“Sequencing of alpha- and beta2- toxin-genes from *C. perfringens* strains isolated from rabbits”

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1. Introduction

During the First World War, it was estimated that hundred thousands of soldiers died of gas gangrene as a result of battlefield injuries, and *C. perfringens* was widely recognized as being the most important causal organism of the disease (T. Shimizu et al., 2002).

The genus *Clostridium* consists of a diverse group of Gram-positive anaerobic bacteria distinguished by their ability to form heat-resistant endospores. These organisms cause diseases such as botulism, tetanus, gas gangrene and pseudomembranosus colitis, diseases generally characterized by the involvement of potent extracellular toxins (Rood, 1998).

Clostridium perfringens is a normal inhabitant of the gastrointestinal tract of humans and animals and is commonly found in the soil. It grows vigorously at temperatures between 20℃ and 50℃, with an optimum of 45℃ for most str ains. The organisms are straight rods with blunt ends that occur singly in pairs, 0.6 to 2.4 μm wide by 1.3 to 19.0 μm long. They are non motile, reduce nitrate, ferment glucose, lactose, maltose, sucrose and other sugars, and liquefy gelatine (Hatheway, 1990).

C. perfringens was the first Gram-positive bacterium for which a genetic map was elucidated, strain CPN50 (Rood, 1998). Its chromosome contained a 3.031.430-bp sequence that had a pronounced C+G content, the replicon origin was designated upstream of *dnaA*. The strain also contains a plasmid of 54.310 nucleotides with a low C+G content than in the chromosomal DNA. The genetic information of *C. perfringens* and the comparison with other *Clostridium* genomes elucidates its way of life as a pathogen. In an environment, such as in soil, it may not grow and survives in spore form. When the organism enters human tissues through an injury and once the preferred anaerobic environment is established, it grows rapidly, using various host materials. *C. perfringens* can degradate large carbohydrate compounds into simpler sugars by using various saccharolytic enzymes and the resulting products are efficiently imported into the cells, where they are finally fermented by the anaerobic glycolysis pathway to generate energy and abundant gas. The anaerobic atmosphere will be enhanced by the production of gas (CO_2), making the environment more suitable for *C. perfringens* (T. Shimizu et al., 2002).

McDonel (1980) has provided a thorough review of all the major and minor toxins produced by *Clostridium perfringens*. The four main lethal toxins: alpha, beta, epsilon and iota, are the basis of strain differentiation (see 1.2). Nine minor toxins were found as well

that may or may not play some role in pathogenicity: delta, theta, kappa, lambda, mu, nu, gamma, eta, neuraminidase and one enterotoxin which is responsible for *C. perfringens* food borne illness (Hatheway, 1990).

The cytolytic toxins, including alpha-toxin, theta-toxin and putative enterotoxins, can destroy the host cells to release various materials. The released proteins and host structural proteins are degraded by proteases. The resulting amino acids and peptides are efficiently imported into *C. perfringens* cells via various transporters, and they are essential for the organism to synthesize proteins because they lack many enzymes for amino acid biosynthesis. Thus, *C. perfringens* actively degrades and imports various substances from the host tissues to grow and survive in the host, which in turn causes massive destruction of the host tissue and several myonecrotic lesions (T. Shimizu et al., 2002).

1.1 Main diseases caused by the *C. perfringens* toxins

Clostridial myonecrosis or gas gangrene: one of the classical bacterial diseases of humans. The pathogenesis of the disease involves the entry of vegetative cells or spores of *C. perfringens* into the body cavity via a major injury or a surgical wound. These organisms originate from soil that has contaminated the wound or from the leakage of intestinal contents as a result of damage to the gastrointestinal tract. Vascular damage resulting from the injury facilitates establishment of the anaerobic conditions required for germination of the bacterium spores and growth of the resultant vegetative cells. Growth is accompanied by the production of extracellular toxins and leads to gas production, extensive necrosis and tissue damage. If not controlled quickly by a combination of antibiotics and surgical intervention, the patient rapidly develops systemic toxaemia and shock; death results from the effects of the toxins on the hemodynamic system of the body. The primary toxin involved in the disease is the alpha-toxin, the first studied bacterial toxin showing an enzymatic activity.

Food poisoning: *C. perfringens* is also one of the major bacterial causes of human nutritional poisoning. Improper heating, storage, or reheating of the meat leads to the germination of *C. perfringens* spores and the rapid growth of the resultant vegetative cells. Ingestion of this contaminated food leads to infection of the gastrointestinal tract. The *C.*

perfringens isolates that cause human poisoning carry the *cpe* gene, which encodes a sporulation-associated enterotoxin. Sporulation in the intestine leads to the production of the enterotoxin, which causes fluid secretion into the lumen and subsequent diarrhoea. The disease is usually fairly self-limiting and in healthy individuals resolves within one or two days.

Enteritis necroticans: a very rare form of enteritis. The disease is characterized by vomiting, diarrhoea, severe abdominal pain and the presence of blood in the stools. Death may occur from intestinal obstruction or systemic toxaemia. The disease was known as "Darmbrand" in Germany at the end of the second World War and as endemic children's disease "Pig Bel" in Papua New Guinea in the 1950s and 1960s. It has been reported more recently in Vietnam. Nutritional and social factors are very important in the epidemiology of the disease. Enteritis necroticans is associated with the ingestion of highly contaminated meat meals by undernourished or protein deficient individuals. The toxin implicated in enteritis necroticans is the *C. perfringens* beta-toxin, an extracellular toxin that is inactivated rapidly in the gastrointestinal tract by trypsin.

Diseases of domestic animals: *C. perfringens* strains cause a variety of economically significant diseases in domestic animals. These diseases include several enteric syndromes such as flow necrotic enteritis, bovine and ovine enterotoxaemia and lamb dysentery. Both alpha-toxin and beta-toxin have been involved in necrotic enteritis (Rood, 1998).

1.1.1 Rabbit enterocolitis

Since the end of 1996 in the west part of France a new and serious gastrointestinal syndrome has appeared in rabbit breeding colonies. This new disease was designated with the term of "syndromes de colite enterique de Lapin", or simplified "enterocolitis" (Heinrich Kleine Klausing, 2005). The disease is characterized by small quantities of watery diarrhoea following a decrease in food intake. It has high mortality rates (30-80%) and spreads very rapidly. Routine antibiotic treatments have been completely ineffective. It was only in the mid-1998 that mortality comes under control, after establishing very strict

hygiene and sanitation measures, combined with the use of certain antibiotics (bacitracin, tiamulin). At the present time it is estimated that 90-95% of breeding colonies are or have been affected by this disease, whatever the rabbit's race and strain. The enterocolitis mainly affects young fattening rabbits, between six and eight weeks of age. The problems usually occur after weaning but have also been observed in adult rabbits.

The enterocolitis can be distinguished from other rabbit intestinal diseases by the specific clinical pattern and lesions. In addition to the diarrhoea and mortality, there is usually a pronounced abdominal swelling due to dilatation of all segments of the gastrointestinal tract, including the stomach, the contents of which are very liquid. These symptoms are sometimes associated with caecal paresis and the presence of mucus, especially in the colon and sometimes in the small intestine (Coudert et al., 1997). However there are no macroscopic congestive or inflammatory lesions, particularly in the caecum, whereas this is the usual site of typical lesions of acute enteritis in cases of caecal coccidiosis, klebsiellosis and colibacilloses (Licois et al., 2000).

The first research studies, coordinated by the "Association Scientifique Francaise de Cuniculture", had three main aims:

a. Search for alimentary factors which might have a direct or predisposing effect on the development of the disease
b. reveal the possible involvement of one or several pathogens in the genesis of the enterocolitis, and to identify the(se) agent(s)
c. set up an investigation to follow the development of the disease in the field.

The first report on these studies were presented during the 7th Journées de la Recherche Cunicole in Lyon in 1998 and then in Paris.

Foodstuffs were the first to be suspected. The studies performed by F. Lebas and his colleagues (IRNA, Toulouse) focused on several aspects, including the nature and levels of principal ingredients, premix, mycotoxins, pesticides (Maverick, Karaté, Gaucho). All these factors were excluded as source of the disease. Studies of gastrointestinal transit did not show any differences between feeds suspected of reproducing enterocolitis and the same feeds treated by irradiation. Three years later there was general belief that feeds were not the direct cause. However, a passive vector can be involved. Feed taken from a feeder in a contaminated breeding colony may transmit the disease. Virulence of contaminated feeds appears to be conserved no longer than 3 or 4 months.

The first results in the second round of rapidly set up studies showed that the disease associated with the above symptoms was reproducible and contagious, confirming the existence of one or several infectious pathogens. Several arguments supported a viral hypothesis, particularly the epizootic nature and the diffusion of the pathogen, the transmissibility of the disease and the existence of histologically-proven lung lesions suggesting a viral infection (Wyers, 1998). In addition there was the ineffectiveness of the majority of common antibiotics used on the field at the onset of the disease.

Thus, up to the end of 2000, the search for a virus as the etiological agent for the disease has been carried on. However, all methods used to identify viruses as causative agents of enterocolitis, failed. The only virus sometimes found in field samples was rotavirus (Ceré et al., 2000; Marlier et al., 2003) but it was not possible to reproduce the disease with rotavirus strains isolated from sick animals. The searches for other enterotopic viruses were all negative: calicivirus, pestivirus, circovirus, adenovirus, coronavirus, parvovirus and that's why the research the search for viruses was suspended.

On the other hand studies on bacteria were carried on as well: the active role of enteropathogenic strains of *E. coli* has been excluded since the beginning of the studies on enterocolitis. Even if this bacterium was found in some sick rabbits, the serotypes of the strains were not constant (Licois, 2004). In April 2001, an inoculum (TEC1) was produced by mixing several selected samples originating from 5 trials carried out on SPF (Specific Pathogen Free) rabbits inoculated with TEC (original inoculum constituted by virulent intestinal contents without opportunistic pathogens). TEC1 comes from the intestinal contents of 19 sick or dead animals. A second inoculum (TEC2) was composed of the intestinal contents of 27 moribund or a dead rabbit infected with TEC1 and then a third inoculum (TEC3) was obtained from animals inoculated with TEC2. On direct examination, TEC1, TEC2 and TEC3 were controlled free of coliform; the flora was poor and unbalanced, Gram positive bacteria like *Clostridium* were dominant but *C. spiriforme* was not detected. On the contrary, *C. perfringens* belonging to type A was identified. The search for enterotropic viruses (calicivirus, pestivirus, circovirus, adenovirus, coronavirus, and parvovirus) was negative. No intestinal parasites were detected (Licois et al., 2005). Therefore, it is possible to assert that *C. perfringens* is the causative agent of rabbit enterocolitis.

1.2 Toxin typing scheme

Clostridium perfringens produces an intimidating arsenal consisting of at least 15 different toxins. Individual strains produce only portions of this toxin repertoire, forming the basis for a commonly used toxin typing scheme that assigns *C. perfringens* isolates to one of five types (from A to E) based upon their presence of alpha-, beta-, epsilon-, iota-toxin genes.

C. perfringens type	Toxin genes			
	alpha	beta	epsilon	iIota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Table 1: **Classification scheme for *C. perfringens* isolates according to toxin gene presence**.

As shown in table 1 the **type A** is defined as strains possessing the gene for the alpha-toxin, **type B** as strains with alpha-, beta and epsilon toxin genes, **type C** as strains with the genes for alpha- and beta toxins, **type D** as strains with the genes for alpha- and epsilon toxins, **type E** as strains with the genes for alpha- and iota toxins. In addition, these types may or may not have the cpe-toxin gene or the beta2-toxin gene (Archambault et al., 2006). Only alpha-toxin is present in all strains. This indicates a chromosomal localization. All the others genes are present on plasmids The strain variability is probably produced by variability in plasmid-types and sequences.

C. perfringens Type	Major toxin	Diseases
A	α	Human myonecrosis (gas gangrene), necrotic enteritis in flows, enterotoxaemia of cattle and lambs, mild necrotizing enteritis of piglets
	α, CPE	Human food poisoning, human non-food-borne gastrointestinal disease, veterinary diarrhoea
	α, CPE, β_2	Human non-food-borne gastrointestinal disease
	α, β_2	Porcine enteritis
B	α, β, ε	Dysentery in newborn lambs, chronic enteritis in older lambs, haemorrhagic enteritis in neonatal calves and foals, haemorrhagic enterotoxaemia in sheep
C	α, β	Human necrotizing enteritis, enteritis in haemorrhagic/necrotic enterotoxaemia in neonatal pigs, lambs, calves goat and foals, acute enterotoxaemia in adult sheep
	α, β, β_2	Porcine enteritis
D	α, ε	Enterotoxaemia in lambs and calves, enterocolitis in neonatal and adult goats and cattle,
E	α, ι	Canine, bovine, porcine enteritis

Table 2: **Diseases associated with *C. perfringens* types**

Each *C. perfringens* toxin type is associated with particular human or animal diseases, as table 2 shows, indicating that variations in toxin production profoundly influence the virulence properties of *C. perfringens* isolates. These isolate-to-isolate differences in toxin production also help explain the pathogenic versatility of *C. perfringens*, which causes both enteric and histotoxic infections and has a disease spectrum ranging from low incidence/high mortality, clostridial myonecrosis, to high incidence/low mortality, food poisoning (Smedley et al., 2004).

1.3 Properties of the major toxins

1.3.1 alpha-toxin

The alpha- toxin is the sole toxin produced by type A strains. It is a phospholipase C and is believed to be responsible for the tissue pathology in myonecrosis (gas gangrene) caused by this organism. In addition alpha- toxin has been shown to affect myocardial function, causing hypotension and bradycardia, resulting in shock, a common and often fatal feature of gas gangrene (Hatheway, 1990). It has also been shown to cause vasoconstriction and platelet aggregation and to modulate the host immune response by causing the mistrafficking of neutrophils. Collectively, these effects reduce the oxygen content of the local environment and perturb the host response to infection, thus allowing the growth and proliferation of the bacterium. It is proposed that these effects are elicited as a consequence of the action of the alpha- toxin on host cell membrane phospholipids (Eaton et al., 2002). The hydrolysis of membrane phospholipids by alpha-toxin results in the activation of cell wall pathways which contribute to the cytotoxic effect. Hydrolysis of membrane phospholipids results in the accumulation of diacylglycerol which can subsequently activate the arachidonic acid pathway. The activation of this pathway can lead to the production of potent mediators of the inflammatory response. Diacylglycerol is also known to activate protein kinase C. Protein kinase C may in turn activate cellular poliphosphases C and D which would increase membrane phospholipids turnover, resulting in extensive cell damage (M. Jepson and R. Titball, 2000).

The gene encoding the alpha- toxin, *plc*, has been shown to be chromosomally located (Titball, 1993). The genes for several putative virulence factors that have been cloned, were used as hybridization probes to test the hypothesis that a particular area of the genome might be associated with bacterial virulence. In this way the chromosomal locations of the *plc* and other genes, encoding the alpha- and others toxins, were established (Conrad et al., 1992).

The crystal structure of *C. perfringens* alpha- toxin shows that the mature protein is organized into two domains. The N-terminal domain has phospholipase C activity whilst the C-terminal domain forms a calcium-dependent putative phospholipids binding domain.

The putative active site is located in the amino terminal domain and the presence of three zinc ions are detected in the active site cleft. Site-directed mutagenesis of the zinc-coordinating residues results in the abolition of phospholipase C, haemolytic and lethal activities. These findings confirm the essential role of zinc ions for the enzymatic activity of the toxin and that the phospholipase C activity of the N-terminal domain is essential for all of the biological activities of the toxin.

The C-terminal domain has been shown to be comprised of eight β-sheets organised into a Greek key fold. Moreau et al. (1988) showed that calcium ions were required for binding of alpha- toxin to lipid films and one of the features of the C-terminal domain is the existence of three calcium-binding sites, termed Ca1, Ca2 and Ca3. All of them are located in the putative membrane-binding surface of the protein and may therefore be involved in binding of alpha- toxin to the membrane, allowing the active site to be optimally positioned to bind phospholipids. Several studies have shown that, although the C-terminal domain plays an important role in haemolysis, this domain alone did not provoke toxic or haemolytic activity as alpha- toxin. Therefore the N-terminal domain must also play a role in toxicity and haemolytic activity (M. Jepson and R. Titball, 2000).

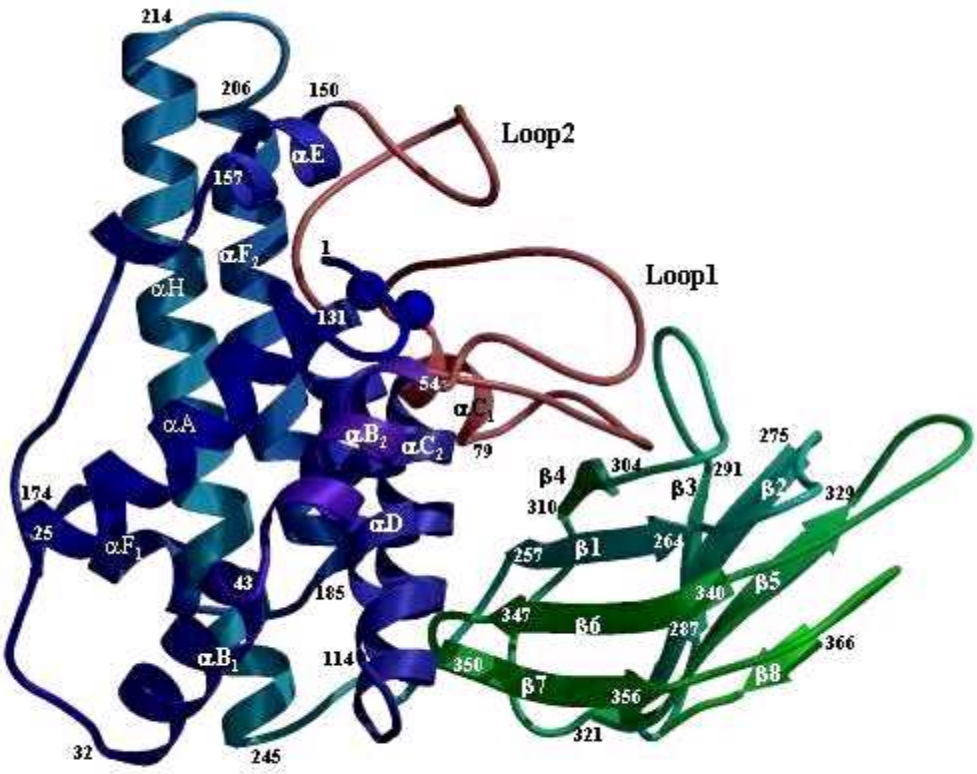


Figure 1: **Diagram of the closed structure of alpha- toxin**. In blue is the N-terminal domain, residues 1-245. In green is the C-terminal domain, residues 250-370. In brick red are the two loop regions, which differ between the closed and open structures. The active site zinc ions are shown in dark blue.

Comparing the structures at pH 4.6, in which the N-terminal domain of the toxin adopts an inactive conformation with the active site being no longer accessible to substrate, with that at 7.5, the active one, was possible to localize the substrate binding site on the two loops, 1and 2 (fig 1). In triacyglycerol lipase enzymes with similar structure one loop movement uncovers the active site and a second, smaller loop change results in the formation of the enzyme's oxyanion hole. These changes allow lipid access to the active site. A similar situation has been described for the alpha- toxin, where the activation of the toxin involves calcium ions binding to the C-terminus. The activation is associated with conformational changes.

Given that one purpose of alpha- toxin is to lyse cells, it seems reasonable that the toxin would benefit from a mechanism that allowed it to discriminate between phospholipids in intact membrane and that from already lysed cells (Eaton et al., 2002).

The knowledge on the regulation of the alpha-toxin gene expression is still very incomplete, but there is a sequence, 77bp immediately upstream of the plc gene, which is supposed to be primarily responsible for the repression of the toxin. This region contains three adjacent AT-rich regions just upstream of the *plc* promoter which result in an intrinsic DNA curvature thought to be responsible for the repression of transcription of the gene. However, the functional role in *C. perfringens* of this region of DNA curvature remains to be determined.

Comparison of the region upstream the *pcl* form *C. perfringens* type A and C strains, which produces different levels of alpha toxin, reveals that they have similar upstream sequences and that they result in similar levels of alpha-toxin when expressed in *E. coli*. However, the high-level alpha-toxin-producing type A strain produces much more *pcl*-specific mRNA than does the type C strain, which produces only low levels of alpha-toxin. These results suggest that other factors are involved in the regulation of the *pcl* gene in these strains. The lower level of *pcl*-gene expression in type C strain may be related to the absence of a protein that specifically binds to *pcl* gene. These data suggest that regulation of alpha-toxin production may involve both a protein that is present in both type A and C strains and that binds to the *pcl* promoter region, and a protein that binds to the *pcl* gene, but is present only in type A strains. The latter protein, which is postulated to be the product of a gene designed as *pclR*, might be an activator that is responsible for the higher level of alpha-toxin production in type A strains.

The expression of many clostridial genes is controlled by a two-component signal transduction system. This system consists of two genes *virR* and *virS* that appear to form

an operon, which encodes for the protein VirR and VirS. The amino-terminal domain of VirS appears to contain six or seven transmembrane domains, suggesting that this portion of the molecule acts as the receptor or sensor of the biochemical signal that leads to the autophosphorylation of the cytoplasmic carboxyterminal VirS domain. VirR contains aspartate residues that are conserved in response regulators and probably act as the site of phosphorylation by the VirS protein. On studies of Rood and Lyristis, 1995, the model for gene expression regulation is based on an unknown environmental or growth-stage stimulus which is detected by the transmembrane domain of the VirS protein. The signal transduction may involve the transport of a solute across the cell membrane; this results in an alteration in the conformation of VirS, enabling it to autophosphorylate an His residue. Subsequently the cytoplasmatic domain of the phosphorylated VirS protein phosphorylates an Asp residue of the cytoplasmic VirR protein. Activated VirR then either activates the transcription of *pfoA* directly, or more likely, activates the transcription of *pfoR*, which, in turn, leads to increased transcription of *pfoA*. This model is also valid for the alpha- toxin, but the phosphorylated VirR only partially activates genes involved in the production of the toxin, this suggests that there should be an additional regulatory protein, which could be the plcR, as told before (Rood and Lyristis, 1995).

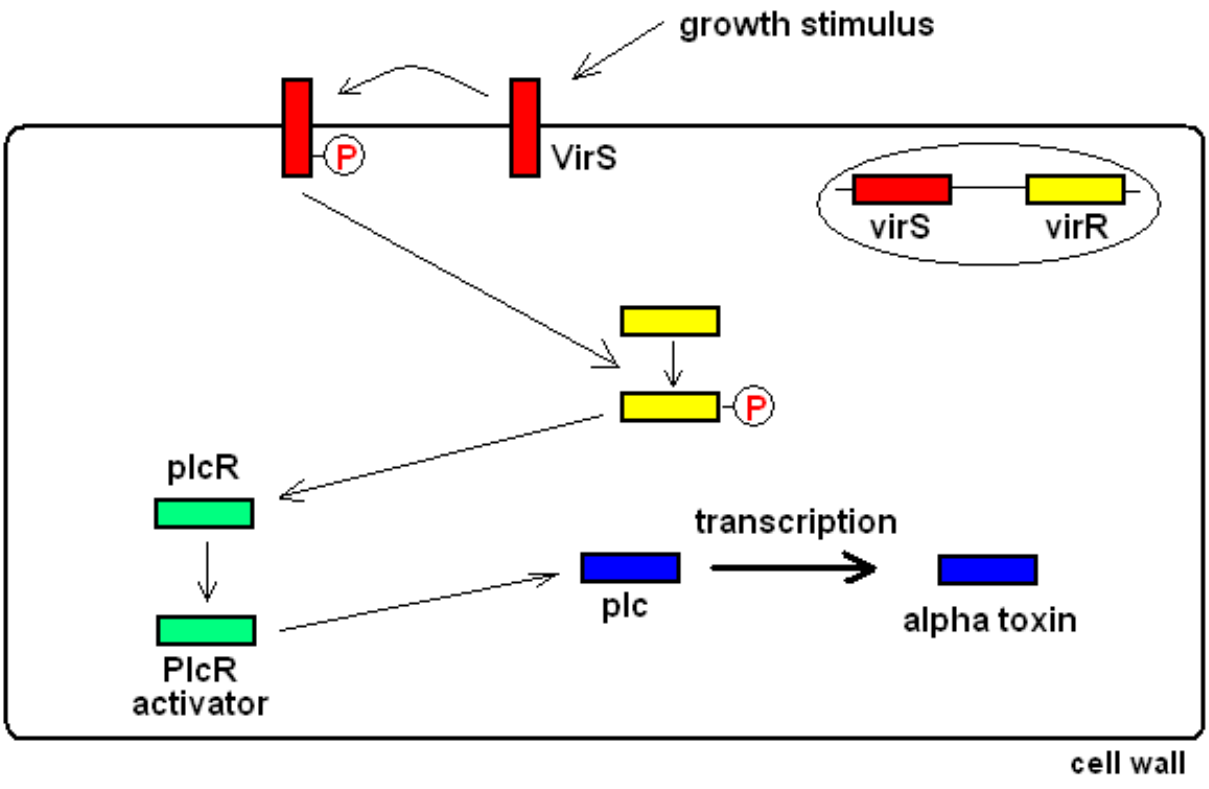


Figure 2: **Model for the transcription of alpha-toxin**. The figure shows the structural genes for alpha- toxin (*plc*) with the regulatory genes *plcR*, *virR* and *virS*.

1.3.2 Beta-toxin:

The beta-toxin is a lethal toxin produced by *C. perfringens* type B and C strains which causes a necrotic enteritis characterized by haemorrhagic mucosal ulceration or superficial mucosal necrosis of the small intestine in humans (type C only) and in animals (type C and B). Beta toxin is secreted in late log growth phase, is thermo-labile, and highly-sensitive to proteases. The molecular mass of the beta-toxin polypeptide is 35 kDa. The gene *cpb* is carried on a plasmid, which reveals an open reading frame encoding a 336 amino acid polypeptide that includes a 27-amino acid signal sequence being absent in the mature beta-toxin. Comparison of *cpb* sequence between *C. perfringens* type C and B isolates detected 1-6 base differences within the open reading frame. Some of these changes result in amino acid substitutions, but the significance of those changes for toxicity is unclear. Chemical modification experiments identified certain residues likely to be essential for beta-toxin lethal activity: the arginine at position 212 if changed into glutamic acid resulted in an 11.5-fold reduction in toxicity. The thiol group on beta toxin's single cysteine residue is sensitive to modification. Changes in residues at the beta-toxin C-terminal region containing the cysteine residue caused the complete loss of activity. That can be due to the importance of the C-terminus either for beta-toxin binding to its receptor or for formation of beta-toxin oligomers.

The toxin forms cation-selective pores in bilayer lipid membranes composed of approximately 50% phosphatidylcholine and cholesterol; these pores were shown to be selective for monovalent cations like Na^+ and K^+, and the insertion rate of the beta-toxin into membranes is stimulated by the presence of divalent cations. The beta toxin is a pore-forming toxin that oligomerizes to form channels in susceptible membranes. The resultant membrane permeability changes result in cell death. The role of beta-toxin in infections is still poorly understood (Smedley III, 2004).

1.3.3 Epsilon-toxin

This toxin primarily affects the intestine by increasing the permeability of the gut wall, thus enhancing its own uptake, and acts systemically as a lethal toxin. After entering the circulation, it causes swollen hyperaemic kidneys, edema in the lungs and excess pericardial fluid. Both type B and D strains of *C. perfringens* produce this toxin (Hatheway, 1990). Epsilon toxin is synthesized and secreted as a relatively inactive proto-toxin, which is then converted to an active toxin by removing 13 residues from the N-terminus and 22 residues from the C-terminus. The proteolytic activation can be catalysed by intestinal proteases, such as trypsin and alpha-chymotrypsin (Hunter et al., 1992; Miyata et al., 2001).

The epsilon-toxin gene, *etx*, is carried on a poorly-studied large plasmid (Katayama et al., 1996); the toxin appears to be composed primarily of beta sheets. When the nucleotide sequence of the *ext* open-reading-frame from a type B isolate and a type D isolate were compared, nucleotide changes were found at position 762 and 962. While the nucleotide difference at position 762 results only in a silent mutation, the second difference codes for a serine in the type B strain and for a tyrosine in the D strain. Despite these changes both toxin variants are biologically active.

During the disease the toxin is produced in the gut of the infected animal, then it is absorbed across the mucosal barrier, either by directly inducing damage or as a result of eating a high fibre diet which can also damage intestinal permeability. Once the epsilon-toxin has crossed the mucosal barrier, it is rapidly carried through the bloodstream to target organs (brain, heart, lung, lever and the kidneys).

Upon reaching target organs, the epsilon toxin binds to cells containing the appropriate receptors (probably glycosylated-proteins). After binding, the toxin forms a non selective pore by ologomerizing into a heptamer. This pore results in loss of transepithelial resistance, and ultimately in net water movement that creates edema within intoxicated tissues (Smedley III et al., 2004).

1.3.4 Enterotoxin (CPE)

When identified, this protein of 35 kDa, was quickly linked to *C. perfringens* type A food poisoning. The *cpe* gene can be present on either the chromosome or on a large plasmid.

Specifically, *C. perfringens* type A food isolates usually carry a chromosomal *cpe* gene, whereas CPE-positive *C. perfringens* type A isolates causing sporadic diarrhoea always posses a plasmid-borne *cpe* gene.

Early studies determined that CPE biosynthesis is temporally associated with sporulation. Promoter mapping of the *cpe* gene identified three functional promoters, each exhibiting homology to a consensus binding sequence for the sporulation-specific sigma factors. During sporulation, CPE is strongly expressed by many CPE-positive type A isolates. After synthesis, CPE is not secreted but instead accumulates in the cytoplasm of sporulating cells. CPE is eventually released into the intestinal lumen at the completion of sporulation, when the mother cell lyses to release its spores (Smedley III et al., 2004).

The mechanism of action of the enterotoxin seems to involve direct binding of the toxin to receptors on the surface of intestinal epithelial cells. Binding is followed by insertion of the entire molecule into the cell membrane, but not internalization into the cell. A sudden change of ion flux occurs, affecting cellular metabolism and macromolecular synthesis. As intracellular calcium ion levels increase, morphological damage occurs, resulting in greatly altered membrane permeability and loss of cellular fluid and ions and moderate-size molecules up to 3.5 kDa. Under some conditions a loss of protein molecules may occur, but this may reflect cell death (Hatheway, 1990).

1.3.5 Iota-toxin:

Iota-toxin has dermonecrotic activity and is implicated in animal enterotoxaemia. Recently, it was demonstrated that it consists of two separate proteins that are immunologically and biochemically distinct, designated as iota-a and iota-b (Heathway, 1990). The active 47.5 kDa component iota-a is encoded by the *iap* gene and has actin-specific ADP-ribosyltransferase activity, whereas the 71.5 kDa binding component iota-b is encoded by the *ibp* gene. The *iap* and *ipb* genes are separated by 40 nt and appear to be transcribed from a single promoter located upstream of *iap* (Rood, 1998). Iota-b (Ib) has cell surface binding capabilities and is produced as an inactive monomer that sheds a 20 KDa N-terminal peptide after proteolysis by serine-type proteases. Cell-bound complexes of Ib then act as a docking platform on the cell surface, which facilitates the entry of a unique enzymatic protein into the cytosol via endosomal trafficking of iota-a (Ia) which ADP-

ribosylates monomeric actin and subsequently prevents formation of cytoskeletal filaments. Functional mapping of Ib reveals that the C-terminus binds to different cell-surface protein receptors and the N-terminus subsequently docks with an enzymatic component (Stiles et al., 2002).

1.3.6 Beta2 toxin

The nucleotide sequence encoding the beta2 toxin (*cpb2*) was first reported in 1997 (Gilbert et al., 1997); the gene is carried on a large plasmid. Several workers have noted an association of *cpb2*-positive strains of *C. perfringens* type A and the occurrence of enteric disease in domestic animals, particularly in piglets, horses in which it may lead to typhlocolitis, dogs, adult cattle (diarrhoea) and a recent study implicated the beta2 toxin as being responsible for neonatal, bovine enterotoxaemia (Bueschel et al., 2003; Schotte, 2004). Moreover, the toxin is toxic to cultured epithelial cells, lethal to mice and is cytotoxic for CHO cells.

The predicted product of cpb2 gene is a 265 amino acid protein. The 30 N-terminal residues correspond to a signal peptide, which is post-translationally removed, since it is not present in the mature protein. The mature protein comprises of 235 amino acids with a molecular mass of 27.7 KDa (Gilbert et al., 1997). The expression of *cpb2* is affected in *C. perfringens* strain 13 by both the two-component VirR/VirS system and its secondary RNA regulator, VR-RNA. Northern-blot analysis demonstrated that in a *VirR* mutant strain the amount of transcript derived from the beta2-toxin gene is low, but in the strain which has intact *VirR/VirS* genes the expression is clearly restored. In order to verify the involvement of the secondary RNA regulation, other analyses were performed on mutant VR-RNA, which confirmed that (Ohtani, 2003).

Despite of its name the beta2-toxin has no significant amino acid similarity with the beta toxin or with other known protein sequences. Antibodies raised against beta2-toxin recognized purified beta2-toxin, but reacted only weakly with beta toxin. Conversely, anti-beta toxin antibodies reacted with beta, but not with beta2-toxin. These results indicate that beta and beta2 toxins are only weakly immunologically related. Since it was reported that beta toxin is highly sensitive to proteolysis, the effect of trypsin on the beta2-toxin was analyzed. Beta2 was degraded to a 24 KDa fragment after treatment with 16 ng/ml trypsin.

Treatment with higher trypsin concentrations, resulted in cleavage to two polypeptides of 13 and 15 KDa and in complete loss in cytotoxic activity. There is also a different distribution of the two toxin genes in *C. perfringens* strains. Analysing 57 strains from different origins it was found that all type B and C strains contained the gene *cpb* (beta toxin). In contrast, *cpb2* (beta2 toxin), was not found in any type B strain, but was found in two type C strains. Among 26 strains isolated from piglets with necrotic enteric lesions, 2 had *cpb*, 12 had both and 12 only had the *cpb2* gene. All the strains isolated from horses that died with colitis symptoms and in which *C. perfringens* was recovered in high number in the intestinal content, had only the *cpb2*. In contrast, only three *C. perfringens* strains out of 15 isolated from food had *cpb2* (Gilbert et al., 1997).

Nevertheless, both beta and beta2 toxins have comparable biological activities: they cause haemorrhage and necrosis of the intestinal wall in the experimental ileal loop test and they are lethal for mice (Gilbert et al., 1997).

Recent studies demonstrated that the beta2 toxin may be differentially expressed in *C. perfringens* strains isolated from different host species. There was a strong correlation between beta2 toxin phenotype (= expression) and genotype only in type A and C isolates obtained from diseased pigs (Jost et al., 2005). For isolates of non porcine origin the correlation of phenotype and genotype was only 50%. A PCR product, amplified with beta2-gene specific primers taken from a bovine type E isolate, was sequenced and found to diverge significantly from cpb2 from a porcine type C isolate. This beta2-gene was called “atypical”, contrary to that of porcine origin, which was desoignated as “consensus”.

PCR analysis was performed to screen 154 *cpb2*-positive isolates from a variety of host sources. No porcine type C isolates and only 3.3% of porcine A isolates carried the atypical *cpb2* gene; only 2.6% of all porcine isolates carried the atypical *cpb2* gene. However, the predominant allele carried by non porcine isolates was the atypical *cpb2* gene, which was present in 88.7% of these isolates.

A total of 23 isolates, type A to E from different origin, carrying the atypical gene were selected for nucleotide analysis: all the *cpb2* atypical genes, if compared as a group, displayed 93% DNA identity; five of the six type E *cpb2* genes are identical and carried a frameshift mutation at position 178, which leads to a production of a protein of only 73 amino acids. There are other mutations, but most of them conferred either no amino acid change or conservative substitutions. Western-blotting was performed, in order to test the *cpb2* toxin expression, on 33 isolates from all five *C. perfringens* types. As expected, beta2-toxin expression was not detected in any of the type A and E carrying frameshift

mutations. However, beta2-toxin was also not detected in the 25 isolates that carried full-length atypical *cpb2* genes. Consensus *cpb2* genes were not common in non porcine isolates, with only 11,5% of non porcine isolates carrying the consensus *cpb2* gene. Interestingly, only five of the nine non porcine isolates carrying the consensus *cpb2* genes expressed beta2-toxin. Sequence analysis was performed and a frameshift mutation was found resulting in the synthesis of a truncated protein of 4.5 KDa, explaining the absence of the beta2 expression in these strains (Jost et al., 2005).

Therefore, it seems that in general, consensus genes from porcine isolates are expressed and atypical genes from non porcine *C. perfringens* are not expressed. Furthermore, consensus genes from non porcine *C. perfringens* isolates are expressed at different levels. Given that the sequences upstream of consensus and atypical cpb2 gene were similar but not identical, the defect in expression of atypical genes could occur at the transcriptional level.

Reverse transcription was used to identify *cpb2* transcripts in *C. perfringens* isolates carrying the atypical gene. Strains from bovine type A and C, did not contain *cpb2* specific transcripts, indicating that very little or no transcription occurred in these isolates. Unexpectedly, however, RNA from a particular strain, bovine type E, contained *cpb2* specific transcript, but expression of beta2 toxin was not detected due to a frameshift mutation in the *cpb2* gene. The putative –35 promoter sequence in type E atypical *cpb2* genes varies slightly from the –35 promoter sequence found in type A, B, C and D atypical genes, and these –35 promoter sequences are slightly different from those found for consensus genes. The absence of transcription in the type A and C isolates examined may be a result of this divergent –35 sequence, whereas the type E *cpb2* –35 sequence may still be able to promote transcription. However, further experiments will be necessary to confirm these hypotheses.

The presence of apparently non-expressed, atypical *cpb2* genes in C. perfringens strains isolates from non-porcine hosts raises the question of whether this protein plays the same role in enteritis in other animal species (B. H. Jost et al., 2005).

1.4 Aim of the work:

The rabbit enterocolitis, as described above, is different from the normal dysentery but, above all, there is still no cure. There is clear experimental evidence that the disease is produced by *Clostridium perfringens*. However, nothing is known about the strain types and toxin sequences circulating in infected rabbits in Germany. This work is intended to:

- genotype the Clostridium strains circulating in rabbits
- clone and sequence the major toxin genes

The data raised should give information on the degree of sequence variability in the respective toxin genes (and their products). They hopefully could form a basis for the development of a toxoid-vaccine. This, certainly, would be the effective way to control the disease.

2. Materials and methods

2.1 Materials

2.1.1 *C. perfringens* samples

C. perfringens samples were isolated from rabbits displaying symptoms of enterocolitis. Samples from gut content and samples of mucosa from the gut walls, were heated for two seconds in the microwave and cultured on Schaedler blood agar (5%, Biomerioux) and grown over night at 37℃, anaerobically.
Single colonies, chosen because of swarming haemolysis were transferred to Schaedler broth and grown over night at 37℃.

The following table shows provenance and numbering of the samples

Provenance	Number	Provenance	Number	Provenance	Number
Regensburg	1	Hamm	26	Essen	50
Torgau	2	Zittau 2	27	Ettlingen	51
Borkheide	3	Zwiesel	28	Herbstein	52
Auerbach	4	Beelitz	29	Tuningen	53
Kleve	5	Mettingen	30	Haigerloch	55
Wallenhorst	6	Freiburg	31	Bückeburg	56
Haldensleben	7	Köpenick	32	Sulzfeld	57
Rathenow	8	Dresden	33	Steinfurth	58
Triberg	9	Stuttgart	34	Krefeld	59
Herne	10	Dittmannsdorf	35	Ehrenberg	60
Euskirchen	12	Schorfheide	36	Großenhagen 1	61
Langen	13	Wandlitz	37	Großenhagen 2	62
Laichtlingen	14	Zerbst	38	Dudenhofen	63
Ahlbeck	15	Duderstadt Hi9	39	Glindow	65
Forbach	16	Südlohn	40	Rudow	66
Saarbrücken	17	Lübars	41	Stolberg	67
Lichtenau	18	Burghaun	42	Crailsheim	68
Hagen	19	Wriezen 2	43	Karlsruhe	69
Düren	20	Poppenricht	44	Kaiserslautern	70
München	21	Templin	45	Aschaffenburg	71
Wriezen	22	Zeulenroda	46	Treviso 1	72
Meiningen	23	Rüdersdorf	47	Treviso 2	73
Hannover	24	Stücken	48	Treviso 3	74
Büttstedt	25	Neucharell	49		

Table 3: **Provenance and numbering of samples**.

2.1.2 Reagents

Substance	Company	City
30% Acrylamid/0,8% Bisacrylamid	Roth	Karlsruhe
Acetic acid (glacial) 100%	Merck	Darmstadt
Agarose Multi-Purpose	Bioline	Luckenwalde
Ammonium peroxidisulfat (APS)	Merck	Darmstadt
Ampicillin	Sigma	Deisenhofen
Anhydrotetracycline (AHT)	IBA	Göttingen
BSA (Bovine Serum Albumin)	Merck	Darmstadt
Butanol	Merck	Darmstadt
Chloroform	Merck	Darmstadt
Cetyltrimethylammonium bromide (CTAB)	Sigma	Deisenhofen
Comassie Brilliant Blue R-250	Sigma	Deisenhofen
Ethylenediaminetetraacetic acid (EDTA)	Merck	Darmstadt
Ethanol ≥ 99,8%	Roth	Karlsruhe
Ethidiumbromide	Roth	Karlsruhe
Glucose	Merck	Darmstadt
Glycerol	Merck	Darmstadt
pd(N)6 Random Hexameres	Amersham Bioscences	Freiburg
Isoamyl alcohol	Merck	Darmstadt
Isopropanol	Merck	Darmstadt
Kanamycin	Sigma	Deisenhofen
KCl (Potassium Chloride)	Merck	Darmstadt
LB medium (Luria/Miller)	Roth	Karlsruhe
β-Mercaptoethanol	Merck	Darmstadt
$MgCl_2$ (Magnesium Chloride)	Merck	Darmstadt

$MgSO_4$ (Magnesium Sulphate)	Merck	Darmstadt
NaCl (Sodium Chloride)	Merck	Darmstadt
dNTPs, 100 mM	Bioline	Luckenwalde
Orange G	Sigma	Deisenhofen
25:24:1 Phenol/chloroform/ isoamyl alcohol	Sigma	Deisenhofen
SDS (Sodium Dodecyl Sulfate)	Merck	Darmstadt
Trichloroacetic acid (TCA)	Merck	Darmstadt
10x TEA Buffer	Roth	Karlsruhe
TEMED (Tetramethyl-ethylendiamin)	Merck	Darmstadt
Tris	Sigma	Deisenhofen

Table 4: **Reagents table**. Reagents used during work.

2.1.3 Solutions

Solution	Recipe (stock solutions)
1% Agarose gel	3 g Agarose; 300 ml 1xTEA buffer
Ampicillin	50 mg ampicillin; 1 ml distilled water
APS	0.1 g; 1 ml water
24:1 chloroform/isoamyl alcohol	24 ml chloroform; 1 ml isoamyl alcohol
CTAB/NaCl (Sodium Chloride)	10% CTAB in 0.7 M NaCl
Destaining Solution	500 ml methanol; 100 ml acetic acid; 400 ml water
70% ethanol	70 ml ethanol; 30 ml distilled water
Ethidium Bromide	5 mg; 1 ml distilled water
LB medium (for plates)	15 g LB Medium; 1 l water
Kanamycin	50 mg; 1 ml distilled water

dNTPs mix	20 µl 100 mM dATP; 20 µl 100 mM dCTP; 20 µl 100 mM dGTP; 20 µl 100 mM; 920 µl distilled water
(Phosphate-Buffer Saline)-PBS	8.55 g NaCl; 1.33 g $Na_2HPO_4x2H_2O$; 0.34 g $NaH_2PO_4xH_2O$; 1 l water
Primers	The stock solution is 2. µM
Separation gel	10 ml 30% Acrylamid/0.8% Bisacrylamid; 7.5 ml 1.5 M Tris, pH 8.8; 0.3 ml 10% SDS; 12.2 ml water
10% (Sodium dodecyl sulfate)-SDS	10 g SDS; 100 ml distilled water
SOC medium	LB Medium; 10 mM $MgCl_2$; 20 mM glucose
Stacking Gel	1ml 30% Acrylamid/0.8% Bisacrylamid; 0.84 ml 1,5 M Tris, pH 6.8; 0,1 ml 10%SDS; 8.06 ml water
Staining Solution	50% (v/v) methanol; 0.05 % (w/v) comassie brilliant blue; 10% (v/v) acetic acid; 40% water
10% (Trichloroacetic acid)-TCA	250 g; 2.5 l water

Table 5: **Solution table**. Stock solution recipes used during work.

2.1.4 Buffer recipes

Buffer	Recipe
1x NEB Buffer 2	50mM NaCl, 10 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9
1x NEB Buffer 4	50mM potassium acetate, 20mM Tris acetate, 10 mM magnesium acetate, 1mM DTT, pH 7.9
5x RT buffer	250 mM Tris/HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT
10x Ligation Buffer	660 mM Tris/HCl, 50 mM $MgCl_2$, 50 mMDTT, 10 mM ATP, pH 7.5
10x NEB Buffer 3	100 mM NaCl, 50 mM Tris/HCl, 1mM DTT, pH 7.9
10x NH_4 Buffer	160 mM $(NH_4)_2SO_4$, 670 mM Tris/HCl (pH 8.8), 0.1% Tween-20

10x TEA Buffer	40 mM Tris/Acetate, pH 7.9 ; 5 mM Sodium Acetate, 1 mM EDTA
Electrophoresis buffer (agarose gel)	200 ml 10x TEA Buffer; 20 µl Ethidium Bromide solution
Electrophoresis buffer (protein gel)	3 g Tris; 14.4 g Glycine; 10 ml 10% SDS; water up to 1 l
Stop Buffer	25 mg Orange G; 5 ml glycerine; 40 µl EDTA; 1 ml 10xTEA buffer; 3.96 ml water
5x SDS Sample buffer	0.4 g SDS; 1.667 ml Tris/HCl (1.5 M, pH 6.8); 7.10 ml Glycerin; 1 ml β-Mercaptoethanol; 7.1 ml water
TE (Tris, EDTA) Buffer	10mM Tris-Cl, pH 8; 1mM EDTA, pH 8

Table 6: **Buffer recipes table**. Buffers used during work.

2.1.5 Enzymes

Method	Enzyme	Company
DNA-digestion (RNA isolation)	RQ1 RNase-free DNase (1U/µl)	Promega (Mannheim)
DNA-digestion (restriction enzyme)	Bsa I (20U/µl)	NewEngland Biolabs (Frankfurt am Main)
	EcoR I (20U/µl)	NewEngland Biolabs (Frankfurt am Main)
	EcoR V (20U/µl)	NewEngland Biolabs (Frankfurt am Main)
	Hind III (20U/µl)	NewEngland Biolabs (Frankfurt am Main)
Ligation	T4 DNA ligase (1U/µl)	Roche (Mannheim)
PCR (Polimerase Chain Reaction)	BioTAQ-DNA polymerase (5U/ µl)	Bioline (Luckenwalde)
Proofreading DNA-polymerase	PFU Turbo HotStart Polymerase (2.5U/µl)	Stratagene (Amsterdam/ Netherlands)

Protein digestion	Proteinase K (20mg/ml)	Bioline (Luckenwalde)
Reverse transcription	M-MLV-Reverse Transcriptase (200U/µl)	Promega (Mannheim)

Table 7: **Enzyme table**. Enzymes used during work.

2.1.6 Primers

The primers used for PCR procedures are listed in the following table. They were derived either from published sequences (EMBL accession number and sequences positions are given) or were derived experimentally in this work.

All primers were synthesized by MWB, Biotech, Ebersberg.

Table 8: **Primer table**. Primers used

Primer Name		Sequence 5'→3'	EMBL Ac. No.	Position
Palp 1	For	GATGGAACAGGAACTCATGC	CP000312	48256-48275
	Rev	CCATCCTTTGTTTTGATTCC		49094-49075
Palp 2	For	CATAAGCCTGTGCAATTGGT	BA00016	48013-48032
	Rev	TAGTTTTGCCAGCTCCTAGG		50019-50000
Palp 3	For	GCGCTAGCAACTAGCCTATG	Sample 1	560-579
	Rev	CCCAATCATCCCAACTATGAC	Alpha-toxin gene	1259-1239
Palp 4	For	GATGGAAAAACTCAAGAATGG	Sample1	1475-1477
	Rev	CACATCTAACCTTACCCGCTC	Alpha-toxin gene	419-399
Pbe	For	CAACCTCTAAAGCTTCATGG	L13198	940-959
	Rev	GTCCTACCCAGTTAGCACCA		1245-1226
Peps	For	TTAGCAATCGCATCAGCGGTG	AY858558	29-49
	Rev	CTTGTATCGAAGTTCCCACAG		579-472
Pcpe	For	TTGGATATTAGGGGAACCCTCAG	Y16009	528-550
	Rev	GGGTATGAGTTAGAAGAACGCCA		1079-1057
Piot	For	TGAACCAGGTAATGGCGATG	X73562	2013-2032
	Rev	TCCTGCATAACCTGGAATGG		2718-2699
Pbe2.1	For	CTCAAGTTTGTACATGGGATG	AY609182	451-471
	Rev	TCACCAAATACTTTAATCGATGC		782-760
Pbe2.2	For	TTTAAGGTGTCCCACGGGGAC	AP003515	13504-13524
	Rev	AGTCCACTTTTACATGGGAGC		1481114791
Pbe2.3	For	ATGAAAAAAATTATTTCAAAGTTTACTGTA	L77965	268-297
	Rev	TCACCAAATACTCTAAATGATGC		1094-1027
Pbe2.4	For	TAGCCAATTAATGTCCCCAAC	AB236337	33727-33749
	Rev	ATTTCCAATACTACTAATGGTGGTG		35359-35383
Pbe2.5	For	CAGGGGGGAATATAAATGAA	---------	
Pbe2.6	For	AAATATGAATAAAATTTAGATAAAAGTG	AP003515	13546-13573
PPCR1RC		CTTTCATTGGACTTATTGCTCC	Sample 8 Beta2-toxin gene	61-82
PPCR1RC-1		CACAATCAATTGGGGGAGTTG	Sample 8 Beta2-toxin gene	482-502
PPRC2RC		TGGACTTATTGCTCCAACAATAAG	Sample 8 Beta2-toxin gene	52-75
PPCR2RC-1		GGGGGAGTTGATCCAAAAAC	Sample 8 Beta2-toxin gene	493-512
PRNA	For	ATTCCTACACCAAGTTTCAGAACTC	AY609183	417-441
	Rev	TCCGGATTTTCACCATATACC		664-644
PRNA1	For	GAGACGCTGTTAGTTTTACACG	AY609183	481-502
M13-40		GTTTTCCCAGTCACGACGTTGTA	pZerO	428-450
M13 Rev		CAGGAAACAGCTATGACCATG	PZerO	205-225
IBAG		GCTAGCTGGAGCCACCCGCAG	pASK-IBA6	202-222
IBALinks		CACTCCCTATCAGTGATAGAG	pASK-IBA6	69-89
PexpA1	For	ATGGTAGGTCTCAGCGCATGAAAAGAAAGATTTGTAAGGC	Sample 1	578-600
	Rev	ATGGTAGGTCTCATATCATTATTTTATATTATAAGTTGAATTTCC	Sample1	1748-1771
PexpB1	For	ATGGTAGGTCTCAGCGCAAGGAAATCGACGCTTATAGAAAG	Sample 3	158-181
	Rev	ATGGTAGGTCTCATATCATTAATAACAATAACCCTCACCAAATAC	Sample 3	845-862
PexpB2	For	ATGGTAGGTCTCAGCGCAATGAAGTGAATAAATACCAATCTG	Sample 2	536-560
	Rev	ATGGTAGGTCTCATATCACTATGCACAATATCCTTCACC	Sample 2	1217-1240

2.1.7 Apparatus

Apparatus	Name	Company
Centrifuge (table)	Biofuge-Fresco	Heraeus (Hanau)
Centrifuge	Sorvall RC 5B Refrigerated Superspeed Centrifuge	Du Pont Instruments
Electrophoresis Power supply	Power Pack P25	Biometra (Göttingen)
Electroporator	Invitrogen Electroporator II	Invitrogen (Karlsruhe)
Gel Documentation System	Gene Genius – Bio Imaging System	Synegene (Cambridge, UK)
Nano Drop	Nano Drop ND100 spectrophotometer	PeQLab (Erlangen)
PCR cycler	Biometra TGradient	Biometra (Göttingen)
Spectrophotometer	Bio Photometer	Eppendorf (Hamburg)

Table 9: **Apparatus table**. Apparatus used during work.

2.1.8 Markers

Marker	Company	City
1 kb DNA Ladder	New England BioLabs	Frankfurt am Main
100 bp DNA-Leiter, extended	Roth	Karlsruhe
λ DNA/*Hind* III Fragments	Invitrogen	Karlsruhe
MassRuler DNA Ladder, Mix	Fermentas	St. Leon-Roth
Precision Plus Protein Standards (Dual color)	Bio Rad	München
GeneRuler 50bp DNA Ladder	Fermentas	St. Leon-Roth

Table 10: **Marker table**. Marker used during work.

2.1.9 Kits

Method	Kit name	Company	Country company
Cloning of the alpha-toxin gene	Zero Background/Kan Cloning Kit	Invitrogen	Karlsruhe
DNA sequencing	ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystem	Darmstadt
Gel extraction	QIAquick Gel Extraction Kit	Qiagen	Hilden
Plasmid isolation	Nucleo Spin Plasmid QuickPure Kit	Macherey-Nagel	Düren
Protein expression	Strep-tag Recombinant Protein Expression and Purification Kit	IBA	Göttingen
Recovering PCR product	Invisorb Spin PCRapid Kit	Invitek	Berlin
RNA extraction	Invisorb Spin Cell RNA Mini Kit	Invitek	Berlin

Table 11: **Kit table**. Kits used during work.

2.2 Methods

2.2.1 Preparation of genomic DNA

Bacteria from a saturated liquid culture are lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides and remaining proteins are removed by selective precipitation with CTAB, and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Protocol

1. Spin 2 ml of the culture in a micro centrifuge for 5 min at 8000 rpm. Discard the supernatant.
2. Resuspend the pellet in 576 µl of TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ml protease K to give a final concentration of 100 µg/ml protease K in 0.5% SDS. Mix thoroughly and incubate 1 hour at 37℃.
3. Add 100 µl of 5 M NaCl and mix thoroughly.
4. Add 8 µl of CTAB/NaCl solution. Mix thoroughly and incubate 10 min at 65℃.
5. Add an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol. Mix thoroughly and spin in a micro centrifuge, for 5 min at 8000 rpm.
6. Transfer aqueous, viscous upper phase to a clean micro centrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, mix thoroughly and spin for 5 min at 13,000 rpm. Transfer the upper phase to a fresh tube. Add 0.6 vol of isopropanol to precipitate the nucleic acids. Shake the tube back and forth until a string of precipitated DNA becomes clearly visible.
7. Spin the tube for 5 min at 13000 rpm and discard the supernatant. Add 1ml of 70% ethanol and spin for 5 min at 13000 rpm. Discard the supernatant and let the pellet dry at room temperature.
8. Resuspend the pellet in 100 µl TE buffer and quantify the DNA (Nano Drop).
9. Verify the extracted DNA with an agarose gel electrophoresis.

2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying and purifying DNA fragments. The agarose concentration is adjusted to the size of the DNA fragments to be separated.

Protocol:

1. Prepare the electrophoresis gel buffer.
2. Prepare the agarose gel and pour it into the gel chamber and insert the gel comb.
3. After the gel has solidified, remove the gel comb and pour the buffer until the gel is covered.
4. Prepare the DNA samples:
    a. pipet in a 1.5 ml tube 10 µl of the sample
    b. add 2 µl of 6x STOP buffer
    c. vortex and centrifuge
5. Load the samples into the wells with a micropipette.
6. Turn on the power supply and let it run for about 1 hour (70 V).
7. Visualize the DNA by placing it under a UV light source.

2.2.3 Typing of the samples

All samples were typed for the major *C. perfringens* toxin genes, via PCR (polymerase chain reaction). The primers were selected from the database (see primer table).

2.2.3 a) PCR reaction

To perform the PCR reaction the genomic DNA has to be diluted in order to have a concentration between 1 and 2 ng/µl.

1. Prepare the master-mix solution:

10x Buffer	2.5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTPs (2 mM)	2.5 µl
Primer 1 (2.5 µM)	2.5 µl
Primer 2 (2.5 µM)	2.5 µl
Taq polymerase (5U/µl)	0.1 µl
Water	0.9 µl

2. Aliquot 12.5 µl master-mix into the 0.2 ml tubes add the DNA samples and water up to a final volume of 25 µl.
The PCR machine (Biometra, TGradient) was already switched-on in order to warm-up the cover.

Cycle conditions

denaturation	94℃	3 min
30 cycles	53℃	30 sec (annealing)
	72℃	2.5 min (extension)
	94℃	1 min (denaturation)
extension	72℃	10 min
	10℃	

All PCR products were analyzed on 1% agarose gel.

2.2.4 Cloning of the alpha-toxin gene

It is often desirable to clone PCR products to establish a permanent source of cloned DNA either to obtain uniform DNA for high-quality sequencing results, or to be able to subclone a specific DNA fragment into other vectors.

PCR cloning consists of the following steps:

1. Prepare the PCR-product of interest. If necessary, polish the ends in order to get it ligated with the vector.
2. Prepare the cloning vector and linearise it with a restriction enzyme.
3. Ligate the PCR product with the linearised vector.
4. Transform competent bacteria with the ligation product.
5. Plate the bacteria on a selective agar and identify recombinant bacteria

Two vectors were used, (a) pZerO-2 (Invitrogen) which allows the insertion of the blunt PCR product into the Eco RV site of the multicloning site or (b) pASK-IBA6 (IBA, Göttingen) which allows the protein expression of the PCR product.

2.2.4 a) Preparation of the Toxin-gene-specific PCR-Products

The toxin gene-specific part of the *C. perfringens* DNA was amplified with primer pairs annealing upstream and downstream of the respective gene. For the alpha-toxin gene the following primers were used (Palp2, for and rev). For the beta2-toxin genes primers taken from database did not work in the first try. The correct primer pairs had to be developed experimentally (see results). PFU (Stratagene) was used as proofreading DNA-polymerase. Cycle conditions were as described in (2.2.3 a). Usually 100 µl reactions were performed.

After the reaction, aliquots were tested on 1% agarose gel and in case the desired PCR product was obtained the PCR product was isolated for the reaction assay by a spin column kit (Invitek, Berlin, see kits).

Protocol:

1. Mix 300 µl Buffer P with the PCR reaction mixture in a 1.5 ml reaction tube. Transfer the suspension completely on a Spin Filter. Incubation for 1 minute.
2. Centrifuge for 1 minute at 10000 rpm.
3. Add 700 µl Wash Buffer to the Spin Filter and centrifuge for 1 minute at 10000 rpm.
4. Discard the filtrate. Remove the residual ethanol of the Wash Buffer by centrifugation for 3 minutes at maximum speed (13000 rpm).

5. Transfer the Spin Filter into a new 1.5 ml receive tube. Add at least 30 µl Elution buffer directly onto the centre of the spin filter. Incubate at room temperature for 3 minutes.
6. Centrifuge for 1 minute at 10000 rpm.
7. Quantify the DNA in the eluate (Nano Drop).

2.2.4 b) Digestion of pZErO-2 for ligation

pZErO-2 is a 3297 bp cloning vector that allows direct selection of positive recombinant clones via disruption of the lethal gene, *ccd*B. The pZErO-2 contains the *ccd*B gene fused to the C-terminus of LacZα. Insertion of a DNA fragment disrupts expression of *LacZα-ccd*B gene fusion permitting growth of only positive recombinant. Cells that contain non-recombinant vector are killed. PZErO-2 vector also contains the Kanamycin resistance gene for selection on agar plates.
The digestion of pZErO-2 is performed with the restriction enzyme EcoRV, which provides blunt ends:

pZErO –2	x µl (2 µg)
10x NEB Buffer 3	2 µl
BSA (200 µg/ml; dil 1:10)	2 µl
EcoRV (20 U/µl)	1 µl
H_2O	up to 20 µl

Incubate at 37℃ for 30 minutes and then inactivate the restriction enzyme by heating the reaction at 80℃ for 20 minutes.

The digested vector is purified by a preparative 1% agarose gel. It was isolated from the gel with the gel extraction kit (QIAquick Gel extraction Kit, see kits).

Protocol:

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel silice in a colourless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50℃ for 10 minutes (or until the gel has completely dissolved). To help dissolution, mix by vortexing the tube every 2-3 min during the incubation.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the column, and centrifuge for 1 min.
8. Discard the flow-through and place the column back in the same collection tube.
9. Recommended: add 0.5 ml of Buffer QG to the column and centrifuge 1 min.
10. To wash, add 0.75 ml of Buffer PE to the column and centrifuge for 1 min.
11. Discard the flow-through and centrifuge the column for an additional 1 min.
12. Place the column into a clean 1.5 ml micro centrifuge tube.
13. To elute DNA, add 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5). to the centre of the membrane and centrifuge the column for 1 min.
14. Quantify the DNA in the eluate using Nano Drop.

2.2.4 c) Ligation of the amplified fragment and vector

Set up the ligation reaction to have a molar ratio of 2:1 (insert : vector)

Digested vector (pZErO –2)	x µl (up to 25 µg)
DNA insert (alpha toxin)	y µl (up to 15 µg)
10x ligation Buffer (with ATP)	1 µl
T4 DNA ligase (1U/µl)	1 µl
Water	Up to 10 µl

Incubate at 16℃ overnight and inactivate the enzym e by heating the reaction at 65℃ for 10 minutes.

2.2.4 d) Transformation

1. Preparation of electrocompetent *E. Coli*

Extra material:

- Single colony of *E. coli* cells (strain Top 10, Invitrogen)
- LB plates, containing ampicillin (final concentration: 50 µg/ml)
- Rotor GSA 3 (DuPont)

Protocol:

1. Inoculate a single colony of E. coli cells into 50 ml of LB medium. Let grow at 37℃ overnight with moderate shaking.
2. Divide 1 litre LB in 4 portions of 250 ml and add 12.5 ml of the overnight culture into each. Let grow at 37℃ shaking at 300 rpm, to an OD_{600} of 0.5 to 0.6.
3. Chill centrifuge tubes in an ice-water bath 30 minutes and transfer to pre-chilled centrifuge rotor.
4. Centrifuge cells 15 min at 1400 x g in the Sorvall centrifuge.
5. Discard supernatant and resuspend the pellet in 1 litre ice-cold water. Centrifuge cells as in step 4.
6. Discard supernatant and resuspend the pellet by swirling in remaining liquid.
7. Add another 500 ml ice-cold water, mix well, and centrifuge again as in step 4.
8. Discard the supernatant and resuspend the pellet in 15-20 ml of 10% glycerol. Centrifuge the cells as in step 4.
9. Discard the supernatant and resuspend the pellet in 2 ml 10% glycerol.
10. Aliquot the cells into pre-chilled 1.5 ml tubes and freeze by liquid nitrogen. Store at –80℃.

2. Electrotransformation of cells

Extra material

- LB plates, containing kanamycin (final concentration 50 µg/ml).

Protocol

1. Set the electroporation apparatus to 2.5 kV.
2. Add 2 µl of the ligation mix to tubes containing 40 µl electrocompetent cells (on ice). Mix by tapping the tube.

3. Transfer the DNA and cells into a electroporation cuvette (Gene Pulser Cuvette 0.1 cm). that has been chilled 5 min on ice, shake slightly to settle the cells to the bottom.
4. Place the cuvette into the sample chamber and set the electrical pulse (voltage 1.8 kV, capacitance 50µF, load resistance 150 Ohm).
5. Remove the cuvette. Immediately add 450 ml SOC medium and transfer to a sterile culture tube. Incubate 30 min with moderate shaking at 37℃.
6. Plate aliquots of the transformation culture on LB plates containing kanamicin and let grow overnight at 37℃.

2.2.4 e) Analysis of recombinants

Plasmid preparation:
Aliquot 5 ml of LB medium, containing 50 µg/ml kanamycin, into a sterile culture tube then add one colony per each tube and shake them overnight at 37℃.

Protocol (Nucleo Spin Plasmid Quick Pure Kit, see kits):

1. Using 1-5 ml of a satured *E. coli* culture, pellet cells in a standard benchtop micro centrifuge for 1 minute at 11000 x g. Discard the supernatant.
2. Cell lysis: add 250 µl buffer A1
    1. resuspend the cell pellet by vigorous vortexing
    2. add 250 µl buffer A2. Mix gently by inverting the tube 6-8 times. Do not vortex. Incubate at room temperature for a maximum of 5 minutes
    c. add 300 µl buffer A3. Mix gently by inverting the tube 6-8 times. Do not vortex
3. Clarification of lysate: centrifuge for 5-10 minutes at 11000 x g at room temperature
4. Bind DNA: place a NucleoSpin Plasmid column in a 2 ml collecting tube and load the supernatant from step 3 onto the column. Centrifuge for 1 min at 11000 x g. Discard flow-through.

5. Wash silica membrane:
    a. add 500 µl of pre-warmed buffer AW (50℃) and centrifuge for 1 min at 11000 x g

b. place the NucleoSpin Plasmid column back into the 2 ml collecting tube and add 600 µl buffer A4 (with ethanol). Centrifuge for 1 min at 11000 x g. Discard the flow-through

6. Dry silica membrane: to dry the silica membrane completely, reinsert the NucleoSpin Plasmid column into the empty 2 ml collecting tube. Centrifuge 2 min at 11000 x g.

7. Elute highly pure DNA: place the NucleoSpin Plasmid column in a 1.5 ml micro centrifuge tube and add 50 µl buffer AE. Incubate 1 min at room temperature. Centrifuge for 1 min at 11000 x g.

In order to detect the insert, the plasmid DNA was analyzed on 1% agarose gel (size increase) and/or a PCR-reaction was performed using the M13 universal primer pair (M13-40, M13- reverse). The PCR product is a little longer than the *Clostridium* specific PCR product due to added vector sequences.

2.2.5 DNA sequencing

a) Sequencing reaction

The PCR products obtained by amplification of the insert DNA of the recombinant plasmids using the M13 universal primer pair were sequenced by the dideoxy method (Sanger et al., 1977), using the BigDye Terminator sequencing kit. The major advantage is that the sequencing can be performed in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with a different fluorescent dye, each fluorescing at a different wavelength.

Protocol (ABI PRISM Big Dye Terminator, see kits)

1. Prepare the samples for the sequence reaction:

Extra material

- Half dye Mix (reduces the amount of Big Dye Terminator needed in a reaction. Bioline, Luckenwalde).

Big Dye (DNA polymerase, dNTPs, ddNTPs, Buffer)	0.8 µl
Half Dye	3.2 µl
template	x µl (90-100 ng)
Sequencing primer (3.2 pmol)	2 µl
H_2O	up to 10 µl
Mineral oil	20 µl

2. Perform the following PCR cycles:

denaturation	96℃	10 sec
25 cycles	50℃	5 sec (annealing)
	60℃	4 min (extension)
	96℃	10 sec (denaturation)
termination	10℃	pause

b) Sequencing of the products of the sequencing reaction

The samples were sequenced by GATC Biotec AG (Jakob-Stadler Plz, 7, 78467 Konstanz, Germany) using a Genome Sequencer FLX instrument (Roche, Mannheim). The transmitted sequences were analyzed using the Vector NTI software (version 10, Invitrogen).

2.2.6 Inverse PCR

Inverse PCR is used to find out informations on unknown sequences surrounding a fragment of DNA on which informations are already available.
A restriction enzyme which should not have a cutting site inside the known region, is used. After the digestion of the entire DNA, a ligation reaction is performed. The DNA fragments are circularized. From the known region two pair of primers are selected, amplifying parts of the known and the outside unknown regions being fused together by the circularizing ligation. The second primer pair was used in a nested PCR, using the reaction product from the first amplification as template. After that the DNA is gel purified, sequenced and analyzed.

2.2.6 a) Restriction

Reaction:

C. perfringens DNA	x µl (up to 5 µg)
Hind III (10 U/µl)	2 µl
1x NEB Buffer 4	2 µl
Water	up to 20 µl

Incubate at 37℃ for three hours. Inactivate the en zyme by heating the reaction at 80℃ for 10-15 minutes. Analyse the restricted DNA on 1% agarose gel, to confirm the restriction.

2.2.6 b) Purification

Protocol

1. Add water to 250 µl.
2. Add 250 µl of phenol/ chloroform/isoamyl alcohol and mix inverting the tube for two min.
3. Centrifuge at 13000 rpm for 1 min.
4. Transfer the upper phase to a fresh 1.5 ml tube.
5. Add 500 µl of chloroform/isoamyl alcohol.

6. Mix inverting the tube for 1 min.
7. Centrifuge at 13000 rpm for 1 min.
8. Transfer the upper phase to a fresh tube.
9. Repeat the steps 5 to 8.
10. To 300 µl of solution add 30 µl of NaAc (2.5 M) and 330 µl of isopropanol. Incubate at room temperature for 1 hour.
11. for 30 sec and centrifuge at 13000 rpm for 25 min. Discard the supernatant
12. Add 300 µl of 70% ethanol.
13. Centrifuge at 13000 rpm for 25 min. Discard the supernatant and let the pellet dry at room temperature.
14. Redissolve the pellet in 20 µl TE buffer.
15. Quantify the sample by Nano Drop.

2.2.6 c) Ligation

Set up the following ligation reaction

sample	x µl (5.1 ng)
ligase buffer	2.5 µl
Ligase (1U/ µl)	1 µl
H_2O	up to 25 µl

Incubate at 25℃ for 4 hours and inactivate the enz yme by heating the reaction at 65℃ for 10 minutes; proceed with the nested PCR.

2.2.7 Reverse Transcription PCR (RT-PCR)

In order to analyse the gene-expression of the toxins, RT-PCR was performed. Total RNA from *C. perfringens* was isolated, retrotranscribed into cDNA, which subsequently was used as template in a toxin-specific PCR reaction.

2.2.7 a) Extraction of total RNA from *C. perfringens*

Protocol (Invisorb, SPin, Cell RNAMinikit, see kits)

1. Harvest bacteria. Spin up $1x10^9$ bacteria cell for 5 min at 5000 rpm in a centrifuge tube. Discard the supernatant and completely remove media.
2. Lysis of the bacteria cell wall with lysozyme:
   a. IMPORTANT: immediately before use, dissolve lysozyme in TE buffer (10 mM Tris/HCl; 1 mM EDTA pH 8). For Gram-positive bacteria the lysozyme concentration in TE buffer should be 3000 µg/ml
   b. loosen the bacteria pellet by flicking the tube and add 100 µl lysozyme containing TE buffer. Mix gently by vortexing and incubate for 10-30 min at room temperature.
3. Disrupt bacteria cell by adding lysis solution
   Add 350 µl solution R to the lysozyme-containing sample. Mix thoroughly by pipetting up and down. No cells clumps should be visible before proceeding with the next step
4. Binding of genomic DNA to the DNA-Binding-Spin filter. Pipette the lysate from step 3 (450 µl), including any precipitate which may have formed, directly onto the DNA-Binding-Spin filter sitting in a receiver tube, incubate for 1 min and centrifuge at 12000 rpm for 2 min. Discard the DNA-Binding-Spin filter.
5. Adjust RNA binding conditions. Add 250 µl 99.8% ethanol to the RNA-containing lysate and mix by pipetting up and down.
6. Binding of the total RNA to the RNA-Binding-Spin filter. Pipette the RNA-containing sample resulting from step 5 directly onto the RNA-binding-Spin filter placed in a receiver tube. Incubate for 1 min and centrifugate 10000 rpm for 30 sec. Discard the flow-through and reuse the receiver tube.
7. First washing of the RNA-Binding-Spin filter. Pipette 600 µl Wash Buffer R1 onto the RNA-Binding-Spin filter and centrifuge for 30 sec at 10000 rpm. Discard the flow-through and reuse the receiver tube.

8. Second washing. Pipette 500 µl Wash Buffer R2 onto the RNA-Binding-Spin filter and centrifuge for 30 sec at 10000 rpm. Discard the flow-through and reuse the receiver tube.
9. Drying of the RNA-Binding-Spin filter. To eliminate any traces of ethanol, centrifuge for 3 min at 13000 rpm. Discard the receiver tube.
10. Elution of total RNA. Transfer the RNA-Binding-Spin filter into a RNase-free elution tube and pipette 40-100 µl of elution buffer R directly onto the membrane of the RNA-Binding-Spin filter. Incubate for 2 min and centrifuge for 1 min at 10000 rpm. Discard the RNA-Binding-Spin filter and place the eluted total RNA immediately on ice.
11. Quantify the total RNA in the eluate by Nano Drop and collect an aliquot for the RT-PCR.
12. Aliquot the total RNA eluate and store it at –20℃.

2.2.7 b) Reverse Transcription

Before performing the reverse transcription the samples are treated with RNase-free DNase, in order to remove even traces of DNA:

Protocol

1. Prepare tubes (0.1 ml).

2. Prepare RNA samples:
    a. 2 ng RNA in 10 µl PCR-water
    b. store on ice
3. Prepare DNase-Premix:

5x RT-Buffer	4 µl
PCR water	5 µl
DNase (1U/µl)	1 µl

Add 10 µl of Premix into RNA-sample, store on ice.

4. Mix samples and centrifuge at 13000 rpm for 1 min
5. Put samples in cycler:

37℃	30 min (DNase reaction)
75℃	5 min (DNase inactivation and RNA denaturation)

After the 75℃ step, place samples immediately on i ce for 5 min.

6. Prepare RT-Premix

5x RT-Buffer	4 µl
dNTPs (2.5 µM)	4 µl
Random hexamers	3 µl
PCR water	8 µl
M-MLV-Reverse Transcriptase (200 U/µl)	1 µl

Add 20 µl of premix to samples.

7. Mix samples and centrifuge at 13000 rpm for 1 min.
8. Put samples in the cycler (reverse transcription).

25℃	10 min
37℃	50 min
90℃	2 min
4℃	pause

9. Put samples on ice. Centrifuge at 1300 rpm for 1 min; mix samples and repeat the centrifugation step.
10. Aliquot samples and freeze at –20℃.

2.2.7 c) PCR with the cDNA

prepare the master mix solution

Buffer	2.5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTPs (2 mM)	2.5 µl
Primer 1 (2.5 µM)	2.5 µl
Primer 2 (2.5 µM)	2.5 µl
H_2O	0.9 µl
Taq-polymerase (5U/ µl)	0.1 µl

Add to the master-mix solution 5 µl of undiluted cDNA (ca. 20 ng/µl) and water up to a final volume of 25 µl.

Cycle conditions:

denaturation	94℃	3 min
30 cycles	53℃	30 sec (annealing)
	72℃	2.5 min (extension)
	94℃	1 min (denaturation)
extension	72℃	10 min
	10℃	

Analyse the PCR product on 1% agarose gel.

2.2.8 Expression-cloning of the alpha- and beta2-gene PCR products

Beta2-PCR product is cloned into the pASK-IBA6 vector (IBA, Göttingen). By this vector a fusion protein is produced which contains an OmpA signal sequence mediating the export of the protein into the periplasmic space where it can be cleaved by signal peptidases. In

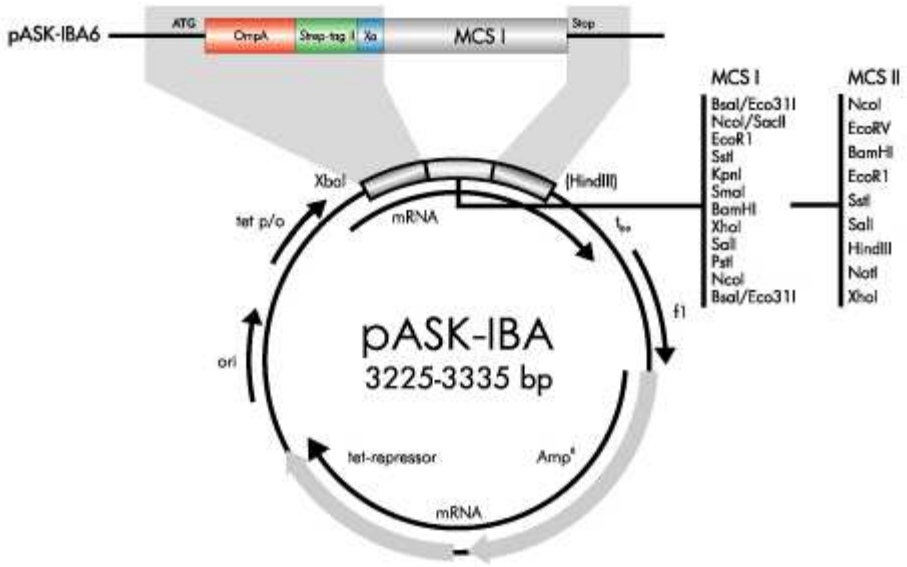


Figure3: **Features of the pASK-IBA vector**.

addition it contains a sequence Strep-Tag which can be used to isolate the fusion protein by affinity chromatography with Strep-Tactin (IBA, Göttingen). Finally, part of the fusion protein can be cleaved off exactly in front of the fusion protein by blood clotting factor Xa.
The polylinkers of the of the pASK-IBA expression plasmid carry 2 BsaI restriction sites allowing the precise fusion of the structural gene with the vector-encoded functional elements (*Strep*-tag, OmpA-signal and start codon). To accomplish this, it is necessary to adapt the structural gene at both ends of the coding region via PCR.
The primers should have the following feature:

1. Forward primer: ATGGTAGGTCTCAGCGCAAGGAAATCGACGCTTATAGAAAG, where GGTCTC is the recognition site of Bsa1, and AAG is the start codon of mature Beta2-toxin. Bsa1 belongs to enzymes which cleave the DNA double strand outside their recognition site. Thereby, the digestion with one single enzyme can generate two different independent sticky ends with 4-base 5'-overhangs allowing directional cloning.
2. Reverse primer: ATGGTAGGTCTCATATCACTAAAAACAATATCCTTCACC, where GGTCTC is the recognition site of Bsa1, and CTA is the reverse complementary of end codon of the gene (UAG).

2.2.8 a) Preparation of the beta2 gene PCR –product and Bsa I treatment

The PCR is performed as follows

Buffer 10x	2.5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTPs (2 mM)	2.5 µl
Forward primer (2.5 µM)	2.5 µl
Reverse primer (2.5 µM)	2.5 µl
PFU (2.5 U/µl)	0.5 µl
DNA	x µl (up to 2 µg)
Water	up to 25 µl

The cycle conditions were as described in (2.2.3 a, extension time 1.5 min). The PCR product was analyzed on 1% agarose gel.

The restriction step with Bsa I is performed for both vector and insert:

Vector	x µl (600 µg)	Insert	x µl (600 µg)
Bsa I (20 U/µl)	1 µl	Bsa I (20 U/µl)	1 µl
NEB Buffer 2	2 µl	NEB Buffer 2	3 µl
Water	y µl	Water	y µl
Total	20 µl	Total	30 µl

Incubate at 50℃ for 1 hour and then 20 minutes at 65℃. Only the vector is subsequently incubated with Pst I for further 30 min. By this, the religation of vector molecules cut only once by Bsa I is prevented, resulting in less background colonies.

After restriction both the vector and the insert are purified using a preparative agarose gel with subsequent spin purification; then the fragments are ligated in a typical assay:

- 100 ng of digested vector fragment
- digested PCR fragment in 2 times molar excess
- buffer for ligation 1 unit of T4 DNA ligase
- water to 10 µl

Incubate overnight at 16℃ and then at 65℃ for 10 minutes, store at –20℃. Simultaneously perform the same ligation assay without the addition of PCR fragment for quantifying background reactions.

2.2.8 b) Electrotransformation and analysis of recombinants

1. Using the electrocompetent cells E. coli, strain Top 10, proceed for the Electrotransformation as described in (2.2.4 d).
2. Prepare the mini-prep by aliquoting 5 ml of LB medium containing 100 µg/ml ampicillin into a sterile culture tube and then add one colony per each tube and shake overnight at 37℃.
3. As described in (2.2.4 e) isolate the plasmid and analyse the plasmid on 1% agarose gel in order to detect the insert and perform a PCR reaction using IBAG and IBALinks primer pair (selected from the plasmid sequence).

2.2.8 c) Cell growth, gene expression and preparation of the cell extract:

Preculture: inoculate 2 ml of LB medium containing 100 µg/ml ampicillin with a fresh colony of the strain harbouring the expression plasmid and shake overnight at 37℃.

Culture for expression: inoculate 100 ml LB-medium containing 100 µg/ml ampicilin with the preculture and shake at 25℃. Monitor the optic al density (OD) of the culture at 550 nm. When OD_{550} equals 0.5, add 10 µl of anhydrotetracycline solution (AHT, 2 mg/ml anhydrotetracycline in DMF) and continue shaking overnight.

Cell harvest, preparation of the periplasmic and seroplasm extract: after harvesting the cells by centrifugation (4℃, 4500 g, 12 minutes) d iscard the supernatant and resuspend

the pellet in 1 ml of pre-cooled buffer P (100 mM Tris/HCL pH 8,0, 500 mM sucrose, 1 mM EDTA) at 4℃, transfer it into a 1.5 ml tube and in cubate on ice for 30 minutes. Under these conditions, the outer membrane of *E. coli* will usually be sufficient permeabilized to release the soluble periplasmic components. Centrifuge at 14000 rpm for 5 minutes and separate the pellet from the supernatant, which is ready fore the SDS gel.

2.2.8 d) SDS gel electrophoresis

Protocol:

Extra material

- electrophoresis apparatus: glasses, clamps, glass plates, buffer chambers
- 1 mm spacers
- 25 ml Erlenmeyer side-arm flask
- vacuum pump
- 1-mm Teflon comb with 25 teeth

Prepare the gel:

1. Assemble the glass-plate sandwich using two clean glass plates and three 1-mm spacers. Lock the sandwich.
2. Prepare the separating gel solution, degassing using a rubber-stopper 25 ml erlenmeyer side-arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding the specific amount of 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.
3. Apply separating gel solution to the sandwich.
4. Using a Pasteur pipet, slowly cover the top of the gel with a layer (~ 1 cm) of butanol.
5. Allow the gel to polymerize 30 to 60 min at room temperature. Then discard the butanol and wash with water.
6. Prepare the stacking gel, degas it and apply it to the sandwich.
7. Insert a 1 mm Teflon comb into the layer of staking gel solution.
8. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

In the mean time prepare the samples by diluting a portion of the protein sample to be analyzed adding the 5xSDS sample-buffer. Heat 3 to 5 min at 100℃.

Prepare the gel chamber by filling it with the electrophoresis buffer. Place the sandwich into the gel chamber and the apply the samples.
Switch on the power supply connect it to the cell and run at 150 V until the bromophenol blue tracking dye enters the separating gel. Then increase the current to 170 V.
After the bromophenol blue tracking dye has reached the bottom of the separating gel, switch off voltage.

2.2.8 e) Staining

Protocol

Extra material

-plastic or glass container with tight-fitting lid of size appropriate for gel
-platform shaker

1. Add ~ 10 gel volumes of 10 % TCA solution to a plastic container.
2. Remove polyacrylamide gel from the electrophoresis unit and place it in 10% TCA solution, then place container on a platform shaker and agitate slowly for 30 min
3. Remove TCA solution by washing with water.
4. Add ~10 gel volumes of Coomassie blue R-250 staining solution to the same container containing the gel. Shake over night.
5. Remove excess staining solution by washing with sterile water.
6. Add 5 to 10 gel volumes distaining solution and place the container with gel on shaker for 1 or 2 hours.
7. Remove distaining solution and add 10% acetic acid solution and shake for 1 hour.
8. For a permanent record, scan or photograph the gel.

3. Results

3.1 Extraction of DNA and typing of *C. perfringens* samples

C. perfringens single colonies were transferred to Schaedler broth and grown overnight at 37℃ in CO_2-saturated atmosphere. The saturated liquid culture was then centrifuged, washed twice with PBS and used for DNA extraction. The extracted DNA was first quantified and then analyzed on 1% agarose gels.

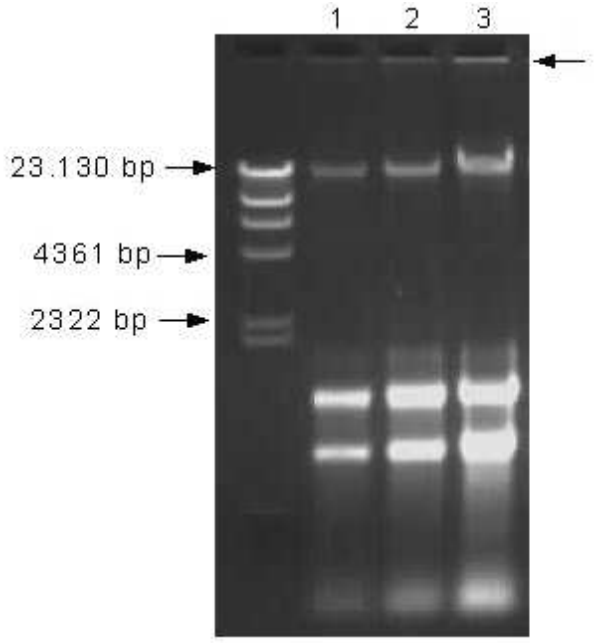


Figure 4: **DNA extraction.** Lines 1-3 extracted DNA, sample n°2 (Torgau) at different concentrations (line 1: 1,5 µg; line 2: 4,5 µg; line 3: 7,5 µg). **Arrow**: chromosomal DNA; bands at 23.130 bp: plasmid DNA; lower bands: ribosomal RNA. **Marker**: λ-DNA, Hind III fragments.

The first step in strain characterization was the typing of the samples according to their toxin gene composition. All *C. perfringens* samples were tested, via PCR reaction, for the presence of the major toxins genes (alpha, beta, epsilon, iota, cpe and beta2.). The PCR

products were analyzed on 1% agarose gels. Figs. 5-7 show the gel analysis of representative samples.

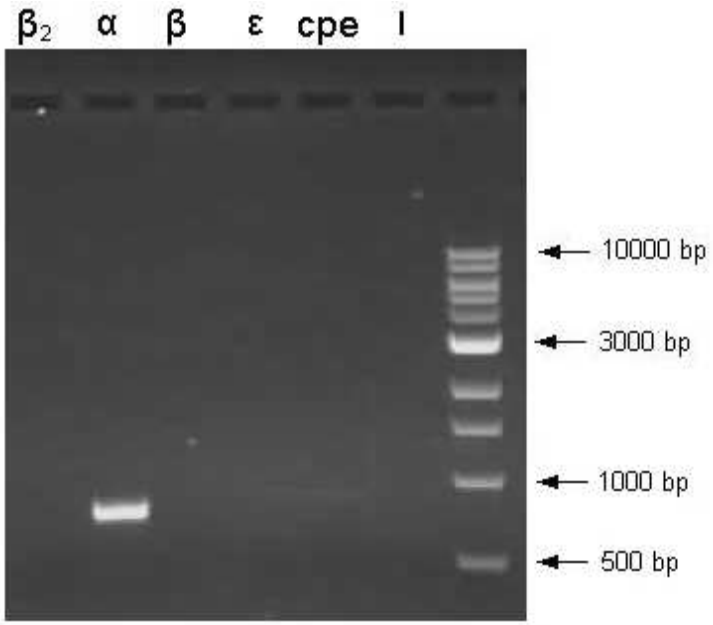


Figure 5: ***C. perfringens*** **strains-typing**. Sample 9 (Triberg). Line1: beta2-toxin primer pair Pbe2.1, for-rev (expected 309 bp); line 2: alpha-toxin gene primer pair Palp1, for-rev (expected 819 bp); line 3: beta-toxin gene primer pair Pbe, for rev (expected 286 bp); line 4: epsilon-toxin gene primer pair Peps, for-rev (expected 443 bp); line 5: cpe-toxin primer pair Pcpe, for-rev (expected 529 bp); line 6: iota-toxin gene prime pair Piot, for-rev (expected 686 bp). **Marker**: 1kb DNA ladder.

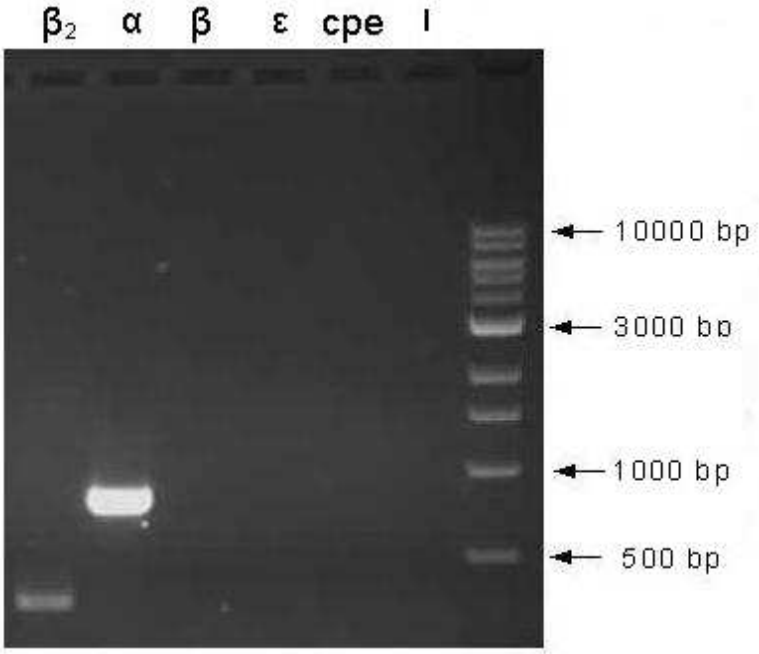


Figure 6: ***C. perfringens* strains typing**. Sample 8 (Rathenow) presenting in addition to the alpha-toxin-also the beta2-toxin gene. **Marker**: 1kb DNA ladder.

All samples can be classified into 3 categories:

a. only alpha-toxin-gene-containing (Fig. 5)
b. alpha- and beta2-gene-containing (Fig. 6)
c. alpha- beta2- and cpe-gene-containing (Fig. 7)

Feature c was shown only by the strain n° 6 (Wallen horst). Taken together, all samples belong to the so called A-type (see table1 in Introduction).
The distribution of the alpha- and beta2-genes in all samples analyzed is shown in table 12. From a total of 71 strains, all contain the alpha-gene and 25 additionally the beta2-gene.

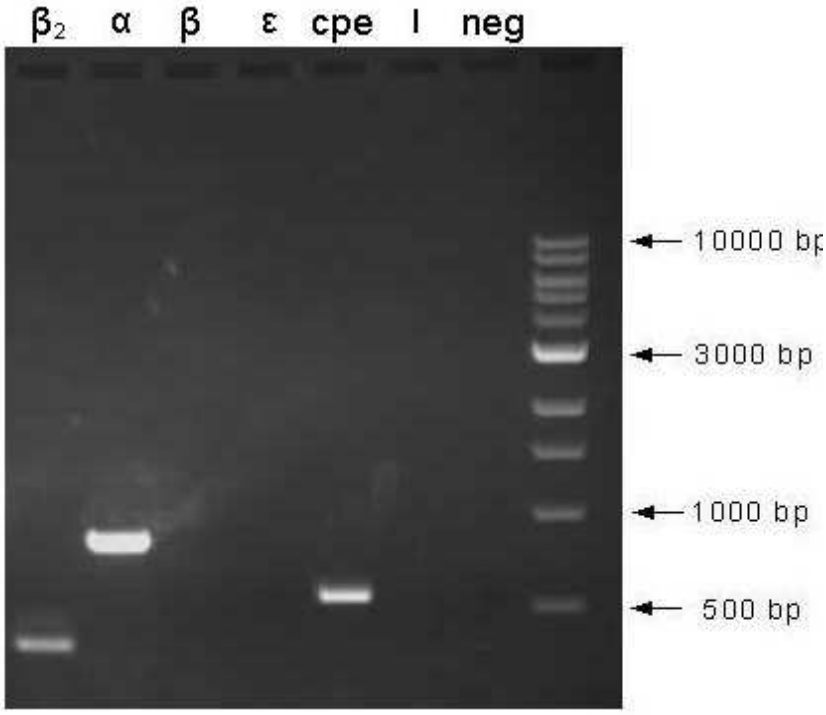


Figure 7: ***C. perfringens*** **strains typing**. Sample 6 (Wallenhorst) presenting in addition to the alpha- and beta2- also the cpe-toxin gene (line 5). Line 8: negative sample (no template added to the reaction). **Marker**: 1kb DNA ladder.

Name	Number	Alpha	Beta2	Name	Number	Alpha	Beta2
Regensburg	1	+	-	Zerbst	38	+	+
Torgau	2	+	+	Duderstadt Hi9	39	+	-
Borkheide	3	+	+	Südlohn	40	+	+
Auerbach	4	+	-	Lübars	41	+	-
Kleve	5	+	-	Burghaun	42	+	-
Wallenhorst	6	+	+	Wriezen 2	43	+	+
Haldensleben	7	+	-	Poppenricht	44	+	-
Rathenow	8	+	+	Templin	45	+	-
Triberg	9	+	-	Zeulenroda	46	+	+
Herne	10	+	-	Rüdersdorf	47	+	-
Euskirchen	12	+	-	Stücken	48	+	-
Langen	13	+	-	Neucharell	49	+	+
Laichtlingen	14	+	+	Essen	50	+	+
Ahlbeck	15	+	-	Ettlingen	51	+	-
Forbach	16	+	+	Herbstein	52	+	-
Saarbrücken	17	+	-	Tuningen	53	+	-
Lichtenau	18	+	-	Haigerloch	55	+	+
Hagen	19	+	+	Bückeburg	56	+	-
Düren	20	+	-	Sulzfeld	57	+	+
München	21	+	-	Steinfurth	58	+	-
Wriezen	22	+	-	Krefeld	59	+	-
Meiningen	23	+	-	Ehrenberg	60	+	+
Hannover	24	+	-	Groβenhagen 1	61	+	-
Büttstedt	25	+	-	Groβenhagen 2	62	+	-
Hamm	26	+	+	Dudenhofen	63	+	-
Zittau 2	27	+	+	Glindow	65	+	+
Zwiesel	28	+	-	Rudow	66	+	-
Beelitz	29	+	+	Stolberg	67	+	-
Mettingen	30	+	-	Crailsheim	68	+	-
Freiburg	31	+	-	Karlsruhe	69	+	-
Köpenick	32	+	+	Kaiserslautern	70	+	-
Dresden	33	+	-	Aschaffenburg	71	+	-
Stuttgart	34	+	+	Treviso 1	72	+	+
Dittmannsdorf	35	+	-	Treviso 2	73	+	+

Schorfheide	36	+	-	Treviso 3	74	+	+
Wandlitz	37	+	-				

Table 12: **alpha- and beta2-toxin-genes in *C. perfringens* strains**.

3.2 Alpha-toxin genes

From the Genebank nucleotide database, containing the complete genome of *C. perfringens* strain 13 (human isolate, Accession Nr. BA00016) two primers were selected annealing in the upstream and downstream region of the alpha-toxin-gene (Palp2 for and rev). Theoretically, a PCR-product of 2007 bp should be expected. A product of approx. 2 kb size was obtained also with our *C. perfringens* samples isolated from rabbits. This is exemplified in Fig. 8 for strain n° 9 (Triberg) at different template concentrations and 22 or 23 cycles respectively. All the other 71 strains behave identically (data not shown). Therefore, it was possible to isolate the alpha-toxin-gene, including its immediate upstream and downstream surrounding, for all *C. perfringens* isolates from rabbits.

3.2.1 Nucleotidic sequence

The PCR-products of the first 15 isolates (see table 12) were cloned into pZerO-2. Several recombinant bacterial clones were picked from the agar plates, and, after plasmid isolation and PCR-amplification of the insert using the universal primers M13rev and M13-40, the alpha-toxin gene area was sequenced. All different clones from the same isolate had identical gene sequences (data not shown).

For the remaining isolates (16-71) the cloning step was omitted. Instead the alpha-gene-specific-PCR product was directly sequenced. The sequencing chromatograms were easily readable, irrespectively, if they were produced from cloned or non-cloned PCR products. This is illustrated in Fig 9 where the same parts of the alpha-gene sequence are shown for a cloned sample, n°7 (Haldensleben), and a non-cloned sample, n° 17 (Saarbrücken).

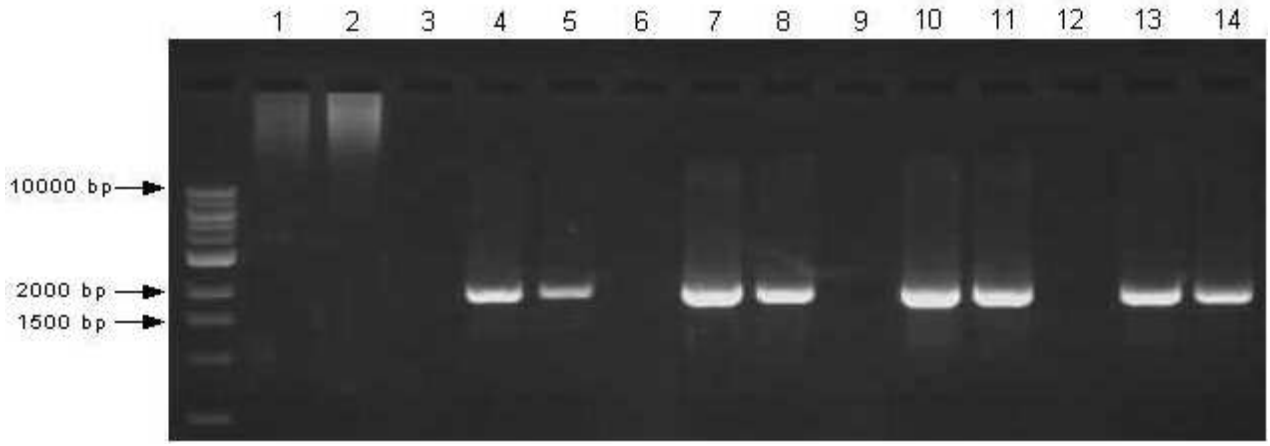


Figure 8: **Alpha-toxin-gene-detection**. Sample n° 9 (Triberg). Line 1, 2: negative samples. Line 4,7,10, 13: 22 cycles with 0.6, 1.2, 1.8, 2.4 µg/µl of sample respectively. Line 5,8,11,14: 23 cycles same sample concentration. **Marker**: 1kb DNA ladder.

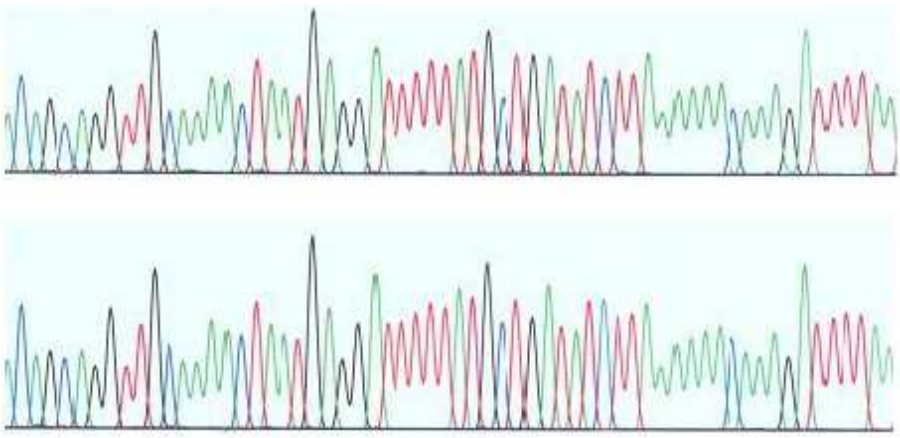


Fig 9: **Sequencing patterns of cloned and non-cloned alpha-gene-specific PCR products**. Upper sequencing gel: sample n° 7 (Haldensleben) cloned; lower sequencing gel: sample n° 17 (Saarbrücken) non-cloned sample.

Fig. 10 shows the complete sequence of the PCR product representing the alpha toxin gene-region of strain n°2 (Torgau)

```
   1 CATAAGCCTG TGCAATTGGT ACAATTCCTG GGAAAGTTTC ATTTCCTGCA AATATATTAA GACCAAAATC
  81 GCTGTTTAAA ACTTCTTCAT TAAGCCCAAG TTCTTCAGCA AGAGAAGTAT TAAACTTAAT AAGTTTAGGA
 161 TTTTTTGAAC CCTTTGGATT TTGTTCACTA AAGAATATAT TTGGAAGAGT TAAATAAGTG TTTTCTAGGT
 241 TAAAACCTGT TTTTGATTGA AAATTTTTAT TATCCATATT AAAATCCTTT GCCTTATAAT TTATTTCAAA
 321 TTTTATTCCA TCCCTTATAT TATGCGTAAA AATTCTTATT AAATTAAAAA ACAATATTTA ACTTATTATA
 401 GCACTAATAA TTGTAAATTT TCATATTAAA AATAAGTTTA ACAATTTAGA GTGGGTAAGG TTAGATGTGT
 481 TTAATTGAAA TTTGAATTGT ATTCAAAAAT ATTTTAAAAA ATATTCAAAA ATTTAGTGAG CTTATGGTAA
 561 TTATATGGTA TAATTTCAGT GCAAGTGTTA ATCGTTATCA AAAAAGGGGA GATTAATACT TGAAAAAAAT
 641 TAACGGGGGA TATAAAAATG AAAAGAAAGA TTTGTAAGGC ACTTATTTGT GCTACGCTAG CAACTAGCCT
 721 ATGGGCTGGG GCATCAACTA AAGTCTACGC TTGGGATGGA AAAATTGATG GAACAGGAAC TCATGCTATG
 801 ATTGTAACTC AAGGGGTTTC AATCTTAGAA AATGATCTGT CCAAAAATGA ACCAGAAAGT GTAAGAAAAA
 881 ACTTAGAGAT TTTAAAAGAG AACATGCATG AGCTTCAATT AGGTTCTACT TATCCAGATT ATGATAAGAA
 961 TGCATATGAT CTATATCAAG ATCATTTCTG GGATCCTGAT ACAGATAATA ATTTCTCAAA GGATAATAGT
1041 TGGTATTTAG CTTATTCTAT ACCTGACACA GGGGAATCAC AAATAAGAAA ATTTTCAGCA TTAGCTAGAT
1121 ATGAATGGCA AAGAGGAAAC TATAAACAAG CTACATTCTA TCTTGGAGAG GCTATGCACT ATTTTGGAGA
1201 TATAGATACT CCATATCATC CTGCTAATGT TACTGCAGTT GATAGCGCAG GACATGTTAA GTTTGAGACT
1281 TTTGCAGAGG AAAGAAAAGA ACAGTATAAA ATAAACACCG CAGGTTGCAA AACTAATGAG GATTTTTATG
1361 CTAATATCTT AAAAAACAAA GATTTTAATT CATGGTCAAA AGAATATGCA AGAGGTTTTG CTAAAACAGG
1441 AAAATCAATA TACTATAGTC ATGCTAGCAT GAGTCATAGT TGGGATGATT GGGACTATGC AGCAAAGGTA
1521 ACTTTAGCTA ACTCTCAAAA AGGAACAGCA GGATATATTT ATAGATTCTT ACACGATGTA TCAGAGGGTA
1601 ATGATCCATC AGTTGGAAAG AATGTAAAAG AACTAGTAGC TTACATATCA ACTAGTGGTG AAAAAGATGC
1681 TGGAACAGAT GACTACATGT ATTTTGGAAT CAAAACAAAG GATGGAAAAA CTCAAGAATG GGAAATGGAC
1761 AACCCAGGAA ATGATTTTAT GACTGGAAGT AAAGATACTT ATACTTTCAA ATTAAAAGAT GAAAATCTAA
1841 AAATTGATGA TATACAAAAT ATGTGGATTA GAAAAAGAAA ATATACAGCA TTCCCAGATG CTTATAAGCC
1921 AGAAAACATA AAGGTAATAG CAAATGGAAA AGTTGTAGTG GACAAGGATA TAAATGAGTG GATTTCAGGA
2001 AATTCAACTT ATAATATAAA ATAATAATAA TAAAAAATA ATTATTGGTT TTGGTGGTAT TTACAAAATA
2081 AAAGCTTAGG AAAGATAAAG TCTTTCTTAA GCTTTTATTT TACTTATTTT TAGTGATTTA GGGATTATTA
2161 CTTTAAGTAA TAATCCTTTT GTTTTAAGAA TATAATCAAT AAGAATATGG TTTCTATATT AGGAGGATTA
2241 AATGAAAGTA GATATAATTT CAGGATTCCT AGGAGCTGGC AAAACTA
```

Figure 10: **Nucleotide sequence of the alpha-toxin gene region**. ORF in boldface beginning with the translational start codon (red ATG) and ending with the stop codon (red TAA). Sample n° 2 (Torgau). Underlined nucleotides: regulatory sequences.

The total PCR product is 2287 bp long and includes an open reading frame (ORF) of 1194 nt which encodes for a protein of 45 KDa. The ORF starts with the ATG codon at position 657-59 (red marked) and ends with the TAA codon at position 2022-24 (red marked). The

stop codon (TAA) is followed by two consecutive TAA stop codons (positions 2025-2027 and 2031-2033 respectively).

Although the main aim of this work is to get informations about the amino acid sequence, analysis at the nucleotide level can help to understand the pattern of protein expression.

In the upstream region, the five consecutive G residues, located 9 bases upstream of the ATG codon, could form a hybrid with the 3' end of the *E. coli* 16S rRNA subunit thus forming a putative Shine-Dalgarno sequence necessary to position the following AUG of the mRNA exactly into the ribosomal P-site. 46 nt upstream of the proposed Shine-Dalgarno region there is the sequence, TATAAT (position 568-73, underlined), probably forming one of the two elements of the *C. perfringens* promoter (the so-called "Pribnow box"). The second bacterial promoter element, TGAGC (position 527-531, underlined), is probably located 17 bases further upstream, corresponding to the so-called –35 consensus recognition sequence, typically found in *Escherichia coli* promoters. Although both promoter elements seem to be present in the upstream region of the alpha-toxin gene, their distance relative to the ATG start codon is different compared to other bacterial promoters.

Further upstream of the putative –35-box equivalent of the promoter there is a TA-rich, tandemly repeated sequence, TATTCAAAAAT (position 500-10 and 522-32, underlined) which may play a role in the expression of the alpha-toxin gene.

Without the presence of the two additional translational TAA stop codons no other regulatory sequences can be detected in the downstream part of the gene

In order to see if all the regulatory sequences are maintained in all the 71 samples, a comparison of the upper flanking region and of the downstream surrounding of the gene was made (see attachments 1,2).

Concerning the upper region, as is shown, only the Shine-Dalgarno (five Gs) and the so called "TATA box" (TATAATT) are always maintained. Other promoterlike sequences present mutations: in the TGAGC the last C is often a G (23 samples). In the first tandem repeat (TATTCAAAAAT) samples n°69 (Karlsruhe) and n° 60 (Ehrenberg) contain a C instead of an A, and only in sample n°60 the fifth A is substituted with a T. Concerning the second tandem repeat there are five samples in which an A is substituted with a T, and in two samples, n°69 and n°60, the last A is substitut ed with a G.

The termination codons are conserved, except for 7 samples in which the second stop codon, TAA, is replaced with TGA.

3.2.2 Amino acid sequence

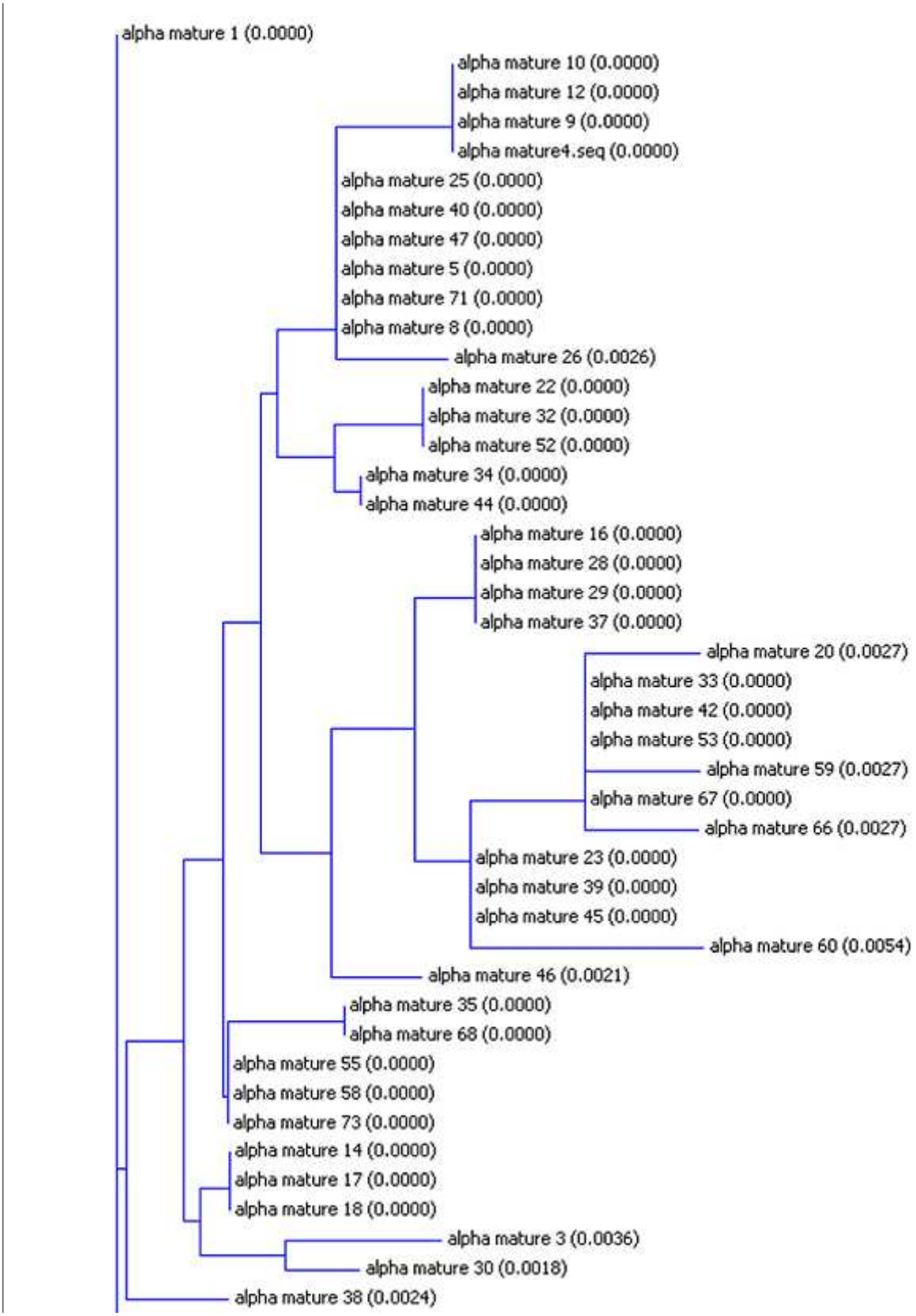
From the nucleotide seqence the amino acid one was deduced. In fig. 11 the amino acid sequence of sample n°2 (Torgau) is shown.

```
  1 MKRKICKALI CATLATSLWA GASTKVYAWD GKIDGTGTHA MIVTQGVSIL ENDLSKNEPE SVRKNLEILK ENMHELQLGS
 81 TYPDYDKNAY DLYQDHFWDP DTDNNFSKDN SWYLAYSIPD TGESQIRKFS ALARYEWQRG NYKQATFYLG EAMHYFGDID
161 TPYHPANVTA VDSAGHVKFE TFAEERKEQY KINTAGCKTN EDFYADILKN KDFNAWSKEY ARGFAKTGKS IYYSHASMSH
241 SWDDWDYAAK VTLANSQKGT AGYIYRFLHD VSEGNDPSVG KNVKELVAYI STSGEKDAGT DDYMYFGIKT KDGKTQEWEM
321 DNPGNDFMTG SKDTYTFKLK DENLKIDDIQ NMWIRKRKYT AFPDAYKPEN IKIIANGKVV VDKDINEWIS GNSTYNIK
```

Figure 11: **Alpha amino acid sequence**. The first 28 amino acids (bold, underlined) represent the putative signal peptide; the mature protein starts with the red marked amino acid, (W) at position 29.The underlined region (position 243-51) form the so called flexible linker region.

The total sequence is 398 amino acids long, the first 28 amino acid forming the signal sequence necessary to transport the alpha-toxin across the cell membrane.

The comparison between the mature deduced amino acid sequences of all 71 samples, allow the setting up of a phylogenic tree (Fig. 12). As the picture shows the samples cluster in 25 different groups, the members of each group being identical. The biggest one contains 16 samples while all the other groups are smaller (some represent even only one strain). In such trees the distance to the right is an index of difference. In order to get an impression on the maximal size of sequence differences two samples, n°1 (Regensburg) and sample n°60 (Ehrenberg) which, according to the phylogenic tree, represent the most distant ones, are compared in fig.14.


alpha mature 1 (0.0000)
alpha mature 10 (0.0000)
alpha mature 12 (0.0000)
alpha mature 9 (0.0000)
alpha mature4.seq (0.0000)
alpha mature 25 (0.0000)
alpha mature 40 (0.0000)
alpha mature 47 (0.0000)
alpha mature 5 (0.0000)
alpha mature 71 (0.0000)
alpha mature 8 (0.0000)
alpha mature 26 (0.0026)
alpha mature 22 (0.0000)
alpha mature 32 (0.0000)
alpha mature 52 (0.0000)
alpha mature 34 (0.0000)
alpha mature 44 (0.0000)
alpha mature 16 (0.0000)
alpha mature 28 (0.0000)
alpha mature 29 (0.0000)
alpha mature 37 (0.0000)
alpha mature 20 (0.0027)
alpha mature 33 (0.0000)
alpha mature 42 (0.0000)
alpha mature 53 (0.0000)
alpha mature 59 (0.0027)
alpha mature 67 (0.0000)
alpha mature 66 (0.0027)
alpha mature 23 (0.0000)
alpha mature 39 (0.0000)
alpha mature 45 (0.0000)
alpha mature 60 (0.0054)
alpha mature 46 (0.0021)
alpha mature 35 (0.0000)
alpha mature 68 (0.0000)
alpha mature 55 (0.0000)
alpha mature 58 (0.0000)
alpha mature 73 (0.0000)
alpha mature 14 (0.0000)
alpha mature 17 (0.0000)
alpha mature 18 (0.0000)
alpha mature 3 (0.0036)
alpha mature 30 (0.0018)
alpha mature 38 (0.0024)

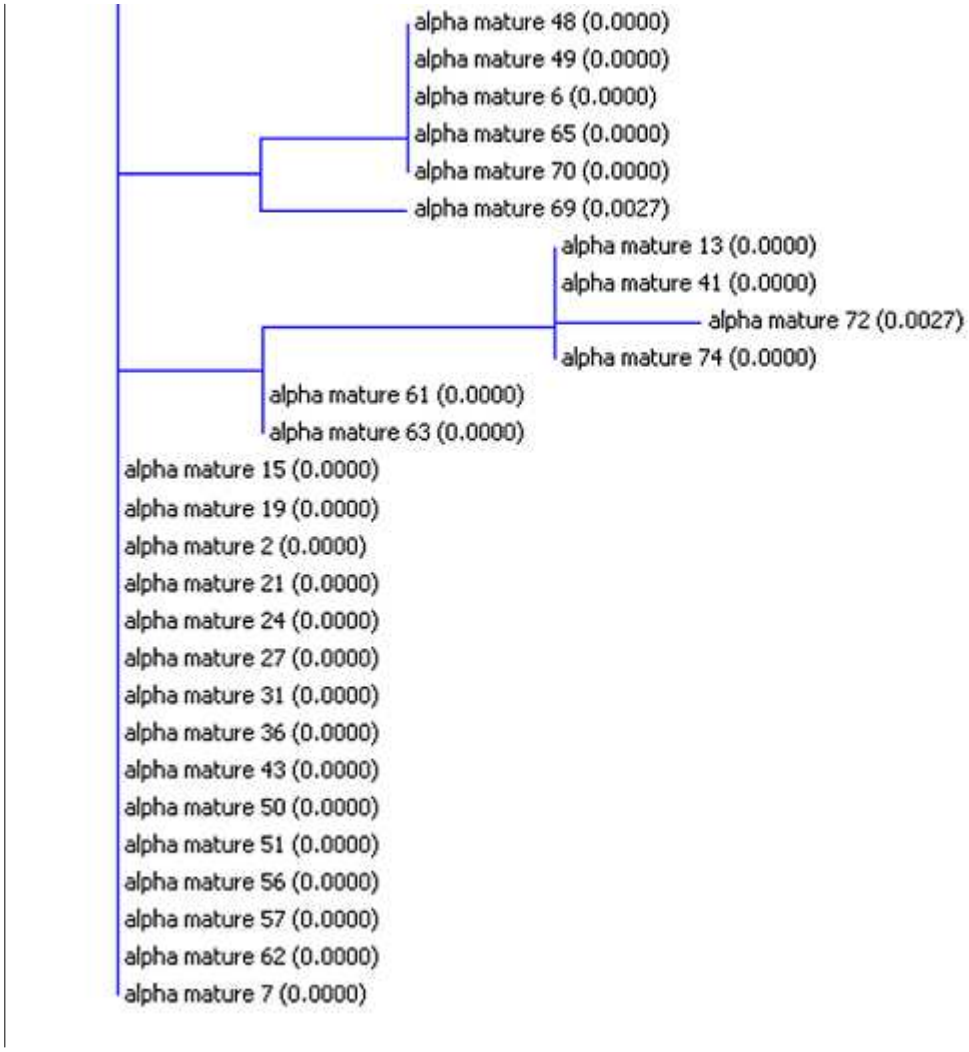


Fig 12: **Phylogenic tree**. The tree was set up considering all 71 samples. There are 25 groups.

sample 1	1	WDGKIDGTGTHAMIVTQGVSILENDLSKNEPESVRKNLEILKENMHELQLGSTYPDYDKNAYDLYQDHFW
sample 60		WDGKIDGTGTHAMIVTQGISILENDLSKNEPESVRKNLEILKENMHELQLGSTYPDYDKNAYDLYQDHFW
sample 1	71	DPDTDNNFSKDNSWYLAYSIPDTGESQIRKFSALARYEWQRGNYKQATFYLGEAMHYFGDIDTPYHPANV
sample 60		DPDTDNNFSKDNSWYLAYSIPDTGESQIRKFSALARYEWQRGNYKQATFYLGEAMHYFGDIDTPYHPANI
sample 1	141	TAVDSAGHVKFETFAEERKEQYKINTAGCKTNEDFYANILKNKDFNSWSKEYARGFAKTGKSIYYSHASM
sample 60		TAVDSAGHVKFETFAEERKEQYKINTAGCKTNEAFYADILKNKDFNAWSKEYARGFAKTGKSIHYSHASM
sample 1	211	SHSWDDWDYAAKVTLANSQKGTAGYIYRFLHDVSEGNDPSVGKNVKELVAYISTSGEKDAGTDDYMYFGI
sample 60		SHSWDDWDYAAKVTLANSQKGTAGYIYRFLHDVSEGNDPSVGKNVKELVAYISTSGEKDAGTDDYMYFGI
sample 1	281	KTKDGKTQEWEMDNPGNDFMTGSKDTYTFKLKDENLKIDDIQNMWIRKRKYTAFPDAYKPENIKIIANGK
sample 60		KTKDGKTQEWEMDNPGNDFMTGSKDTYTFKLKDENLKIDDIQNMWIRKRKYTAFPDAYKPENIKVIANGK
sample 1	351	VVVDKDINEWISGNSTYNIK
sample 60		VVVDKDINEWISGNSTYNIK

Figure 13: **Amino acid sequence comparison of sample n° 1 (Regensburg) and sample n° 60 (Ehrenberg)**. The comparison is restricted to the mature protein.

The comparison shows that the two proteins differ only in 5 amino acids with 3 I/V replacements which are considered as conservative ones because these 2 amino acids are very similar in structure. Besides that fig. 14 is representative for the general distribution of the location of the sequence differences within the alpha-toxin. Comparing all 71 samples (see attachment 3) a clustering of the amino acid exchanges can be observed (fig. 14).

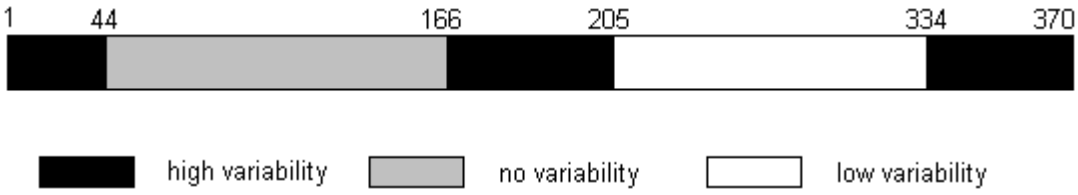


Figure 14: Location of amino acid differences within the alpha-toxin. Only the mature proteins are compared.

Most differences are found within the N-terminal 50 amino acids, a stretch of 40 aa in the middle and near the C-terminus. However, as mentioned previously, these as highly variable designated parts do not contain many amino acid exchanges. This is even more true for the so-called low variability region between positions 206 and 333. Here, within all of the 71 samples, only two display amino acid differences: sample n°20 (Düren), position 324: glutamic acid instead of glycine (Gly→Glu) and sample n° 35 (Dittmannsdorf), position 277: serine instead of glycine (Gly → Ser). Between positions 45 and 165 there exists a stretch of 120 conserved amino acids. Taken together, these data indicate that the alpha-toxin is a protein which in most of its amino acids is conserved.

3.3 Beta2-toxin genes

In order to get the beta2-toxin gene sequences a similar strategy as for the alpha-toxin gene sequences was followed. At the beginning of this work only 1 complete beta2-toxin gene sequence together with upstream and downstream parts was available in the database (Gilbert et al., 1997). From this sequence (L77965) 2 external primers were selected (Pbe2.2 for and rev) and were tested on sample n° 2 (Torgau). Several different parameter combinations were tested (i.e., different dilution, annealing temperature, cycle numbers). All results were negative. This means that none of these primer annealed successfully on our sample. In order to see if at least one of them works, the 2 primers were tested in combination with the successfully annealing internal ones (see fig. 7)

No other than the “internal” primer combination worked (fig.15 lane 4).

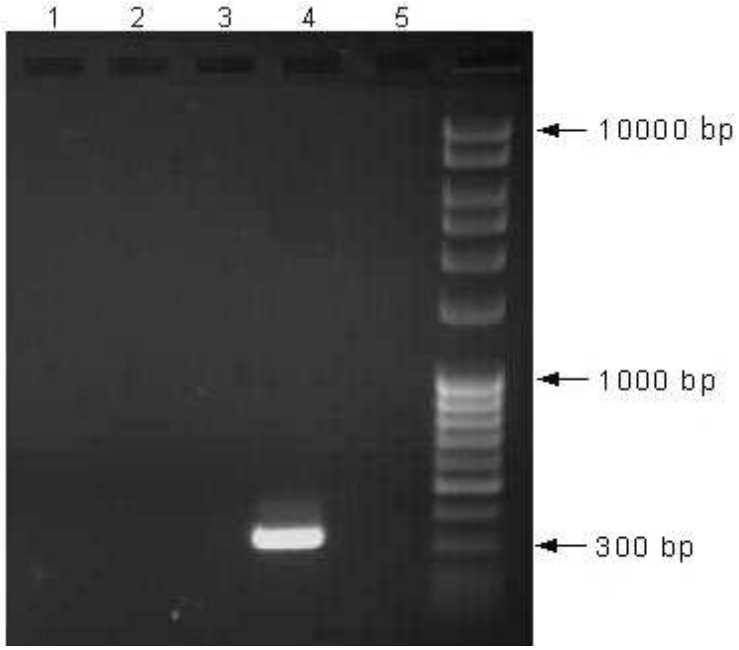


Figure 15: **Gel analysis of different primer pair combinations (beta2-toxin)**. Line 1: primer pair Pbe2.1 For and Pbe2.2 Rev; line 2: primer pair Pbe2.2 For and Pbe2.1 Rev; line 3: primer pair Pbe2.2 for and rev; line 4: primer pair Pbe2.1 for and rev; line 5: negative sample. **Marker**: 1kb DNA ladder.

From these data it had to be concluded that the ß2-gene of isolate 2 was embedded in a different surrounding than the L77965 gene.

3.3.1 “Porcine” and “non-porcine” beta2-toxin genes

Due to the negative results obtained with the external primer pair and, because the internal primer pair combination (Pbe2.1 for and rev) gave positive results, it was decided to sequence this part of the gene. The sequence obtained was used as a reference for a blast search in the nucleotide database, resulting in the fact that there two different kinds of beta2-toxin genes exist. One was the so called “porcine”-type (Accession Nr. L77965) from porcine *C. perfringens* isolates and the other, called “non porcine”-type (Accession Nr. AB236337) from non porcine *C. perfringens* isolates. The comparison between “porcine” and non “porcine” beta2-genes and sample n°2 (Torgau) revealed that it belongs to the “non porcine” group (data not shown). As the primers Pbe2.2 for and rev, were selected from the “porcine” flanking region (L77965) it seemed now clear why the PCR results were negative. From the “non porcine”-type beta2-group only the coding part of the gene sequence was delivered to the database. Therefore, no upstream and downstream sequences were available for primer selection. Therefore, suitable primers had to be found.

3.3.2 ”non porcine”-beta2 toxin genes

At the beginning of 2006 the total sequence of a *C. perfringens* plasmid from a human isolate (plasmid pCPF4013) containing the “non porcine” beta2-toxin gene was published. From this sequence a new external primer pair, Pbe2.4, for and rev, was selected and tested on sample n°2 (Torgau). The expected product should be 1654 bp long. Results were positive (fig. 16).

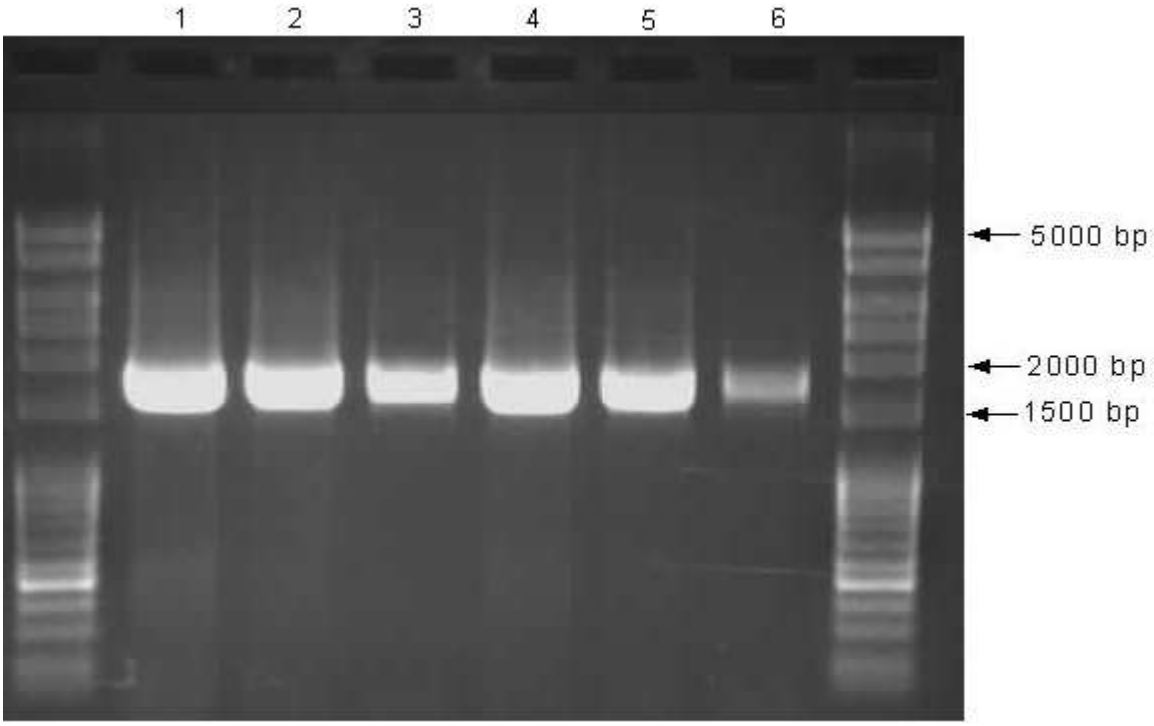


Figure 16: **Pbe2.4, for and rev, primer test**. Line 1-6: different sample (sample n°2, Torgau) concentrations (1.5 ng; 3 ng; 4.5 ng; 7.5 ng; 10.5 ng; 15 ng). **Marker**: 1kb DNA ladder

Since the PCR results were now positive the new primer pair was tested with all 25 samples containing the beta2-toxin gene. Unexpectedly only the following 9 samples were positive:

sample 2	Torgau	sample 49	Neucharell
sample 14	Laichtlingen	sample 50	Essen
sample 20	Düren	sample 55	Haigerloch
sample 26	Hamm	sample 57	Sulzfeld
sample 46	Zeulenroda		

Despite the lack of information on the other 16 samples it was first decided to sequence these 9 beta2-toxin genes. Unfortunately, due to sequencing problems only for 5 of them (n°s 2, 14, 20, 26, 46) the complete sequence could be determined. As an example follows the nucleotide sequence of sample n°2 (Torgau):

```
   1 GTTACGAAAG TTTATAATAC TTCAAATATA AAGAGAGGAG ATATTTTAGT CTTTAATTCT GGTTAATATA
  71 TAAGGTTAAT ATTTTTTATT AACCTTATTT TTTCTTGCAG AATATAAAAC CCAATTAATA TTACTTATAA
 141 TAACTAATAT TATAAGTAAA GGAATCCATA AAAATTTAGG AATCATTATT AACATAATAA ATCCTATAAC
 211 CCAAATTATT ATTGAATATA TCAAATATCT AAGAAATAGT TTCAAATAAT CACCTACTTA AAATTAAATT
 281 TATTATTAAT TTAATTAATC TAAAAATCAA CCTTTCTAAT ATTATAATCT TCCTTTTGAT AAATTAACAT
 351 AATTCTAGAT AATAATTCTT AAAAAGTATC TTTATTTAAA AAATGTTAAA AATTTGATA TA TAAT AGAATTG
 421 TAAAAAAATT CA GGGGGG AA ACCAAATGAA AAAATTAATA GTAAAAAGTA CAATGATGC TTTTATTTTCT
 491 TGCTTATTAT GTTTAGGAAT ACAGTTACCT AATACAGTTA AAGCAAATGA AGTGAATAA ATACCAATCTG
 561 TAATGGTACA GTATTTAGAA GCTTTTAAAA ATTATGATAT TGATACGATA GTAGATATT TCTAAAGATAG
 631 TAGAGCTGTT ACTAAAGAAG AATATAAAAA CATGTTAATG GAATTTAAAT ATGATCCTA ACCAAAAACTT
 701 AAATCATATG AAATAACAGG TTCAAGAAAA ATTGATAATG GCGAAATTTT TTCTGTGAA AACAGAGTTTT
 771 TAAATGGTGC TATATACAAT ATGGAATTTA CAGTATCATA TATTGATAAT AAATTAATG GTAAGTAATAT
 841 GAATAGAATA TCAATAGTAA ATGAAGGTAA ATGTATTCCT ACACCAAGTT TCAGAACTC AAGTTTGTACA
 911 TGGGATGACG AATTAAGTCA ATATATTGGA GACGCTGTTA GTTTTACACG TTCTAGTAA ATTTCAATATA
 981 GTTCTAATAC GATTACATTA AACTTTAGAC AATATGCAAC TTCTGGATCA AGATCCTTA AAGGTAAAATA
1051 CAGTGTAGTA GACCATTGGA TGTGGGGGGA TGACATTAGA GCTTCTCAAT GGGTATATG GTGAAAATCCG
1121 GATTATGCTA GACAGATAAA ATTATATCTA GGTTCAGGAG AAACTTTCAA AAATTATAG AATTAAAGTAG
1191 AAAATTATAC TCCAGCATCG ATTAAAGTAT TTGGTGAGGG TTATTGTTAT TAA AAAAAT GAGGC TAC GCC
1261 TCATTTTT TC TTTTGATTTT TAATATTTTA ATAATATAAA AACCTAGAAT GGATGATAT TATTCTAAAAA
1331 TTGTTAAATC AAAATTAAAA GAATAAAAAT GTAACAATGA AATAATAACA TTAAATAAT AAAAAAATAAT
1401 TAAACTTGGT TTTAGAGAAT TATATTTATT TATAACAATA GGTATTAAAA TACCAAAAG GTATAAATAAT
1471 AATAGAATAA ATAATTGATC TAATACAAAT ACATTATCTA ATAACGATTT ATTACTATT AAATAAATTAT
1541 ATAAAAATTT AAAAGGTAAA TATGAACCTT TTGATAAATA GCCACCACCA TT
```

Figure 17: **ß2-toxin gene sequence of n°2 (Torgau)** Complete beta2-toxin gene (boldface) plus flanking region. ATG red marked: start codon; TAA red marked: stop codon. Underlined nucleotides: putative regulatory sequences.

The total gene is 795 nt long and encodes for a protein of 30 KDa. The open reading frame (ORF) starts with the ATG codon at position 494-496 (red marked) and ends with the TAA codon at position 1289-91 (red marked).

As for the alpha-toxin-gene flanking region, the sequence was examined for regulatory sequences: 7 bp in front of the start codon the ribosome binding site (RBS), GGGGGG (position 48-53) is present. 35 bp upstream the typical “Pribnow”-box sequence TATAAT is found. Regarding the downstream part, immediately behind the stop codon, an inverted repeat, position 1292-1303/1305-1317 (underlined), followed by few T nucleotides is located. This situation is typical for a Rho independent transcription termination.

The beta2 upstream- and downstream-flanking regions were compared (see attachments 4,5). They both show nearly any variability with some exception in sample n°20 (Düren).

Fig. 18 displays the delineated amino acid sequence of the Torgau ß2-toxin:

```
  1 MKKLIVKSTM MLLFSCLLCL GIQLPNTVKA NEVNKYQSVM VQYLEAFKNY DIDTIVDISK DSRAVTKEEY
 71 KNMLMEFKYD PNQKLKSYEI TGSRKIDNGE IFSVKTEFLN GAIYNMEFTV SYIDNKLMVS NMNRISIVNE
141 GKCIPTPSFR TQVCTWDDEL SQYIGDAVSF TRSSKFQYSS NTITLNFRQY ATSGSRSLKV KYSVVDHWMW
211 GDDIRASQWV YGENPDYARQ IKLYLGSGET FKNYRIKVEN YTPASIKVFG EGYCY
```

Figure 18: **Complete amino acid sequence of the beta2-toxin (Torgau).** Aspargine red marked: start of the mature protein.

The first 30 amino acids represent a putative signal peptide which likely is cotranslationally removed. Therefore the mature protein is 235 amino acids long. The mature proteins of the five “non porcine” samples were compared (see attachment 6).
The amino acid sequence variability, at least in the 5 samples available, is extremely low. Only at 4 of the 235 sites differences exist. Two of them represent conservative exchanges (valine, V versus isoleucine, I at position 10 and glutamic acid, E, versus aspartic acid, D at position 30 respectively). There remains, strictly speaking, only 1 strong difference, i. e. tyrosine, Y versus cysteine, C at position 114. Asparagine, N and aspartic acid, D at position 46 are related to each other. Three of the 5 samples, i.e. n°2 (Torgau), n°14 (Laichtlingen) and n°46 (Zeulenroda) are ident ical.

3.3.3 ”porcine” beta2-toxin genes

Since the first selected primer pair, Pbe2.2, for and rev, was delineated from sequence L77965, a member of the “porcine” group and, because there were samples clearly not belonging to the “non porcine” group, it was decided to try these primers on the remaining 16 samples. Unexpectedly the PCR results were negative.
In order to see if both or only one of these primers did not anneal correctly internal/external primer combinations, similar as at the beginning of the previous chapter were used.

The PCR product were verified on 1% agarose gel (fig. 19).

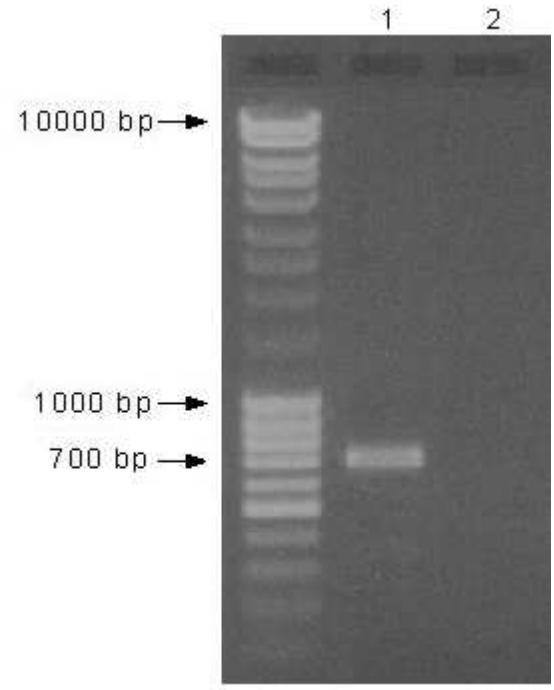


Figure 19: **Primer test on porcine sample (n°3, Borkheide)**. Line 1 Pbe2.1 For and Pbe2.2 Rev; line 2: Pbe2.2 For and Pbe2.1 Rev. **Marker**: MassRuler DNA Ladder Mix.

From fig. 19 it is clear that only the external primer Pbe2.2 rev, worked and resulted in a PCR product of approx. 700 bp, yielding approx. 350 downstream nucleotides. Despite of this success the upstream region of the “porcine”-type beta2-gene and the complete ORF was still not available. Assuming that the “porcine”-type beta2-toxin genes are similarly well conserved as the “non porcine”-type we decided to select from L77965 the gene-start and –end sequences as basis of a new “porcine”-type PCR primer pair (primers Pbe2.4for and Pbe2.4rev). These new primer pair was tested on sample n°3 (Borkheide). The expected product of 759 bp was obtained, subsequently sequenced and analyzed. An ORF was obtained encoding a 253 amino acid long protein.

Despite this positive result, the situation was still not satisfying for two reasons. First, the upstream sequences with their regulatory elements were still not available. Second, the start and end primers were taken from L77965 and do not necessarily represent the actual sequences of **our** “porcine”-type beta2 genes. Therefore, we tried to determine upstream parts of **our** “porcine”-type beta2-genes experimentally.

3.3.4 Inverse PCR

Inverse PCR is a method allowing to determinate unknown sequences starting from a known one.

Normally, the template DNA is treated with a restriction enzyme cutting outside the gene of interest. The only enzyme suitable in our case was AccI, which however did not work. As we already had information on the downstream flanking-gene region and we only needed the upstream part we modified the standard inverse PCR procedure as follows. Instead of using an “outside-gene”-cutting restriction enzyme we treated our template DNA with HindIII which has a single cutting site within the known beta2-gene sequence. The modified inverse PCR procedure is illustrated in fig. 20. After HindIII restriction and circular ligation a nested PCR was performed with primer pairs of known parts of the gene (black and grey arrows in fig. 20). After the second PCR a product should be obtained, containing pieces of the known sequence (white area in fig. 20) and unknown upstream sequences (grey area in fig. 20)

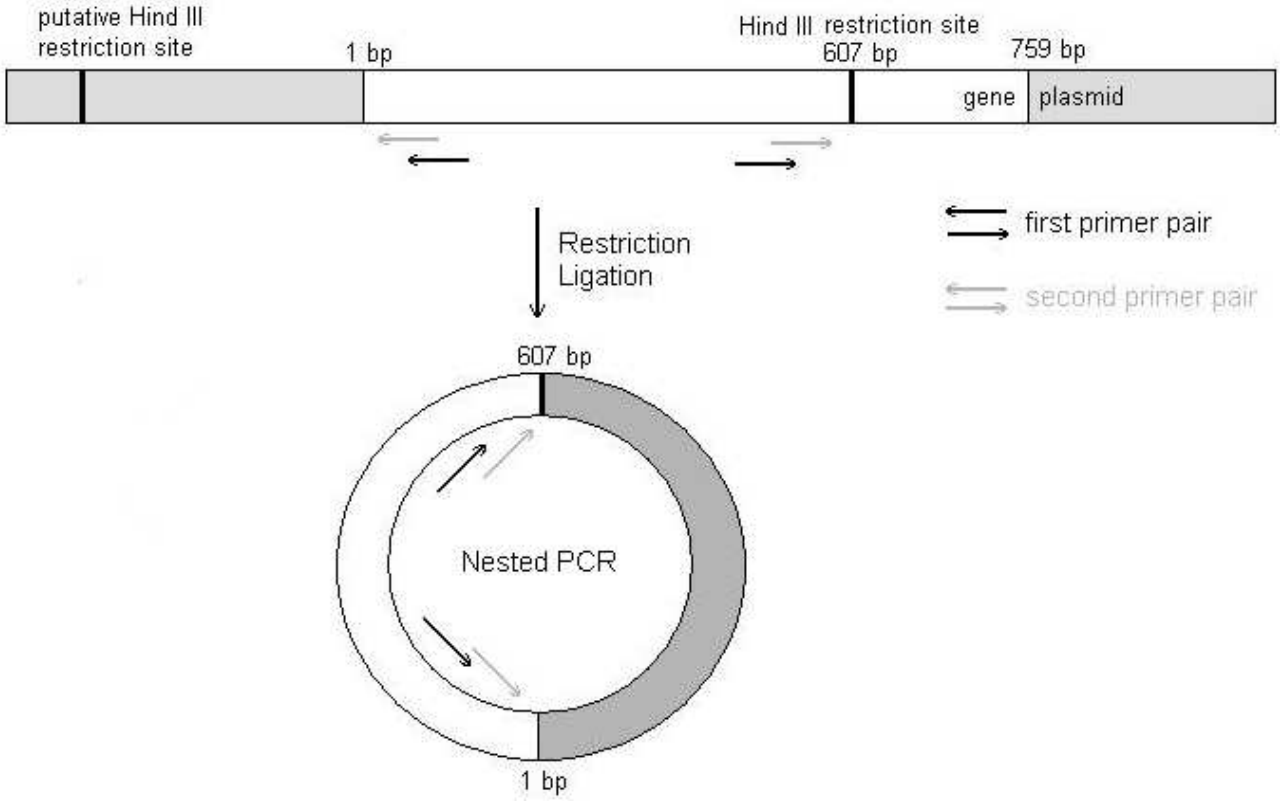


Figure 20: **Inverse PCR**. For the nested PCR two set of primer were used: 1°primer (PPCR1RC and PPCR1RC-1) and 2°primer (PPCR2RC and PPCR2RC-2)

After the second PCR we got a weak band of approx. 300 bp on the agarose gel (fig. 21) and we decided to sequence it directly.

Probably due to the low amount of PCR product available, the sequencing gel was not good with roughly 200 nt readable approx. 100 of them being unknown upstream ones (fig. 22).

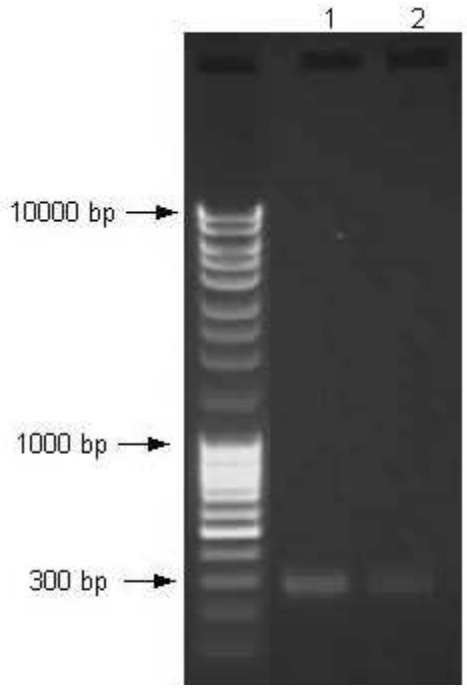


Fig. 21: **Gel analysis of Inverse PCR**. Different applied quantities. Line 1: 10 µl. Line 2: 5 µl.

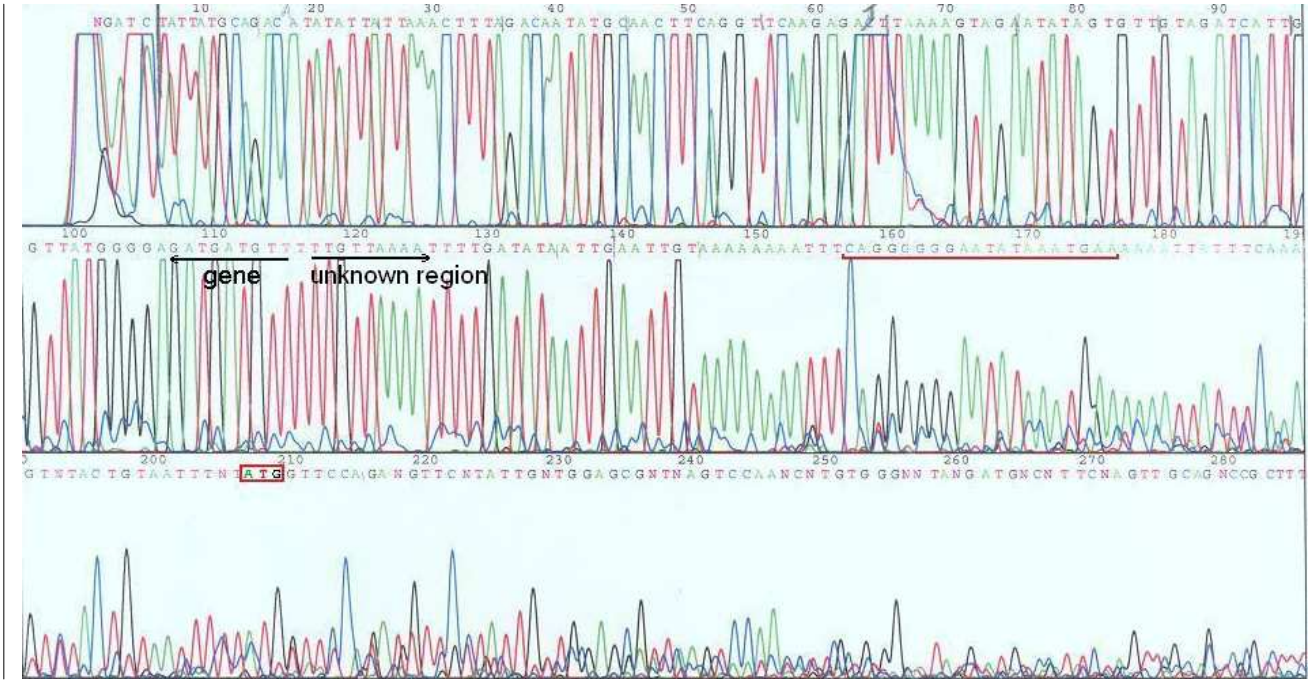


Fig 22: **Sequencing gel of the 2° PCR product** . The gel is not good but is possible define the gene region and the unknown one (black arrows). Red box: gene start ATG codon. Red line: selected new primer.

From them we selected a new PCR primer, Pbe2.5 (red underlined in fig. 22) and tested it in combination with the corresponding downstream one , Pbe2.2 rev. As f. 23 shows the theoretically expected product of 1170 bp is present. Sequencing of this product however, revealed that primer Pbe2.5 is still too close to the start codon (first readable nucleotide on the sequencing gel is approx. 50 nt distant from the primer). Then the primer sequence Pbe2.5 was used as a reference for a “BLAST”-search in the genebank database. The result was surprising: it fits with two different sequences:

- AP003515 (cCP13), from which the first external primer pair was selected (Pbe2.2)
- L77965 from which the start and end primers for the “porcine” group were selected (Pbe2.3)

This result was unexpected as both primer Pbe2.2 and primer Pbe2.3 did not work in experiments described previously. Therefore, the two sequences were compared (fig. 24).

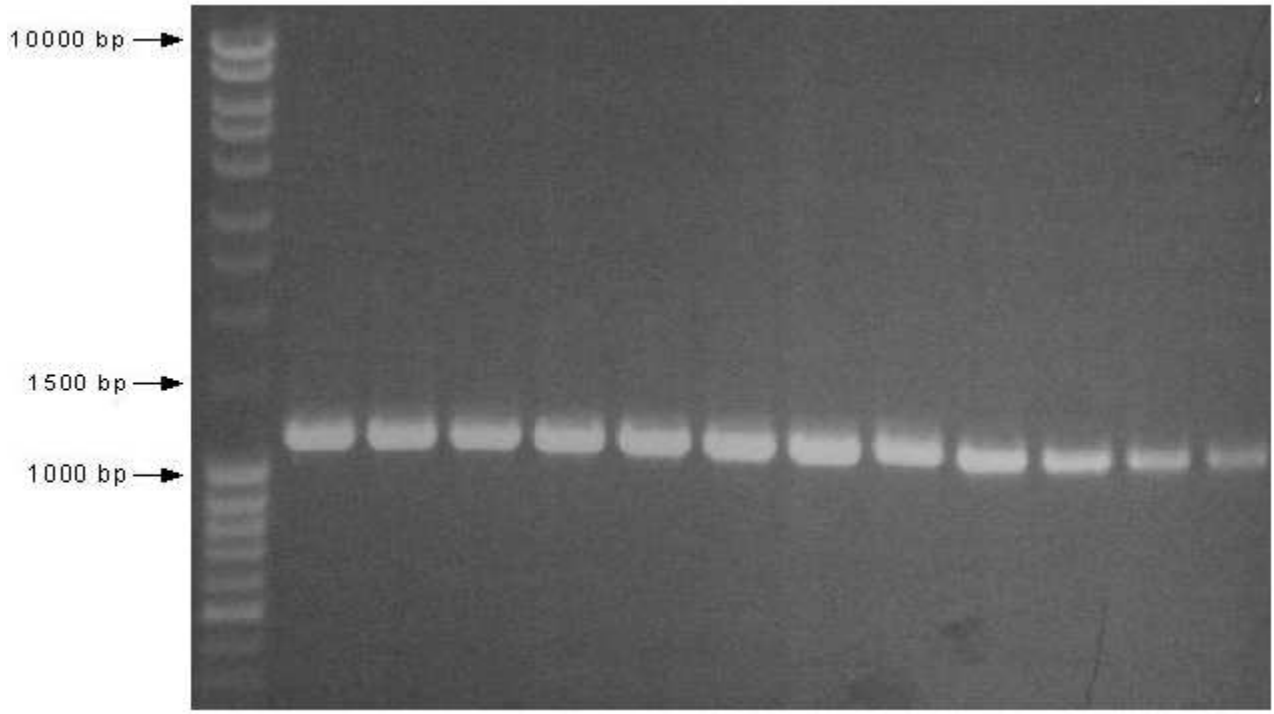


Figure 23: **Test with new primer**. T-cycle of sample n°3 (Borkheide) using the new primer Pbe2.5 and pbe2.2 rev. (T= 40, 40.5, 41.9, 44, 46.4, 48.8, 51.2, 53.6, 55.9, 58.1, 59.5, 60°C). **Marker**: MassRuler DNA Ladder Mix.

```
cp13     ATTTATTATTGATTTAATTTTCCATTATAGTGCTAGTTATTTTTACTATTATAAAGTTTAATAATTTACATTGTTTAA
L77965   ATTTGGGATATCTTAAATTTAGCAC-AGAAG--AAT---GTTTAAATGAAATAAAGAT-AATAAAA-AGATATATTAA

cp13     ACTAATTGCTAAATAGAATTTTAACTCATGTTTTAAAAGTTAAATATATATTTTAAATTTAAGGTGTCCCACGGGGAC
L77965   TTATATAGCTGAA---AATTTATAATTTATAT-------GATAAGTATAGTTAATAAATAAAAGTGTTCTCGGGGGAC

cp13     ATCTTTTTGTTTTTAAAAGGTAAATATGAATAAAATTTAGATAAAAGTGTAAAAGAATTATTTTTATTTTAAATTTGT
L77965   ACTTTTTTGTTTTAAAAAGGAAAATATAAATAAAATTTAGATAAAAGTGTAAAATAATTATTTTTATTTTAAATTTGT

cp13     TAAAATTTTGATATAATTGAATTGTAAAAAAAATTTCAGGGGGGAATATAAATGAAAAAAATTA
L77965   TAAAAATTTGATATAATTGAATTGTAAAAAAAATTTCAGGGGGGAATATAAATGAAAAAAATTA
```

Figure 24: C**omparison between cCP13 and L77956**. ATG blue marked: start codon. Yellow marked: new selected primer. Black underlined: primer selected with the Inverse PCR.

The two sequences are very similar only up to 100 nt upstream the start ATG. Further upstream cp13- and L77965 sequences differ completely. This explains, why the previously designed external primers Pbe2.2 and Pbe2.3 did not work. In fig. 24 the primer selected using the Inverse PCR is black underlined: it includes the start ATG codon. It is clear that the use of this primer to sequence the gene will not help to get informations about the ORF of the “porcine” gene. It was decided to select from the cp13 sequence a new primer, Pbe2.6 (yellow marked in fig. 24), further upstream than the previous one. This one was chosen because here both sequences are still similar enough to obtain annealing in both cases. Pbe2.6 was then tested in combination with the external upstream primer, Pbe2.2 rev, and the expected product of 1263 bp was obtained and sequenced (see Fig 25).

Figure 25: **Beta2-toxin gene sequence of n°6 (Wallenhorst)** Complete beta2-toxin gene (boldface) plus flanking region. ATG red marked: start codon; TAG red marked: stop codon. Underlined nucleotides: putative regulatory sequences.

```
   1 AAATATGAAT AAAATTTAGA TAAAAGTGTA AAAGAATTAT TTTTATTTTA AATTTGTTAA AATTTTGATA
  71 TAATTGAATT GTAAAAAAAA TTTTAGGGGG GAATATAAAT GAAAAAAATT ATTTCAAAAT TTACTGTAAT
 141 TTTTATGTTT TCATGTTTTC TTATTGTGGG AGCAATAAGT CCAATGAAAG CAAGTGCAAA GGAAATCGAC
 211 ACTTATAGAG AGGTAATGGA AAATTATCTT AATGCTTTTA AAAACTACGA TATTAATACG ATTGTAAACG
 281 TATCAGAAGA TGAAAGAGTA AATAGTGATG AAAAGTATAA AGAAATGTTA GAGGAGTTCA AATATGATCC
 351 TAACCAACAA CTAAAATCTT TTGAAATACT TAATTCACAA AAGAGTGATA ATAAAGAAAT ATTTAATGTA
 421 AAAACTGAAT TTATGAATGG TGCAATTTAT GATATGAAAT TTACTGTATC ATCTAAAGAT GGAAAATTAA
 491 TAGTATCTGA CATGGAAAGA ACAAAAATTG AAAATGAGGG AAAATATATT TTAACACCAT CATTTAGAAC
 561 TCAAGTTTGT ACATGGGATG ATGAATTATC ACAATCAATT GGGGGAGTTG ATCCAAAAAC ATATTCTACT
 631 AGATTTACAT ATTATGCAGA CAATATATTA TTAAACTTTA GACAATATGC AACTTCAGGT TCAAGAGATT
 701 TAAAAGTAGA ATATAGTGTT GTAGATCATT GGGTATGGGG AGATGATGTT AAAGCTTCTC AAATGGTATA
 771 TGGTCAAAAC CCTGATTCTG CTAGACAAAT AAGATTATAT ATAGAAAAAG GACAATCTTT CTATAAATAT
 841 AGAATAAGAA TACAAAACTT TACACCTGCA TCAATTAAAG TATTTGGTGA AGGATATTGT GCATAGAAAA
 911 AAATATGAAG TGACTTAGTC ACTTCATATT TTTTTTACTA TTAATTTTAT TATATAAAAA CCTAACACAC
 981 ATGAAAGTAT TCTTAATATA GTTATATCAA AATTAAAGTA TGGGAAATAA AATAAAAGGC TAAAAACTAT
1051 ATTAAAAACT ATAAAAATTA TTAAATTAGG TTTTAAGTTG TTATATCTAT TTATGATTAT AGGAATAAAT
1121 ATTCCAAATG GAATAAATAA AAGCAATATT AATAATTGGT CTAAAAAGTA TACATCATTG ATAAAAGAAA
1191 AATTACCAGT AAAAATTGAG CTTAAAAAAT TAAATGAAAA TTTAAAGGGG ATGAAGCTCC CATGTAAAAG
1261 TGGACT
```

Similar as for the alpha-toxin- and the “non-porcine” beta2-toxin genes the nucleotide sequences were examined for regulatory elements. Concerning the upstream region there can be found in both beta2-toxin gene types both the ribosomal binding site (RBS) (stretch of 5Gs at position –7 to the start codon) and the “Pribnow box” (TATAAT at position –40 relative to the start codon). Analysis of the downstream region revealed, similar to the non-porcine beta2-toxin gene, the presence, immediately after the stop codon TAG, of an inverted repeat, these sequences being, however a little different from each other (attachments 7,8).

All samples, besides n° 6 (Wallenhorst), are out of frame. This leads to a stop signal 9 codons later (fig. 26). Theoretically, all porcine-type beta2-toxin genes, besides n°6 should not be translated (but see discussion).

```
sample3   AAATTTCAGGGGGGAATATAAATGAAAAAA-TTATTTCAAAGTTTACTGTAATTTTTTATGTTTTCAT
sample 6  AAATTTTAGGGGGGAATATAAATGAAAAAAATTATTTCAAAATTTACTGTAATTTTTTATGTTTTCAT
```

Fig 26: **Gene beginning differences within the “non porcine” group**. TAA red marked: Stop codon. ATG red marked: “new” start codon.

Therefore, a comparison between the deduced porcine amino acid sequence does not make sense. However, it had been shown, that these types of genes, despite of their mutation, can be translated under special circumstances (Vieli et al., 2005) Therefore, we nonetheless compared the delineated porcine-type beta2 toxins on their amino acid level, omitting the putative signal peptide (see attachment 9).

The phylogenic tree for this group of proteins (fig. 27) shows, of the total 16 sequences 10 are identical (n°s 8, 16, 27, 32, 34, 38, 40, 43, 7 2, 74). Four other groups exist, n°s 3/29, 60/65, 73 and 6. From fig. 27 it is clear that the most different sample is n° 6 (Wallenhorst), the one without A- deletion in the signal peptide region. Generally, as seen from the attachment 9, the amino acid differences are extremely low.

3.3.5 Comparison of Porcine- and Non porcine- type beta2-toxin sequences

Fig. 28 presents the comparison of 2 beta2-toxins of the two types. As examples are taken n°2 (Torgau) representing the nonporcine type and n°6 (Wallenhorst) representing the porcine type. Despite quite more of the amino acids are identical (red) there are still a lot of them being different (black). These differences are far more pronounced as those within each single subtype. Nevertheless, the PCR primers used for toxin typing (see Chapter 2.2.3) can detect both types of genes, because they are delineated from identical nucleotide sequence stretches.

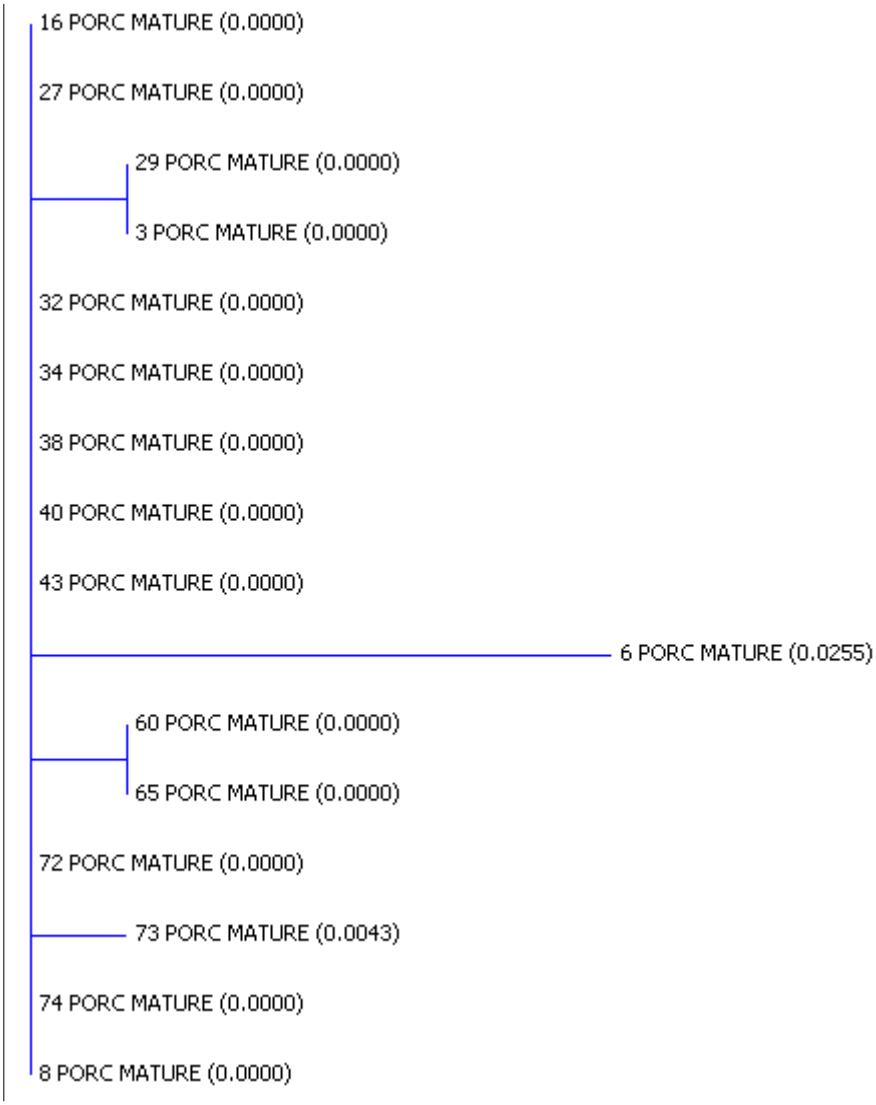


Figure 27: **Phylogenic tree of porcine ß2-toxins**. The tree was set up considering all 16 samples. There are 5 groups.

Figure 28: **Amino acid sequences of porcine and non-porcine beta2-toxins**. Red amino acid: same. Green amino acid: similar. Black amino acid: different.

3.4 Expression-cloning of the toxin genes

According to the toxin gene distribution within the *Clostridium perfringens* isolates from sick rabbits it is highly probable that the alpha-toxin is responsible for the disease. A possible participation of the beta2-toxins, however, cannot be completely excluded, considering the unclear expression of these genes (see discussion). In order to prove or disprove the translation of the toxin genes both in vitro and in vivo specific antibodies would be a helpful tool. Experimentally, the easiest way to get them would be the preparation of recombinant toxins followed by subsequent immunizing of animals. This project, however, would be far beyond the scope of this work. Nevertheless, some experiments were made to clone the toxin genes into bacterial expression vectors and test if this system is suited to isolate the recombinant proteins. As vector we used pASK-IBA6 which contains all elements necessary to express and secrete the recombinant protein and, in addition, should allow isolating the protein easily.

3.4.1 alpha-toxin-gene expression

The procedure is started with PCR amplification of the toxin genes using plasmids containing the respective genes of interest. Special primers have to be used, which at their 5'-ends contain additional sequences necessary for restriction with Bsa I being the prerequisite for in-frame cloning into pAsk-IBA6. The primers used were PexpA1 for and rev.

After cutting with Bsa I the PCR product was ligated with the vector and *E.coli* cells were transformed with the ligation product. Many recombinant colonies were obtained on selective agar plates. Before starting with the gene expression some selected colonies were tested for the in frame presence of the right product using PCR amplification and sequencing (data not shown).

One colony was transferred into liquid medium and, after growing to an OD^{600} to 0.5, anhydrotetracycline was added in order to induce gene expression. Subsequently, the outer membrane of the *E. coli* cells was permeabilized allow the soluble periplasmic components to be released in the supernatant. After centrifugation, an aliquot of both, supernatant and pellet, was then analyzed on an SDS-gel (see Fig. 29). On the gel was also applied a sample taken before induction, this would help the identifying of the recombinant protein.

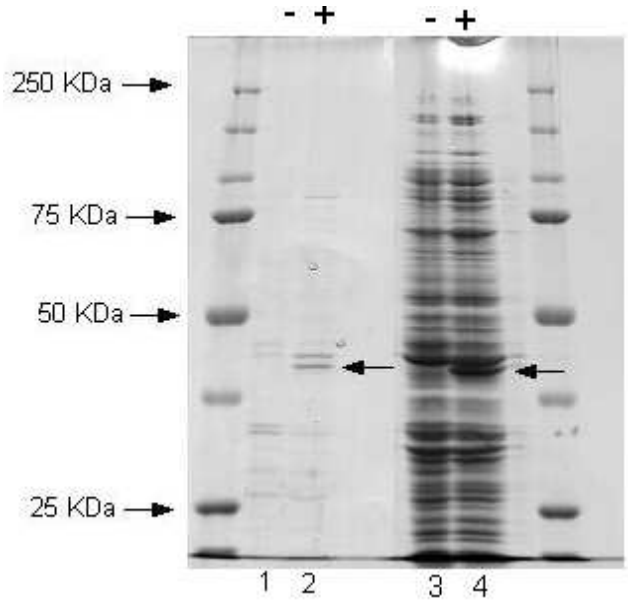


Figure 29: **Protein gel of *E. coli* extracts transformed with alpha-toxin-gene containing pASK-IBA6**. Expected molecular weigh: 45 KDa. **Arrow**: putative recombinant protein. +: induced; -: non induced. **Marker**: Precision Plus Protein Standards (Dual color).

As the picture shows, a recombinant protein with the expected size has been produced. However, most of the recombinant protein is not secreted into the periplasma and still resides within the cells. Nevertheless, the expression clone is suited for the isolation of the recombinant alpha-toxin.

3.4.2 Beta2-toxin-gene expression

Concerning the beta2-toxin-gene expression the DNA sequences at least of the porcine type, raise some doubts on their expression (out of frame, see Results 3.3.4). As mentioned previously and described in more detail in the discussion, these genes can nonetheless be expressed under certain circumstances. Therefore, we decided to clone both ß2-toxin gene-types.

3.4.2 a) Non-porcine ß2-toxin-gene expression

We started this part with the expression of the non-porcine beta2-toxin genes, as all of them are oriented in frame. Before cloning, we checked if these genes are transcribed within the Clostridia cells looking for the respective mRNA. Total RNA was extracted from isolate Torgau (n°2) and transcribed reversely into cDNA as described in Materials and Methods. The cDNA was then used in a PCR reaction with 2 beta2-toxin-gene specific primer pairs, selected from the known region

PRNA for and rev, expected product size 278 bp
PRNA1 for and PRNA rev, expected product size 184 bp

The PCR products were verified on 1% agarose gel.

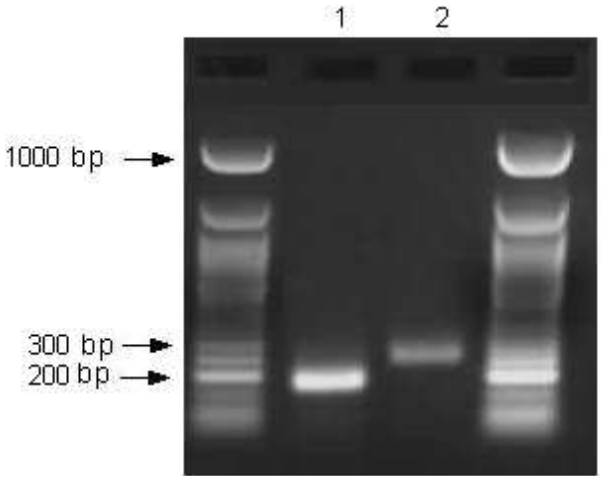


Figure 30: **cDNA analysis**. Line 1: PRNA1 and PRNA rev; line 2: PRNA, for and rev. **Marker**: GeneRuler 50 bp DNA Ladder.

As Fig. 30 shows, the expected PCR products were found, indicating that beta2-toxin-gene-specific mRNA is present within the Clostridia isolate. For the expression-cloning of this gene the primer pair, PexpB1 for and rev was selected, containing, in addition to the pAsk-IBA6 necessary sequences, those representing the beginning and end of the mature protein. Cloning and expression was performed as described for the alpha-toxin. An aliquot of supernatant and sediment before and after induction was applied on the gel (Fig. 31).

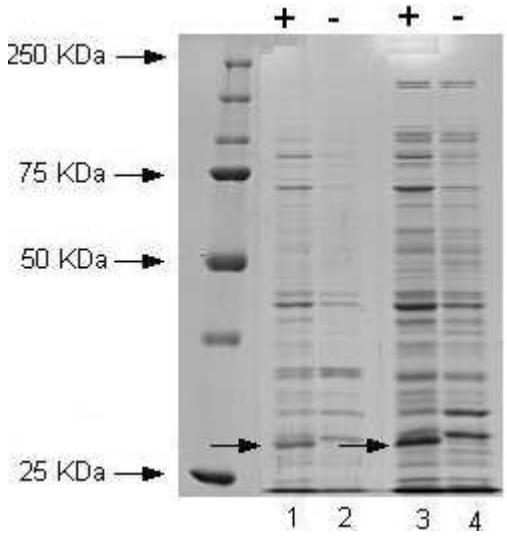


Figure 31: **Protein gel of *E. coli* extracts transformed with non-porcine beta2-toxine-gene-containing pASK-IBA6**. Expected 30 KDa. Arrow: putative mature recombinant protein. +: induced; -: non induced. Line 1, 2: 10 µl; line 3, 4: 15 µl. **Marker**: Precision Plus Protein Standards (Dual color).

The gel picture shows the presence of the expected recombinant protein (28 kDa). Again most of the protein remains within the cell and is not secreted into the periplasma.

3.4.2 b) Porcine-type beta2-toxin gene expression

From this group sample n°6 (Wallenhorst) was used, the only one which does not contain the stop codon at the beginning of the sequence (see Fig. 25). The standard procedure for the expression-cloning of this gene was used, as described in the previous chapters. As shown in fig. 32 the recombinant protein with the expected size (28 kDa) was produced.

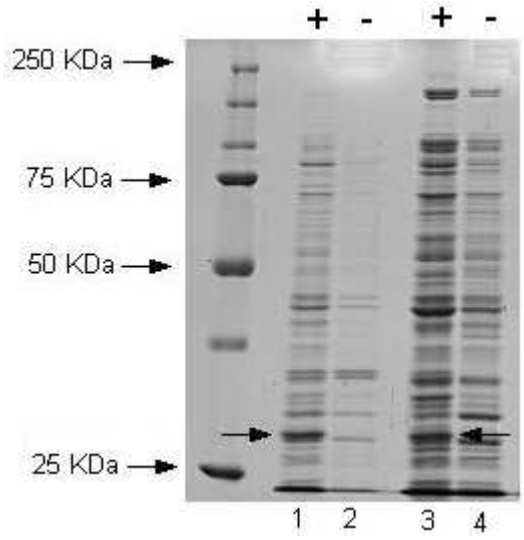


Figure 32: **Protein gel of *E. coli* extracts transformed with the Wallenhorst beta2-toxine-gene-containing pASK-IBA6**. Arrow: putative mature recombinant protein. +: induced; -: non induced. Line 1, 2: 10µl; line 3, 4: 15µl. **Marker**: Precision Plus Protein Standards (Dual color).

Taken together, all 3 toxin genes were successfully transferred into the expression vector pAsk-IBA6. Principally, it should now be possible to isolate the respective recombinant

proteins for antibody production and toxin expression-checking both within the Clostridia isolates and sick rabbits.

4. Discussion

4.1 Typing of the *Clostridium perfringens* samples

The rabbit enterocolitis is a disease caused by *C. perfringens*. This bacteria produces about 15 different toxins, and four of them (alpha, beta, epsilon, iota) are used to form a toxin-type typing scheme, which divide *C. perfringens* into five types (A-E; see table 1, Introduction). By now it was still unknown to which of these five types the isolates from infected rabbits do belong.

Therefore, the first part of this work was the typing of the 71 *C. perfringens* isolates originating from many different rabbit-breeding sources. After DNA extraction all 71 samples were analyzed for the presence of the four major toxins genes (alpha, beta, epsilon, iota) plus the cpe (enterotoxin)-toxin gene and the beta2 one, which might be involved in rabbit enterocolitis as well. From this analysis, performed by PCR, results that all 71 samples contain the alpha-toxin-, 25 the beta2- and only one the cpe-gene as well. Therefore it is clear that all 71 samples are of A type. The fact that 46 of the 71 isolates contain only the alpha-toxin gene can be regarded as a strong hint towards the alpha-toxin being the major causative agent of enterocolitis in rabbits. However, at least an accompanying role of the beta2-gene product cannot be ruled out.

Therefore, our work focused both on the alpha- and beta2-toxin genes. Their nucleic acid sequences were determined and the deduced amino acid sequences were compared to have an idea of the degree of sequence variability. These results are going to be discussed in this section.

4.2 alpha-toxin-genes

4.2.1 alpha-toxin-gene regulatory sequences

At least as important as the sequence itself is the gene’s surrounding as this tells how it is transcribed and translated. Therefore, the PCR primers selected to amplify the gene annealed far enough up- and downstream to detect ptomoter-like and termination sequences.

As already mentioned in Results (3.2.3), there were found the -10 and -35 recognition sites being typical for bacterial promoters. Although the mRNA sequence was not determined one would predict transcription to start at the G 10 nucleotides downstream of the –10 sequence TATAAT, assuming that the clostridial RNA polymerase behaves similar to that of *Escherichia coli*. Accordingly, the sequence TGAGC situated –32 nucleotides upstream of the putative first nucleotide to become transcribed, is in a distance typical for promoters of *Escherichia coli*. The TGAGC sequence is not strictly conserved. In 23 of the 71 samples the last C is changed to a G. Titball et al. (1989) mentioned that usually the alpha-toxin -10 sequences are identical to the TATAAT consensus sequence, but they show only a low similarity with the -35 ones. Therefore, the changed nucleotide within the –35 sequence would not represent a problem for the recognition by the RNA polymerase. Moreover, the 17 nucleotide spacing between -10 and -35 recognition sequences is similar to the optimal distance for strong gene expression in *E. coli* and is similar to the reported spacers lengths in other clostridial gene. There is another strictly conserved element in the upstream part of the alpha-toxin gene, i.e. an inverted repeat, 11 nucleotides long with an 11 nucleotide spacer in between (see fig. 10). Currently, it is not known if it has a regulatory role in alpha-toxin gene transcription. However, due to its high symmetry a regulatory role is likely.

Regarding transcription termination signals at the end of the alpha-toxin gene, none were found. Especially, the common GC-containing inverted repeat with following AT sequences is absent. This might be an indication that the alpha-toxin gene is the first in a multi-gene operon. However, as the corresponding mRNAs have not been analyzed yet, this point can not be clarified at the moment.

As far as translation signals are concerned, 5 consecutive G residues (position 645, fig. 10) form the ribosomal binding site being the equivalent of the Shine-Dalgarno sequence in *E. coli*. Thus, the first ATG, 7 nucleotides apart, can be considered as the start codon for translation. The translation termination point of the alpha-toxin is marked by repeated stop signals which are maintained even if with different stop codons (in seven samples the second stop codon is TGA instead of TAA) in all 71 samples. This particular feature is rarely observed in prokaryotic genes, the real significance being unclear.

Taken together, the alpha-toxin gene presumably is the first of several within an operon and contains all elements necessary to become translated. It has indeed been shown that all strains do express the alpha–toxin (Dr. Rossi, personal communication).

4.2.2 Deduced alpha-toxin amino acid sequences

From the nucleic acid sequences the amino acid ones were deduced. The total sequence is 398 amino acids long. The amino acids encoded between the start codon and nucleotide 661 (corresponding to the first 30 amino acids) can be considered to function as a signal sequence directing transport of the alpha-toxin across the cell membrane.
Von-Heijne, 1984 described such a signal sequence characterized by a short charged N-terminal amino acid stretch followed by a hydrophobic core, which in turn would be followed by the processing site. This description fits to the first amino acids sequence of the alpha-toxin gene:

amino acids 2-6: short charged stretch
amino acids 8-22: hydrophobic core
amino acids 28-29: cutting site

Thus, the alpha-toxin is synthesized as a precursor which subsequently is processed and exported. Therefore, the calculated molecular mass of the mature toxin would be 42 KDa.
Regarding the amino acid sequence variability within the 71 alpha-toxins, the comparison (attachment 3 and results 3.2.2) reveals at least two points. First, the degree of sequence variability is rather low (the 2 most distant alpha-toxins differ only in 6 amino acids). Second, the amino acid differences are not distributed uniformly along the sequence, but instead restricted to certain areas. Three regions could be defined as having no, low and high variability (see results fig. 14). However, as mentioned, one must keep in mind that the absolute variability is rather low. The crystal structure reveals the alpha-toxin to be a two-domain protein with the amino-terminal domain (residues 1-246) composed by alpha-helices and a carboxy-terminal domain (residues 243-251) composed by β sheet. These domains are joined by a flexible linker region (243- 251) (Eaton et al., 2002). As the alpha-toxin has enzymatic properties (see Introduction) those residues being essential for its function should not be changed significantly. This is going to be discussed in the following chapters.

4.2.3 Metal ion-binding residues in the N- and C-terminal domains

The putative active site is located in the N-terminal domain within a cleft which is also occupied by three catalytically essential zinc ions co-ordinated by a tryptophan, a glutamic acid, two aspartic acid and five histidine residues. One of the three ions, designed as Zn1, is most likely to be Zn^{2+} while the other two, designed as Cd2 and Cd3, are most likely to be Cd^{2+} ions. The Cd^{2+} ions can replace the zinc ions without any loss of toxin activity. Each of the three ions is bound to different residues:

Zn1	Trp1, His 11, Asp 130
Cd2	His 136, His 148, Glu 152
Cd3	Asp 56, His 68, His 126, Asp130

Site directed mutagenesis of these residues results in proteins which have reduced zinc ions content and are devoid of phospholipase C-, haemolytic and lethal activity (M. Jepson and R. Titball, 2000). Looking at the comparison of all 71 alpha-toxin sequences (see attachment 3), all amino acids being involved in the coordination of the metal ions are conserved. From this one could therefore conclude that the alpha-toxins of all 71 *Clostridia* isolates are fully active. However, residues in the C-terminal domain are important for alpha-toxin activity as well.

The function of the C-domain is not fully elucidated. However, several lines of evidence suggest that this domain plays a key role in the calcium-requiring interaction of the alpha-toxin with membrane phospholipids (M. Jepson and R. Titball, 2000). One feature of the carboxy-terminal domain is the presence of three calcium-binding sites, termed Ca1, Ca2 and Ca3.

Each of them is co-ordinated with different amino acids:

Ca1: Asp 269, Glu 271, Asp 273, Asp 274 and Asp 336
Ca2: Asp 293 and Asp 298
Ca3: Asp 273 and Asp 298

Concerning Ca1, residues Asp 269 and Asp 336 are of influence for the toxin activity. When substituted with Asn the mutated toxins showed a 20% reduction in pholiphospase C activity, and they required a higher calcium concentration for full activity. All the calcium-binding sites are located at the putative membrane-binding surface of the protein and may

be involved in the binding of alpha-toxin to the membrane, allowing the active site to be optimally positioned to bind phospholipids (M. Jepson and R. Titball, 2000). Again, the sequence comparison (see attachment 3) reveals that none of these calcium-binding residues is mutated within the 71 samples.

Taken together, all amino acids being involved in metal ion binding are conserved. Before concluding all alpha-toxins to be equally active, the interaction of the protein with the target cell membrane has to be looked at, as this plays an important role in alpha-toxin activation.

4.2.4 alpha-toxin interaction with the target cell membrane

The alpha toxin at pH 7.5 is in a two-domain conformation where the active site is accessible to substrate. At pH 4.7 the N-terminal domain adopts a conformation, designed as “closed form”, in which the active site has been obscured by a loop movement, and is no longer accessible to substrates. In addition, one zinc ion has been lost from the active site. In the closed form there are considerable changes in two loops close to the third zinc-binding site. These two loops are on either side of the active site cleft, on the face of the toxin molecule that is believed to interact with the membrane surface. The first loop, residues 55-93, lies between the active site cleft and the cleft between the N-terminal and the C-terminal domain. The second loop (residues 132-149) contains residues involved in the binding of the third zinc in the open form and is on the opposite side of the active site to loop1 (Eaton et al., 2002).

The mechanism of toxin activation is generally proposed to need calcium ions and is associated with conformational changes in the protein. Mutagenesis studies reveal that residues within the loop1 play a key role in the biological activity of the alpha toxin. A mutation in amino acid 74 (Thr → Ile) of the alpha-toxin results in a 250-fold reduction of haemolytic, lecithinase- and sphingomyelinase-activity and a mutation at amino acid 69 (Phe → Cys) leads to a complete loss of haemolytic and sphingomyelinase activity and results in a 280-fold reduction in lecithinase activity (Eaton et al., 2002). When looking at the sequence comparison (see attachment 3) both the Phe 69 and the Tyr 74 are conserved.

A detailed analysis of the crystal structure has suggested a mechanism by which the alpha-toxin interacts with cell membranes (Titball et al., 1999). Exposed hydrophobic

residues, like Trp 214 and Phe 334, are located on one face of the protein in the N- and C-domains respectively, and are appropriately positioned to interact with the fatty-acyl tail groups of the phospholipids.

A second element of alpha-toxin-membrane interaction are the calcium ions of the C-domain which is similar to eukaryotic C2 domains being involved in calcium-mediated phospholipid recognition (Titball et al., 1999). The calcium ions are partially co-ordinated by acidic amino acid side chains and partially by the phosphate groups of membrane phospholipids. On the basis of enzyme kinetic studies it appears that the calcium ions first bind loosely to the alpha toxin and the protein/Ca^{2+} complex is then able to bind to phospholipids. The mutation of residues Asp 269 and Tyr 275, which are strongly implicated in calcium binding, results in proteins with reduced activities (Titball et al., 1999). All amino acid residues considered to be involved in membrane-binding of the alpha-toxin leading to its subsequent activation , i.e. Phe 69, Ile74, Trp 214, Tyr 275 and Phe 334, are conserved within the 71 samples.

4.2.5. Concluding remarks on the alpha-toxin

Taken together, all the functionally important amino acid residues, both involved in metal ion-, membrane-binding and conformational activation are conserved in the 71 alpha–toxins. Therefore, it can be concluded that all the toxins are equally well active. This can be taken as confirmation for the alpha-toxin to be the major causative agent in rabbit enterocolitis. Contrary speaking, mutated amino acids leading to severe reduction of activity, would rule out these Clostridia as being responsible for the disease.

4.3 Beta2-toxin gene sequences

In 1997 Gilbert et al., reported the finding of a novel toxin produced by *C. perfringens*. This new toxin, designed as beta2, was isolated from a piglet that died of necrotizing enterocolitis. Beta2-toxin is considered to be involved in the pathogenesis of digestive diseases in type A strain–infected animals. The alpha toxin, which is the major toxin produced by type A strains, is not considered as the primary cause of digestive lesions. Studies carried on by Gilbert et al., 1997, showed that beta2 toxin-producing strains are associated with animal diseases such as necrotic enteritis in piglets and enterocolitis in horses. Its possible role in rabbit enterocolitis is completely unclear. Therefore, the beta2 toxin gene, *cpb2*, was analyzed in the *C. perfringens* isolated from sick rabbits as well.

4.3.1 Beta2-toxin gene-types

As already pointed out (results 3.3.1) there exist two different beta2-genes: the “porcine” and the “non-porcine” one. The strategy applied to obtain the beta2-gene sequences was analogous to that for the alpha-genes, i.e. PCR amplification of the DNA region of interest including upstream and downstream parts of the gene.

However, finding the correct PCR primers for the amplification of the two beta2-toxin gene types was difficult. This was due to the fact that primers delineated from at that time published sequences either did not work or were not available because only the coding parts of the genes were delivered to the nucleic acid sequence databases. The reason that primers taken from the published sequence did not work is the plasmid-localization of the beta2-toxin gene. There, its exact deposition site seems to be variable.

The first step towards the beta2-toxin gene sequences therefore, was to find correctly annealing primers outside the beta2-toxin gene. Some time after initiation of this work total sequences from several *C. perfringens* plasmids (from human origin) containing the beta2-toxin gene was published. As described in Results, taking primers from these plasmids resulted in the amplification of the “non-porcine” beta2-toxin genes, the upstream primer also working in the amplification of the 3’-part of the “porcine”-type beta2-toxin genes. However, the downstream primers, annealing at the 5’-part of the gene were still not available. Finally, by reverse PCR (see results 3.3.4) a piece of the upstream region of the “porcine”-type beta2-toxin gene was obtained. However, this piece was rather short (90 nucleotides) and, after recircularization, did not contain, as to be expected, a HindIII recognition site. This indicates that during ligation nucleotides had been removed,

probably caused by minimal nuclease activities within our DNA ligation lots. Insofar, it was luck to get a religation at all. Anyhow, this short upstream stretch of the “porcine”-type beta2-toxin gene was long enough to get, by another Database search, downstream primers annealing far enough outside the gene.

4.3.2 Beta2-toxin genes regulatory sequences

Within the upstream region of both beta2-toxin genes typical bacterial promoter sequences exist, which are slightly different in both types:

Sample n°2 (Torgau), “non-porcine”-type: TTTAAA-N_{17}-TATAAT
Sample n°3 (Borkheide), “porcine”-type: TTTTAA-N_{17}-TATAAT

The first hexamer represents the –35, the second the –10 or “Pribnow box”. The likely transcription start is at the G at position +1 relative to the “Pribnow box”. This is in coincidence with the alpha-toxin gene promoter where the same GT element is found downstream of the “Pribnow box” (chapter 4.2.1).
Regarding transcriptional termination signals both ß2-toxin genes contain, contrary to the alpha-toxin one, one inverted repeat immediately past the translational stop codon:

sample n°2 (Torgau), “non-porcine”-type: A_5TGAGGC-N_3-GCCTCAT_5
sample n°3 (Borkheide), “porcine”-type : A_7TATAGAAGTGAC-N_3-GTCACTTCATAT_7

Therefore, a hairpin structure in the transcript can be formed, leading to a halt of the RNA polymerase followed by dissociation of the transcription complex due to the weak base pairing of the AT-stretch behind the repeat. This is the typical characteristic of a rho-independent transcription termination. In addition, this feature indicates that the beta2-toxin gene is not member of an operon.
Regarding translational signal sequences, similar as in the alpha-toxin gene, the ribosomal binding site (5 Gs) is present 8 nucleotides upstream of the putative initiation codon ATG. The translational stop codon is TAA for all non-porcine genes and TAG for the porcine-type ones. This, however, does not seem to be important.

4.3.3 Beta2-toxin gene coding sequences

4.3.3 a) “Porcine”-type beta2-toxin genes and their expression

From the 25 beta2-toxin gene-positive *C. perfringens* isolates 16 contain the “porcine” type. Porcine-type beta2-toxin genes differ in their coding sequences dependent on the animal source the bacteria were isolated from. In 2005 Vieli et al. published a paper on the sequence of the porcine-type cpb2 gene of beta2-toxigenic *C. perfringens* isolates from horses. All these, being 94.5% identical to the porcine-type beta2 gene from a piglet display one marked difference. Because of deletion of an A-residue in the oligo-A stretch following the start codon ATG the open reading frame is interrupted leading to a stop codon 19 nucleotides further downstream. Therefore, translation stops already within the signal peptide region and the beta2-toxin gene is not expected to become translated. Exactly the same feature is found in our porcine-type beta2-toxin genes (isolated from the non-porcine rabbits), besides one exception. In sample n°6 (Wallenhorst) the “missing A” is present, the open reading frame is not interrupted and the beta2-toxin gene should be translated as in swine. This is the first time that such an observation has been made leading to the prediction that a porcine-type beta2-toxin gene should be expressed in “non-porcine” animals.

The situation can even be more complex. Despite the A-deletion within the early gene region, these genes, under certain circumstances, can nevertheless be expressed. This has been shown by Vieli et al. (2005) who demonstrated by immunoblotting with anti-beat2-toxin antibodies both in total antigen preparations and in cell culture supernatants the presence of the beta2-toxin typical 28 kDa band. This, however, was only observed when the *Clostridia* were put under stress conditions, like antibiotic-treatment (gentamycin > 10 µg/ml) or temperature shock (10 min, 50°C). Under normal circumstances the beta2-toxin was not present. These effects, observed *in vitro*, could be seen *in vivo* as well. Immunohistology in tissue samples of small and large intestine from horses with severe typhlocolitis, that had been treated before with gentamycin revealed the presence of the beta2-toxin. Obviously, under antibiotic and heat stress, a frame shifting occurs during translation, eliminating the A-deletion by repositioning the ribosome on the mRNA. This might become possible by the AT-rich “slippery codon”-sequence AAA-AAA-TTA-TTA

allowing the ribosome to move 1 nucleotide backwards and by this the last A of the oligo-A stretch being read twice. This has the consequence as if an additional A would be present at this position in the mRNA. Whatever the exact mechanism is, the presence of the A-deletion in porcine-type beta2-toxin genes from *C. perfringens* isolated from non-porcine animals does not necessarily rule out the production of the mature beat2-toxin.

4.3.3 b) Non-porcine-type beat2-toxin genes and their expression

Non-porcine-type beta2-toxin genes have been detected in *C. perfringens* from all types isolated from several “non-porcine” species (bovine, avian, canine, caprine, corvine, equine feline, human, ovine) (Jost et al. 2005). Some of these genes contain mutations leading to premature termination of translation resulting in the synthesis of truncated, inactive proteins. However, 25 of the total 71 strains tested carried full-length beat2-toxin genes (Jost et al. 2005). None of them seemed to be expressed. 5 of their 25 strains were tested for the presence of beta2-toxin-specific mRNA. In 3 of these 5 isolates no cpb2-derived mRNA was found by RT-PCR. The authors speculated that a too low promoter strength would have been responsible for this effect. The remaining 2 strains produced cpb2-specific mRNA but still no beta2-toxin was detectable.
Strain NCIB 10784 contained a frameshift mutation preventing translation but strain 13 not. Why its mRNA was untranslated remained obscure. Generalizations from these data on the ineffectiveness of beta2-gene-expression towards all the 25 strains with full-length beta2-toxin genes should be made with caution. In addition, the experiments on protein expression in this study must be looked at critically. The antibody used in the Western-blotting experiments was a monoclonal one raised against the **porcine**-type beta-toxin. The authors regarded this antibody being suitable for the detection of **non-porcine** beta2-toxin as well. This they concluded from a control experiment where they got a positive reaction with a recombinant non-porcine beta2-toxin produced by cloning of the corresponding gene into an expression vector. However, as marked amino acid differences exist between porcine- and non-porcine beta2-toxins (see Results fig. 28) it can not be ruled out that this monoclonal antibody detects epitopes not being displayed by all of the non-porcine beta2-toxins. A better approach would be the use of monospecific, polyvalent antibodies against non-porcine beta2-toxins.

Regarding the expression of our 5 non-porcine-type beta2-toxin genes, cpb2-specific mRNA could be demonstrated by RT-PCR (data not shown) in one of the isolates (sample n°2, Torgau). Because of the high similarity in the promoter region of the 5 genes it is highly probable transcription to occur with all of them. In bacteria protein expression is regarded to be controlled on the transcriptional level. It must therefore be concluded that the beta2-toxin is produced by all the 9 isolates containing the non-porcine-type beta2-toxin genes. If this happens can only be proven by immunodetection with anti-beat2-toxin-specific antibodies. As these are not available yet, no definite answer can be given on the beta2-toxin-gene expression by these *Clostridia* isolates.

4.4 Expression cloning of the alpha- and beta2-toxin genes

Because of the unresolved problem of beta2-toxin gene expression described in the previous chapters, preliminary experiments were performed to develop toxin-specific antibodies. As already mentioned, this project would be beyond the scope of this work. Nevertheless, a first step was made into this direction, i.e. the expression-cloning of the respective genes. Summarizing these experiments, it is obvious that the cloning system used, worked for both the alpha- and beta2-toxin genes. Recombinant clones were obtained, containing the full-length coding sequence and producing new proteins of the theoretically expected size. From them it should be easily possible to isolate the toxins, first as fusion proteins and after removal of the vector part the pure toxins as well. By subsequent immunization of animals with the purified toxins monospecific antibodies would be obtained. By their use both *in vitro* in immuno western-blotting experiments and *in vivo* by immunohistology the problem of the up to now unclear expression at least of the beta2-toxin genes should be definitely be resolved.

4.5 Concluding remarks on the toxin-gene sequence data

The major objective of this work was to collect sequence data of the major toxins of those *C. perfringens* strains circulating in German rabbit stables and being made responsible for the enterocolitis disease, up to now not being curable. From these data it is expected to decide if the development of a toxoid vaccine against the disease is feasible.

The amino acid variability within the 71 alpha-toxins is rather low, being restricted to functionally inactive regions of the protein. Therefore, it should be possible to produce toxoid vaccines with broad protection by immunizing animals with a mixture of alpha-toxin preparations (obtainable by culture supernatants) representing the complete antigenic repertoire of all the 71 alpha-toxins. Alternatively, alpha-toxin peptides from the conserved parts of the protein might be suitable as well. For that, C-terminal part-derived peptides are most promising. Such antibodies could block the protein's binding to the target cell membrane leaving the alpha-toxin inactive.

As described, 25 of the 71 *C. perfringens* isolates contain the beta2-toxin gene (*cpb2*) as well. The beta2-toxin, first demonstrated in 1997 to become produced by a *C. perfringens* strain isolated from a piglet suffering from enteric disease, is toxic to cultured epithelial cells and lethal to mice when administered intravenously (Gibert et al., 1997). *Cpb2*-positive *C. perfringens* strains are associated with enteric disease in domestic animals, notably pigs (2, 3, 6, 13), horses (1, 5) and dogs (12). However, apart from this epidemiologic association, there is little experimental evidence to support the role of beta2-toxin in pathogenesis. Here, it has been shown for the first time that cpb2-containing *C. perfringens* are circulating in rabbits as well. From the 25 beta2-toxin genes analyzed, surprisingly the majority (16) belong to the porcine-type. Thus, in a non-porcine animal as the rabbit more porcine- as non porcine-type *C. perfringens* circulate. Again, this is the first time to become reported, and it might be better to redefine the nomination of the two beta2-gene types.

As described, it is completely unclear whether or not these beta2-genes are expressed. Therefore, in the development of a toxoid vaccine against rabbit enterocolitis one should not exclude the possibility of the participation of the beta2-toxins in the pathology. Although only alpha-toxin-producing C. perfringens strains are sufficient to induce the disease, at least an accompanying role of the beta2-toxins must be kept in mind as long as their production is unresolved.

English summary

Rabbit enterocolitis is a serious gastrointestinal syndrome appearing in France since the end of 1996. The disease is characterized by small quantities of watery diarrhoea followed by a decrease in food intake and finally resulting in intestinal paralysis. It has high mortality rates (30-80%) and spreads very rapidly.

There is clear experimental evidence that this disease is produced by *Clostridium perfringens*. Aim of this work is to characterize the genotype of the Clostridium strains circulating in Germany and sequence the major toxin genes.

C. perfringens strains were collected from different rabbit livestocks throughout Germany (including 3 from Italy), and isolated from the intestine of sick rabbits. All samples were typed via PCR using consensus primers of the major toxin genes (alpha, beta, epsilon, iota, cpe and beta2). From a total of 71 strains, all contain the alpha-gene and 25 additionally the beta2-gene. Therefore all are type A *Clostridium perfringens*.

The alpha-toxin genes were isolated and sequenced, including their immediate upstream and downstream surroundings. The upstream region contains all elements necessary for expression, i.e. typical bacterial promoter elements for transcription initiation and a Shine-Dalgarno-sequence necessary for translation. No transcriptional termination signals were found at the end of the coding region, suggesting the alpha-toxin gene to be member of an operon. The deduced amino acid differences within the 71 alpha-toxins are low and restricted to certain areas of the protein. All the functionally important amino acid residues, i.e. the ones involved in metal ion-, membrane-binding and in the conformational activation, are conserved in all the 71 alpha–toxins. Therefore, it can be concluded that all alpha- toxins are equally well active.

Studies carried on by Gilbert et al., 1997, showed that beta2 toxin-producing strains are associated with animal diseases such as necrotic enteritis in piglets and enterocolitis in horses. Therefore, the beta2-toxin gene, *cpb2*, was analyzed in the *C. perfringens* isolated from sick rabbits as well. The strategy applied to obtain the beta2-gene sequences was analogous to that for the alpha-toxin genes, i.e. PCR amplification of the DNA region of interest including upstream and downstream parts of the gene. After finding the correct PCR primers for the amplification of the beta2-toxin gene types it could be shown that from the 25 beta2-toxin genes 16 belong to the so-called “porcine”- and 9 to the “non-porcine”-type. All the “porcine”- and 5 of the “non-porcine”-type genes were sequenced. They contain all regulatory elements necessary for transcription, i.e. promoter elements

and “hairpin”-termination signals indicationg that monocistronic mRNAs are produced. 15 of the 16 “porcine”-type beta2-toxin genes display an A-deletion within the putative signal peptide region, expecting an immediate downstream translational stop to occur. Only n°6 (Wallenhorst) has an undisturbed open reading frame and should therefore be expressed as a complete protein. This is for the first time, that a “porcine-type” beta2-toxin gene isolated from *Clostridia* grown in non-porcine animals has this feature.

Due to non-solvable sequencing problems, only 5 of the 9 non-porcine beta2-toxin genes could be sequenced completely. All display an undisturbed open reading frame. Similar as within the alpha-toxin samples, the deduced amino acid differences within each beta2-toxin-type are very low, although being marked between the two groups.

From only the nucleotide sequence data no prediction can be made for the expression of the two beta2-toxin genes. Therefore, they were cloned into the bacterial expression vector pASK-IBA6. The cloned genes were expressed as fusion proteins, offering the possibility to purify the recombinant beta2-toxins. After immunizing animals, anti-beta2-toxin-specific antibodies should become available to clarify the problem of the expression of the beta2-toxin genes.

Summarizing all the data on all toxin-specific genes, due to their low sequence variability, the development of a toxoid vaccine against rabbit enterocolitis should become feasible.

Zusammenfassung

Die sogenannte “Enterocolitis” des Kaninchens wurde zuerst Ende 1996 in Frankreich beschrieben. Die Erkrankung ist charakterisiert anfangs durch geringfügigen wässrigen Durchfall, gefolgt von reduzierter Nahrungsaufnahme mit am Ende völligen Zusammenbruchs intestinaler Aktivität (Darmlähmung). Mortalitätsraten (30-80%) und die Ausbreitungsgeschwindigkeit sind sehr hoch. Klare experimentelle Evidenz weist auf *Clostridium perfringens* als den Erreger der Erkrankung hin. Ziel dieser Arbeit ist die Charakterisierung der Toxin Genotypen möglichst vieler in deutschen Kaninchenbeständen kursierender *C.* perfringens Stämme und die Sequenzierung ihrer Haupttoxin-Gene.

71 *C.perfringens* Isolate aus infizierten Kaninchenbeständen (68 aus Deutschland, 3 aus Italien) wurden gesammelt und mittels PCR unter Verwendung von „consensus“ Oligonukleotiden, charakteristisch für die Haupttoxin Gene (alpha, ß, ε, i, cpe and beta2), typisiert. Alle 71 Proben enthalten das alpha-Toxin-, 25 zusätzlich das ß2-Toxin-Gen. Daher gehören alle zum *Clostridium perfringens* Typ-A.

Die alpha-Toxin Gene wurden amplifiziert, zum Teil kloniert und anschließend sequenziert, einschließlich der strom-aufwärts und –abwärts Regionen. Die Stromaufwärtsregionen enthalten alle zur Transkription erforderlichen Elemente, wie bakterielle Promotoren und das für die Translation notwendige Shine-Dalgarno-Äquivalent. Am Ende des kodierenden Berichs waren keine Transkriptionsterminationselemente vorhanden, was darauf hinweist, dass das alpha-Toxin Gen Bestandteil eines Operons ist. Was den kodierenden Bereich betrifft, ist zu konstatieren, dass die aus der Nukleotidsequenz abgleiteten Aminosäure-Unterschiede in den 71 alpha-Toxinen gering sind und zudem auf bestimmte Bereiche des Proteins beschränkt bleiben. Alle funktionell wichtigen Aminosäure-Reste, also diejenigen, die involviert sind in Metallionen-, Membranbindung und Aktivierung, sind konserveirt. Daraus ist zu schließen, dass alle 71 alpha-Toxine ähnlich hoch aktiv sind.

Studien von Gilbert et al (1997) zeigten, dass beta2-Toxin-Gen enthaltende *C. perfingens* Stämme mit Enteritiden bei Ferkeln und Pferden assoziiert sind. Deshalb wurden die beta2-Toxin Gene von *C.perfringens*-Isolaten aus erkrankten Kaninchen ebenfalls analysiert. Die dabei angewandte Strategie entsprach der bei der Charakterisierung der alpha-Toxin Gene verwendeten, d.h., PCR-Amplifizierung der entsprechenden DNA-Region unter Einbeziehung strom-aufwärts und-abwärts liegender Genregionen. Nach zum Teil experimenteller Ermittlung der geeigneten PCR-Starter konnte gezeigt werden,

dass von den 25 beta2-Toxin Genen 16 zum sog. „Schwein“- und 9 zum „Nicht-Schwein“-Typ gehören. Alle „Schwein“-Typ- und 5 der 9 „Nicht-Schwein“-Typ beta2-Gene wurden sequenziert. Sie enthalten alle die für die Transkription notwendigen Promoter- und Terminations-elemente, was darauf hinweist, dass eine monocistronische mRNA synthetisiert wird. 15 der 16 „Schwein“-Typ beta2-Toxin Gene zeigen eine A-Deletion innerhalb der Signalpeptid-kodierenden Region, was zu einem sehr schnellen Translationsstop führen sollte. Nur die Probe 6 (Wallenhorst) besitzt einen nicht unterbrochenen offenen Leserahmen, sodass erwartet werden kann, dass hier das komplette beta2-Toxin synthetisiert wird. Dies ist das erste Mal, dass ein „Schwein“-Typ beta2-Toxin Gen von Clostridien, die aus Nicht-Schwein-Tieren isoloiert wurden, eine solche Charakterisitik besitzt. Auf Grund nicht lösbarer Sequenzierprobleme konnten nur 5 der 9 „Nicht-Schwein“-Typ beta2-Toxin Gene komplett sequenziert werden. Alle weisen einen kompletten offenen Leserahmen auf. Ähnlich wie bei den alpha-Toxin Genen sind die Aminosäure-Unterschiede innerhalb der Mitglieder jedes beta2-Toxin-Typs sehr gering, allerdings deutlich zwischen Mitgliedern verschiedener beta2-Toxin-Typen.

Aus den Nukleinsäuresequenzdaten allein kann keine Voraussage über die Synthese der beiden beta2-Toxine getroffen werden. Deshalb wurden die Gene in den bakteriellen Expressionsvektor pASK-IBA6 kloniert. Alle drei Toxin-Gene ließen sich als Fusionsproteine exprimieren. Dadurch bietet sich die Möglichkeit die rekombinanten Toxine aufzureinigen und durch anschließende Immunisierung anti-beta2-Toxin-spezifische Antikörper zu erhalten. Mit ihrer Hilfe sollte sich das Problem der beta2-Toxin-Expression zweifelsfrei klären lassen.

Fasst man alle Sequenzdaten über die Toxin-spezifischen Gene zusammen, so sollte auf Grund der geringen Sequenzvariabilität die Entwicklung einer Toxoid-Vakzine gegen die Enterocolitis des Kaninchens machbar sein.

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APPENDIX

Attachment 1

Sequence comparison of the upstream region of the alpha-toxin genes

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541 taacgggggga tataaaaa
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Attachment 2

Sequence comparison of the downstream region of the alpha-toxin genes

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Attachment 3

Sequence comparison of the mature alpha-toxin protein

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  alpha_mature_10  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_12  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
   alpha_mature_9  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
alpha_mature4.seq  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_25  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_40  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_47  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
   alpha_mature_5  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_71  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
   alpha_mature_8  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_26  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_22  WDGKIDGTGT HAMIVTQGVS ILENDMSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_32  WDGKIDGTGT HAMIVTQGVS ILENDMSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_52  WDGKIDGTGT HAMIVTQGVS ILENDMSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_34  WDGKIDGTGT HAMIVTQGVS ILENDMSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_44  WDGKIDGTGT HAMIVTQGVS ILENDMSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_16  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_28  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_29  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKENMHELQL
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  alpha_mature_53  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKQNMHELQL
  alpha_mature_59  WDGKIDGTGT HSMIVTQGIS ILENDLSKNE PESVRKNLEI LKQNMHELQL
  alpha_mature_67  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKQNMHELQL
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  alpha_mature_60  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_46  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKENMHELQL
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  alpha_mature_68  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
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  alpha_mature_38  WDGKIDGTGT HAMIVTQGIS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_48  WDGKIDGTGT HAMIVTQGVS ILENDMSKSE PESVRKNLEI LKENMHELQL
  alpha_mature_49  WDGKIDGTGT HAMIVTQGVS ILENDMSKSE PESVRKNLEI LKENMHELQL
   alpha_mature_6  WDGKIDGTGT HAMIVTQGVS ILENDMSKSE PESVRKNLEI LKENMHELQL
  alpha_mature_65  WDGKIDGTGT HAMIVTQGVS ILENDMSKSE PESVRKNLEI LKENMHELQL
  alpha_mature_70  WDGKIDGTGT HAMIVTQGVS ILENDMSKSE PESVRKNLEI LKENMHELQL
  alpha_mature_69  WDGKIDGTGT HAMIVTQGVS ILENDMSKNE PESVRKNLEI LKDNMHELQL
  alpha_mature_13  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_41  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_72  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_74  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_61  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_63  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_15  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
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   alpha_mature_2  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_21  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_24  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
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  alpha_mature_31  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_36  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_43  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_50  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
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  alpha_mature_57  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
  alpha_mature_62  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
   alpha_mature_7  WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL

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  alpha_mature_22  GSTYPDYDKN AYDLYQDHFW DPDTDNNFSK DNSWYLAYSI PDTGESQIRK
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  alpha_mature_62  GSTYPDYDKN AYDLYQDHFW DPDTDNNFSK DNSWYLAYSI PDTGESQIRK
   alpha_mature_7  GSTYPDYDKN AYDLYQDHFW DPDTDNNFSK DNSWYLAYSI PDTGESQIRK

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  alpha_mature_10  FSALARYEWQ RGNYKQATFY LGEAMHYFGD IDTPYHPANV TAVDSAGHVK
  alpha_mature_12  FSALARYEWQ RGNYKQATFY LGEAMHYFGD IDTPYHPANV TAVDSAGHVK
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  alpha_mature_45  FETFAEERKE QYKINTAGCK TNEAFYADIL KNKDFNAWSK EYARGFAKTG
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  alpha_mature_35  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_68  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_55  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_58  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_73  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_14  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_17  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
  alpha_mature_18  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
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  alpha_mature_38  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
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   alpha_mature_6  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
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  alpha_mature_69  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
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  alpha_mature_41  FETFAEERKE QYKINTAGCK TNEAFYTDIL KNKDFNAWSK EYARGFAKTG
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  alpha_mature_62  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
   alpha_mature_7  FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG

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  alpha_mature_12  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
   alpha_mature_9  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
alpha_mature4.seq  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
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  alpha_mature_40  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
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  alpha_mature_52  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
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  alpha_mature_62  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
   alpha_mature_7  KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS

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  alpha_mature_12  VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
   alpha_mature_9  VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
alpha_mature4.seq  VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
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  alpha_mature_59  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_67  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_66  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_23  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_39  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_45  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_60  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_46  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_35  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_68  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_55  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_58  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_73  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_14  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKLIANGK
  alpha_mature_17  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKLIANGK
  alpha_mature_18  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKLIANGK
   alpha_mature_3  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKVIANGK
  alpha_mature_30  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKLIANGK
  alpha_mature_38  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_48  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_49  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
   alpha_mature_6  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_65  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_70  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_69  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_13  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFSDAYKP ENIKIIANGK
  alpha_mature_41  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFSDAYKP ENIKIIANGK
  alpha_mature_72  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFSDAYKP ENIKIIANGK
  alpha_mature_74  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFSDAYKP ENIKIIANGK
  alpha_mature_61  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFSDAYKP ENIKIIANGK
  alpha_mature_63  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFSDAYKP ENIKIIANGK
  alpha_mature_15  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
```

```
  alpha_mature_19  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
   alpha_mature_2  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_21  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_24  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_27  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_31  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_36  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_43  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_50  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_51  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_56  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_57  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
  alpha_mature_62  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
   alpha_mature_7  TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK

                   351                 370
   alpha_mature_1  VVVDKDINEW ISGNSTYNIK
  alpha_mature_10  VVVDKDINEW ISGNSTYNIK
  alpha_mature_12  VVVDKDINEW ISGNSTYNIK
   alpha_mature_9  VVVDKDINEW ISGNSTYNIK
alpha_mature4.seq  VVVDKDINEW ISGNSTYNIK
  alpha_mature_25  VVVDKDINEW ISGNSTYNIK
  alpha_mature_40  VVVDKDINEW ISGNSTYNIK
  alpha_mature_47  VVVDKDINEW ISGNSTYNIK
   alpha_mature_5  VVVDKDINEW ISGNSTYNIK
  alpha_mature_71  VVVDKDINEW ISGNSTYNIK
   alpha_mature_8  VVVDKDINEW ISGNSTYNIK
  alpha_mature_26  VVVDKDINEW ISGNSTYNIK
  alpha_mature_22  VVVDKDINEW ISGNSTYNIK
  alpha_mature_32  VVVDKDINEW ISGNSTYNIK
  alpha_mature_52  VVVDKDINEW ISGNSTYNIK
  alpha_mature_34  VVVDKDINEW ISGNSTYNIK
  alpha_mature_44  VVVDKDINEW ISGNSTYNIK
  alpha_mature_16  VVVDKDINEW ISGNSTYNIK
  alpha_mature_28  VVVDKDINEW ISGNSTYNIK
  alpha_mature_29  VVVDKDINEW ISGNSTYNIK
  alpha_mature_37  VVVDKDINEW ISGNSTYNIK
  alpha_mature_20  VVVDKDINEW ISGNSTYNIK
  alpha_mature_33  VVVDKDINEW ISGNSTYNIK
  alpha_mature_42  VVVDKDINEW ISGNSTYNIK
  alpha_mature_53  VVVDKDINEW ISGNSTYNIK
  alpha_mature_59  VVVDKDINEW ISGNSTYNIK
  alpha_mature_67  VVVDKDINEW ISGNSTYNIK
  alpha_mature_66  VVVDKDINEW ISGNSTYNIK
  alpha_mature_23  VVVDKDINEW ISGNSTYNIK
  alpha_mature_39  VVVDKDINEW ISGNSTYNIK
  alpha_mature_45  VVVDKDINEW ISGNSTYNIK
  alpha_mature_60  VVVDKDINEW ISGNSTYNIK
  alpha_mature_46  VVVDKDINEW ISGNSTYNIK
  alpha_mature_35  VVVDKDINEW ISGNSTYNIK
  alpha_mature_68  VVVDKDINEW ISGNSTYNIK
  alpha_mature_55  VVVDKDINEW ISGNSTYNIK
  alpha_mature_58  VVVDKDINEW ISGNSTYNIK
  alpha_mature_73  VVVDKDINEW ISGNSTYNIK
  alpha_mature_14  VVVDKDINEW ISGNSTYNIK
  alpha_mature_17  VVVDKDINEW ISGNSTYNIK
  alpha_mature_18  VVVDKDINEW ISGNSTYNIK
   alpha_mature_3  VVVDKDINEW ISGNSTYNIK
  alpha_mature_30  VVVDKDINEW ISGNSTYNIK
  alpha_mature_38  VVVDKDINEW ISGNSTYNIK
  alpha_mature_48  VVVDKDINEW ISGNSTYNIK
  alpha_mature_49  VVVDKDINEW ISGNSTYNIK
```

```
 alpha_mature_6  VVVDKDINEW ISGNSTYNIK
alpha_mature_65  VVVDKDINEW ISGNSTYNIK
alpha_mature_70  VVVDKDINEW ISGNSTYNIK
alpha_mature_69  VVVDKDINEW ISGNSTYNIK
alpha_mature_13  VVVDKDINEW ISGNSTYNIK
alpha_mature_41  VVVDKDINEW ISGNSTYNIK
alpha_mature_72  VVVDKDINEW ISGNSTYNIK
alpha_mature_74  VVVDKDINEW ISGNSTYNIK
alpha_mature_61  VVVDKDINEW ISGNSTYNIK
alpha_mature_63  VVVDKDINEW ISGNSTYNIK
alpha_mature_15  VVVDKDINEW ISGNSTYNIK
alpha_mature_19  VVVDKDINEW ISGNSTYNIK
 alpha_mature_2  VVVDKDINEW ISGNSTYNIK
alpha_mature_21  VVVDKDINEW ISGNSTYNIK
alpha_mature_24  VVVDKDINEW ISGNSTYNIK
alpha_mature_27  VVVDKDINEW ISGNSTYNIK
alpha_mature_31  VVVDKDINEW ISGNSTYNIK
alpha_mature_36  VVVDKDINEW ISGNSTYNIK
alpha_mature_43  VVVDKDINEW ISGNSTYNIK
alpha_mature_50  VVVDKDINEW ISGNSTYNIK
alpha_mature_51  VVVDKDINEW ISGNSTYNIK
alpha_mature_56  VVVDKDINEW ISGNSTYNIK
alpha_mature_57  VVVDKDINEW ISGNSTYNIK
alpha_mature_62  VVVDKDINEW ISGNSTYNIK
 alpha_mature_7  VVVDKDINEW ISGNSTYNIK
```

Attachment 4

Sequence comparison of the upstream region of the beta2-“non porcine“-toxin genes

```
  1 tagccaatta atgtccccaa cattaaaagt tggaaatcaa ctatttgtta cgaaagttta
 61 taatacttca aatataaaga gaggagatat tttagtcttt aattctgcaa gaaaaaataa
121 ggttaatatt ttttattaac cttatttttt cttgcagaat ataaaaccca attaatatta
181 cttataataa ctaatattat aagtaaagga atccataaaa atttaggaat cattattaac
241 ataataaatc ctataaccca aattattatt gaatatatca aatatctaag aaatagtttc
301 aaataatcac ctacttaaaa ttaaatttat tattaattta attaatctaa aaatcaacct
361 ttctaatatt ataatcttcc ttttgataaa ttaacataat tctagataat aattcttaaa
421 aagtatcttt atttaaaaaa tgttaaaaat ttgatataat agaattgtaa aaaaaattca
481 ggggggaaac caa
  1 tagccaatta atgtccccaa cattaaaagt tggaaatcaa ctatttgtta cgaaagttta
 61 taatacttca aatataaaga gaggagatat tttagtcttt aattctggtt aatatataag
121 gttaatattt tttattaacc ttatttttтc ttgcagaata taaaacccaa ttaatattac
181 ttataataac taatattata agtaaaggaa tccataaaaa tttaggaatc attattaaca
241 taataaatcc tataacccaa attattattg aatatatcaa atatctaaga aatagtttca
301 aataatcacc tacttaaaat taaatttatt attaatttaa ttaatctaaa aatcaacctt
361 tctaatatta taatcttcct tttgataaat taacataatt ctagataata attcttaaaa
421 agtatcttta tttaaaaaat gttaaaaatt tgatataata gaattgtaaa aaaattcagg
481 ggggaaacca a
  1 tagccaatta atgtccccaa cattaaaagt tggaaatcaa ctatttgtta cgaaagttta
 61 taatacttca aatataaaga gaggagatat tttactcttt aattctgcaa gaaaaaataa
121 ggttaatatt ttttattaac cttatttttt cttgcagaat ataaaaccca attaatatta
181 cttataataa ctaatattat aagtaaagga atccataaaa atttaggaat cattattaac
241 ataataaatc ctataaccca aattattatt gaatatatca aatatctaag aaatagtttc
301 aaataatcac ctacttaaaa ttaaatttat tattaattta attaatctaa aaatcaacct
361 ttctaatatt ataatcttcc ttttgataaa ttaacataat tctagataat aattcttaaa
421 aagtatcttt atttaaaaaa tgttaaaaat ttgatataat agaattgtaa aaaaaattca
481 ggggggaaac caa
  1 tagccaatta atgtccccaa cattaaaagt tggaaatcaa ctatttgtta cgaaagttta
 61 taatacttca aatataaaga gaggagatat tttagtcttt aattctgcaa gaaaaaataa
121 ggttaatatt ttttattaac cttatttttt cttgcagaat ataaaaccca attaatatta
181 cttataataa ctaatattat aagtaaagga atccataaaa atttaggaat cattattaac
241 ataataaatc ctataaccca aattattatt gaatatatca aatatctaag aaatagtttc
301 aaataatcac ctacttaaaa ttaaatttat tattaattta attaatctaa aaatcaacct
361 ttctaatatt ataatcttcc ttttgataaa ttaacataat tctagataat aattcttaaa
421 aagtatcttt atttaaaaaa tgttaaaaat ttgatataat agaattgtaa aaaaaattca
481 ggggggaaac caa
  1 tagccaatta atgtccccaa cattaaaagt tggaaatcaa ctatttgtta cgaaagttta
 61 taatacttca aatataaaga gaggagatat tttagtcttt aattctgcaa gaaaaaataa
121 ggttaatatt ttttattaac cttatttttt cttgcagaat ataaaaccca attaatatta
181 cttataataa ctaatattat aagtaaagga atccataaaa atttaggaat cattattaac
241 ataataaatc ctataaccca aattattatt gaatatatca aatatctaag aaatagtttc
301 aaataatcac ctacttaaaa ttaaatttat tattaattta attaatctaa aaatcaacct
361 ttctaatatt ataatcttcc ttttgataaa ttaacataat tctagataat aattcttaaa
421 aagtatcttt atttaaaaaa tgttaaaaat ttgatataat agaattgtaa aaaaaattca
481 ggggggaaac caa
```

Attachment 5

Sequence comparison of the downstream region of the beta2-“non porcine“-toxin genes

```
  1 taaaaaaatg aggctacgcc tcatttttc ttttgatttt taatatttta ataatataaa
 61 aacctagaat ggatgatatt attctaaaaa ttgttaaatc aaaattaaaa gaatgaaaat
121 gtaacaatga aataataaca ttaaataata aaaaaataat taaacttggt tttagagaat
181 tatatttatt tataacaata ggtattaaaa taccaaaagg tataaataat aatagaataa
241 ataattgatc taatacaaat acattatcta ataacggttt attactatta aataaattat
301 ataaaaattt aaaaggtaaa tatgcacctt ttgataaata gccaccacca ttagtagtat
361 tggaaat
  1 taaaaaaatg aggctacgcc tcatttttc ttttgatttt taatatttta ataatataaa
 61 aacctagaat ggatgatatt attctaaaaa ttgttaaatc aaaattaaaa gaataaaaat
121 gtaacaatga aataataaca ttaaataata aaaaaataat taaacttggt tttagagaat
181 tatatttatt tataacaata ggtattaaaa taccaaaagg tataaataat aatagaataa
241 ataattgatc taatacaaat acattatcta ataacgattt attactatta aataaattat
301 ataaaaattt aaaaggtaaa tatgaacctt ttgataaata gccaccacca ttagtagtat
361 tggaaat
  1 taaaaaaatg aggctacgcc tcatttttc ttttgatttt taatatttta ataatataaa
 61 aacctagaat ggatgatatt attctaaaaa ttgttaaatc aaaattaaaa gaataaaaat
121 gtaacaatga aataataaca ttaaataata aaaaaataat taaacttggt tttagagaat
181 tatatttatt tataacaata ggtattaaaa taccaaaagg tataaataat aatagaataa
241 ataattgatc taatacaaat acattatcta ataacgattt attactatta aataaattat
301 ataaaaattt aaaaggtaaa tatgcacctt ttgataaata gccaccacca ttagtagtat
361 tggaaat
  1 taaaaaaatg aggctacgcc tcatttttc ttttgatttt taatatttta ataatataaa
 61 aacctagaat ggatgatatt attctaaaaa ttgttaaatc aaaattaaaa gaatgaaaat
121 gtaacaatga aataataaca ttaaataata aaaaaataat taaacttggt tttagagaat
181 tatatttatt tataacaata ggtattaaaa taccaaaagg tataaataat aatagaataa
241 ataattgatc taatacaaat acattatcta ataacggttt attactatta aataaattat
301 ataaaaattt aaaaggtaaa tatgcacctt ttgataaata gccaccacca ttagtagtat
361 tggaaat
  1 taaaaaaatg aggctacgcc tcatttttc ttttgatttt taatatttta ataatataaa
 61 aacctagaat ggatgatatt attctaaaaa ttgttaaatc aaaattaaaa gaatgaaaat
121 gtaacaatga aataataaca ttaaataata aaaaaataat taaacttggt tttagagaat
181 tatatttatt tataacaata ggtattaaaa taccaaaagg tataaataat aatagaataa
241 ataattgatc taatacaaat acattatcta ataacggttt attactatta aataaattat
301 ataaaaattt aaaaggtaaa tatgcacctt ttgataaata gccaccacca ttagtagtat
361 tggaaat
```

Attachment 6

Sequence comparison of the mature beta2 “non porcine“ protein

1 50

```
beta14_mature  ANEVNKYQSV MVQYLEAFKN YDIDTIVDIS KDSRAVTKEE YKNMLMEFKY
 beta2_mature  ANEVNKYQSV MVQYLEAFKN YDIDTIVDIS KDSRAVTKEE YKNMLMEFKY
beta20_mature  ANEVNKYQSV MVQYLEAFKN YDIDTIVDIS KDSRAVTKEE YKNMLMEFKY
beta26_mature  ANEVNKYQSI MVQYLEAFKN YDIDTIVDIS KDSRAVTKDE YKDMLMEFKY
beta46_mature  ANEVNKYQSV MVQYLEAFKN YDIDTIVDIS KDSRAVTKEE YKNMLMEFKY

               51                                                  100
beta14_mature  DPNQKLKSYE ITGSRKIDNG EIFSVKTEFL NGAIYNMEFT VSYIDNKLMV
 beta2_mature  DPNQKLKSYE ITGSRKIDNG EIFSVKTEFL NGAIYNMEFT VSYIDNKLMV
beta20_mature  DPNQKLKSYE ITGSRKIDNG EIFSVKTEFL NGAIYNMEFT VSYIDNKLMV
beta26_mature  DPNQKLKSYE ITGSRKIDNG EIFSVKTEFL NGAIYNMEFT VSYIDNKLMV
beta46_mature  DPNQKLKSYE ITGSRKIDNG EIFSVKTEFL NGAIYNMEFT VSYIDNKLMV

               101                                                 150
beta14_mature  SNMNRISIVN EGKYIPTPSF RTQVCTWDDE LSQYIGDAVS FTRSSKFQYS
 beta2_mature  SNMNRISIVN EGKYIPTPSF RTQVCTWDDE LSQYIGDAVS FTRSSKFQYS
beta20_mature  SNMNRISIVN EGKCIPTPSF RTQVCTWDDE LSQYIGDAVS FTRSSKFQYS
beta26_mature  SNMNRISIVN EGKCIPTPSF RTQVCTWDDE LSQYIGDAVS FTRSSKFQYS
beta46_mature  SNMNRISIVN EGKYIPTPSF RTQVCTWDDE LSQYIGDAVS FTRSSKFQYS

               151                                                 200
beta14_mature  SNTITLNFRQ YATSGSRSLK VKYSVVDHWM WGDDIRASQW VYGENPDYAR
 beta2_mature  SNTITLNFRQ YATSGSRSLK VKYSVVDHWM WGDDIRASQW VYGENPDYAR
beta20_mature  SNTITLNFRQ YATSGSRSLK VKYSVVDHWM WGDDIRASQW VYGENPDYAR
beta26_mature  SNTITLNFRQ YATSGSRSLK VKYSVVDHWM WGDDIRASQW VYGENPDYAR
beta46_mature  SNTITLNFRQ YATSGSRSLK VKYSVVDHWM WGDDIRASQW VYGENPDYAR

               201                                     236
beta14_mature  QIKLYLGSGE TFKNYRIKVE NYTPASIKVF GEGYCY
 beta2_mature  QIKLYLGSGE TFKNYRIKVE NYTPASIKVF GEGYCY
beta20_mature  QIKLYLGSGE TFKNYRIKVE NYTPASIKVF GEGYCY
beta26_mature  QIKLYLGSGE TFKNYRIKVE NYTPASIKVF GEGYCY
beta46_mature  QIKLYLGSGE TFKNYRIKVE NYTPASIKVF GEGYCY
```

Attachment 7

Sequence comparison of the upstream region of the beta2-“porcine“-toxin genes

```
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa ttttaggggg gaatataa
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
  1 aaatatgaat aaaatttaga taaaagtgta aaagaattat ttttatttta aatttgttaa
 61 aattttgata taattgaatt gtaaaaaaaa tttcaggggg gaatataaat gaaaaaatta
121 tttcaaagtt tactgtaatt ttt
```

Attachment 8

Sequence comparison of the downstream region of the beta2-“porcine“-toxin genes

1 tagaaaaaaa tatgaagtga ctaagtcact tcatattttt tttactatta attttattat
61 ataaaaacct aacatacatg aaagtattct taatacagtt atatcaaaat taaagtatgg
121 gaaataaaat aaaaggctaa aaactatatt aaaaactata aaaattatta aattaggttt
181 taagttgtta tatttattta tgattatagg aataaatatt ccaaagggaa taaataaaag
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301 aattgaactt aaaaaattaa atgtaaattt aaaggggata aagctcccat gtaaaagtgg
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61 ataaaaacct aacacacatg aaagtattct taatatagtt atatcaaaat taaagtatgg
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181 taagttgtta tatctattta tgattatagg aataaatatt ccaaatggaa taaataaaag
241 caatattaat aattggtcta aaaagtatac atcattgata aaagaaaaat taccagtaaa
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301 aattgaactt aaaaaattaa atgtaaattt aaaggggata aagctcccat gtaaaagtgg
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301 aattgaactt aaaaaattaa atgtaaattt aaaggggata aagctcccat gtaaaagtgg
361 act
  1 tagaaaaaaa tatgaagtga ctaagtcact tcatattttt tttactatta attttattat
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181 taagttgtta tatttattta tgattatagg aataaatatt ccaaagggaa taaataaaag
241 taatattagt aattggtcta aaaaatatac atcatcgata aaagaaaaat taccagtaaa
301 aattgaactt aaaaaattaa atgtaaattt aaaggggata aagctcccat gtaaaagtgg
361 act
```

Attachment 9

Sequence comparison of the mature beta2 “porcine“ protein

1 50

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16__PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 27_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 29_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
  3_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 32_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 34_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 38_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 40_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 43_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
  6_PORC_MATURE  KEIDTYREVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 60_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 65_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 72_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 73_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
 74_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD
  8_PORC_MATURE  KEIDAYRKVM ENYLNAFKNY DINTIVNVSE DERVNSDEKY KEMLEEFKYD

                 51                                                  100
16__PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 27_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 29_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
  3_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 32_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 34_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 38_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 40_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 43_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
  6_PORC_MATURE  PNQQLKSFEI LNSQKSDNKE IFNVKTEFMN GAIYDMKFTV SSKDGKLIVS
 60_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 65_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 72_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 73_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
 74_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS
  8_PORC_MATURE  PNQQLKSFEI LNSQKIDNKE IFNVKTEFMN GAIYDMKFTV SSKDGELIVS

                 101                                                 150
16__PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 27_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 29_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
  3_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 32_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 34_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 38_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 40_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 43_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
  6_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 60_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 65_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 72_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 73_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
 74_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA
  8_PORC_MATURE  DMERTKIENE GKYILTPSFR TQVCTWDDEL SQSIGGVDPK TYSTRFTYYA

                 151                                                 200
16__PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 27_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 29_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
  3_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 32_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 34_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 38_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 40_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
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 43_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
  6_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWVW GDDVKASQMV YGQNPDSARQ
 60_PORC_MATURE  DNILLNFRQY ATSCSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 65_PORC_MATURE  DNILLNFRQY ATSCSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 72_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
 73_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDFARQ
 74_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ
  8_PORC_MATURE  DNILLNFRQY ATSGSRDLKV EYSVVDHWLW GDDVKASQMV YGQNPDSARQ

                 201                                 235
16__PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 27_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 29_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPVSIRVFG EGYCA
  3_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPVSIRVFG EGYCA
 32_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 34_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 38_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 40_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 43_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
  6_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIKVFG EGYCA
 60_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 65_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 72_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 73_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
 74_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
  8_PORC_MATURE  IRLYIEKGQS FYKYRIRIQN FTPASIRVFG EGYCA
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