recombinant bacteriophage should be expressing P. haemolytica DNA sequences as fusions with beta-galactosidase. Insert DNA containing an internal P. haemolytica promotor would express a non-fused product. Complete representation and expression of any given DNA sequence at the 99% probability level would require 7.4×10^4 recombinant bacteriophage. Our library was calculated to contain 1.04×10^4 recombinant bacteriophage that are expressing the foreign DNA, corresponding to a 97.9% probability that any P. haemolytica DNA sequence is both represented and expressed in our library.

Antibody Screening

The complete library was screened with the 476 serum 36 times (a total of 2.3 x 10^6 plaques), yielding 40 positive plaques. The number of positive plaques was much lower than expected and implies that only a single recombinant bacteriophage in the entire library was expressing a Pasteurella antigen. There are several explanations that could account for this result.

The low number of recombinant bacteriophage identified by the 476 serum may reflect the insensitivity of the detection system that was used. Identification of bacteriophage expressing P. haemolytica antigens using the antibody screening procedure depends on the quantity and affinity of antibody to a given antigen. If the sensitivity of the system is low, the serum would detect only those bacteriophage expressing antigens to which a high concentration of high affinity antibodies are present. Likewise, if antibodies to Pasteurella leukotoxin represented a minor fraction of the total antibody population or if the affinities of anti-leukotoxin antibody for the antigen was low, it is conceivable that recombinant bacteriophage expressing leukotoxin antigens would not be identified.

The lambda gt11 vector, itself, imposes a bias toward viable recombinant bacteriophage during amplification of the library. The lacI gene product does not completely repress transcription of the lacZ gene contained within the vector.

Therefore, expression of beta-galactosidase and the foreign DNA sequence occurs at a low level. If the insert DNA encodes a product that is detrimental or fatal for the E. coli host, representation of that recombinant bacteriophage will be reduced.

The 40 bacteriophage initially identified may not be a good representation of P. haemolytica sequences actually cloned. Most likely, an initial screening of the library with anti-leukotoxin serum rather than 476 serum would have identified a different set of clones.

Six of the 40 recombinant bacteriophage which initially reacted with 476 serum also reacted with the anti-leukotoxin serum (figure 2). It is not known whether this number is representative of an efficient recovery rate. These bacteriophage may not code for true leukotoxin determinants, but could code for shared antigenic determinants that are readily detected with the anti-leukotoxin serum.

Southern Blot

The results of the Southern hybridizations showed that the DNA fragments contained in pRC14 (pUC18/12a) and pRC15 (pUC18/20c) share homology. The pRC15 labeled probe recognized the homologous fragment in the pRC15 EcoRI digest (figure 10, lane 2) and the 12a fragment in the pRC14 EcoRI digest (figure 10,lane 1). The reciprocal reaction with labeled pRC14 did not occur. This result is probably related to the percent incorporation of the radiolabeled

nucleotides. The percent incorporation for pRC15 was four times as high as the percent incorporation for pRC14, indicating that the nick translation of pRC14 was not efficient. Because the radiolabeled nucleotide was the sole source of dATP in the nick translation reaction, the low number of counts per minute indicated that radiolabeled dATP was not incorporated efficiently and nick-translation of the DNA was inhibited (data not shown). Therefore, not only is the amount of labeled pRC14 DNA available for hybridization much lower than the amount of pRC15 DNA available, but also the amount of radioactivity incorporated in pRC14 was much lower than in pRC15.

The labeled pRC15 plasmid also hybridized with a single band in EcoRI digested P. haemolytica chromosomal DNA (figure 10, lane 7). This confirms the origin of the inserted DNA fragments. The pRC14 probe did not detect the band probably for the reasons state above. The results of hybridization of the labeled probes to the EcoRI-digested Y1089 lysogen DNA were uninterpretable.

Molecular Weight Estimations

The size of the P. haemolytica DNA fragments contained in SW12a and SW20c were approximately 0.5 kbp and 1.2 kbp, respectively (figure 9, lanes 5 and 7). Estimates of the molecular weights of the proteins encoded by the DNA fragments were 53 kDa for SW12a and 54 kDa for SW20c (figure 4). Assuming a single amino acid equals approximately 100

kDa, the number of kbp of DNA required to code for a protein of 53 kDa is 1.5. This is three times the number of kbp estimated for 12a. One explanation for the discrepancy is that three identical DNA fragments were ligated end to end in the same vector molecule. It is unlikely that this tandem insertion of the identical fragment into the lambda gt11 vector could occur. First, the DNA used for constructing the library was generated by shear force. The probability is low that this random method of fragmentation would generate identical pieces of DNA. Second, if these identical pieces were generated, it is unlikely that they could be ligated into the same vector molecule, in the proper orientation, and reading frame to generate a tandem fusion protein. A plausible explanation of the discrepancy between the calculated and estimated kbp involves post-translational modification of the fusion proteins. The possible types of modification include hydroxylation, phosphorylation, or attachment of prosthetic groups, etc. The secondary and tertiary structure of the peptide either exposes or protects amino acid residues from the environment. If exposed residues were modified, the modified protein would migrate through a SDS-polyacrylamide gel differently than if it were not modified. The slower migration could be due to the physical size and shape of the attached groups, or could be due to a change in the charge of the protein caused by the addition of acidic, basic, or neutral groups.

Cytotoxicity

Preliminary cytotoxicity experiments using the 12a and 20c induced crude lysates showed no evidence of toxic activity. This is probably due to interference by E. coli cellular constituents. Adherence of cellular debris by electrostatic interactions to the 'active' portion of the fusion protein or to the target cell could prevent binding of the fusion protein.

The results observed in subsequent cytotoxicity assays using the purified 12a and 20c fusion proteins showed immediate, toxic changes in the BL3 cells. The possibility that this toxicity was due to osmolality or pH of the purified fusion preparations can be discounted for two reasons. First, the column fractions were dialyzed against 3 changes of PBS (pH 7.3) to adjust the pH to that of physiological conditions. Second, dilutions of the fusion proteins could be as high as 1:50 and still maintain toxicity.

The dilutions of the fusion proteins used in the cytotoxicity assays varied according to the batch. The variation could be due to the variation in the amount of the starting material and the efficiency of recovery of the purified product. The efficiency of IPTG induction of expression of the beta-galactosidase gene and the foreign DNA sequence during the preparation of the crude lysates could also contribute to the batch variation.

While the toxic effects occurred faster with the

purified fusions proteins than with the leukotoxin control, the pattern of events are similar. The difference in the time required for toxicity to occur could be attributed to one of several mechanisms. The purity and amount of the fusion protein used in the assays was most likely greater than that of the leukotoxin preparations. The leukotoxin used as a positive control was a crude derivative of P. haemolytica culture supernatants and contained fetal bovine serum and bacterial cellular contaminants. Those contaminants may interfere with and slow down the process of leukotoxin binding to the target cell. Therefore, the binding process of toxin to target cell may be a limiting step.

Because of the marked specificity of leukotoxin for ruminant leukocytes, some type of recognition between the toxin and target cell would be expected. The fusion proteins 12a and 20c do not contain the entire leukotoxin molecule because the proteins have been calculated to be 53 kDA and 54 kDA, respectively, and the molecular weight of the intact leukotoxin molecule has been reported to be 105 kDa (14,60). It is possible that the active portion of the toxin and not the portion required for the specific recognition is present in these fusion proteins. Therefore, the toxic activity of 12a and 20c would not be slowed by a prior recognition step and could act more quickly. The concept of a single protein molecule acting as a membrane-damaging entity is substantiated by the work of Bhakdi et

al. (8).

A second explanation for the difference in the rate of toxicity observed between leukotoxin and the fusion proteins could simply be a difference in their relative concentrations. Accurate protein determinations could not be performed because of the limited amount of toxin recovered Therefore, the protein determinations are only estimates, and there may be more toxin present in the fusion preparations than in the crude leukotoxin preparations. Quantitation of leukotoxin in the crude preparations was impossible because of the high concentration of fetal bovine serum in the preparations. There is experimental evidence to support this explanation in that progressive dilutions of the fusion proteins increased the time interval before toxic events occur.

The morphologic changes that follow exposure of target cells to both crude leukotoxin and the fusion proteins (12a and 20c) followed a similar pattern. The cells, prior to exposure, were small and round with a shiny target-like appearance (figure 7). After the addition of toxin, the cells would swell to approximately twice their normal size and lose the shiny, target-like appearance (figure 7, 20 minutes). After this event, trypan blue uptake would occur. Rupture of the cellular membranes and aggregation were the final events (figure 7, 60 minutes). Swelling was more pronounced with the fusion proteins than with leukotoxin. The similarities or differences in the morphological events

cannot be used to postulate a mechanism of action for either the crude P. haemolytica leukotoxin or the fusion proteins.

Neutralization

The toxic activity of the crude leukotoxin preparation and both fusion proteins was neutralized to varying degrees with the anti-leukotoxin serum (figure 6). The negative rabbit serum used as a control illustrates that nonspecific stabilization of the target cell membrane by serum components may reduce the immediate toxic effects observed with the fusion proteins. While some BL3 cells are still intact after 60 minutes, obvious clumping and numerous swollen cells are seen. These swollen cells will eventually die defined by uptake of trypan blue. Trypan blue uptake may not be an indication of irreversible cell damage. The results shown in figure 6 do not take into account that the swollen cells may eventually take up the dye. Therefore, the 38% viable indicated on the graph may be too high.

Preabsorption of the fusion proteins with anti-leukotoxin serum resulted in a much higher percentage of viable cells by 60 minutes. Many cells were still intact and clumping was minor compared to the results seen with the negative rabbit serum. The difference in the ability of anti-leukotoxin serum to neutralize 12a and 20c (figure 6B and figure 6C) may reflect the number or type of antigenic determinants on each protein. The SW12a insert DNA is approximately 700 bp smaller than the SW20c insert DNA and

may not have as many antigenic determinants or may not have the important determinants to which antibody can bind and prevent toxicity. The results of the neutralization experiment do not prove that the cloned DNA contains part of the leukotoxin gene.

Western Blots with Guinea Pig Sera

Western blots using the guinea pig sera confirm that SW12a and SW20c fusion proteins share antigenic determinants. It could be argued that antibodies to beta-galactosidase, rather than antibodies to the P. haemolytica proteins, were recognizing the fusion protein bands. However, both the anti-12a and anti-20c sera recognized a protein band of approximately 100 kDa in P. haemolytica (figure 11). This same band was not recognized by the anti-beta-galactosidase or preinoculated guinea pig control sera and probably represents the native protein molecule.

The intensity of the 100 kDa band with the anti-12a serum is much greater than the intensity of the fusion protein band (figure 11). Because the sensitivity of the Western blot procedure is not known, the difference in intensity may reflect the difference in the amount of protein present in the different bands.

Conclusions

From this work I can make several conclusions. We have succeeded in cloning two separate fragments of P.

haemolytica DNA that share a degree of homology. Both of these are expressed as fusion proteins in the lambda gt11 host and both are toxic when purified away from E. coli cellular constituents. The toxicity to BL3 cells observed is immediate and appears to follow a similar pattern of events seen with crude leukotoxin preparations. The toxicity can be neutralized to an extent by anti-leukotoxin serum. Sera prepared by injection of guinea pigs with the fusion proteins, recognizes a band of approximately 100 kDa in P. haemolytica that is not recognized by the anti-beta-galactosidase or preinoculated serum control.

Unfortunately, conclusions concerning the biological activity of the fusion proteins (Ca^{2+} dependence and target cell specificity, for example) cannot be made since only a portion of a protein is represented. The lack of purified leukotoxin preparations has frustrated our efforts to either confirm or deny that the pieces of protein we have cloned are part of the leukotoxin molecule. We do not have evidence to conclude that the DNA we cloned is part of the leukotoxin gene. Isolation and testing of the native protein for cytotoxicity to bovine leukocytes would resolve this question.

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APPENDIX

DETAILED PROTOCOLS

ISOLATION OF CHROMOSOMAL DNA FROM PASTEURELLA HAEMOLYTICA

1. Start with a late log or early stationary phase culture of Pasteurella haemolytica. Pellet the bacteria in polypropylene Oak Ridge centrifuge tubes.
2. For 1 gram of bacteria, resuspend in 10 ml STE (0.1 M NaCl, 50 mM Tris [pH 8.0], 50 mM EDTA) at room temperature.

   Transfer the cells to a screw cap Corex tube.
3. Add 1 ml lysozyme (200 mg/ml in STE stock) to a final concentration of 100 µg/ml and vortex to mix thoroughly. Chill on ice for 10 minutes.
4. Add 50 ul of proteinase K (25 mg/ml in dH_2O stock) to a final concentration of 100 µg/ml and vortex to mix thoroughly.
5. Add 1.1 ml of 10% SDS and invert gently to mix. Incubate at 55° C for 20 to 30 minutes, then chill on ice.
6. Add an equal volume of buffered phenol:chloroform. Invert gently to emulsify. Keep cool.

NOTE: If the nucleic acid is too thick and viscous to

extract efficiently, shake the tube several times to break the viscosity. Otherwise, avoid shaking vigorously during the following steps. The objective is to minimize shearing of the high molecular weight DNA.

7. Centrifuge 10000 rpm, 4° C for 10 minutes in an SS34 rotor.
8. Remove and save the upper (aqueous) phase and discard the lower (organic) phase.
9. Extract with an equal volume of chloroform, always saving the upper phase.
10. Repeat the chloroform extraction until the interface is clear. Transfer the upper phase to a beaker.
11. To precipitate the nucleic acid, slowly overlay the aqueous phase with 2 volumes of cold ethanol. Use a glass rod to collect the DNA by spooling, first picking up DNA at the interface of the phases and then mixing the phases together.
12. Allow the excess ethanol to drain off. Press the rod against the wall of the beaker to help remove the excess ethanol.
13. Transfer the nucleic acid to a test tube containing 10 mls of TE and allow it to dissolve (overnight if necessary).
14. After the nucleic acid is dissolved, add RNase A (25 ul of a 20 mg/ml stock) to give a final concentration of 50 μg/ml.

15. Incubate at 37° C for 30 minutes.
16. Extract with phenol:chloroform, then with chloroform.
17. Add NaCl (0.2 mls of a 5 M stock) to a final concentration of 0.1 M.
18. Precipitate the DNA with two volumes of ethanol and spool the DNA.
19. Dissolve the DNA in 5 mls of TE.
20. Add sodium acetate (0.25 mls of a 5 M stock) to give 0.25 M.
21. Precipitate the DNA with one volume of isopropanol and spool again.
22. Dissolve the DNA in a minimal amount of TE, add a few drops of chloroform and store at 4° C.

MECHANICAL SHEARING OF DNA AND SUCROSE GRADIENT FRACTIONATION

1. Dilute the quantity of DNA to be sheared in TE buffer (in this case, 2 mls of DNA was diluted to 6 mls total).
2. Place the plunger in the French pressure cell.
3. Add an aliquot of the DNA solution to the cell.
4. Place the handle with the stop-cock into the top of the cell.
5. Place the top on the cell, and turn the entire cell upside down on the cell holder. Push the plunger down to near the mark corresponding to the amount of fluid in the cell.

6. Turn the handle until it just seats in the cell. Depress the plunger and collect the pre-shear fluid in a beaker marked dregs.
7. Place the cell in a Carver press and apply pressure 2500-3500 psi. Turn the handle open while applying pressure.
8. Collect the drops of sheared DNA in a tube or beaker.
9. Dismantle the cell and rinse it with TE buffer. Do not keep the rinse fluid.
10. Precipitate the DNA with 0.1 M NaCl and 2 volumes of ethanol overnight in the freezer.
11. Pellet the DNA by centrifugation at 1500 x g in an SS-34 rotor, for 15 minutes at 4° C. Resuspend the DNA in TE buffer.
12. Layer approximately 1 mg of sheared DNA onto each 10-40% continuous sucrose gradient. Balance the tubes and spin at 40,000 rpms in a SW-41 rotor, 5° C for 16 hours.
13. Set up the fractionating apparatus and standardize it with an unused gradient. Set the pump at 5 mls/minute, the absorbance, 0-2A 25 L/M and the chart speed at 60 cm/hr.
14. Run the collected fractions on a gel to determine which contain the DNA in the correct size range. Estimate which fractions to load by looking where the peaks fall on the chart.
15. Pool the fractions containing the DNA of the desired

size and concentrate by precipitation with 0.1 M NaCl and 2 volumes of ethanol.

PREPARATION OF INSERT DNA

1. Set up the following methylation reaction:

   33 µl concentrated reaction buffer (100 mM Tris-HCL [pH 8.0], 10 mM EDTA, 1 mM s-adenosyl-methionine, 4.4 mg/ml BSA)
   25 µl sheared DNA (0.25 ng)
   12.5 µl EcoRI methylase
   180 µl water

   Place in a 37° C dry bath for 1 hour. Add 1 µl of s-adenosyl-methionine and incubate another hour. Dilute with 6 volumes of water, add NaCl to 0.1 M, 2 volumes of ethanol and precipitate overnight.

2. To phosphorylate the linkers, set up the following reaction:

   5 µl 10X ligation buffer (250 mM Tris-HCL [pH 7.8], 100 mM $MgCl_2$, 40 mM beta-mercaptoethanol)
   5 µl T4 polynucleotide kinase
   14800 picomole ends of linkers
   3 µl water

   Incubate at 37° C for 10 minutes then add 3.7 µl of 10 mM ATP and incubate for 50 minutes at same temperature.

3. For polymerase I treatment of the DNA, use 5 units of polymerase I for every 4 µg of DNA. Also use a mixture of 10 mM of each of dATP, dGTP, dCTP and dTTP. Set up the following reaction:

   3 µl of dXTP mixture (0.9 mM)
   polymerase I
   8.5 µl 10X ligation buffer
   12 µg of DNA
   water to make up reaction volume

to 85 µl.

Incubate for 30 minutes at room temperature then heat at 65° C for 5 minutes.

4. Attachment of linkers:

40 picomole ends of DNA
100-fold excess of linkers
(400 picomole ends)
1 µl T4 ligase per 10 µl of reaction volume
10X ligation buffer
0.02 mM ATP
water to make up reaction volume

Incubate 24 hours at 15° C.

5. Cleavage of excess linkers:

1 µl EcoRI/ul of linkered DNA
0.01 M NaCl
Linkered DNA

Incubate 3 hours at 37° C.

6. Removal of excess linkers:

Make a slurry of 10 g of G50 Sephadex and then autoclave. Wash at least 3 times with sterile TE. Construct a column using a 3cc syringe and angel hair, making the bed volume up to 2.5cc. Wash the column 5-10 times with TE. Apply the sample when the TE volume is just below the bed volume. Collect 10 drop fractions in Eppendorf tubes and run 7 µl of each on a gel to determine which fractions to pool. Precipitate the DNA with 0.1 M NaCl, 20 µg/ml tRNA and 2 volumes of ethanol.

VECTOR LIGATION, PACKAGING AND TITERING

1. Prepare the following ligation reaction:

1µl lambda gt11 DNA (0.5 µg)
2 ul Pasteurella haemolytica DNA (0.2 µg)
1 µl H_2O
0.5 µl 10X ligation buffer
(300 Mm Tris-HCL [pH 7.8], 100 mM $MgCl_2$, 100 mM dithiothreitol, 4 mM ATP)
0.5 µl T4 DNA ligase (0.5 Weiss units)

2. Incubate the reaction mixture for 2 hours at 16° C.
3. Set up a flask of host cells (Y1090) in 25 mls of LB + 418 µl of a 30% stock of maltose. Incubate with shaking 37° C until the OD_{560} = 0.6.
4. Thaw packaging extract(s) and add 5 µl of the ligation reaction and incubate at room temperature for 2 hours.
5. Add 500 µl of phage dilution buffer (0.1 M NaCl, 0.01 M Tris [pH 7.9], 0.01 M $MgSO_4$) and 25 µl of chloroform.
6. Dilute the packaging extract in phage dilution buffer to 10^{-6}.
7. Mix 100 µl of host cells and 100 µl of the diluted bacteriophage and absorb 20 minutes at room temperature.
8. Pour 2.5 mls of 0.7% LB soft agar (held at 45° C) containing 8 µl/ml of a 20 mg/ml IPTG stock, 20 µl of a 20 mg/ml stock of X-gal, 2 mM $CaCl_2$ and 10 mM $MgCl_2$ into each of the bacteriophage/host cell mixtures, and plate on LB bottom agar. Incubate upside down, 37° C, overnight. Plaques can be visualized as early as 6 hours.

PLATE LYSATES

1. Mix 10^5 pfus of bacteriophage with 0.1 ml of plating

bacteria. Incubate at room temperature for 20 minutes.

2. Add 2.5 mls of melted soft agar (held at 45° C) and pour onto hardened bottom agar.
3. Invert the plates and incubate 8-12 hours until lysis is confluent.
4. Add 2.5 mls of SM buffer and scrape the top agar with a bent glass rod into a centrifuge tube. Rinse the plate with 2.5 mls of SM buffer and add that to the centrifuge tube.
5. Add 0.1 ml of chloroform and let sit at room temperature with occasional shaking for 15 minutes.
6. Centrifuge 4000 x g, 10 minutes at 4° C.
7. Recover the supernatant and add chloroform to 0.3%. Store at 4° C.

AMPLIFICATION AND STORAGE OF THE LAMBDA GT11 LIBRARY

1. Mix approximately 450,000 bacteriophage with 14 mls of an overnight culture of Y1090 cells grown in LB broth + 0.2% maltose. Absorb 20 minutes at room temperature.
2. Add 75 mls of 0.7% top agar and pour onto 500 mls of bottom agar in a 23cm x 33cm x 4.5cm glass baking dish. Cover with foil and incubate at 37° C for a maximum of 8-10 hours. Plaques should not be so large that they touch.
3. Overlay the agar with 150 mls of SM (per liter: 5.8 g NaCl, 2 g $MgSO_4$-7 H_2O, 50 mls 1 M Tris-HCl [pH 7.5], 5 mls 2% gelatin) and store at 4° C.

4. Remove the top agar from the dish into sterile polypropylene bottles. Rinse the agar surface with approximately 8 mls of SM and add chloroform to the bottles to 5%. Incubate at room temperature 15 minutes with occasional shaking.
5. Centrifuge 4000 x g, 5 minutes at 4° C in a GSA rotor.
6. Transfer the supernatant to a glass tube or bottle and add chloroform to 0.3%. Store at 4° C.

ANTIBODY SCREENING PROTOCOL

BUFFERS
TBS: 50 mM Tris-HCL (pH 7.5), 150 mM NaCl
TTBS: TBS containing 0.1% Tween 20
Antibody Buffer: TBS containing 1% Bovine Serum Albumin
HRP Color Developer: 60 mg HRP into 20 mls ice cold methanol. Immediately before use, add 100 mls of room temperature TBS containing 60 µl of ice cold 30% H_2O_2.

1. If not already done, filters should be blocked with blotto (5% nonfat dry milk in TBS), 10-12 mls per dish for 15 minutes at room temperature, on a rotary shaker.
2. Remove the blotto by aspiration.
3. If the filters are to be left overnight before screening, they should be washed twice with TBS, 5 minutes each wash on a rotary shaker. Add fresh TBS and let the plates sit overnight.
4. Wash filters 5 times, 5 minutes each wash with TTBS.
5. Add primary antibody (10-12 mls per plate): anti-leukotoxin serum is already at a 1/50 dilution so use without further dilution. 476AS is already at a 1/25 dilution so use without further dilution.

Incubate at room temperature for one hour on a rotary shaker.

6. Wash filters 5 times, 5 minutes each wash with TTBS.
7. Add the biotinylated second 'antibody' (10-12 mls per plate). For rabbit source serum, use the biotinylated Protein A diluted 1/300 in antibody buffer. For bovine source serum, use the biotinylated anti-goat IgG diluted 1/800 in antibody buffer. Incubate at room temperature with shaking for one hour.
8. Wash filters 5 times, 5 minutes each with TTBS.
9. Add the Streptavidin-HRP complex (10-12 mls per plate) diluted 1/400 in antibody buffer. Incubate at room temperature with shaking for 30 minutes.
10. Wash filters 2 times with TTBS and once with TBS, 5 minutes each wash.
11. Make up the color developer reagent as follows: In one flask, add 120 mg HRP reagent to 40 mls of ice cold methanol and keep in the dark. In another flask, add 120 µl of 30% H_2O_2 to 200 mls of room temperature TBS. Mix the two flasks together and add 10-12 mls per plate. Incubate up to 45 minutes.

Special considerations: Care should be taken to insure that all of the undissolved solids in the blotto are rinsed from underneath the filters. The primary antibody can be left on the filters overnight.

PLAQUE LIFT PROTOCOL

1. Streak out Y1090 cells on LB plates containing 50 μg/ml ampicillin. Incubate at 37° C.
2. Inoculate 25 mls of LB + 418 μl of a 30% stock of maltose with a single colony of Y1090. Incubate with shaking at 37° C overnight.
3. Mix 100 μl of the diluted lambda gt11 with 100 μl of cells and adsorb 20 minutes.
4. Add 2.5 mls of LB soft agar and plate out on 2 day old plates to insure that the soft agar sticks to the bottom agar.
5. Incubate the plates at 42° C for 3 hours.
6. One at a time, remove the plates from the incubator and overlay them with nitrocellulose filters saturated with 10 mM IPTG (0.06 g in 25 mls water).
7. Incubate the plates at 37° C for 3 hours longer.
8. Remove the plates from the incubator and mark the orientation of the filters with the plates with an ink pen. Be sure to mark the plates also. Carefully remove the filters from the surface of the agar using forceps and place them in sterile petri dishes with the agar side up (markings face down).
9. Block the filters with Blotto (5% nonfat dry milk in TBS [50 mM Tris-HCL pH 7.5, 150 mM NaCl]) for 15 minutes.
10. Wash the filters 3 times, 5 minutes each with TBS.
11. Move the filters to new petri dishes, cover with TBS

and store overnight at room temperature.

Special considerations: The nitrocellulose overlay step should be done as quickly as possible to prevent the cooling of the plates below 37° C. Special care should be taken when applying the filter--it cannot be moved once placed on the plate. Be sure to remove bubbles from underneath the filter.

DOT BLOT PROTOCOL

1. Pick plaques and suspend them in 1 ml of SM plus 1 drop of chloroform.
2. Spread LB plates with 100 µl of an overnight culture of Y1090 and allow the liquid to soak in. 1 week old plates are best for this procedure.
3. Drop 5 µl of each plaque suspension onto the surface of the agar in rows. 4 drops across and 6 drops down is the maximum number per plate. Care must be taken to provide enough space to prevent the drops from running together.
4. Make sure the drops have soaked in before carrying the the plates through the plaque lift procedure.

PREPARATION OF LAMBDA GT11 LYSOGENS

1. Dilute an overnight culture of Y1089 cells to $1x10^{-5}$ with 10 mM $MgSO_4$ (1 M stock solution diluted 1/100 with LB).
2. Mix 5 µl of a $1x10^5$ pfu/ml stock of recombinant lambda

gt11 with 100 µl of the $1x10^{-5}$ dilution of the Y1089 and allow it to adsorb 20 minutes at 37° C.

3. Meanwhile, spread 100 µl of a 1:1 mixture of the lambda h80 Δ 9 and lambda b221 cI26 onto at least 2 day old plates and allow it to soak in.
4. Spread the bacteria/bacteriophage mixture on top of the previous mixture and allow it to soak in.
5. Incubate 32° C overnight.

NOTE: Include a bacteriophage only control and a cell only control. The number of colonies should be less than that on the cell only control.

6. To test for temperature sensitivity, streak in a straight line half of a suspect colony on one plate labeled 32° C and the other half on a plate labeled 42° C. Incubate overnight at the respective temperatures. Lysogens will grow well at 32° C and poorly at 42° C.

PREPARATION OF INDUCED CRUDE LYSATES

1. Inoculate 100 mls of LB broth in 250 ml flasks with a single colony of the Y1089 recombinant lysogen.
2. Incubate at 32° C with good aeration in a water bath.
3. When the OD_{600}=0.5, shift the flask quickly to a 42° C water bath and incubate 20 minutes with good aeration.
4. Add IPTG to 10 mM (make up a 1 M stock and add 1 ml to each flask), shift the flask quickly to 37° C and incubate 45 minutes. It is important not to let the

temperature of the flask drop below 37° C during the shift.

5. Harvest the cells in a room temperature centrifuge 5000 rpm for 5 minutes in a GSA rotor.
6. Discard the supernatant and resuspend the pellet in 1/50 of the initial culture volume (5 ml) with protease inhibitor buffer (50 mM Tris-HCL [pH 6.8], 1 mM phenylmethylsulfonyl fluoride).
7. Dispense in 0.5 ml aliquots in Eppendorf tubes embedded in dry ice. Store at -70° C.

Special considerations: The air to liquid ratio in the flasks is important. If the cultures are to be scaled up or down, be sure to maintain an equivalent ratio.

WESTERN BLOT PROTOCOL

Tank buffer (500 mls):	Tris base 1.50 g Glycine 7.20 g SDS 0.50 g pH to 8.3 add H_2O to 500 mls.
Sample buffer (2X):	0.125 M Tris-Cl [pH 6.8] 4% SDS 20% Glycerol 10% 2-mercaptoethanol
Transphor buffer (1.5 L):	Tris base 4.54 g Glycine 21.66 g Methanol 300 mls pH to 8.3 add H_2O to 1.5 L

1. Resolving gel:

0.8% Bis, 30% Acrylamide	6.66 mls

Resolving buffer (1.5 M Tris-Cl, [pH 8.8])	5.00 mls
10% SDS	0.20 mls
H_2O	8.00 mls
Degas for 45 seconds	
Ammonium persulfate (0.10 g in 0.90 ml water)	0.10 mls
TEMED	0.04 mls

Overlay with Resolving Buffer saturated butanol and allow it to polymerize for 1 hour.

2. Wash off butanol thoroughly with nanopure water.

3. **Stacking Gel:**

0.8% Bis, 30% Acrylamide	1.34 ml
Stacking Buffer (0.5 M Tris-Cl, pH 6.8)	2.50 ml
10% SDS	0.10 ml
H_2O	3.00 ml
Degas for 45 seconds	
Ammonium persulfate	0.05 ml
TEMED	0.004 ml

Insert combs and make sure there are no air bubbles. Let polymerize 1 hour.

4. To prepare samples, combine 50 µl of sample with 50 µl of 2X sample buffer in an Eppendorf tube and boil for 5 minutes. Spin down the condensation in a microfuge and load 20 µl of sample per well. Run the gel in the Mighty Small apparatus at 35ma for approximately 2 hours. If only one gel is run, be sure to clamp a glass plate to the other side before running the gel.

5. When the dye front has reach the bottom of the gel, turn off the power supply and take the plates apart removing the gel carefully from the surface of the silica plate. Be sure to mark the orientation of the gel by cutting off one or more of the corners. Soak the gel in transphor buffer 3 times, 10 minutes each on a

rocker.

6. In a small container, assemble the cassette under transphor buffer to avoid air bubbles. Place the cassette piece on the bottom and on top of that place the first sponge. Remove any air bubbles that are trapped in the sponge. Next, place one sheet of Whatman filter paper that has been cut to size, then one piece of nitrocellulose filter also cut to size. Place the gel in proper orientation on the nitrocellulose and line up 2 sides. Cut the nitrocellulose to match the cuts on the gel so that the orientation can be determined later. On top of the gel, place 2 sheets of nitrocelluose paper, the second sponge (removing the trapped air bubbles) and finally the second cassette piece. Pick the entire cassette up so that the gel is nearest to you and place in that orientation in the Mini Transphor unit (already half-filled with transphor buffer). Fill the chamber with buffer to just below the banana plugs and move the cassette up and down to release any air bubbles. Start the cooling system and the stir bar. Place the top of the chamber on so that the black plug is closest to you and run for 1.5 hours at 250ma.
7. Remove the cassette from the transfer unit and place the nitrocellulose filter in a small container using forceps. Block with blotto and blot as before using the antibody screening protocol.

BIORAD PROTEIN ASSAY AND BINDING AND ELUTION OF FUSION PROTEINS USING AN IMMUNOAFFINITY COLUMN

Biorad Protein Assay

1. Make up a bovine serum albumin standard (1.5mg/ml) with sterile water.

2. Dilute the standard with water as follows:
   1. 100 µl BSA std. + 0 µl of water = 1.500 mg/ml
   2. 150 µl BSA std. + 50 µl of water = 1.125 mg/ml
   3. 150 µl BSA std. + 100 µl of water = 0.900 mg/ml
   4. 100 µl BSA std. + 100 µl of water = 0.750 mg/ml
   5. 100 µl BSA std. + 200 µl of water = 0.500 mg/ml
   6. 100 µl BSA std. + 300 µl of water = 0.375 mg/ml
   7. 100 µl BSA std. + 400 µl of water = 0.300 mg/ml
   8. 100 µl BSA std. + 900 µl of water = 0.150 mg/ml
   9. 0 µl BSA std. + 100 µl of water = 0.000 mg/ml

3. Transfer 100 µl of each dilution to a 10 ml snap cap tube.

4. Dilute the unknown samples 1/5 with water in duplicate.

5. Make up dye reagent: mix 20 mls of the concentrate with 80 mls of water and filter through Whatman #1 filter paper.

6. Add 5 ml of dye to each of the standards and the samples and allow them to stand 5 minutes.

7. Read the absorbance at 595nm and use linear regression to generate the standard curve and to estimate the value for the unknowns. A_{595} = X, mg/ml = Y. Unknown values should take into account the dilution factor.

Immunoaffinity Columns

1. Thaw the induced lysates and sonicate in bursts until foamy. Spin 15 minutes in a microfuge at 4° C.

2. Collect the supernatant in a 10 ml polypropylene tube and add 3 volumes of saturated ammonium sulfate. Store at 4° C overnight.
3. Spin down the precipitate in a microfuge, 20 minutes at 4° C.
4. Remove the supernatant and resuspend the pellet in cold TEP (100 mM Tris-HCL [pH 7.4], 10 mM EDTA, 1 mM PMSF) to 20 mg/ml protein.
5. Dilute with cold TBSN (10 mM Tris-HCL [pH 8.0], 150 mM NaCl, 1% Triton X-100 or 0.2% NP-40) to 4 mg/ml total protein.
6. Resuspend the column matrix and flick the bottom to get the air bubbles out. Use a 10 ml snap cap or 15 ml screw cap tube buried in ice to catch the fluid.
7. Remove the caps from the columns and allow the liquid to drain to just below the bed volume before equilibrating the column with 5 mls of TBSN.
8. Allow the liquid to drain to just below the bed volume and add the sample. Catch a few drops to get the sample into the column bed. Seal off both ends and allow it to stand 1hr to overnight in ice or in the refrigerator.
9. Allow the flow to continue at a slow rate--if the rate is too fast, a 26 gauge needle can be fitted on the end of the column.
10. Wash the column with 10 mls of TBSN.
11. Elute the protein with 3 successive 1 ml washes with

the 0.1 M carbonate/bicarbonate buffer, pH 10.8 followed by 1 ml of TBS (10 mM Tris-HCL [pH 8.0], 150 mM NaCl).

12. Re-equilibrate the column with 3-5 mls of TBS + 0.02% sodium azide.
13. Read the absorbance of the 4 fractions at 280nm (optional).
14. Run a Western blot to detect the protein in the fractions.

TRYPAN BLUE EXCLUSION PROTOCOL

1. Count the BL3 cells on a hemocytometer, then spin down the cells, 2000 rpm for 5-10 minutes. Resuspend the pellet in RPMI to give a final concentration of 1 X 10^7 cells/ml.
2. Dilute the samples to be tested in RPMI.
3. Reconstitute lyophilized leukotoxin + 7% fetal bovine serum in 1 ml of RPMI.
4. Combine the cells and the samples one at a time as follows:

   Cells only control- in an Eppendorf tube, combine 180 µl of RPMI with 90 µl of cells and immediately set the timer for 1 hour. For 0 time, immediately remove 100 ul of sample to another Eppendorf tube and place the remainder at 37° C. To the 100 µl aliquot, add 50 µl of trypan blue dye and count live versus dead cells on a hemocytometer. Read the large squares at the corners

of the grid and count 200 total cells.

Samples- in an Eppendorf tube, combine 60 µl of the sample with 120 µl of RPMI and 90 µl of cells. Repeat procedure above to count the 0 time point.

For 1 hour time point- mix 100 µl of the mixture in a new tube with 50 µl of trypan blue dye and count as before.

5. To calculate % viable: live - dead/live X 100
6. For neutralization experiments, the dialyzed fusion protein preparations were diluted 1:4 with PBS and preincubated with anti-leukotoxin serum (diluted 1:8 in PBS) 2 hours at room temperature.
7. The preincubated mixtures were then combined with B13 cells and carried through the trypan blue assay as previously described.

SOUTHERN BLOT AND NICK TRANSLATION PROCEDURES

1. Wash out gel boxes to be used to get rid of any extraneous DNA.
2. Run the DNA samples on 0.7% agarose minigels until they are approximately 3/4 of the way down the gel.
3. Take a picture of the gel using a transilluminator.
4. Soak the gel 15 minutes at room temperature in 0.25 M HCL on a rocker. The dye front should turn yellow.
5. Wash 2X with nanopure water.
6. Soak gels in 0.5 M NaOH + 1 M NaCl for 15 minutes at room temperature on a rocker. Dye front should turn

back to a dark blue.

7. Wash 2X with nanopure water.
8. Soak the gel in 0.5 M Tris [pH 7.0-7.5] + 3 M NaCl for 15 minutes at room temperature on a rocker.
9. Make a foil pan with the sides turned up and place 1 blotting pad in the middle. Saturate with 20X SSC pH 7.0 (175.3 g NaCl, 88.2 g Sodium citrate, 800 mls of water) until it runs out the sides of the blotting pad.
10. Place 1 piece of 3MM paper on top of the blotting pad.
11. Put the gel on top and make sure there are no air bubbles.
12. Wet a piece of nitrocellulose with water and place on top of the gel. Work out the air bubbles with gloved fingers.
13. Put 1 piece of 3MM paper on top followed with lots of paper towels cut just smaller than the nitrocellulose and gel. Finally, place a glass slide on top and put a beaker of water weighing 200 g on the slide. Let sit 1.5 to 5 hours.
14. Take the blot apart with gloves on and leave the gel stuck to the nitrocellulose. Turn the gel and filter on the glass plate so that the gel is next to the glass and mark the wells on the filter with a ball point pen. Also mark the number of the gel if necessary.
15. Soak the filter in 6X SSC (30 mls of 10X + 70 mls of H_2O) for about 1 minute then blot dry with Whatman No. 3 filter paper. Place the nitrocellulose between a

folded,oversized piece of Whatman filter paper and place in foil to be folded larger than the filter.

16. Bake in a vacuum oven 1-2 hours at 80° C under vacuum. Store the bake filters in ziplock bags until you are ready to use them.

HYBRIDIZATIONS

1. Float the baked filter on the surface of 6X SSC; once it is wet, immerse the filter in the buffer for 2 minutes.
2. Slip the wet filter into a plastic seal-a-meal bag and double seal 3 sides leaving 1/2 inch clearance on each side. Seal the top of the bag except for 1 corner, allowing a 1 inch clearance.
3. Add 5 mls of prehybridization solution (5X SSC, 0.5% SDS, 5X Denhardt's solution, 1 mM EDTA, 100 µg/ml denatured salmon sperm DNA, 45% deionized formamide)(Denhardt's solution: 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin, 500 mls water. Filter sterilize.) and double seal the corner. Incubate overnight in a 42° C waterbath.

   In the meantime, start the nick translation.

REAGENTS: **10X Nick Translation Buffer**
- 0.5 M Tris Cl [pH 7.2]
- 0.1 M $MgSO_4$
- 1m M dithiothreitol
- 500 µg/ml BSA

DNase 1
Stock: 1 mg/ml DNase 1 in 0.15 M NaCl and 50% glycerol. Dilute in cold nick translation buffer with 50% glycerol and store at -20° C.

Deoxynucleotide triphosphate stock solution
1 mM dATP (10 µl of 20 mM)
1 mM dGTP (10 µl of 20 mM)
1 mM dCTP (10 µl of 20 mM)
70µl of H_2O)
100µl total

1. Set up the following reaction:
   1µg of DNA
   H_2O to make up to 25 µl
   2.5 µl 20 mM dNTPs
   2.5 µl 10X NTB

Add these reagents in hood:

   11 µl [α^{32}P]dATP (approx. 100uCi)
   0.2 µl polymerase 1 (1 unit)
   1 µl DNase (0.1 µg/ml diluted to 10^{-4})

1. When setting up the reaction, use 4 pairs of gloves and make sure the bottle of nucleotides never point at you. Wear goggles for extra protection. Peel off gloves after touching radioactive fluid.
2. Incubate the mixture 2.5 hours at 15° C.
3. Check the percent incorporation by diluting 1 µl of probe in 9 µl of TE and then spotting 0.5 µl of the diluted probe onto individual pieces of Whatman DE-81 filter paper stuck on pins. Spot 2 filters/probe. Put one of each into a petri dish and into separate vials with caps to put in the scintillation counter. 30% incorporation is considered very good. Store the probe in the freezer.
4. Thaw the probe and transfer 200 µl to a new Eppendorf tube. Tape the tube shut and hold the lid closed with

pliers while boiling for 5 minutes. Immediately place on ice.

5. Use a 1 ml syringe with a 26.5 gauge needle to inject the probe. Pick a corner of the bag and use the needle to pull up one side so that the needle is inserted into and not completely through the bag. After injection, get rid of excessive bubbles and seal the hole with scotch tape. Double seal the corner.
6. Place the bags as flat as possible in a 42° C incubator on absorbent paper and incubate for 48 hours.
7. In the hood, cut off the corner of the bag and pour the hybridization fluid into a 50 ml conical tube. Add 5 ml of buffer A (2X SSC, 0.5% SDS) to the bag and let it sit 5 minutes. Discard buffer in the liquid disposal bottle.
8. Cut open the bag and place the filter in a large weigh boat and add 10 mls of buffer A. Let sit 15 minutes with occasional shaking. Discard the liquid as before. Repeat the wash.
9. Place the filters into 500 ml beakers already filled with 250 mls of buffer B (0.2X SSC, 0.5% SDS) and heated to 68° C. Then put the beakers into a 68° C waterbath and leave the filters there for 30 minutes.
10. Move the filters to a fresh beaker and repeat.
11. Blot the filter dry with a paper towel, wrap with Saran wrap and place on top of an intensifying screen.
12. Preflash the film and make sure the white side of the

screen is facing the film. Next to the film, put the filters and finally another intensifying screen if desired. Close up the packet, place 2 aluminum plates on the out side and clip them in place. Incubate in the freezer.

Y1089 CHROMOSOMAL DNA ISOLATION

1. Place 0.5 ml aliquots of an overnight culture of Y1089 lysogens grown at 31° C into Eppendorf tubes and spin in a microfuge for 2 minutes.
2. Wash the pellet(s) with 1 ml of STE (10 mM Tris-Cl [pH 8.0], 100 mM NaCl, 1 mM EDTA [pH 8.0])
3. Pellet as before in a microfuge and resuspend in 0.5 ml of Solution 1 with lysozyme (50 mM glucose, 25 mM Tris [pH 8.0], 10 mM EDTA [pH 7.5], 4-5 mg/ml lysozyme added just before use) at room temperature.
4. Add 2.5 µl of proteinase K (20 mg/ml stock) to give a final concentration of 100 µg/ml.
5. Add 50 µl of 10% SDS to give a final concentration of 1% and mix.
6. Incubate at 55° C for 20-30 minutes, then chill on ice.
7. Extract twice with phenol/chloroform.
8. Extract twice with chloroform.
9. Add ethanol (0.5 ml) and precipitate overnight in the freezer.
10. Pellet the DNA in a microfuge for 15 minutes and redissolve in 0.5 ml of TE.

11. Add 0.5 µl of concentrated RNase and incubate at 37° C for 30 minutes.
12. Precipitate the DNA with 25 µl of NaoAC and 0.5 ml of ethanol.
13. Pellet the DNA 15 minutes on a microfuge and redissolve in 0.5 ml of TE.
14. Precipitate the DNA with 100 µl of NH_4oAC and 0.5 ml of isopropanol.
15. Pellet the DNA 15 minutes on a microfuge and redissolve in 0.5 ml of TE overnight.
16. Load approximately 2 µl on a 0.7% agarose gel.

VITA

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