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1 INTRODUCTION

Microbial catabolism of bilirubin in the gut lumen contributes importantly to the serum bilirubin homeostasis. In the absence of bilirubin-reducing microflora, such as in the early newborn period, or in patients treated with systemic antibiotics, unconjugated bilirubin may undergo substantial enterohepatic and enterosystemic circulation. Unconjugated hyperbilirubinaemia in neonates remains of concern because of the potential danger for the central nervous system. The current therapeutic options are phototherapy or exchange transfusion in severe cases, but these procedures are costly and accompanied with potential adverse effects. Utilization of the reduction properties of intestinal microflora might possibly represent a new therapeutic approach for neonatal jaundice.

Despite the importance of the catabolic pathway of bilirubin only little is known about precise mechanisms of bilirubin reduction onto urobilinoids and the particular microbial species involved in this process. In our recent studies bacterial strains with bilirubin-reducing activity were isolated from the stools of 5 days old neonates. Such activity was detected in two novel strains of *Clostridium perfringens* and *Clostridium difficile.* The detailed analysis of metabolic products of bilirubin conversion and identification of genes encoding proteins responsible for the reduction of bilirubin is presently of major interest.

2 LITERATURE REVIEW

2.1 Neonatal hyperbilirubinemia

Neonatal hyperbilirubinemia with peak serum bilirubin levels above 220 μM occurs in approximately 8% to 20% of healthy, full-term newborn infants (Maisels 1988, Gies and Roy 1990). Such high bilirubin levels are cytotoxic, and during the neonatal period hyperbilirubinemia poses a direct threat to the central nervous system because of a possibility of bilirubin deposition in the neurons, and the development of kernicterus.

2.1.1 Metabolism of bilirubin

2.1.1.1 Bilirubin chemistry

Bilirubin is a linear derivate of a cyclic tetrapyrrol heme. The predominant isomer in the serum is bilirubin-IXα (Fig. 2-1A), so designated because its production involves oxidative cleavage of α-methene bridge in the heme ring. On account of its two carboxyl groups of the propionic acid side chains, bilirubin-IXα reacts as a slightly acid and its solubility is pH dependent in aqueous media. In the naturally occurring internally hydrogen-bonded structure, formally known as 4Z,15Z-bilirubin-IXα (Fig. 2-1B), the hydrophilic carboxyl groups are masked by hydrogen bonds involving the pyrrole nitrogens and lactam oxygens (Bonnett *et al.* 1976). This configuration is responsible for the poor water solubility of bilirubin. The other known bilirubin isomers, β, γ and δ, which are found in traces amounts in bile, are not capable of forming intramolecular hydrogen bonds and are water soluble (Onishi *et al*. 1980).

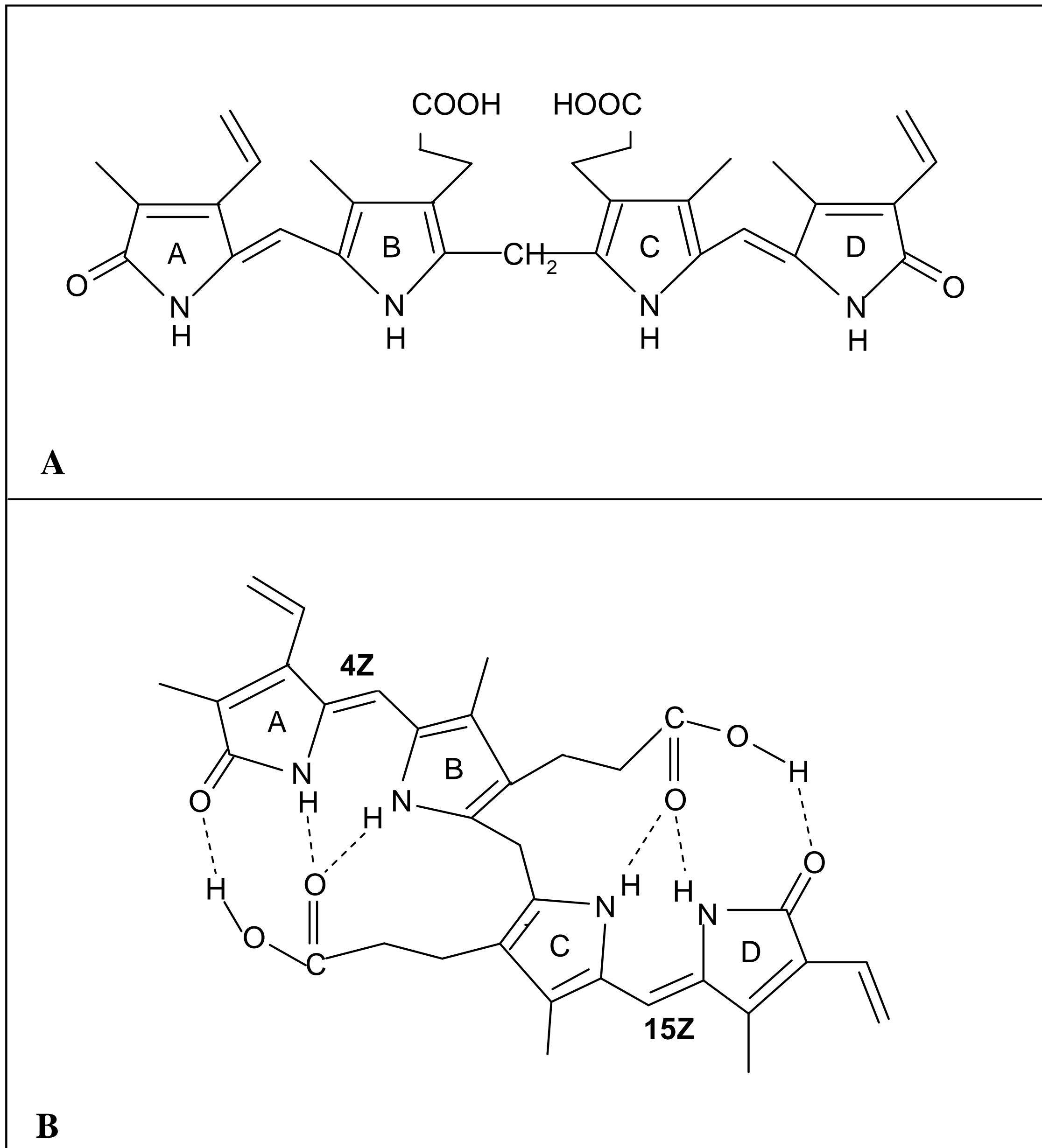

Fig. 2-1. (A) Conventional formula of bilirubin-IXα. **(B)** The hydrogen-bonded structure of 4Z,15Z-bilirubin-IXα.

2.1.1.2 Formation of bilirubin

Bilirubin, yellow, poor water-soluble pigment, is formed during the process of heme degradation. The predominant source of bilirubin is the breakdown of hemoglobin from senescent or hemolyzed red cells. Other heme-containing proteins metabolized to

bilirubin include cytochromes, catalases and muscle myoglobin (Fig. 2-2) (Schmid and McDonagh 1975). Heme is degraded by microsomal heme oxygenase, resulting in release of iron and the formation of carbon monoxide and biliverdin. Microsomal heme oxygenase is located mainly in reticuloendothelial tissues, but also is present in tissue macrophages and intestinal epithelium (Gourley and Odell 1989). Biliverdin is green, water-soluble, nontoxic molecule and represents the major degradation product in birds. In mammals, the biliverdin is immediately reduced to bilirubin by biliverdin reductase and iron is reutilized by the body (Fig. 2-3). Carbon monoxide plays important role as a gaseous messenger in neuronal signaling and modulation of vascular tone via cGMP (Marilena 1997).

2.1.1.3 Hepatic clearance of bilirubin

Due to water-insolubility, a carrier molecule, plasma albumin, is necessary to transport bilirubin from sites of its formation via circulation into the liver for excretion. The hepatic clearance of bilirubin can be divided into three stages: (a) the initial uptake of bilirubin by the hepatocyte from the sinusoids, (b) the detoxification of bilirubin by biotransformation into more water-soluble derivatives, and (c) its secretion via bile into the intestinal lumen. Bilirubin uptake across the hepatocyte sinusoidal membrane is mediated by OATP2 transporter (Cui *et al*. 2001), a member of organic anion transporting polypeptide family (Wolkoff 1996). Within hepatocyte, bilirubin is bound by two cytosol anion-binding proteins, ligandin and protein Z (Levi *et al*. 1969). Ligandin is identical to glutathione S-transferase B, a multiple enzyme that catalyze a large number of reactions in which glutathione participates as a nucleophil (Habig *et al.* 1974). Ligandin carries bilirubin to the endoplasmic reticulum (ER) of hepatocytes, where the biotransformation of bilirubin takes place. Bilirubin is conjugated with UDP-glucuronic acid to form excretable, water-soluble bilirubin monoglucuronide or diglucuronide (BMG and BDG, respectively). The glucuronic acid moiety reacts with the propionic acid side chains and the reaction is catalyzed by bilirubin UDP-glucuronosyltransferase (UGT1A1) (Peters and Jansen 1986). The excretion of bilirubin glucuronides across the canalicular membrane of hepatocytes into the bile is mediated by ABC transporter, a multidrug resistance protein (MRP2) (Kamisako *et al*. 1999).

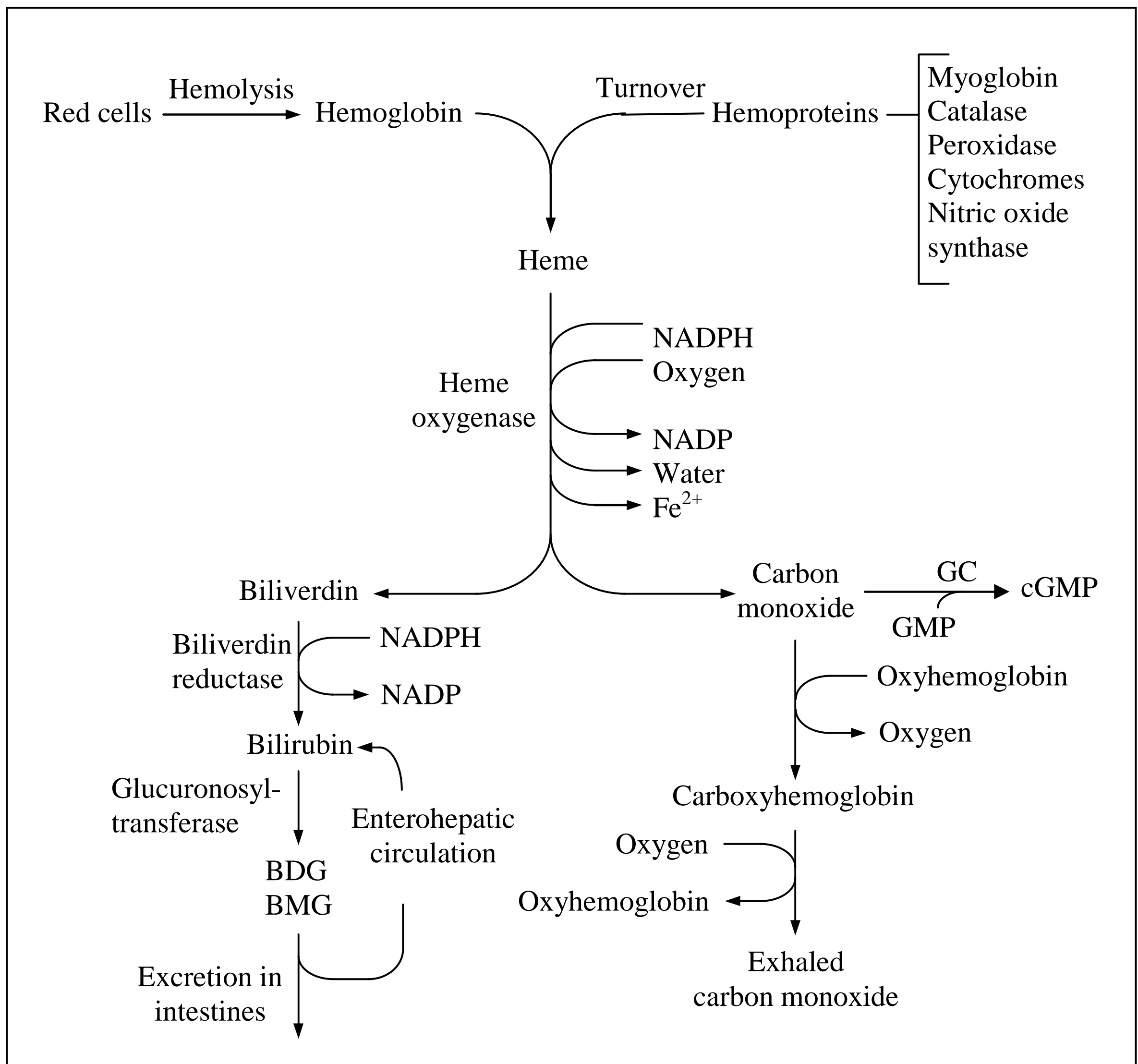

Fig. 2-2. Metabolic pathway of the degradation of heme and the formation of bilirubin.

Heme released from the hemoglobin of red cells or from other hemoproteins is degraded by an enzymatic process involving heme oxygenase, the first and rate-limiting enzyme in a two-step reaction requiring NADPH and oxygen, and resulting in the release of iron and the formation of carbon monoxide and biliverdin. Biliverdin is reduced to bilirubin by the enzyme biliverdin reductase. Carbon monoxide can activate guanyl cyclase (GC) and lead to the formation of cyclic guanosine monophosphate (cGMP). It can also displace oxygen from oxyhemoglobin to be finally exhaled. The bilirubin that is formed is taken up by the liver and conjugated with glucuronides to form bilirubin monoglucuronide or diglucuronide (BMG and BDG, respectively), in reactions catalyzed by uridine diphosphate and monophosphate glucuronosyltransferase. The bilirubin glucuronides are then excreted into the intestinal lumen but can be deconjugated by intestinal bacteria or enzymes, allowing the formed bilirubin to be reabsorbed into the circulation, as shown (From Dennery *et al*. 2001).

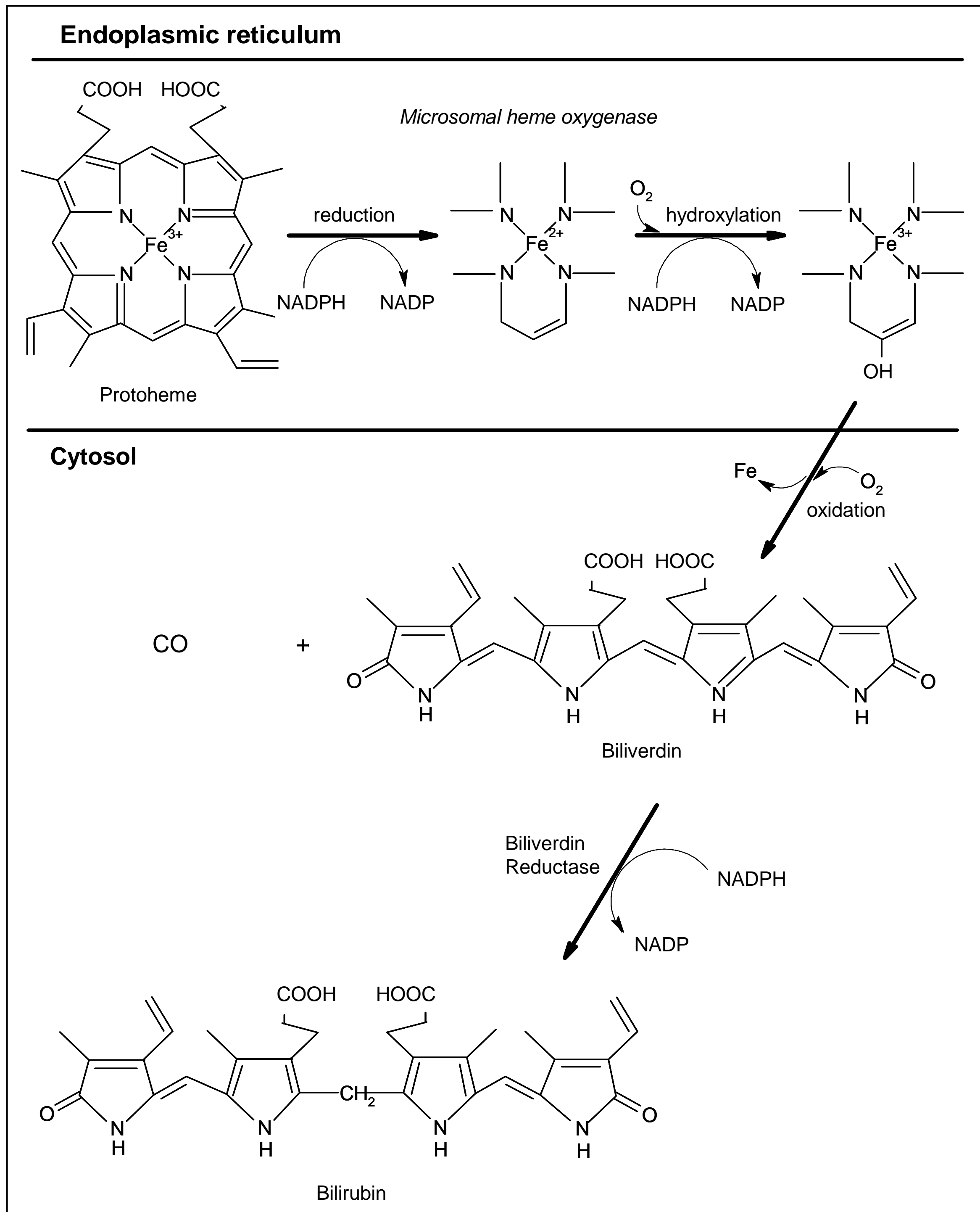

Fig. 2-3. Schematic representation of the catabolism of heme to biliverdin-IXα by coupled oxidation in the microsomal heme oxygenase system and the reduction of the biliverdin to bilirubin-IXα by the cytosolic biliverdin reductase (From Gourley and Odell 1989).

2.1.1.4 Intestinal metabolism and enterohepatic circulation of bilirubin

Conjugated bilirubin then enters the intestinal lumen where it is deconjugated by β-glucuronidase, an enzyme commonly occurring among intestinal bacteria (Rod and Midtvedt 1977, Gadelle *et al*. 1985). Enzymatic activity of β-glucuronidase was also demonstrated in hepatocytes, epithelium of biliary duct and in human breast milk (Whiting *et al.* 1993, Ho *et al*. 1986, Gourley and Arend 1986). Under specific conditions, unconjugated bilirubin undergoes enterohepatic circulation (Brink *et al*. 1996) by passive reabsorption from the intestinal lumen into portal circulation and partial uptake by hepatocytes. Because of a low first pass clearance (approximately 30%) (Bloomer and Zaccaria 1976), a significant portion of reabsorbed bilirubin reaches systemic circulation (so-called enterosystemic circulation of bilirubin) and leads to the elevation of serum bilirubin levels. Bilirubin transported to the liver is reconjugated in the hepatocytes and resecreted in the bile which results in the elevation of biliary levels of conjugated bilirubin.

Reduction of bilirubin to urobilinoids by the intestinal microflora represents a natural detoxification mechanism in adults. The term urobilinoids covers the group of reduction products of bilirubin. Among them urobilinogen and stercobilinogen with their respective oxidation products, urobilin and stercobilin, are the most important compounds (Moscowitz *et al*. 1971). The substances are believed to be nontoxic due to their increased polarity. It is generally accepted that bilirubin is reduced by multiple sequential reactions, into a series of urobilinogens; these colorless chromogens may in turn be oxidized to respective yellow oxidation products, urobilins (Fig. 2-4). It is also believed that vinyl side-chains of the A and D rings of bilirubin are reduced prior to the reduction of any double bonds within the end pyrrolic rings or the 1- and 3-methene bridges. A small part of urobilinoids undergoes enterohepatic circulation and is extracted in the urine or in the bile.

Only negligible amounts of fecal urobilinoids are present in the intestinal lumen of infants during the first months of life due to undeveloped intestinal microflora capable of reducing bilirubin (Norin *et al*. 1985, Midtvedt *et al*. 1988). This presumably contributes importantly to the pathogenesis of neonatal jaundice (Vítek *et al*. 2000). In adults, urobilinoids production is highly efficient. Under normal conditions, only small amounts of bilirubin can be found in the stools of adults (5 – 20 mg/day), whereas urobilinoids are the predominant bile pigments (50 – 250 mg/day) (With 1968). Nevertheless, only few bacterial strains have been isolated, those are unequivocally capable of reduction of bilirubin to urobilinogens: *Clostridium ramosum* (Gustafsson and Lanke 1960),

Bacteroides fragilis (Fahmy *et al.* 1972) and *Clostridium perfringens* and *Clostridium difficile* (Vítek *et al.* 2000). Previously, such catalytic activity had been already suggested to be an attribute of the *Clostridium* genus (Passini and Czakes 1923). This is consistent with previous findings by Weimer *at al.* mentioned by Watson (1977) who isolated strains of *C. perfringens*, capable of reducing bilirubin, from ileostomy content and other human fluids.

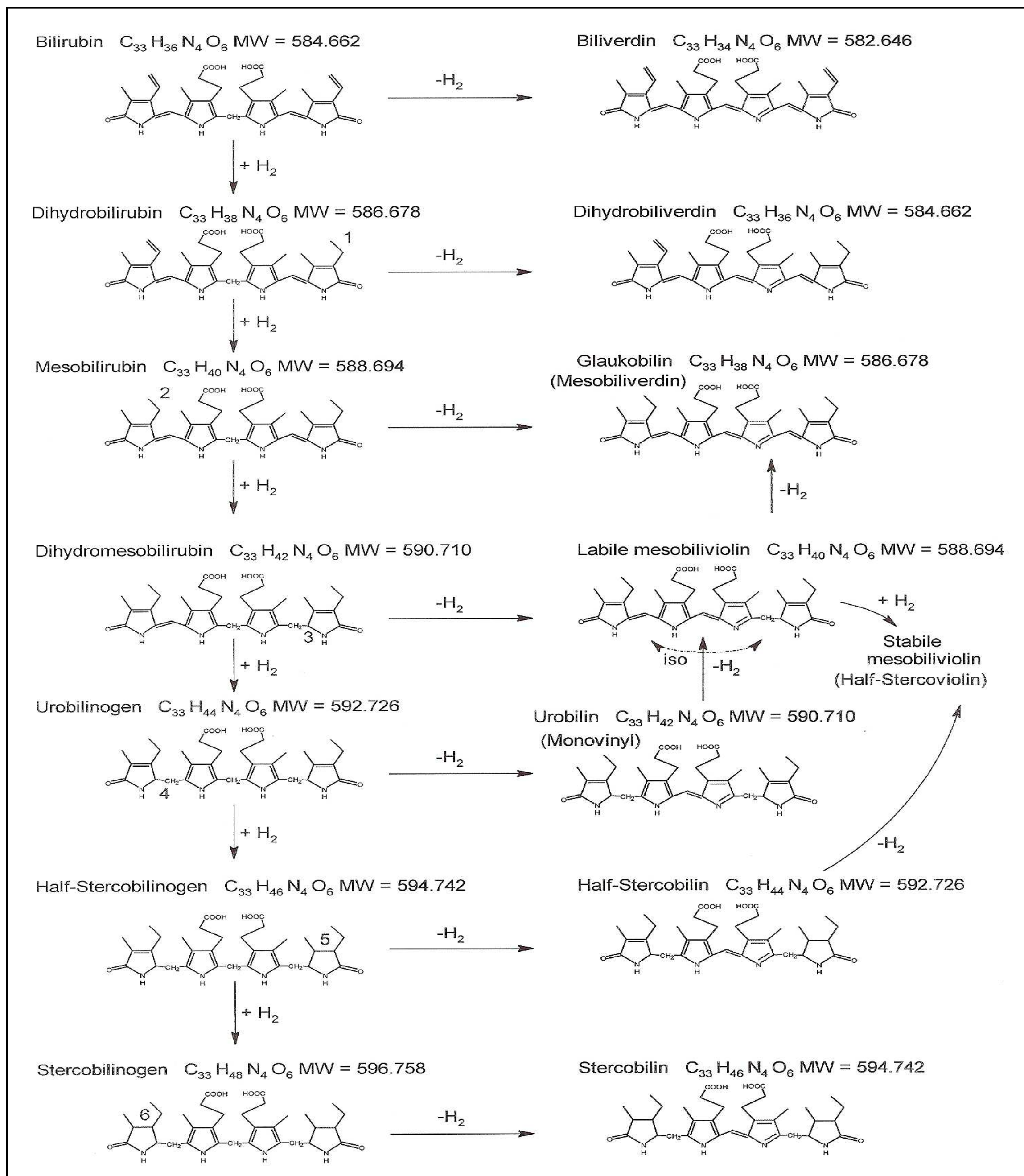

Fig. 2-4. Multiple steps in the reduction of unconjugated bilirubin by intestinal microflora showing the chemical structure of products formed.

Vítek *et al.* (2000) studied the fecal excretion of bilirubin and urobilinoids in healthy newborns and infants, as well as the intestinal bacteria capable of reducing bilirubin, to assess the influence of early urobilinoids production on enterohepatic circulation of bilirubin and on serum bilirubin concentrations. Urobilinoids were detected in stools of 57% of newborns at day 5 after delivery. However, the urobilinoids production on that day was only a fraction of that observed in adults (0.07 vs. 0.7 - 3.6 mg/kg per day (With 1968)), and was quantitatively insufficient to contribute significantly to the removal of bilirubin. At the sixth week of life fecal urobilinoinds production approached adult values (0.9 vs. 0.7 - 3.6 mg/kg per day). Cultured microbial strains from the stools of neonates with positive fecal urobilinoids were tested for their bilirubin-reducing activity. Such activity was found in two novel strains of *C. perfringens* and *C. difficile*.

The role of intestinal microflora on bilirubin homeostasis was directly demonstrated in Gunn rats, a hyperbilirubinemic strain with congenital bilirubin UDP-glucuronyltransferase deficiency, whose intestinal microflora was eradicated by wide-spectrum antibiotics. Oral administration of clindamycine plus neomycine to hyper-bilirubinemic Gunn rats resulted in disappearance of fecal urobilinods indicative of the eradication of intestinal microflora capable of reducing bilirubin (148±48 vs. 0 nm/d/100 g b.wt). Simultaneously, serum bilirubin increased dramatically (186±31 vs. 289±35 µmol/l). Intestinal colonization with a strain of *C. perfringens* reducing bilirubin led to reappearance of urobilinoids in feces to 26% of the initial values (0 vs. 39±28 nmol/d/100 g b. wt) and to a partial decrease of serum bilirubin (by 17.3%; from 289±35 to 239±17 µmol/l) (Vítek *et al.* 2005a).

2.1.2 Causes of neonatal hyperbilirubinemia

Several mechanisms are implicated in the pathogenesis of neonatal jaundice. Hepatic bilirubin clearance is decreased at birth, mainly by low bilirubin UDP-glucuronosyltransferase activity, especially in premature newborns (Kawade and Onishi 1981). Neonates have delayed maturation of bilirubin UDP-glucuronosyltransferase expression with normal activity attained by 3 month of age (Onishi *et al.* 1979). In addition, bilirubin production in neonates is more than twice the rate observed in adults, because of larger hemoglobin mass and enhanced degradation of fetal hemoglobin (Gies and Roy 1990). The fate of bilirubin in the intestine is important as well, because

deconjugated bilirubin can be reabsorbed. This can further enhance serum bilirubin levels (Poland and Odell 1971). A number of risk factors which are assumed to modify or potentiate bilirubin toxicity by either enhancing tissue uptake of bilirubin or affecting its binding to albumin have been proposed. These factors include prematurity, immaturity of the blood-brain barrier, anoxia, hypothermia, acidosis and sepsis (Walker 1987).

Other reasons for pathologic neonatal hyperbilirubinemia are inborn errors of hepatic bilirubin conjugation. These congenital errors of *UGT1A1* expression are commonly qualified as the syndromes of unconjugated hyperbilirubinemia, and include the Crigler-Najjar type I and II and Gilbert's syndromes.

Crigler-Najjar (CN) type I and II syndromes are caused by genetic lesions in any of the five exons or flanking regions of the *UGT1A1* gene (Watchko *et al.* 2002). Newborn infants with CN I type, the most sever form of *UGT1A1* deficiency, develop bilirubin encephalopathy in the first days or month of life. CN I type is typically caused by nonsense or stop mutations in the structural gene (Bosma *et al.* 1992). In contrast, the CN II, characterized by more moderate levels of indirect hyperbilirubinemia, as well as low but detectable hepatic bilirubin UDP-glucuronosyltransferase activity, appears in the majority of cases to be mediated by a missense mutation in the *UGT1A1* gene (Moghrabi *et al.* 1993).

Infants with Gilbert's syndrome also have mildly decreased bilirubin UDP-glucuronosyltransferase activity. This decrease has been attributed to an expansion of thymidine-adenine (TA) repeats in the promotor region of the *UGT1A1* gene (Bosma *et al.* 1995).

Finally, several other factors such as breast-feeding can influence neonatal jaundice. Despite many advantages of breast-feeding, there is ample documentation of a strong association between breast-feeding and an increase in the risk of neonatal hyperbilirubinemia (American Academy of Pediatrics 2001). Breast-fed infants have higher bilirubin levels than formula fed infants and there is recent confirmation that breast-feeding is significant risk factor for hyperbilirubinemia. Suggested mechanisms for these findings include poor fluid and caloric intake, inhibition of hepatic excretion of bilirubin, and intestinal absorption of bilirubin (enterohepatic circulation) (Gourley 2002).

2.1.3 Clinical features of neonatal hyperbilirubinemia

Neonatal hyperbilirubinemia can become a threat to the nervous system, presumably because of deposition of the pigment in neurons. The clinical features of kernicterus vary, and up to 15 percent of infants have no obvious neurological symptoms. The disease can be divided into an acute and a chronic form (Table 2-1). The toxicity can lead to extrapyramidal disturbances, hearing loss and, less often, to intellectual deficits (Walker 1987).

Clinical features of kernicterus
Acute form
Phase 1 (first days): poor sucking, stupor, hypotonia, seizures
Phase 2 (middle of the first week): hypertonia of extensor muscles, opisthotonus, retrocollis, fever
Phase 3 (after the first week): hypertonia
Chronic form
First year: hypotonia, active deep-tendon reflexes, obligatory tonic neck reflexes, delayed motor skills
After first year: movement disorders (choreoathetosis, ballismus, tremor), upward gaze, sensorineural hearing loss

Tab. 2-1. Clinical features of neonatal hyperbilirubinemia (Dennery *et al.* 2001).

2.1.4 Cellular toxic effects of bilirubin

The primary concern with respect to exaggerated hyperbilirubinemia is the potential for neurotoxic effects, but generalized cellular injury may also occur. *In vitro* bilirubin inhibits mitochondrial enzymes and can interfere with DNA synthesis, induce DNA-strand breakage, and inhibit protein synthesis and phosphorylation (Chuniaud *et al.* 1996).

Bilirubin has an affinity for membrane phospholipids and inhibits the uptake of tyrosine, a marker of synaptic transmission. Bilirubin may also inhibit the function of N-

methyl-d-aspartate-receptor ion channels (Hoffman *et al.* 1996). This suggests that bilirubin can interfere with neuroexcitatory signals and impair nerve conduction (particularly in the auditory nerve) (Bratlid 1990). Bilirubin can inhibit ion exchange and water transport in renal cells, which may explain the neuronal swelling that occurs in the bilirubin encephalopathy associated with kernicterus. The pigment has widespread inhibitory effects on protein phosphorylation as well (Hansen *et al.* 1996). In immature rats, increased levels of lactate, decreased levels of cellular glucose, and impaired cerebral glucose metabolism were associated with hyperbilirubinemia (Roger *et al.* 1995).

2.1.5 Treatment of neonatal hyperbilirubinemia

2.1.5.1 Phototherapy

Phototherapy has remained the standard of care for the treatment of hyperbilirubinemia in infants for four decades (Fig. 2-4). Blue-light phototherapy rapidly reduces the serum bilirubin concentration. The formation of lumirubin, a water-soluble compound, is the rate-limiting step in the elimination of bilirubin by phototherapy (Ennever *et al.* 1987). Two factors determine the rate of lumirubin formation: the spectrum (Ennever 1990), and the total dose of light delivered (Tan 1989). Because bilirubin is a yellow pigment, it is likely to absorb blue light (with a wavelength of approximately 450 nm) (Tan 1989). Thus, blue lamps are most effective in reducing hyperbilirubinemia. Longer (green) wavelengths penetrate the skin more deeply and may interact more effectively with albumin-bound bilirubin (Tan 1989), but fluorescent blue light remains the most common form of phototherapy. This therapy is widespread, but not regarded as fully satisfactory since there are many as yet unanswered questions concerning its safety and effectiveness (Gies and Roy 1990).

2.1.5.2 Exchange transfusion

Exchange transfusion was the first successful therapy for severe neonatal jaundice. This technique rapidly eliminates bilirubin from the circulation. Circulating antibodies that target erythrocytes are also removed. Exchange transfusion is especially

beneficial in infants who have ongoing hemolysis from any cause. One or two central catheters are placed, and small aliquots of blood are removed from the infant and replaced with similar aliquots of red cells from a donor, mixed with plasma. This procedure is repeated until twice the blood volume has been replaced (Dennery *et al*. 2001). Many complications of exchange transfusions have been reported, including thrombocytopenia, portal vein thrombosis, necrotizing enterocolitis (Livaditis *et al.* 1974), electrolyte imbalance, graft-versus-host disease (Lauer *et al*. 1982), and infection.

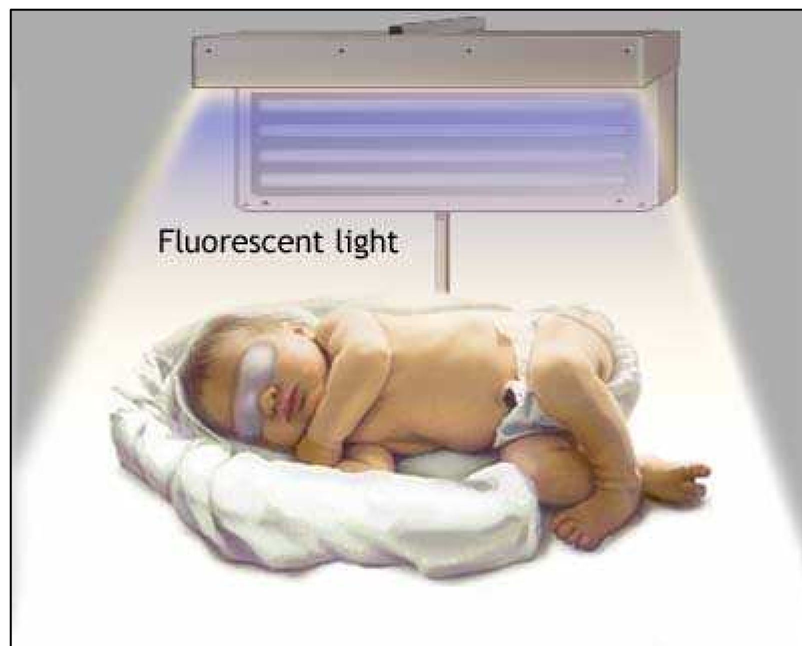

Fig. 2-5. Phototherapy. From SouthWest Washington Medical Center. http://www.swmedicalcenter.com/.

2.1.5.3 Pharmacologic therapies

Another therapeutic approach includes interruption of the enterohepatic circulation of bilirubin in the intestinal lumen. A favorable effect was described in newborn infants with neonatal jaundice treated with oral agar (Odell *at al*. 1983, Caglayan *et al*. 1993) activated charcoal (Ulstrom and Eisenklam 1964), cholestyramine (Nicolopoulos *et al*. 1978), and chitosan (Nagyvary 1982). Other effective bilirubin binders include zinc sulfate (Méndez-Sánchez *et al*. 1997) and calcium phosphate (Van der Veere *et al*. 1997). The effect of zinc salts on serum bilirubin levels was demonstrated in Gunn rats. Oral

administration of zinc salts efficiently decreased serum bilirubin levels, presumably as a result of inhibition of enterohepatic circulation of bilirubin (Vítek *et al*. 2005 b). There is also a possibility to use metalloporphyrins to inhibit bilirubin production which results in a decrease of serum bilirubin levels (Martinez *et al*. 1999). Even though this method was shown to be effective, it is not widely used in routine practice, mainly because of potential side-effect. Oral administration of immobilized bilirubin oxidase was also shown to decrease serum bilirubin levels in Gunn rats (Soltys *et al*. 1992). Bilirubin oxidase degrades serum bilirubin levels into not precisely defined catabolic dipyrrolic products. However, under physiological circumstances this enzyme does not occur in the human body.

2.2 *Clostridium perfringens*

2.2.1 C. perfringens - Introduction

The genus *Clostridium* consists of a diverse group of Gram-positive, rod-shaped bacteria which do not grow in the presence of oxygen and have the ability to form heat-resistant endospores. Many of these anaerobes are pathogenic for both humans and animals, and most of the resultant diseases, such as tetanus and botulism, are mediated by the production of potent extracellular toxins.

C. perfringens (Fig. 2-5) is different from many other clostridia in that it is non-motile, relatively aerotolerant and, *in vitro*, forms spores only in specialized culture media. The organism is fermentative and grows rapidly in media containing carbohydrates. Under these conditions it produces copious amounts of H_2 and CO_2, which help to maintain anaerobic environment. It could be commonly found in the gastrointestinal tract of humans and animals, as well as in soil and sewage. *C. perfringens* can cause human diseases such as gangrene (clostridial myonecrosis), food poisoning, necrotizing enterocolitis of infants and enteritis necroticans (McDonel 1980). On the other hand *C. perfringes* is a natural part of the normal human intestinal microflora, which plays a considerable role in the metabolism of endogenous and exogenous substances (Simon and Gorbach 1986). For example *C. perfringens* is involved in the metabolism of bile acids (Wells and Hylemon 2000), and in the metabolism of bilirubin as mentioned above.

C. perfringens is the first Gram-positive anaerobic pathogen that was completely sequenced (Shimizu *et al*. 2002). Sequenced *C. perfringens* strain 13 is a natural isolate from the soil. It is classified as a type A strain that commonly causes gas gangrene in humans (Mahony and Moore 1976). Its chromosome contains 3.031.430-bp sequence that has pronouncedly low G+C content (28.6%). The chromosome has 10 rRNA genes, 96 species of tRNA genes and 2.660 predicted ORFs. The ORFs cover 83.1% of the whole chromosomal sequence with an average size of 946 bp. The biological roles were assigned to 56.1% (1.492) of the ORFs; 18.9% (502) of the ORFs are similar to hypothetical genes of unknown function and 25.2% (666) are unique hypothetical genes with no significant similarities to putative or demonstrated genes in other organisms.

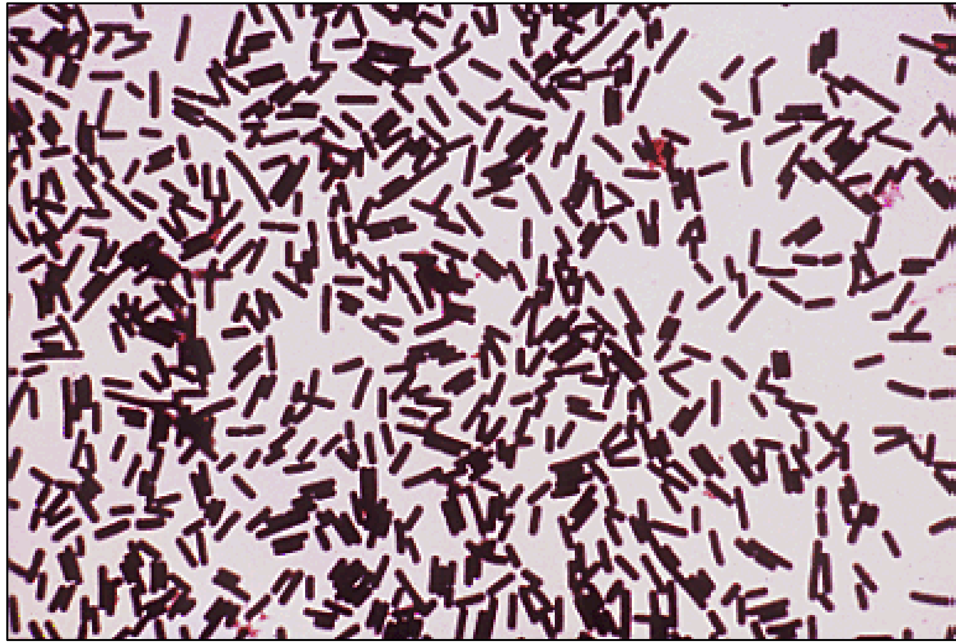

Fig. 2-6. *C. perfringens* Gram staining. (From Todar's Online Textbook of Bacteriology http://www.textbookofbacteriology.net/).

2.2.2 Experimental approach

In the past years dramatic progress has been made in the development of genetic methods for the analysis of *C. perfringens,* to the extent that *C. perfringens* is now the pathogenic clostridium most amenable to detailed genetic analysis (for review see Rood and Cole 1991). In the following section transposon-based mutagenic techniques suitable for isolation of gene(s) involved in bilirubin metabolism are reviewed. Obviously, a prerequisite for such study is means of introducing transposons into *C. perfringens*. Therefore, the second part of this chapter is focused on transformation/electroporation of *C. perfringens.*

2.2.2.1 Transposons and transposon mutagenesis

Transposons were identified over fifty years ago and subsequently became powerful tools for molecular-genetic studies. Transposon is DNA fragment that can jump to new locations within genome. Such insertion can alter regulation and expression of target gene, even more the gene could be disrupted. Transposon-based mutagenic strategies are therefore often used for identification of essential and pathogenicity-related genes in both pathogenic and nonpathogenic bacteria.

***C. perfringens* transposons**

The only transposons which have been detected to date in *C. perfringens* are the Tn*4451* and Tn*4452* (Abraham and Rood 1987) located on the conjugative tetracycline-resistance plasmids pIP401 and pJIR27, respectively. Heteroduplex analysis showed that they are very closely related, differing only in a 0.4 kb region at the end of each transposon (Abraham and Rood 1987). Well before they were characterized as transposable genetic elements, it was observed that these DNA segments, which were normally very stable in *C. perfringens*, were lost after conjugative transfer from *C. perfringens* strains containing the conjugative plasmids pIP401 and pJIR27. Subsequent studies carried out in *E. coli* showed that Tn*4451* excises precisely from multicopy plasmids (Abraham and Rood 1988); later it was shown that Tn*4451* also excises precisely from a multicopy plasmids in *C. perfringens* (Bannam *at al*. 1995). Analysis of the nucleotide sequence of Tn*4451* showed that the element is 6.338 bp in size and encodes 6 genes (Fig. 2-6), one of which, *catP*, encodes chloramphenicol resistance (Bannam *et al*. 1995). The excision is mediated by the Tn*4451*-encoded site-specific recombinase, TpnX. A transposition model (Fig. 2-6) in which the resolvase/integrase domain of TpnX excises Tn*4451* as a circular transposition intermediate has been postulated (Crellin and Rood 1997), and low frequency, intracellular transposition has been demonstrated in *E. coli* (Abraham and Rood 1987), but not in *C. perfringens.* The *tpnZ* encodes a Mob protein, TpnZ, which mediates transposon mobilization by nicking the element at the RS_A site located upstream of this gene (Crellin and Rood 1998). In the presence of an appropriate conjugative element, TpnZ and RS_A allow Tn*4451*, or plasmids on which these elements are located, to be mobilized to recipient cells (Crellin and Rood 1998, Lyras and Rood 2000). Tn*4451* carries three other genes, *tpnV*, *tpnY* and *tpnW*, the function of which is unknown, but deletion analysis

showed that they are not essential for excision or integration (Lyras *et al*. 2004). Closely related transposons, Tn*4453a* and Tn*4453b*, were identified from the chromosome of a chloramphenicol-resistant *C. difficile* isolate (Lyras *et al.* 1998). These unusual non-conjugative but mobilizable elements are grouped together as the Tn*4451/3* family of integrative mobilizable elements (Adams *et al*. 2002).

Even though Tn*4451* and Tn*4452* represent the unique group of mobilizable transposons, they are still not suitable for transposon mutagenesis, because the conditions for transposition in *C. perfringens* should be more deeply examined.

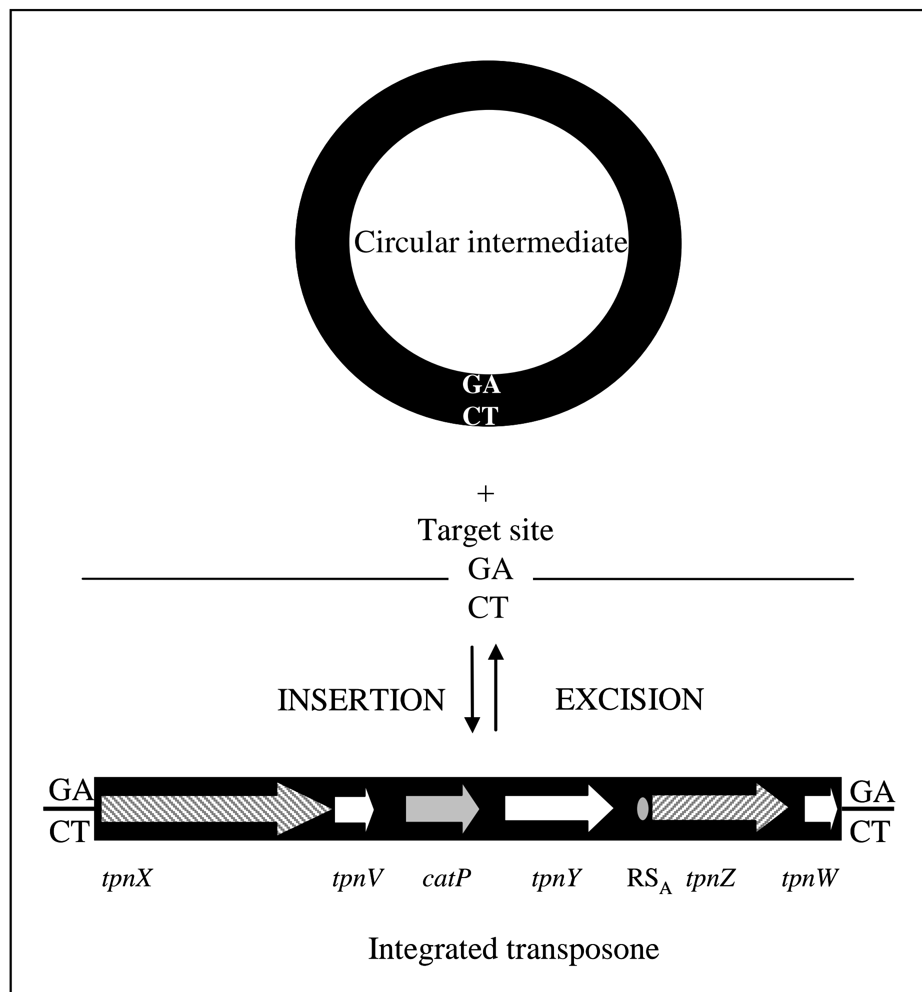

Fig. 2-7. Mechanism of transposition of Tn*4451.* The Tn*4451* is excised by TpnX to form a non-replicating circular intermediate. One copy of the duplicated GA dinucleotides is present in the circular intermediate; the other remains at the site of excision. Transposition represents the reverse of this process, with TpnX-mediated site-specific recombination leading to the integration of the element onto the chromosome, with the GA residues residing at each end of the element (From Adams *et al*. 2002).

Tn*916* mutagenesis

Tn*916*, a member of Tn*916* - Tn*1545* transposon family, is an 18.032 bp conjugative tetracycline-resistance transposon that was originally isolated from *Enterococcus* (formerly *Streptococcus*) *faecalis* (Franke and Clewell 1981). Members of Tn*916* - Tn*1545* family exhibit a broad host range and have been found in or introduced into more than 50 different gram-positive and -negative species (Clewell *et al*. 1995). As a consequence Tn*916* has been extensively used for transposon mutagenesis (Nida and Cleary 1983, High *et al*. 1996, Briolat and Reysset 2002). The element bears 24 ORFs grater than 25 codons, which encode putative proteins ranging in molecular mass from 2.9 to 93.7 kDa (Flannagan *et al*. 1994). The first step in conjugative transposition of Tn*916* is excision of the transposon from the donor DNA (Fig. 2-7). This process has been compared with excision of the bacteriophage λ (Poyart-Salmeron *et al*. 1990), as both proceed by a mechanism that include staggered cleavages on both ends of the element, circularization, transfer to a new host and insertion at target site. A difference between the excision of λ and Tn*916* is that recombining sites of λ are homologous, while the excision of Tn*916* usually occurs in the absence of sequence homology at the ends of the transposon. Tn*916* excision involves staggered cleavages at both ends of the element resulting in 6 bp 5' end overhangs, which usually differ in the sequence (Manganelli *et al*. 1996, Rudy and Scott 1994). The transposon then circularizes and the ends are ligated, resulting in a heteroduplex at circle joint (Caparon and Scott 1989). One strand of circularized transposon is transferred via a functional origin of transfer (*oriT*) to a new host where the complementary strand is synthesized (Scott *et al*. 1994). The transposon inserts at sites characterized by AT richness (Lu and Churchward 1995) and does not duplicate the target sequence (Clewell *et al*. 1988). The only Tn*916*-encoded proteins known to be involved in transposition are integrase (Int) and excisionase (Xis). Int is a site-specific recombinase (Lu and Churchward 1994, Rudy *et al*. 1997) which performs the standard cleavage and joining reactions through a phosphotyrosine intermediate. The Xis protein enhances excesive recombination (Rudy *et al*. 1997).

Tn*916* has been shown to transfer to many different clostridial species: In *C. difficile*, insertion of Tn*916* has been shown to be site specific (Mullany *et al*. 1991, Mullany *et al*. 1994), whereas in *Clostridium tetani* (Volk *et al*. 1988), *Clostridium botulinum* (Lin and Johnson 1991, Lin and Johnson 1995), and *Clostridium acetobutylicum* (Bertram *et al*. 1990, Babb *et al*. 1993) insertion appears to be relatively random.

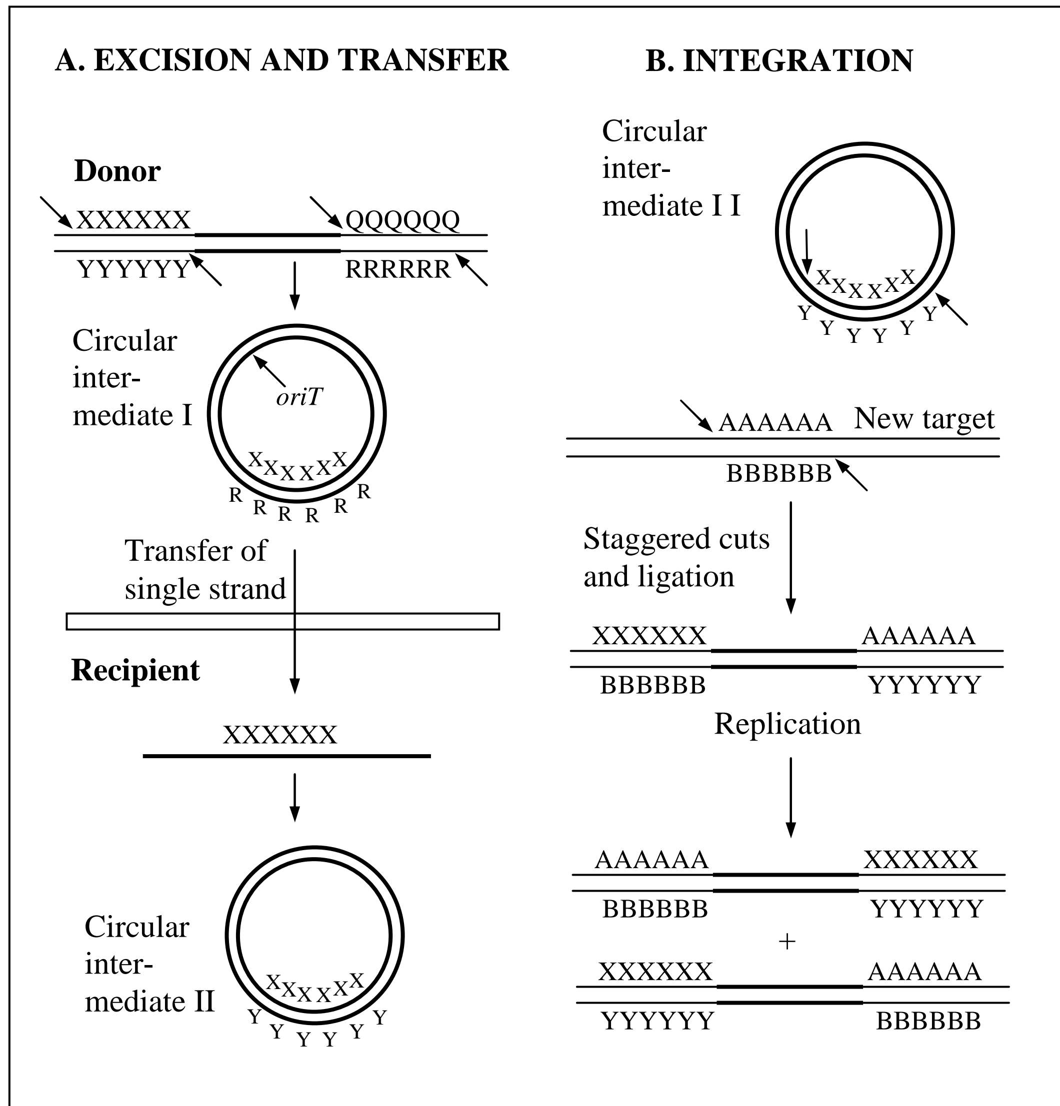

Fig. 2-8. A model for excision and integration of Tn*916* (From Scott and Churchward 1995)

In *C. perfringens* Tn*916* insertion was demonstrated by Allen and Blaschek (in Rood and Cole 1991). *C. perfringens* cells were transformed with a suicide plasmid carrying Tn*916* and transformants were selected on the basis of their ability to grow in medium containing tetracycline. Hybridization analysis showed that Tn*916* had transposed to different sites on the chromosome. Transfer of Tn*916* from *E. faecalis* and *E. coli* into *C. perfringes* was also achieved by mating (Kaufmann *et al.* 1996). The frequency of Tet^r transfer from *E. coli* to *C. perfringens* was up to 3.0 x 10^{-7} per donor and from *E. faecalis* to *C. perfringens* 2.9 x 10^{-7} per donor. Tn*916*-mutagenesis has been used for identification of a locus that regulates extracellular toxin production (Lyristis *et al.* 1994), for the

isolation of α-toxin, θ-toxin and κ-toxin mutants (Awad and Rood 1997) and for the identification of genes involved in the adaptive response to oxidative stress (Briolat and Reysset 2002). Even thought Tn*916*-mutagenesis is the only tool for random generating of *C. perfringens* mutants, it has some limitations that were encountered in studies mentioned above. Although Tn*916* can insert itself into numerous chromosomal sites, the insertion is not entirely random and tends to occur in A+T - rich regions (Scott *et al.* 1994). Tn*916* can also insert itself at multiple sites within the same strain, which complicates analysis of the mutants obtained by this method (Lyristis *et al.* 1994, Awad and Rood 1996, Briolat and Reysset 2002).

Tn*917* mutagenesis

The second transposon, that has been reported to be used in *Clostridium* genus, is Tn*917*. Tn*917* was originally isolated from *E. faecalis* and it is a member of the Gram-negative Tn*3* family, very similar in the sequence and the genetic organization to Tn*551* of *Staphylococcus aureus* (Perkins and Youngman 1984). Tn*3* family members transpose using a replicative mechanism. This involves formation of cointegrates promoted by transposase, which are then resolved by the site-specific recombination between the two directly repeated transposon copies. Recombination is mediated by the product of a gene encoding a site-specific recombinase, resolvase. Tn*917* is 5.257 bp long and 6 ORFs were identified within the element (Shaw and Clewell 1985). ORFs 1 to 3 were identical to the erythromycin resistance determinant from *Streptococcus sanguis* (Horinouchi *et al.* 1983), the ORF4 encodes resolvase (Shaw and Clewell 1985). Sequence corrections later revealed that what was formerly thought to be two open reading frames (ORF5 and ORF6, (Shaw and Clewell 1985)) is actually one reading frame (ORF5), which encodes transposase (An and Clewell 1991). Like other members of the Tn*3* family, Tn*917* generates 5 bp target site duplications and exhibits very low frequency of precise excision (Youngman *et al.* 1983, Perkins and Youngman 1984). High transposition frequency and the formation of stable insertions make Tn*917* a suitable insertion mutagenesis agent that has been used in several *Bacillus* species (Chen *et al.* 1995, Barlass *et al.* 2002) as well as in other Gram-positive bacteria, including *Staphylococcus* (Gruter *et al.* 1993, Lim *et al.* 2004) and *Streptococcus* (Gutierrez *et al.* 1996, Slater *at al.* 2003) species and *Listeria monocytogenes* (Camilli *et al.* 1990, Gardan *et al.* 2003). To the best of our knowledge, there have been no reports of the use of Tn*917* in *C. perfringens*, but the transposon was used for isolation and

characterization of solvent deficient and metronidazole resistant mutants in *C. acetobutylicum* (Babb *et al*. 1993).

2.2.2.2 Transformation methods

The first report of DNA transformation in *C. perfringens* involved the polyethylene glycol-mediated transformation of protoplasts (Heefner *et al*. 1984). The maximal transformation efficiency achieved with this method was only 10^2 transformants per µg DNA. Moreover, the technique proved to be tedious and time-consuming to perform, and sometimes gave inconsistent results. One of the more efficient and rapid techniques reported recently for many species of Gram-positive and Gram-negative bacteria is that of electroporation. The technique involves subjecting the cells to high voltage, high current electric pulse. The pulse is thought to create pores in cell membrane allowing passive influx of the DNA molecules (Calvin and Hannawalt 1988). Significant advances in development of this transformation method have been made for pathogenic *C. perfringens* strains (Allen and Blaschek 1988, 1990; Kim and Blaschek 1989, Scott and Rood 1989, Phillips-Jones 1990, Chen *et al.* 1996). Scott and Rood (1989) achieved the maximal transformation efficiency (3×10^5 transformants per µg DNA).

A notable feature of clostridial electroporation is the variety of conditions required for different species and strains. The only *C. perfringes* strain that yields enough transformants for direct cloning of genes is *C. perfringens* strain 13 (Scott and Rood 1989). Other strains are also transformable but the frequencies obtained are lower and their use requires optimization of transformation conditions. Some strains are not transformable (Scott and Rood 1989, Allen and Blaschek 1990).

The most important factors affecting transformability and transformation efficiency (transformants per µg of DNA) of *C. perfringens* are:

I. The point in the growth curve at which the cells are harvested.
II. DNase and restriction enzyme activity. DNase and restriction enzyme activity are common barriers in different *Clostridium* species (Lin and Blaschek 1984, Chen *et al*. 1996). It was demonstrated that cell associated nucleases disable plasmid transformation of *C. perfringens* type B (Chen *et al*. 1996).

III. Electric parameters. The small size of bacterial cells necessitates high initial electric field strengths for transformation, typically in the range of 5 – 20 kV/cm. The electric field strength and time constant are primary determinants of electrotransformation efficiency (Miller 1994). The maximal field strength gives the highest levels of *C. perfringens* transformants (Kim and Blaschek 1989, Scott and Rood 1989, Phillips-Jones 1990). On the other hand high values of field strength often result in unacceptable levels of cell killing and increase the occurrence of arcing during the pulse (Miller 1994). A similar effect on electroporation efficiency was found for the prolongation of the pulse (time constant).

IV. Gram-positive cell wall. In general, gram-positive bacteria appear to be more difficult to transform by electroporation than gram-negative bacteria are, although the technique is widely used for both classes of microorganisms. These difficulties are probably coupled with the cell wall composition of gram-positive bacteria (Miller 1994). Although the evidence is fragmentary, the density and thickness of the peptidoglycan layer appear to interfere with electrotransformation. Scott and Rood (1989) described electroporation of *C. perfringens* using cells that were pretreated with lysostaphin. Lysostaphin is an enzyme normally used to digest the cell wall of *Staphylococcus aureus*. It is a peptidase that cleaves the pentaglycine bridge of staphylococcal peptidoglycan (Browder *et al*. 1965) and *C. perfringens* is one of four clostridial species which contain glycine in the cell wall (Schleifer and Kandler 1972).

3 AIMS

The major way to eliminate bilirubin pigments out of the body is the degradation to urobilinogens followed by their excretion in stools and somewhat in urine. Bilirubins are transformed into urobilinogens by bacterial enzymes. The bacterial flora is almost absent in the neonates, rendering them at risk for hyperbilirubinemia.

The final aim of this project was to identify the enzyme(s) responsible for the bilirubin breakdown in the intestinal bacterium *C. perfringens*. The enzymes could then be given to neonates at risk, either directly via enema in the colon or via small, acid-resistant, capsules within milk. Alternatively, the gene(s) coding for these enzyme(s) might be possibly incorporated into probiotic microorganisms that are now given to neonates for the prevention or treatment of certain intestinal infections.

The direct aims of the present study were:

I. Detailed analysis of bile pigments metabolised by *C. perfringens* and their respective reduction products.

II. Identification of gene(s) involved in bilirubin metabolism by using the principle of transposon mutagenesis, or alternatively by construction and screening *C. perfringens* genomic library in *E. coli* for clones able to reduce bilirubin. The classical approach, purification of the bilirubin-reducing enzyme has been performing in cooperating laboratory (L.Vítek, 1st Medical Faculty, Charles University of Prague).

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Bacterial strains and plasmids

Strain or plasmid	Characteristic	Reference/source
Strains		
C. perfringens		
Bilirubin-reducing (BR1)	Wild type, isolated from neonates with positive fecal urobilinoid excretion	Vítek *et al*. 2000
13	Wild type	Mahony and Moore 1976
P90.2.2.	Wild type	Smart *et al*. 1979
E. coli		
DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(r_K^-, m_K^+), *phoA*, *supE*44, λ–, *thi*-1, *gyrA*96, *relA*1	Sambrook *et al.* 1989
JIR2348	DH5α strain carrying *E. coli – C. perfringens* shuttle vector pJIR750	Bannam and Rood 1993
S17-1	*recA* derivative of *E. coli* 294 (F^- *thi pro hsdR*) carrying a modified derivative of IncPα plasmid pRP4 (Ap^s Tc^s Km^s) integrated in the chromosome, Tp^r	Simon *et al*. 1983
Plasmids		
pJIR750	*E. coli-C. perfringens* shuttle vector, 6.568 kb, Cm^r	Bannam and Rood 1993
pAM120	Derivative of pBR322 carrying Tn*916*, 23.363 kb, Ap^r, Tc^r	Gawron-Burke and Clewell 1984
pTV408	13.9 kb vector carrying Tn*917*, Ap^r, Km^r	Slater *et al.* 2003
pK19	pUC based vector, 2.661 kb, Km^r	Pridmore 1987

4.1.2 Cultivation media and growth conditions

Media for *C. perfringens* cultivation:

FTG (BBL)

TGY (Kim and Blaschek 1989): Trypticase peptone (BBL) 3 %, Yeast extract (Difco) 1 %, L-cysteine (AppliChem) 0.1 %, Glucose (AppliChem) 2 %. For solid-agar plate growth, BactoAgar (Difco) 1.5 % was added to TGY medium.

TGY-expression (Allen and Blaschek 1990): TGY containing $CaCl_2$ 25 mM, $MgCl_2$ 25 mM, and BactoAgar (Difco) 0.075 %, adjusted to pH 6.4

TPG (Rood *et al.* 1978): Trypticase peptone (BBL) 5 %, Proteose peptone (Oxoid) 0.5 %, Sodium thioglycollate (Sigma) 0.1 %

Nutrient agar (Rood 1983): Nutrient broth No2 (Oxoid) 2.5 %, Yeast extract (Difco) 0.3 %, Sodium thioglycollate (Sigma) 0.1 %, Glucose (AppliChem) 0.25 %, BactoAgar (Difco) 2 %

BHI (Difco) For solid-agar plate growth, BactoAgar (Difco) 1.5 % was added to BHI medium.

Sucrose-BHI (Scott and Rood 1989): BHI (Difco) 3.7 %, Sodium thioglycollate (Sigma) 0.1 %, Glucose (AppliChem) 1.5 %, Sucrose (AppliChem) 25 %

Regeneration-BHI: BHI (Difco) 3.7 %, Sodium thioglycollate (Sigma) 0.1 %, Glucose (AppliChem) 1.5 %

Diluent-BHI (Scott and Rood 1989): BHI (Difco) 0.37 %, Sodium thioglycollate (Sigma) 0.1 %

YE medium: Yeast extract (Difco) 2 %, Na_2HPO_4 x $12H_2O$ 65 mM, KH_2PO_4 5 mM, adjusted to pH 8.

C. perfringens was grown under anaerobic conditions using a Gas generation kit (Oxoid) at 37 °C. All manipulations were performed aerobically using media and buffers pre-incubated anaerobically overnight. Agar plates were anaerobically pre-incubated for 24 h. *C. perfringens* cells were maintained in the cooked meat medium (Phillips-Jones 1995). The media were supplemented with chloramphenicol (5 - 25 µg/ml), erythromycin (50 µg/ml) or tetracycline (10 µg/ml) as required.

Media for *E. coli* cultivation:

LB (Sambrook *et al.* 1989): Tryptone peptone (Difco) 1 %, Yeast extract (Difco) 0.5 %, NaCl (Analar) 1 %. For solid-agar plate growth, BactoAgar (1.5 %, Difco) was added to LB medium.

YE medium: Yeast extract (Difco) 2 %, Na_2HPO_4 x $12H_2O$ 65 mM, KH_2PO_4 5 mM, adjusted to pH 8.

E. coli was grown under aerobic condition at 37 °C. For long-time storage *E. coli* was kept at -70 °C in LB medium containing 20 % glycerol. For selective purposes, the LB agar plates were supplemented with ampicilin (50 µg/ml), chloramphenicol (30 µg/ml), erythromycin (150 µg/ml), kanamycine (50 µg/ml), or tetracycline (10 µg/ml).

4.1.3 Chemicals, enzymes, abbreviations

Chemicals

Ampicilin	Ap	Fluka
5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside	X-gal	Alexis Corporation
Chloramphenicol	Cm	Sigma
Erythromycin	Em	Sigma
Ethylenediamine tetraacetic acid	EDTA	Serva
Isopropyl-thiogalactoside	IPTG	Sigma
Lysostaphin (2000 U/mg)		Sigma
Lysozyme		USB
Kanamycin	Km	Fluka
Phenol		Q – BIO gene
Proteinase K		USB
Ribonuclease A	RNase	USB
Shrimp Alkaline Phosphatase	SAP	USB
Sodium dodecyl sulfate	SDS	Serva
T4 DNA ligase		USB
Tetracycline	Tc	USB

Tris(hydoxymethyl)aminomethane	Tris	Serva

Bile pigments

Bilirubin diamide		Gift of Prof. Jirsa
Bilirubin diethylester		Prepared according to Blanckaert 1980
Bilirubin diglucuronide	BDG	Prepared according to Wu *et al*. 1980
Bilirubin dimethylester	BDM	Porphyrin products
Bilirubin ditaurate	BDT	Porphyrin products
Biliverdin		Sigma
Mesobilirubin	MB	Porphyrin products
Unconjugated bilirubin IXα	UCB IXα	Sigma
Unconjugated bilirubin IXβ	UCB IXβ	Prepared according to Heirweg *et al*. 1991
Urobilin		Porphyrin products
[^{14}C]-labeled bilirubin		Prepared according to Ostrow *et al.* 1961

Other chemicals used were of analytical grade.

DNA restriction enzymes

Eco RI	NEB
Bam HI	NEB
*Nde*I	NEB
Pvu I	NEB
Tsp 509I	NEB

4.2 Methods

4.2.1 Determination of urobilinoids, reduction products of bilirubin (modified protocol according to Kotal and Fevery 1991)

The method is based on oxidation of urobilinogens to urobilins and measurement of the characteristic green fluorescence of their Zn^{2+} salts (Schlesinger's reaction).

4 µl of bilirubin solution (2.5 mM bilirubin dissolved in DMSO) were added to 196 µl of a mid-exponential phase *C. perfringens* culture grown in YE medium. Alternatively, *C. perfringens* cells were disintegrated using French mini pressure cell press (4 x, 9000 PSI) and crude protein extract was used. The sample was cultivated anaerobically at 37 °C overnight (24 h). Then it was mixed with 1.2 ml of Zn-acetate solution (1% Zn-acetate dissolved in DMSO). Urobilinogen was oxidized by addition of 100 µl of iodine solution (25 mM I_2 dissolved in 120 mM KI solution in water). After vigorous vortexing the residue of iodine was reduced by addition of 50 µl of 82 mM cysteine in water. Sample was centrifuged at 3000 x g, for 5 min at room temperature and absorption spectrum (400 – 680 nm) was measured in supernatant.

Quantification of urobilins was performed by comparing of absorption maximum of Zn-complex of urobilin (508.5 nm) with a calibration curve of Zn-complex of standard urobilin-i (Fig. 4-1). The bile pigment conversion rate was calculated as a proportion of urobilinoid production to the initial bile pigment concentration.

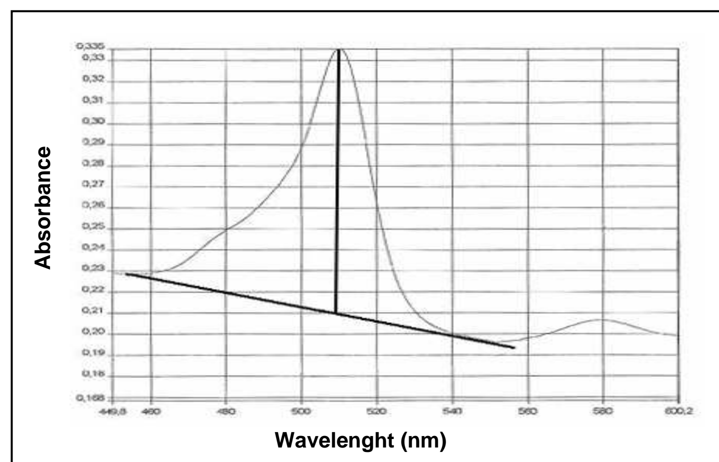

Fig. 4-1. Quantification of urobilinoids according to Kotal and Fevery (1991).

4.2.2 Analysis of bile pigment reduction products

4.2.2.1 Extraction of bile pigments from media (according to Blanckaert 1980)

One ml of the YE medium containing bile pigments produced by the strain BR1 was mixed with 60 mg of sodium ascorbate, 2-3 mg of EDTA and 5 ml of methanol. After vigorous mixing, 6 ml of chloroform and 12 ml of glycine/HCl buffer (0.4 M, pH 2.4) were added sequentially, and the mixture was again shaken and centrifuged. The organic phase was transferred to a dry tube and evaporated under nitrogen at 30 °C.

4.2.2.2 TLC and spectral analysis of the extracted pigments

Extracted pigments were sequentially dissolved in methanol and chloroform (2:1) and separated by thin layer chromatography (TLC) using plates coated with silica gel (HPTLC, silica gel 60 Merck) in a solvent system composed of chloroform:acetic acid (99:1, by vol.). The separated products were examined under visible and UV light, and extracted from silica gel with methanol (urobilinoids) or chloroform (UCB, MB) or with mixtures of both solvents and further analyzed by spectrophotometry (UV/Vis Spectrophotometer Lambda 20, Perkin-Elmer), spectrofluorometry (Luminescence Spectrometer LS 55, Perkin-Elmer) and mass spectrometry (ZAB-EQ, Micromass).

4.2.2.3 Mesobiliviolin reaction (according to Watson *et al*. 1960)

Mesobiliviolin is formed by oxidation of urobilin with $FeCl_3$ in HCl (= mesobiliviolin reaction) and has intensive violet color. Mesobiliviolin reaction is suitable for qualitative differentiation of urobilin and urobilinogen from stercobilin and stercobilinogen in biological materials.

Extracted pigments were dissolved in 2 ml of methanol and 1 ml of 20 % $FeCl_3$ in concentrated HCl was added. The mixture was boiled for 15 min and then adjusted with saturated sodium acetate solution approximately to a pH 4.2. The oxidized pigments were extracted twice with ethyl ether. The extracts derived from urobilin are blue-violet

coloured, whereas that from strercobilin are brownish or salmon coloured, due to unchanged stercobilin.

4.2.2.4 TLC of reduction products formed from [^{14}C]-labeled bilirubin

Direct identification of reduction products of bilirubin was performed using [^{14}C]-labeled bilirubin as a substrate. [^{14}C]-labeled reduction products were separated by TLC (HPTLC, silica gel 60 Merck) in a solvent system composed of chloroform:acetic acid (99:1, by vol.). Separated chromogens were oxidized *in situ* under UV light, followed by development in the second solvent system composed of chloroform:methanol:acetic acid (80:19:1, by vol.) and visualized using Phosphoimager Bas 5000 (Fuji).

4.2.3 DNA manipulations

If not otherwise indicated, all DNA manipulations and DNA gel electrophoresis were carried out according to standard protocols (Ausubel *et al*. 1995). Plasmid DNA intended for electroporation of *C. perfringens* was isolated from *E. coli* by using an alkaline lysis method (Sambrook *et al.* 1989). All restriction endonucleases and DNA modifying enzymes were used according to manufacturer's instructions.

Special techniques, such as isolation of chromosomal DNA from *C. perfringens*, extraction of chromosomal fragments from agarose gel and electroporation induced transformation of *E. coli*, involved in construction of *C. perfringens* genomic library are described in separate chapter 4.2.6.

4.2.4 Electroporation induced transformation of C. perfringens

All electroporation experiments were performed using the Bio-Rad Gene Pulser. Controls for each transformation experiment consisted of an unshocked cell culture without added plasmid DNA, and shocked cell culture without added plasmid DNA. To determinate a percent of cell killing the shocked sample was serially diluted and plated on

nonselective agar plates. The transformation efficiency was defined as a number of Antibiotic-resistant transformants per µg of DNA.

4.2.4.1 Electroporation of late-stationary phase cells (according to Kim and Blaschek 1989)

Cells from a fresh overnight culture of *C. perfringens* grown in TGY broth were inoculated into fresh TGY broth to a starting $O.D._{600} = 0.1$ and incubated at 37 °C for 6 h. A late-stationary phase *C. perfringens* cell culture was harvested by centrifugation at 5000 x g for 10 min at 4 °C. The cell pellet was washed in cold electroporation buffer (15 % glycerol). The washed cell pellet was suspended in 1/20 of a volume of cold electroporation buffer. Cell suspension (0.4 ml) was mixed with plasmid DNA (1 µg per ml) in the 0.2 cm-gap electroporation cuvette and incubated for 10 min on ice. The chilled cell mixture was shocked at 2.5 kV and 25 µF. Following 10 min post-electroporative incubation on ice, the cells were diluted into 9 volumes of TGY medium and incubated for 1 h at 37 °C. The culture was centrifuged and suspended in 0.4 ml of TGY medium. The cell suspension was plated onto TGY selective plates and incubated under anaerobic conditions overnight at 37 °C.

4.2.4.2 Electroporation of lysostaphin-treated cells I (according to Scott and Rood 1989)

C. *perfringens* was grown overnight on nutrient agar. Single colonies were subsequently subcultured into 5 ml of FTG and grown overnight. The FTG culture was then inoculated into 100 ml of TPG broth to a starting $O.D._{600} = 0.02$ and incubated at 37 °C. An early-exponential phase *C. perfringens* cell culture ($O.D._{600} = 0.12 - 0.18$) was harvested by centrifugation at 12000 x g for 15 min at 20 °C, washed twice in 10 ml of SEB (0.272 M sucrose, 1 mM $MgCl_2$, 7 mM Na_2HPO_4, adjusted to pH 7.4) and resuspended in 10 ml of SEB. The cells were then treated for 1 h at 37 °C with lysostaphin (2000 units per mg) at a final concentration of 5 to 10 µg per ml. After two washes in SEB, the cells were suspended in 10 ml of SEB and 0.4-ml aliquots were mixed with 2.5 µg of plasmid DNA, transferred to prechilled cuvettes (0.2 cm gap) and placed for 10 min on ice. The sample was shocked at 2.5 kV, 200 Ω and 25 µF. After a further 10 min on ice, the

entire cell mixture was subcultured into 20 ml of Sucrose-BHI broth. After overnight incubation at 37 °C the cells were harvested by centrifugation, suspended in 1 ml of Diluent-BHI, plated onto selective nutrient agar plates and incubated under anaerobic conditions overnight or longer before transformants were counted.

4.2.4.3 Electroporation of lysostaphin-treated cells II (modified protocol)

C. *perfringens* was grown overnight on nutrient agar. Single colonies were subsequently subcultured into 5 ml of FTG and grown overnight. The FTG culture was then inoculated into 100 ml of TPG broth to a starting $O.D._{600}$ = 0.02 and incubated at 37 °C. An early-exponential phase *C. perfringens* cell culture ($O.D._{600}$ = 0.12 – 0.18) was harvested by centrifugation at 12000 x g for 15 min at 20 °C, washed once in equal volume of 20 °C SEB (0.272 M sucrose, 1 mM $MgCl_2$, 7 mM Na_2HPO_4, adjusted to pH 7.4) and resuspended in 10 ml of SEB. The cells were then treated with lysostaphin (2000 units per mg) at a final concentration of 0.5 to 10 μg per ml for 1 h at 37 °C. Cell suspension was centrifuged at 12000 x g for 15 min at 4 °C and once washed in equal volume of cold SEB. Cells were suspended in 10 ml of cold SEB and 0.4-ml aliquots were mixed with 2.5 μg of plasmid DNA, transferred to prechilled cuvettes (0.2 cm gap) and placed for 10 min on ice. The sample was shocked at 2.5 kV, 200 Ω and 25 μF. Immediately after the pulse delivery a half of transformation mixture was added to 10 ml of Sucrose-BHI medium. The rest was incubated on ice for 10 min, and then added to 10 ml of Sucrose-BHI medium. After overnight incubation at 37 °C the cells were harvested by centrifugation, suspended in 0.5 ml of Diluent-BHI medium and plated.

4.2.4.4 Electroporation of early-exponential phase cells (Jirásková *et al.* 2005)

Ten ml of FTG medium was inoculated with 0.1 ml of stock *C. perfringens* culture and incubated anaerobically overnight at 37°C. The overnight FTG culture was used to inoculate a 100 ml of TPG medium to a starting $O.D._{600}$ = 0.02. The early-exponential-phase cell culture (OD_{600} = 0.20 - 0.25) was harvested by centrifugation at 12,000 x g for 15 min at 20° C, washed once in 10 ml of SMP electroporation buffer (272 mM sucrose, 7 mM Sodium phosphate pH 7.4, 1 mM $MgCl_2$) and resuspended in 10 ml of

SMP. 0.4-ml aliquots were mixed with 2 μg of pJIR750 DNA, transferred to prechilled cuvettes (0.2 cm gap) and placed on ice for 10 min. Electroporation was carried out at 25 μF, 200 Ω and 9 kV · cm $^{-1}$. Immediately after the pulse delivery, transformation mixture was subcultured into 20 ml of Regeneration-BHI medium. Suspension was incubated at 37°C for 3 h to allow gene expression. Then cells were harvested by centrifugation, resuspended in 1 ml of Diluent-BHI medium and plated on selective BHI agar plates.

4.2.5 DNase activity of intact cells of C. perfringens (according to Chen and Blaschek 1996)

C. perfringens competent cells (1 ml) were mixed with 10 μg of pJIR750 DNA and the sample was incubated on ice for different time intervals. Following the incubation, the sample was centrifuged, the cell pellet discarded and the supernatant containing the plasmid DNA was transferred to a microcentrifuge tube. DNA was recovered following precipitation with 2 volumes of cold (-20 °C) 95 % ethanol, pelleted, dissolved in distilled water and assayed by agarose gel electrophoresis.

4.2.6 Plasmid DNA isolation from C. perfringens

Cells (1 ml) of both bacteria transformants grown overnight in appropriate selective media, were centrifuged at 13000 x g for 2 min at room temperature, and subsequently dried for 5 min at room temperature. The cell pellet was suspended in 100 μl of GTE buffer (50 mM glucose, 25 mM Tris, 10 mM EDTA, adjusted to pH 8) containing lysozyme 2 mg per ml and incubated for 30 min at room temperature. 200 μl of alkaline lysis buffer (1% sodium dodecyl sulfate, 0.2 N NaOH) was added to the cell suspension, and the mixture was incubated for 10 min on ice. 150 μl of 5 M potassium acetate buffer (pH 4.8) was added to the lysed cell suspension and the mixture was incubated for 30 min on ice to precipitate chromosomal DNA and proteins. Plasmid DNA was recovered by following centrifugation at 13000 x g for 10 min at 4 °C and extracted once with phenol (Tris buffer saturated, pH8):chloroform:isoamyl alcohol (25:24:1) followed by one chloroform extraction. Plasmid DNA was precipitated by adding 2 volumes of cold ethanol

and incubated for 2 h at -20 °C. After washing with 70 % ethanol and drying at room temperature, the DNA pellet was dissolved in TE buffer containing 50 µg of RNase per ml.

4.2.7 Conjugative transfer of pAM120 plasmid DNA from E. coli S17-1 to C. perfringens (modified protocol according to Simon *et al*. 1983 and Kaufmann *et al*. 1996)

E. coli S17-1 containing conjugative plasmid pAM120 was grown aerobically overnight at 37 °C on LB-agar plate containing tetracycline at concentration of 10 µg per ml. The plate was transferred into anaerobic jar. *C. perfringens* cells were grown anaerobically overnight at 37 °C in BHI medium, diluted 50 times in fresh media and incubated anaerobically until they reached $O.D._{600}$ of 0.6. Cells were once washed in one volume of anaerobic BHI medium and diluted in different volumes of BHI medium. Using a sterile toothpick, a visible amount of *E. coli* S17-1 cells was picked off the agar plate, and carefully transferred into eppendorf tube containing 50 µl of *C. perfringens* cells. Cells were carefully mixed using wide-ended tip, spotted onto the center of very well dried BHI-agar plate and incubated anaerobically overnight at 37 °C. Using a loop, transconjugants were scraped off the BHI-agar plate and resuspended in 200 µl of BHI medium. 20 µl and 180 µl aliquots were plated onto selective BHI-agar plates and incubated anaerobically for 3-4 days at 37 °C.

4.2.8 Construction of a C. perfringens genomic library

4.2.8.1 Chromosomal DNA isolation (modified protocol according to Gerhards *et al*. 1994)

A mid-exponential phase cell culture of *C. perfringens* (10 ml) grown in TGY medium was harvested by centrifugation at 5000 x g for 10 min at room temperature and washed with 10 ml of TEN buffer (30 mM Tris, 10 mM EDTA, 50 mM NaCl). The cell pellet was suspended in 750 µl of TEN buffer containing lysozyme 4 mg per ml and RNase 50 µg per ml, and incubated for 30 min at 37 °C. 20 % sodium dodecyl sulfate solution to a

final concentration of 0.5 % and 10 µl of proteinase K from 10 mg per ml stock solution was added to the cell suspension. Cell suspension was incubated for 1 h at 56 °C until the lysate became clear. Chromosomal DNA was extracted once with phenol (Tris buffer saturated, pH 8):chloroform:isoamyl alcohol (25:24:1) and once with chloroform. Chromosomal DNA was precipitated by adding 2 volumes of cold ethanol and incubated overnight at -20 °C. After washing with 70 % ethanol and drying at room temperature, the DNA pellet was dissolved in distilled water.

4.2.8.2 Extraction of chromosomal DNA fragments from agarose gel using Glass milk

Agarose gel slices containing appropriate chromosomal DNA fragments were excised from a gel and 2-3 ml of NaI buffer (12 M NaI, 20 mM Tris, pH 7.5, Na_2SO_3) per 1 g of gel were added. Mixture was incubated for 5 min at 53 °C, or until gel slices had completely dissolved. 1 µl of Glass milk solution* per µg of DNA was added and mixture was placed for 5-10 min on ice. Suspension was centrifuged at 13 000 x g for 10 s, and pellet was twice washed in 0.5 ml of NEET buffer (100 mM NaCl, 1 mM EDTA, 50 % ethanol, 10 mM Tris, pH 7.5). Pellet was dried for 1 min at room temperature, suspended in 50 µl of distilled water and eluted for 5-10 min at 53 °C. Eluted DNA was extracted once with phenol (Tris buffer saturated, pH 8) : chloroform : isoamyl alcohol (25:24:1), once with chloroform and precipitated by adding 0.1 of a volume of 3 M Na-acetate (pH 4.8) and 0.7 of a volume of isopropanol. After washing in 70 % ethanol and drying at room temperature, the DNA pellet was dissolved in distilled water.

* Two hundred g of glass powder (Silica, surface area 255 g/m^2, Sigma) was suspended in 400 ml of distilled water, stirred for 60 min and settled down for 90 min. Suspension was centrifuged for 10 min and sediment was suspended in 100 - 150 ml of distilled water. HNO_3 to a final concentration of 50 % was added and mixture was warmed up to 90 °C and cooled down. Sediment was washed for 4-6 times in distilled water, until pH 7 was reached. Sediment was finally suspended in distilled water to a final concentration of 50 % and kept at - 70 °C.

4.2.8.3 Electroporation-induced transformation of *E. coli* (according to Dower *et al*. 1988)

Ten ml of an overnight *E. coli* DH5α culture was used to inoculate 1000 ml of LB medium. Culture was vigorously shaking at 37 °C, until $O.D._{600} = 0.5$ was reached. The culture was incubated for 20 min on ice to stop the growth. Cells were harvested by centrifugation at 1200 x g for 15 min at 4 °C, washed once in 500 ml of distilled water and suspended in 100 ml of cold 10 % glycerol. Suspension was incubated for 20 min on ice, centrifuged at 1200 x g for 10 min at 4 °C, suspended in 10 ml of cold 10 % glycerol and kept for 20 min on ice. After centrifugation pellet was suspended in 4 ml of cold 10 % glycerol. 100 µl aliquots were frozen in liquid nitrogen and kept at -80 °C.

Hundred µl of competent cells was mixed with 10 - 20 ng of plasmid DNA or 3 µl of ligation mixture and transferred to pre-chilled cuvette (0.2 cm gap). The sample was shocked using Gene Pulser (Bio-Rad) set at 2.5 kV, 200 Ω and 25 µF. Immediately after the pulse delivery transformation mixture was added to 1 ml of LB medium. After 1 h incubation at 37 °C cells were harvested by centrifugation, suspended in 0.1 ml of LB medium and plated.

4.2.8.4 Construction of *C. perfringens* genomic library

Two hundred µg of chromosomal DNA of *C. perfringens* P90.2.2., isolated according to protocol 4.2.6.1, was partially digested with *Tsp* 509 I (60 U) for 5 min at 65 °C. DNA fragments in the range from 8 to 12 kb were isolated from 0.8% agarose gel (4.2.6.2) and ligated with *Eco* RI digested and SAP dephosphorylated pK19. pK19 is a pUC based vector with a Km resistance gene replacing the ampicilin resistance gene of the pUC series. It also contains a multiple cloning site in a *lacZα* gene fragment for easy selection of inserts on X-gal plates (Fig. 4-2). *E. coli* DH5α electrocompetent cells were transformed with 3 µl of ligation mixture (4.2.6.3). Transformants were selected on LB agar plates supplemented with Km (50 µg/ml), then replica plated on LB agar plates containing IPTG (0.2 mM) and X-gal (40µg/ml) to identify recombinants (Fig. 4-3). The size of inserted fragments from vectors of putative positive clones was determined by double digestion with *Bam*HI and *Pvu*I enzymes. Recombinants were propagated in 96-well plates and stored at -80 °C.

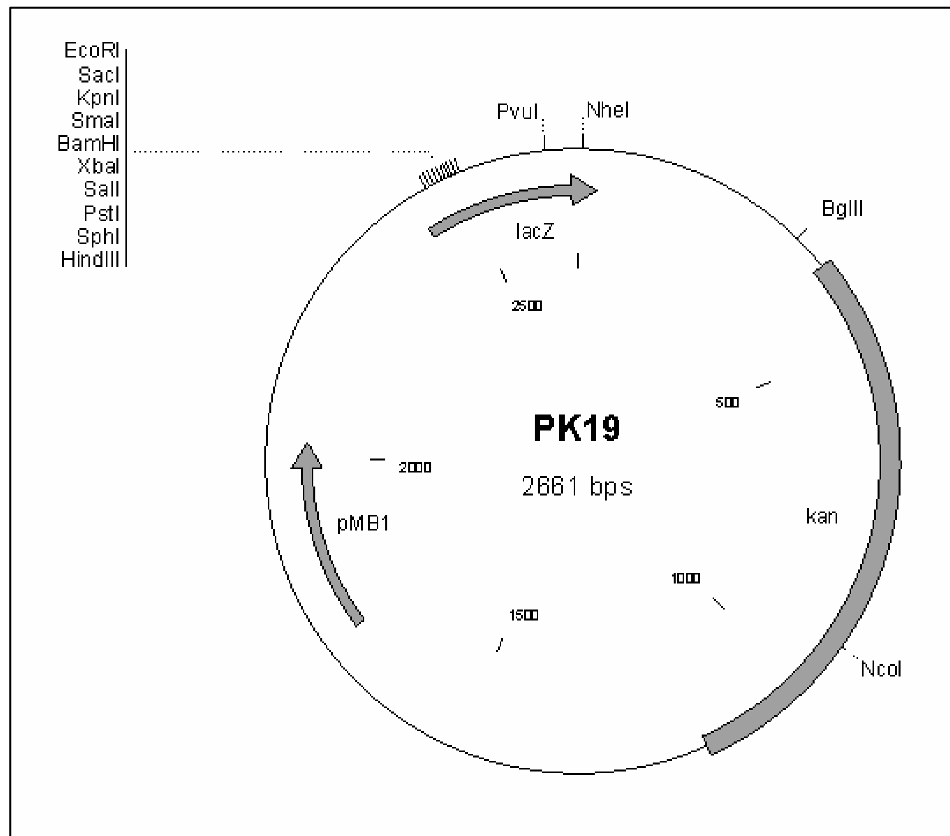

Fig. 4-2. Map of pK19.

4.2.8.5 Screening *C. perfringens* genomic library in *E. coli* for clones able to reduce bilirubin

Forty eight clones from 96-well plate were replica plated on LB agar plate containing Km. Using a metal stamp assured equal representation of all analyzed clones on the plate. Cells were grown for 8 h at 37 °C, scraped off using a sterile loop and inoculated into 100 ml of YE medium containing Km. For the purpose of a negative control *E. coli* DH5α transformed with pK19 was also inoculated into 100 ml of YE medium. After overnight incubation at 37 °C, cells were harvested by centrifugation at 10000 x g for 10 min at 4°C resuspended in 1 ml of Na phosphate buffer (pH 8) and disintegrated using French mini pressure cell press (4 cycles, 9000 PSI). Cell debris was removed by centrifugation at 5000 x g for 10 min at 4 °C and crude protein extract was tested for ability to reduce bilirubin (4.2.4) (Fig 4-4).

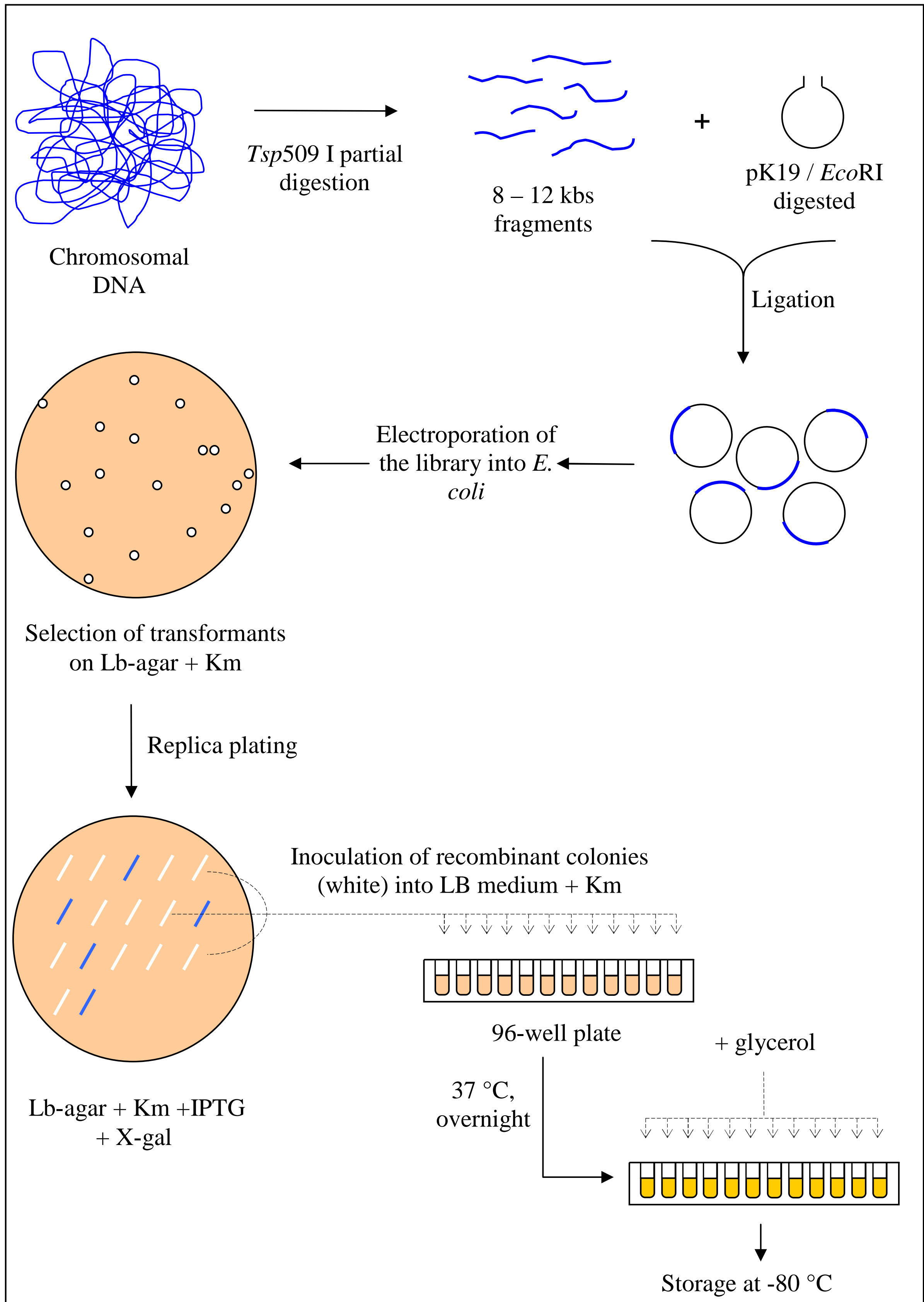

Fig. 4-3. Construction of *C. perfringens* genomic library.

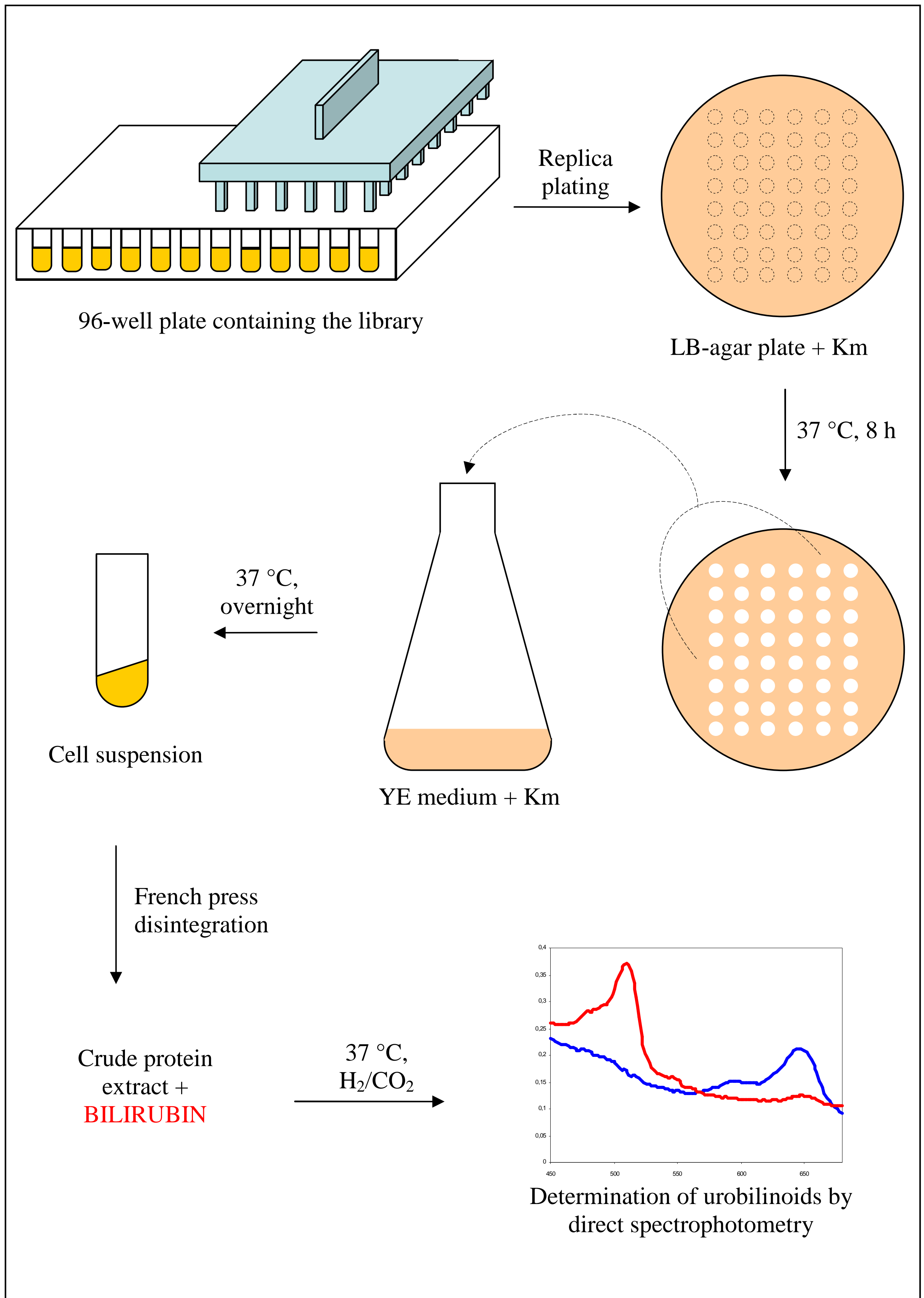

Fig. 4-4. Screening *C. perfringens* genomic library in *E. coli.*

4.3 Software and computer analysis

Chemical formulas were drawn in MDL ISIS Draw 2.5 and ACD/ChemSketch. DNA sequences were analyzed in DNASTAR and plasmid molecules were drawn in CloneManager version 4.01.

5 RESULTS

5.1 Analysis of bile pigments metabolized by *C. perfringens* and their respective reduction products

5.1.1 Analysis of bile pigments metabolized by C. perfringens BR1

Firstly, we undertook analysis of various native as well as synthetic bile pigments metabolized by the strain BR1 of *C. perfringens*. Depending on their polarity, purified pigments were dissolved either in DMSO or distilled water and added to the mid-exponential phase *C. perfringens* culture at a final concentration of 50 μM. The culture medium, 2% YE, was buffered to pH 8 using phosphate buffer, because solubility of the non-polar pigments decreases with decreasing pH in aqueous media. Determination of urobilinoids was performed by direct spectrophotometry of a zinc complex of urobilin in DMSO (4.2.1). Natural *in vitro* studied bile pigments included UCB IXα, UCB IXβ, mesobilirubin, bilirubin diglucuronid and biliverdin. Synthetic bilirubin derivatives, including bilirubin dimethylester, bilirubin diethylester, and bilirubin ditaurate were studied as well. Conversion rates of the bile pigments, normalized to the mass of bacteria are summarized in Tab. 5-1.

Bile pigment	Conversion rate (%)
Mesobilirubin (MB)	24.1
Bilirubin diamide	22.1
Unconjugated bilirubin IXα (UCB IXα)	16.8
Bilirubin dimethylester (BDM)	14.9
Bilirubin diglucuronide (BDG)	13.8
Bilirubin diethylester	8.3
Bilirubin ditaurate (BDT)	5.9
Unconjugated bilirubin IXβ (UCB IXβ)	3.3
Biliverdin	1.0

Tab. 5-1 Reduction of bile pigments by the strain BR1. Each value represents the average of two experiments.

A broad range of bile pigments was reduced by the strain BR1. The highest rate was detected for MB, in which the vinyl groups at both end pyrrolic rings are already reduced to ethyl groups. Slower rates were observed for UCB IXα and BDG, as for BDM. The rates for BDT and bilirubin diethylester were much lower.

We also tested whether the putative bilirubin reductase could be secreted into medium during cultivation. The strain BR1 was grown in YE-medium and the ability to reduce bilirubin was determined in the cell-free medium and French press disintegrated cell suspension. The enzymatic activity was detected only in disintegrated cell suspension indicating that the bilirubin reductase is not secreted into the medium.

5.1.2 Analysis of bilirubin/mesobilirubin reduction products

5.1.2.1 TLC analysis

We performed analysis of reduction products formed from UCB and MB. Both pigments, at a final concentration of 50 µM, were incubated with *C. perfringens* BR1 for 24 h and the reduction products formed were extracted from the media (4.2.2.1) and separated by TLC (4.2.2.2). Separated pigments were oxidized under UV light, which oxidizes the central methene bridge, and visualized using visible and UV light respectively (Fig. 5-1, 5-2). Exposure of the reduction products of MB to UV light yielded distinctive products (Fig. 5-1): A colorless chromogen was converted to a yellow pigment with a greenish UV fluorescence, characteristic of urobilin, identifying the chromogen as urobilinogen. A light yellow pigment was converted to a purple compound with a pink/red UV fluorescence, characteristic of mesobiliviolin, identifying the yellow pigment as dihydromesobilirubin. A darker yellow pigment was converted into a blue compound without fluorescence, characteristic of glaucobilin, characterizing the yellow pigment as unmetabolized MB.

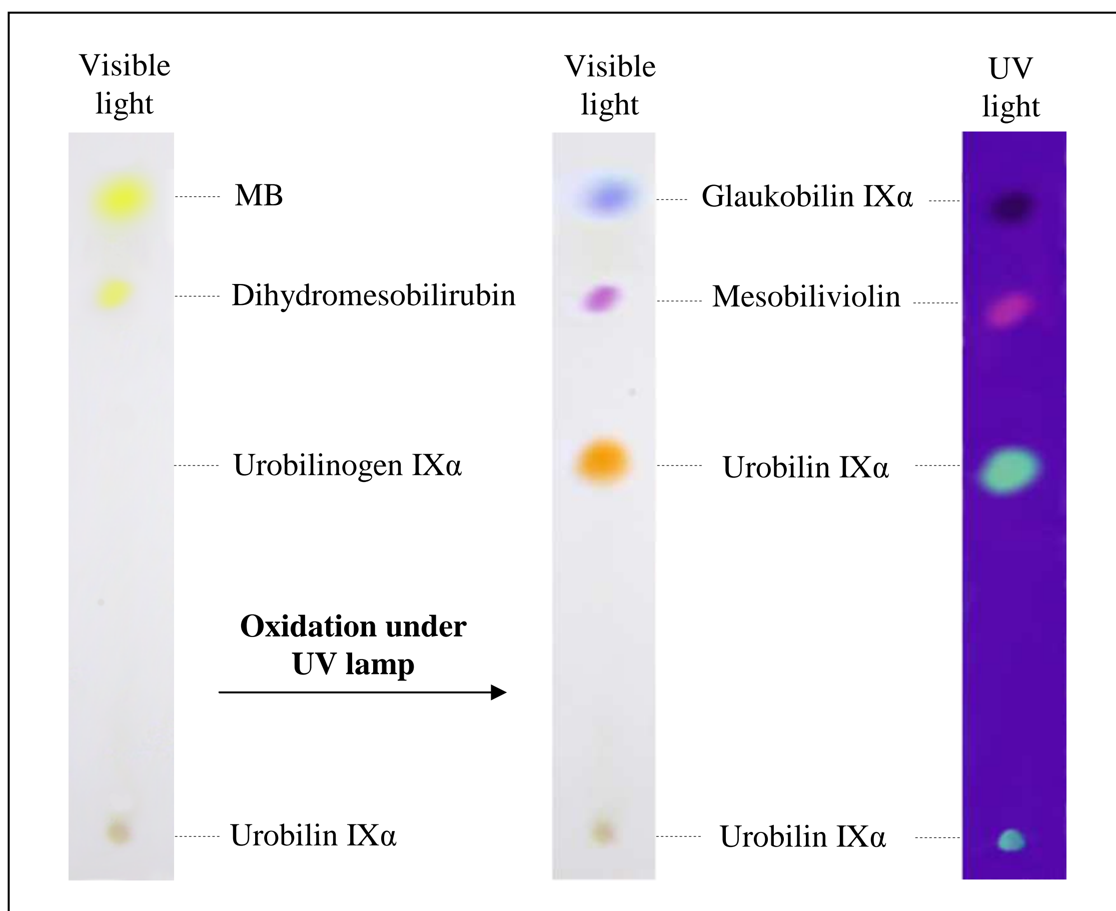

Fig. 5-1. TLC of reduction products formed from MB by anaerobic incubation with *C. perfringens* BR1 in YE medium for 24 h. The initial bile pigment concentration in the broth was 50 μM. Left track represents reduction products. Center and right tracks represent reduction products after oxidation of the middle methylene bridge under UV light visualized under visible and UV light respectively.

As shown in Fig. 5-2, identical products were derived from incubation of UCB and MB with *C. perfringens* BR1, but each product formed from UCB showed three bands. This experiment suggested that MB was an intermediate in the reduction of UCB, but that the substrate UCB had contained three different isomers (IIIα and XIIIα as well as IXα) arising from the molecular scrambling of UCB IXα (No authors listed, Semin. Liver Dis. 1994) and yielded the corresponding isomers of the reduction products.

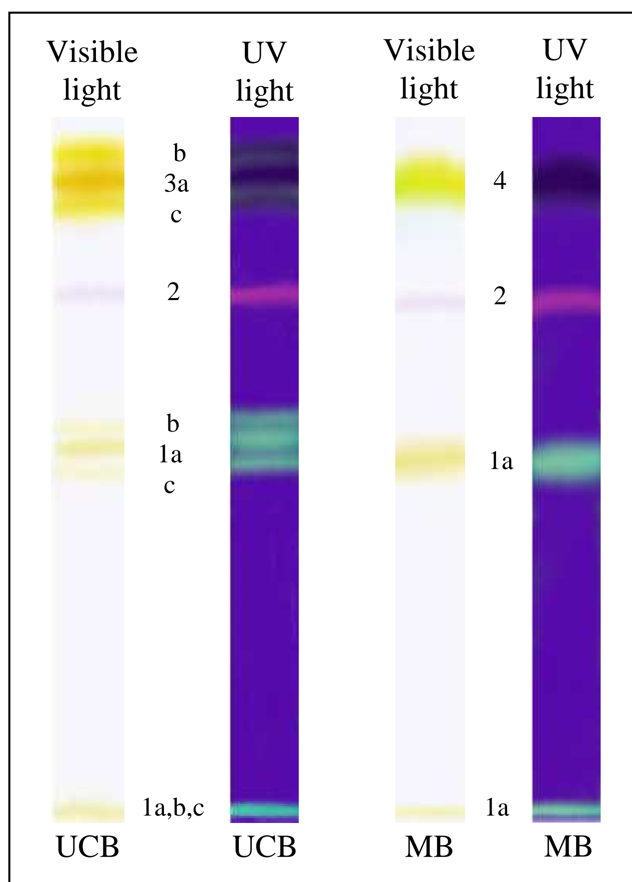

Fig. 5-2. TLC of reduction products formed from UCB and MB by anaerobic incubation with *C. perfringens* BR1 in YE medium for 24 hours. The central methene bridge of the separated urobilinogens was oxidized under UV light after TLC development. The initial bile pigments concentration in the broth was 50 µmol/l. 1a - Urobilin IXα, 1b - Urobilin IIIα, 1c - Urobilin XIIIα, 2 - Labile mesobiliviolin, 3a - UCB IXα, 3b - UCB IIIα, 3c - UCB XIIIα, 4 - MB IXα.

5.1.2.2 Spectral analysis and mesobiliviolin reaction

The three reduction products formed from UCB (1 a,b,c; Fig. 5-2) were extracted from silica gel and further analyzed spectrofluorometrically, spectrophotometrically and mass spectra were determined.

The pigments exhibited identical absorption maxima at 495 nm, and emission fluorescent maxima at 501.5 nm. The spectral analysis of the predominant isomer (1a, Fig. 5-2) is shown in Fig. 5-3, 5-4. Ferric chloride oxidation of the reduction products (mesobiliviolin reaction, 4.2.2.3) resulted in blue-violet color, indicating that only urobilin-

originated mesobiliviolins were produced. This observation was also supported by the absorption spectra measurement, when the peak corresponding to the stercobilin was not detected (Fig. 5-5). Molecular mass of the pigments was determined by mass spectrometry (performed by K. Ubik) and revealed identical $[M+H]^+$ ions at m/z 591 for all three pigments identifying them as different isomers of one compound urobilin. The mass spectrum of the predominant isomer urobilin IXα is shown in Fig. 5-6. The measured accurate mass 613.3024 corresponded to the elemental composition of sodium salt of urobilin IXα molecule $C_{33}H_{42}N_4O_6Na$ (calculated exact mass 613.3002).

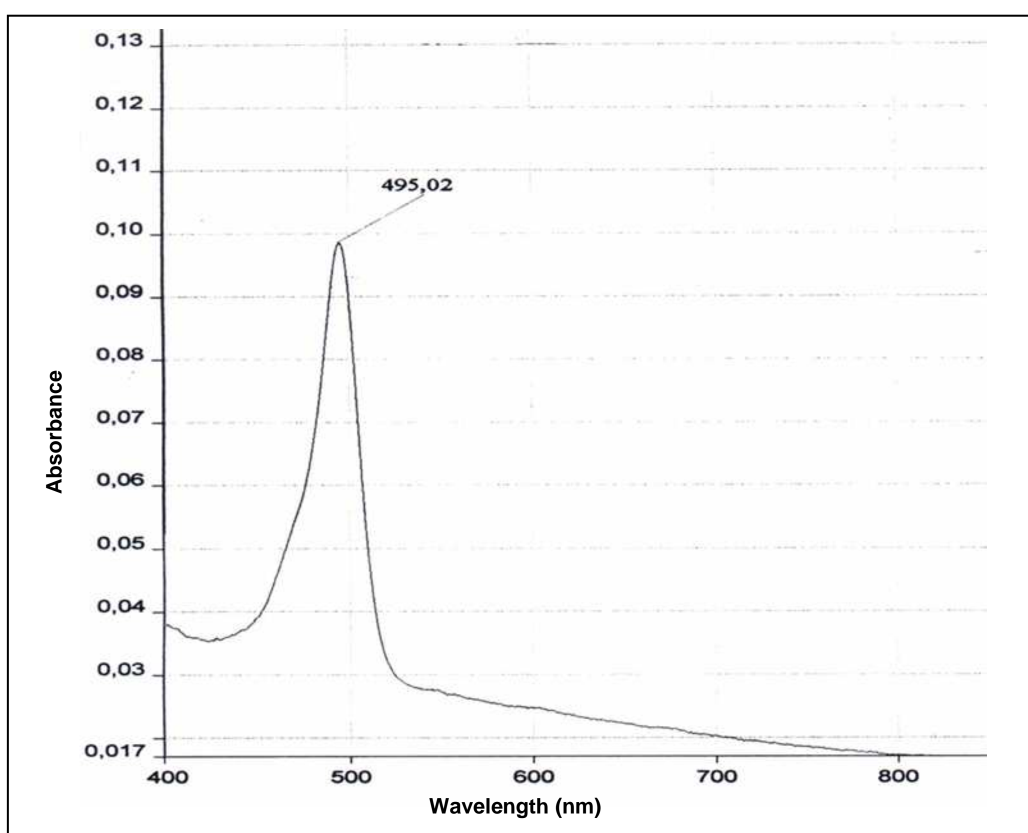

Fig.5-3. Absorption maximum of the predominant reduction product (1a, Fig. 5-2) formed from UCB.

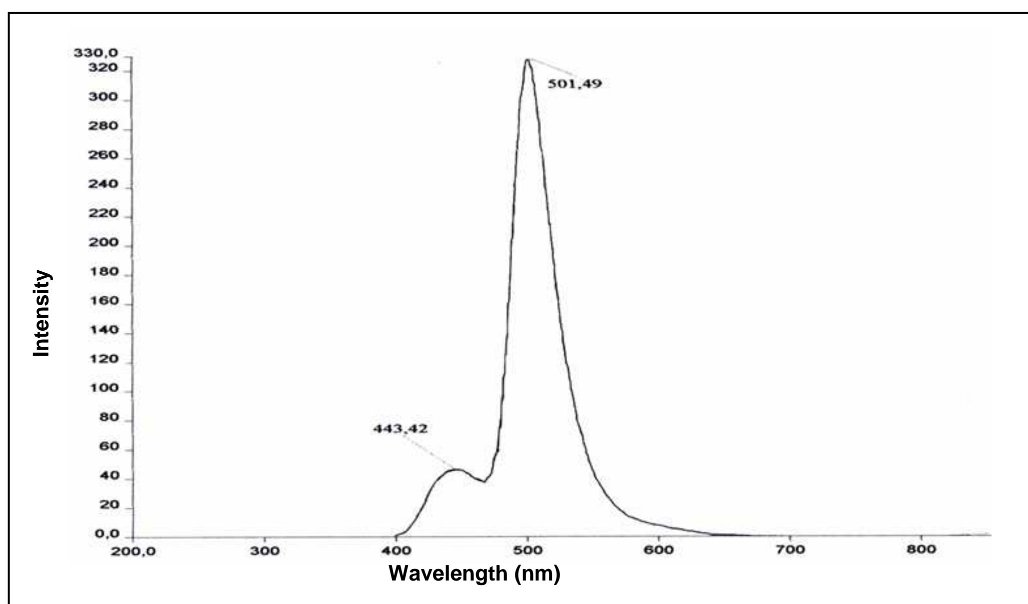

Fig. 5-4. Emission fluorescent maximum of the predominant reduction product (1a, Fig. 5-2) formed from UCB. The molecul was excitated at 345 nm, the wavelength representing the excitation maxima.

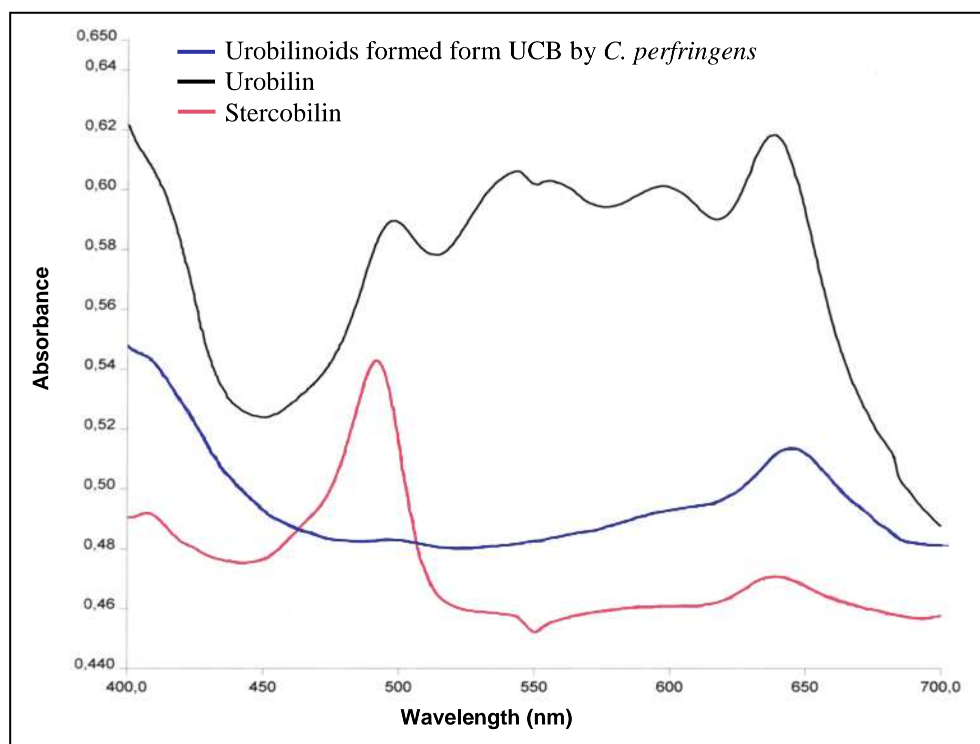

Fig. 5-5. Absorption spectra of ferric chloride oxidation products of urobilin, stercobilin and the predominant reduction product (1a, Fig. 5-2) formed from UCB by the strain BR1.

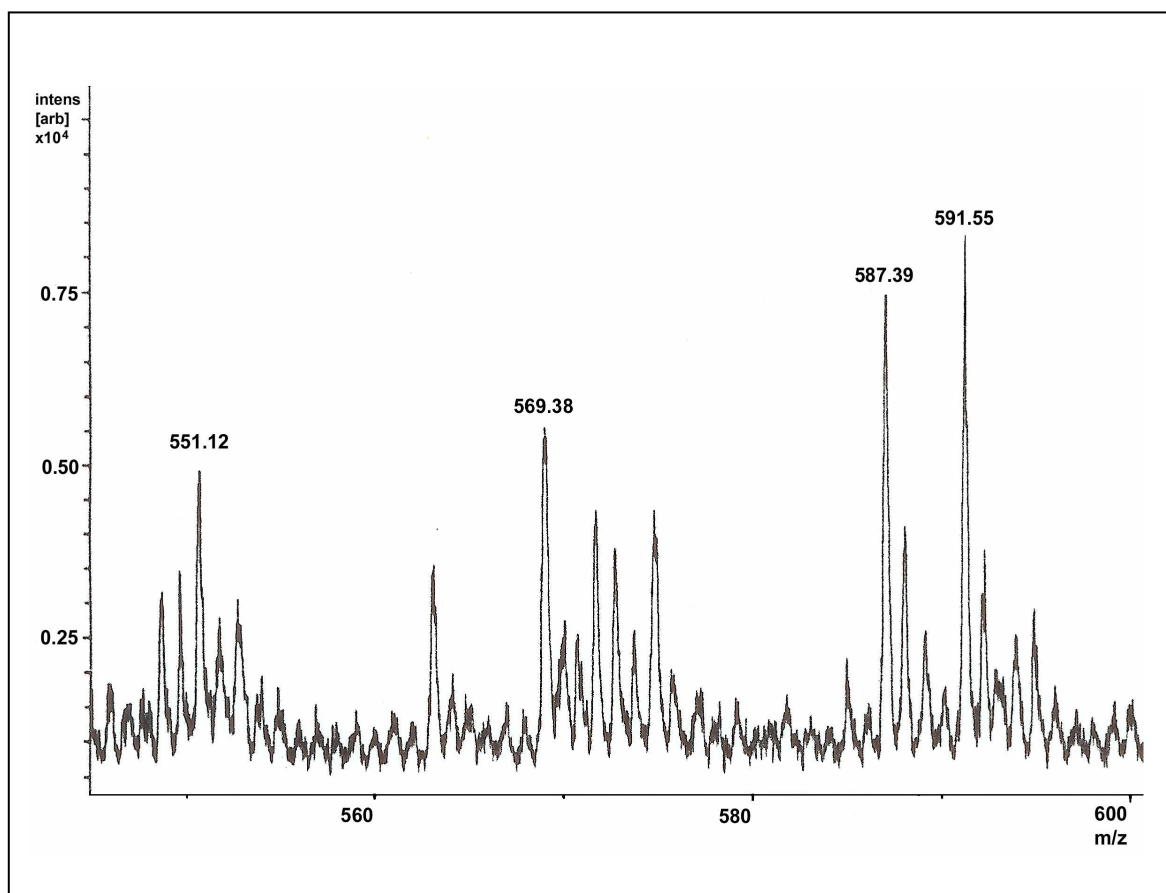

Fig. 5-6. Mass spectra of urobilin IXα, the major UCB reduction product formed by *C. perfringens* BR1.

5.1.2.3 TLC of reduction products formed from [^{14}C]-labeled bilirubin

In order to identify whether urobilinoids detected were produced from UCB [^{14}C]-labeled bilirubin was used as a substrate. The 2D TLC (4.2.2.4) results clearly demonstrated presence of [^{14}C]-labeled bilirubin reduction products produced by *C. perfringens* BR1. The pigment on the start represents most likely urobilin, whereas pigments within the path represent urobilinogen, MB and dihydromesobilirubin oxidized onto respective oxidation products (Fig. 5-7).

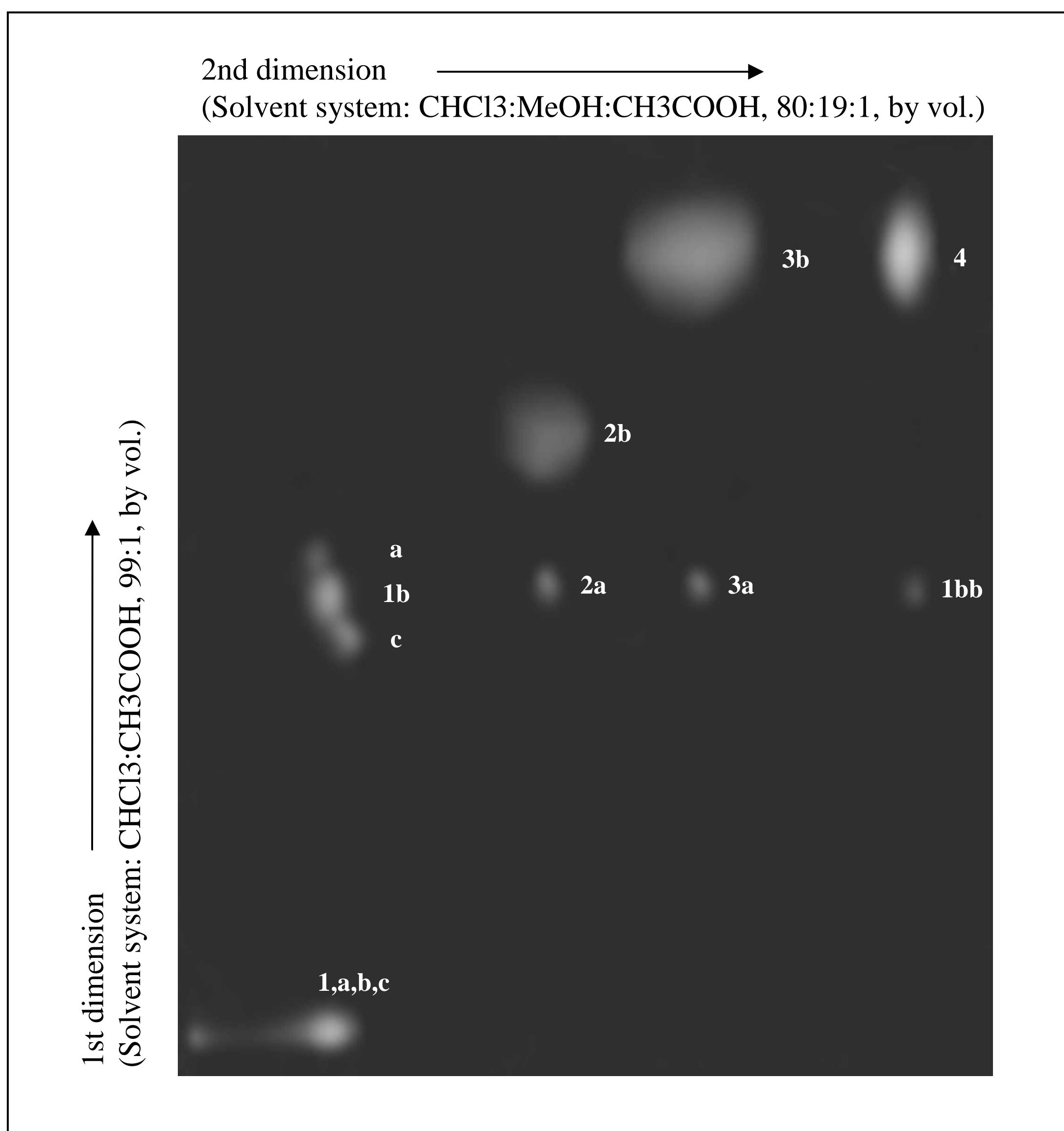

Fig. 5-7. 2D TLC of reduction products formed by anaerobic incubation of ^{14}C-UCB with *C. perfringens* BR1 in broth culture for 24 hours. The central methylene bridge of the separated isomers was oxidized from urobilinogen isomers after TLC development in the vertical dimension. Specific activity of the [^{14}C]-UCB was 13000 dpm/nmol. 1a - Urobilin IIIα, 1b - Urobilin IXα, 1c - Urobilin XIIIα, 1bb - Urobilin IXα oxidized from urobilinogen IXα after development in the second dimension, 2a - Labile mesobiliviolin produced by advanced oxidation of urobilinogen IXα after development in the first dimension, 2b - Labile mesobiliviolin, 3a - Glaukobilin produced by advanced oxidation of urobilinogen IXα after development in the first dimension, 3b - Glaukobilin produced by advanced oxidation of MB after development in the first dimension, 4 - UCB mixture of all three isomers.

5.2 Identification of gene(s) involved in bilirubin metabolism

5.2.1Electroporation of C. pefringens BR1

Essential prerequisite for transposon mutagenesis is the means of introducing transposon into *C. perfringens*. The strain BR1 of *C. perfringens* is a non-pathogenic wild-type strain, isolated from human feces, that has not been genetically manipulated so far. The first step in the process of transposon mutagenesis was therefore electroporation.

E. coli - *C. perfringens* shuttle vector pJIR750 was used in all electroporation experiments. Plasmid pJIR750 was isolated from transformed cells of *C. perfringens* by modified alkaline lysis protocol (4.2.6). The presence of plasmid DNA was confirmed by electrophoretic analysis of *Eco* RI digested samples.

5.2.1.1 Transformation of late-stationary phase cells

Initially we performed elctroporation of late-stationary phase *C. perfringens* cells due to relative simplicity of this technique (4.2.4.1). Late-stationary phase cells were once washed in cold electroporation buffer (15 % glycerol), and shocked at 12.5 kV/cm and 200 Ω with pJIR750 (1 µg/ml). Transformants were selected on TGY-agar plates supplemented with Cm (25 µg/ml). We carried out six independent electroporation experiments (Tab. 5-2), out of which only two were successful, in two attempts *C. perfringens* cells did not survive electroshocking. For that reason we tried to optimize electroporation conditions.

Experiment	O.D.$_{600}$ [a]	Transformation efficiency
1	2.62	9.92×10^3
2	2.80	0
3	2.42	1.62×10^3
4	2.45	0
5[b]	2.80	0
6[b]	2.51	0

Tab. 5-2. Electroporation of late-stationary phase cells of *C. perfringens* BR1. a - absorbance of *C. perfringens* culture at the point of harvesting. b - *C. perfringens* cells did not survive electroshocking, shocked cells were not recovered on nonselective TGY plates after overnight incubation at 37 °C.

DNase activity of intact cells of *C. perfringens* BR1 prior to the electroshocking

At first, we tested whether cell associated nucleases did not digest plasmid DNA during the pre-shock incubation on ice. 1 ml of *C. perfringens* competent cells was mixed with 10 µg of pJIR750 DNA and the sample was incubated on ice for 1, 3, 5 and 10 min. Following the incubation, 100 µl aliquots were centrifuged and the DNA presented in supernatant was ethanol precipitated, pelleted, dissolved in distilled water and assayed by agarose gel electrophoresis (Fig. 5-8). 1 µg of pJIR750 was loaded as a reference, what corresponded to the plasmid amount incubated with competent cells. No losses of plasmid DNA during prolonging incubation of pJIR750 with competent cells (from 1 min to 10 min) were observed, because density of DNA bands remained same as the reference. Therefore the possible negative role of DNases on transformability of the strain BR1 was excluded.

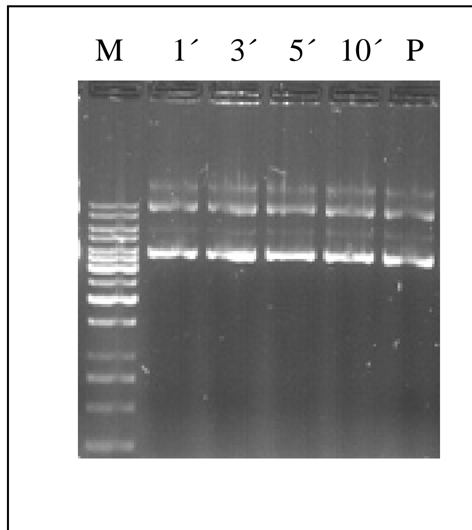

Fig. 5-8. DNase activity of intact cells of *C. perfringens* BR1 prior to the electro-shocking. M – 1 kb Marker (NEB), 1' – 10' pJIR750 after 1, 3, 5 and 10 min incubation with competent cells, P – pJIR750

Optimization of electric parameters of the pulse and regeneration of the transformants after electro-shocking

During the first set of experiments we found that clostridial cells occasionally did not survive electro-shocking (Tab. 5-1). Therefore we tested effect of various pulse parameters on viability of shocked cells and changed medium and conditions for regeneration of transformants after the pulse delivery.

C. perfringens cells were subjected to a single pulse of 6.25 kV/cm, 10 kV/cm, and 12.5 kV/cm. The time constant was changed using parallel resistors set at 200 Ω, 400 Ω, and 600 Ω. After the pulse delivery, a sample (0.1 ml) was serially diluted in electroporation buffer and plated on nonselective TGY agar to determinate percentage of cell killing. The remaining 0.3 ml was added to 15 ml of a rich regeneration BHI medium containing 25% sucrose, and incubated either for 3h or overnight (16h), instead of 1h regeneration in TGY medium. This type of experiment was performed twice (Tab. 5-3 and 5-4).

Electric field strength (kV)/ resistance (Ω)	% of cell killing	O.D.$_{600}$		
		Immediately after pulse delivery	After 3h incubation in sucrose-BHI	After 16h incubation in sucrose-BHI
6.25 / 200	67.7	0.372	0.245	1.752
6.25 / 400	76	0.354	0.236	1.831
6.25 / 600	83.1	0.355	0.149	2.071
10 / 200	62.9	0.355	0.237	1.929
10 / 400	84.7	0.330	0.205	1.834
10 / 600	87.3	0.311	0.136	2.137
12.5 / 200	90.5	0.327	0.126	2.255
12.5 / 400	93.1	0.303	0.051	1.627
12.5 / 600	98.1	0.305	0.023	1.746

Tab. 5-3. Optimization of electric parameters of the pulse and regeneration of the transformants after electro-shocking, experiment 1.

Electric field strength (kV)/ resistance (Ω)	% of cell killing	O.D.$_{600}$		
		Immediately after pulse delivery	After 3h incubation in sucrose-BHI	After 16h incubation in sucrose-BHI
6.25 / 200	64.5	0.411	0.325	1.909
6.25 / 400	70	0.402	0.318	1.711
6.25 / 600	76	0.383	0.298	1.815
10 / 200	75.7	0.382	0.290	1.676
10 / 400	86.1	0.351	0.222	1.902
10 / 600	88.8	0.346	0.148	1.892
12.5 / 200	89.5	0.340	0.125	2.000
12.5 / 400	93.4	0.337	0.072	2.659
12.5 / 600	97.9	0.324	0.034	2.513

Tab. 5-4. Optimization of electric parameters of the pulse and regeneration of the transformants after electro-shocking, experiment 2.

Competent cells electroshocked at 6.25 kV/cm, 10 kV/cm, and 12.5 kV/cm regenerated after incubation in sucrose-BHI medium. The percentage of cell killing ranged from 64 -98 %. Using 200 Ω, 400 Ω, and 600 Ω resistors the time constants were 6 ms, 10.5 ms and 15 ms long. Extension of post-electroporation incubation to 3h was enough for the regeneration of shocked cells. There was no detectable increase in the total number of viable cells during this prolonged incubation period in contrast to overnight regeneration (Tab. 5-3 and 5-4). Electroporation under optimized conditions (the pulse parameters: 6.25 kV/cm / 200, 400, 600 Ω; 10 kV/cm / 200, 400, 600 Ω; 12.5 kV/cm / 200, 400, 600 Ω, and transformants regeneration in sucrose-BHI medium for 3 h with varying amounts of pJIR750 (1, 2.5, 5, and 10 μg/ml) failed to yield any transformants.

5.2.1.2 Transformation of exponential phase cells

Based on repeating problems associated with electroporation of late-stationary phase cells, we examined the effect of growth phase on transformability of the strain BR1. Cells were harvested at different points in the growth curve, washed in cold electroporation buffer (15 % glycerol), and shocked at 12.5 kV/cm and 200 Ω with pJIR750 (1 μg/ml) (Tab. 5-5). However, neither change in growth phase affected transformability of this strain.

Experiment	$O.D._{600}$ at the point of *C. perfringens* culture harvesting	Transformation efficiency
1	0.721	0
	1.134	0
	1.766	0
	2.345	0
2	0.619	0
	1.053	0
	1.632	0
	2.158	0

Tab. 5-5. Growth phase effect on *C. perfringens* BR1 transformability.

5.2.1.3 Electroporation of lysostaphin-treated cells

One explanation of the poor transformability could be that the cell wall of *C. perfringens* is a physical barrier which prevents entry of the plasmid molecules into the cell, even though electroporation might have induced pore formation in the cell membrane. Therefore we carried out transformation experiments with enzymatic pretreated cells of the strain BR1 (4.2.4.2).

Cells were harvested in early-exponential phase, treated with lysostaphin, a peptidase that cleaves the pentaglycine bridge in the cell wall, (10 µg/ml, 1h at 37 °C) and shocked at 12.5 kV/cm and 200 Ω with pJIR750 (2.5 µg/ml). Likewise in case of late-stationary phase cells, electroporation of lysostaphin-treated cells of strain BR1 was not fully reproducible (Tab. 5-6). Nevertheless we tried to optimize this technique, because lysostaphin treatment prior electroporation increased transformation efficiency by more than one order of magnitude.

Experiment	O.D.$_{600}$ at the point of *C. perfringens* culture harvesting	Transformation efficiency
1	0.147	9.62×10^{3}
2	0.151	1.39×10^{4}
3	0.123	0
4	0.172[a]	6.98×10^{4}
5	0.160	6.40×10^{4}
6	0.139[a]	0

Tab. 5-6. Electroporation of lysostaphin treated *C. perfringens* BR1. [a] - *C. perfringens* cells did not survive electroshocking.

5.2.1.4 Optimization of lysostaphin-treated *C. perfringens* BR1 electroporation

We tried to find out the point in the process of competent cells preparation, when cells die, and examine whether the lysostaphin concentration did not affect viability of *C. perfringens* BR1.

One hundred-µl aliquots of competent cells were sampled (see details in Table 5-6 and 5-7) and each sample was regenerated in 5 ml of BHI medium overnight (16 h), then centrifuged, and plated on nonselective nutrient agar plates to determinate the cell survival. We tested three different lysostaphin concentrations: 0.5, 1, and 10 µg/ml. Cells were shocked at 12.5 kV/cm and 200 Ω with pJIR750 (2.5 µg/ml). We carried out two independent electroporation experiments (Tab. 5-7 and 5-8). *C. perfringens* cells died during washing in electroporation buffer (Tab. 5-7) or during pre-shock incubation on ice (Tab. 5-8). In both cases no transformants were obtained.

Competent cells, without lysostaphin treat.		Lysostaphin-treated competent cells	
Sampling after	Cell No. after 16 h regeneration/1 ml	Lysost. conc. [µg/ml]/ sampling after	Cell No. after 16 h regeneration/1 ml
1. wash	4.1×10^5	0.5 / 3. wash	3.2×10^3
2. wash	3.2×10^4	0.5 / 4. wash	0
3. wash	9.8×10^3	0.5 / Pre-shock [a]	0
4. wash	0	0.5 / The pulse[b]	0
Pre-shock[a]	0	0.5 / Post-shock[c]	0
The pulse [b]	0	1 / 3. wash	2.1×10^3
Post-shock[c]	0	1 / 4. wash	0
		1 / Pre-shock [a]	0
		1 / The pulse [b]	0
		1 / Post-shock[c]	0
		10 / 3. wash	7.8×10^2
		10 / 4. wash	0
		10 / Pre-shock [a]	0
		10 / The pulse [b]	0
		10 / Post-shock[c]	0

Tab. 5-7. Electroporation of lysostaphin-treated *C. perfringens* BR1. [a] - sampling after 10 min pre-shock incubation of competent cells with pJIR750 on ice. [b] - sampling immediately after pulse delivery. [c] - sampling after 10 min post-shock incubation on ice.

Competent cells, without lysostaphin treat.		Lysostaphin-treated competent cells	
Sampling after	Cell No. after 16 h regeneration/1 ml	Lysost. conc. [µg/ml]/ sampling after	Cell No. after 16 h regeneration/1 ml
3. wash	2.8 x 10^3	0.5 / 3. wash	840
4. wash	3 x 10^3	0.5 / 4. wash	0
Pre-shock[a]	0	0.5 / Pre-shock [a]	0
The pulse [b]	0	0.5 / The pulse[b]	0
		0.5 / Post-shock[c]	0
		1 / 3. wash	960
		1 / 4. wash	230
		1 / Pre-shock [a]	0
		1 / The pulse [b]	0
		1 / Post-shock[c]	0
		10 / 3. wash	210
		10 / 4. wash	300
		10 / Pre-shock [a]	0
		10 / The pulse [b]	0
		10 / Post-shock[c]	0

Tab. 5-8. Electroporation of lysostaphin-treated *C. perfringens* BR1. [a] - sampling after 10 min pre-shock incubation of competent cells with pJIR750 on ice. [c] - sampling immediately after pulse delivery. [c] - sampling after 10 min post-shock incubation on ice.

In the following experiment we reduced a number of washes and increased a volume of SEB. Cells were washed once in an equal volume of 20 °C SEB prior incubation with lysostaphin. Again we used lysostaphin at concentrations of 0.5, 1, and 10 µg/ml. After lysostaphin treatment a half of the cell suspension was centrifuged at 4 °C and washed in an equal volume of cold SEB, the second half was centrifuged at 20 °C and washed in an equal volume of 20 °C SEB. In the initial protocol the competent cells were washed twice in 10 ml (1/10 of volume) of 20 °C SEB prior lysostaphin treatment and twice after the incubation. Cells were subjected to a pulse of 12.5 kV/cm and 200 Ω.

Immediately, after the pulse delivery 100 µl of transformants was added to 5 ml of BHI medium. The rest was incubated on ice for 10 min, and then 100 µl aliquots were added to 5 ml of BHI medium. After overnight incubation at 37 °C the cells were harvested by centrifugation, suspended in 0.25 ml of diluent (0.37 % BHI, 0.1 % sodium thioglycollate) and plated (Tab. 5-9).

Transformants were obtained when cells in cold (4 °C) electroporation buffer after lysostaphin treatment were washed (= modified protocol 4.2.4.3). Furthermore, when the post-shock incubation on ice was omitted, transformants were obtained at all thee concentrations of lysostaphin. Cells washed in 20 °C buffer were transformed only when they with lysostaphin at the lowest concentration (0.5 µg/ml) were treated. Modified protocol was used in further electroporation experiments, however, the preliminary results were not repeated (Tab. 5-10).

Lysostaphin conc. [µg/ml] / post-shock incubation	SEB-20°C[a] Transformation efficiency	SEB-4°C[b] Transformation efficiency
0.5/BHI[c]	40	2×10^4
0.5/ice-BHI[d]	0	160
1/ BHI[c]	0	13
1/ice- BHI[d]	0	0
10/ BHI[c]	0	200
10/ice- BHI[d]	0	0

Tab. 5-9. Optimization of electroporation of lysostaphin-treated *C. perfringens* BR1. [a] - after lysostaphin treatment the cell suspension was centrifuged at 20°C and washed with an equal volume of 20°C SEB. [b] - after lysostaphin treatment the cell suspension was centrifuged at 4°C and washed with an equal volume of cold SEB. [c] - immediately after pulse delivery transformants were added to a regeneration BHI medium. [d] - after pulse delivery transformants were incubated on ice for 10 min and then regenerated. In all attempts *C. perfringens* cells survived electroshocking.

Lysostaphin conc. [µg/ml] / post-shock incub.	Transformation efficiency				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
0.5/BHI[a]	0	0	0	0	0
0.5/ice-BHI[b]	0	0	0	0	0
1/BHI[a]	580	0	0	1.8×10^3	0
1/ice-BHI[b]	280	0	0	880	0
10/BHI[a]	0	0	0	0	0
10/ice-BHI[b]	220	0	0	0	0

Tab. 5-10. Electroporation of lysostaphin-treated *C. perfringens* BR1 using modified protocol. [a] - immediately after pulse delivery transformants were added to a regeneration BHI medium. [b] - after pulse delivery transformants were incubated on ice for 10 min and then regenerated. In all experiments *C. perfringens* cells survived electroshocking, there was observed continuous *C. perfringens* film on nutrient agar plates.

We found that strain BR1 of *C. perfringens* is transformable, but none of performed optimizations brought a stable electroporation protocol.

5.2.1.5 C. *perfringens* P90.2.2. - transformable bilirubin-reducing strain

Searching for a transformable bilirubin-reducing strain of *C. perfringens*

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By reason of the poor transformability of the strain BR1 we changed the strategy and started to search for the bilirubin-reducing activity among transformable *C. perfringens* strains. Two transformable strains, a pathogenic strain 13, currently the only *C. perfringens* strain used in genetic studies, and α-toxin producing strain, P90.2.2., were tested for ability to reduce bilirubin. The BR1 strain of *C. perfringens* was used as a positive control. Determination of urobilinoids, reduction products of bilirubin, was performed by direct spectrophotometry of zinc complex of urobilin in DMSO (4.2.1, Fig. 5-9). Bilirubin-reducing properties and transformability of analyzed *C. perfringens* strains are summarized in Table 5-11.

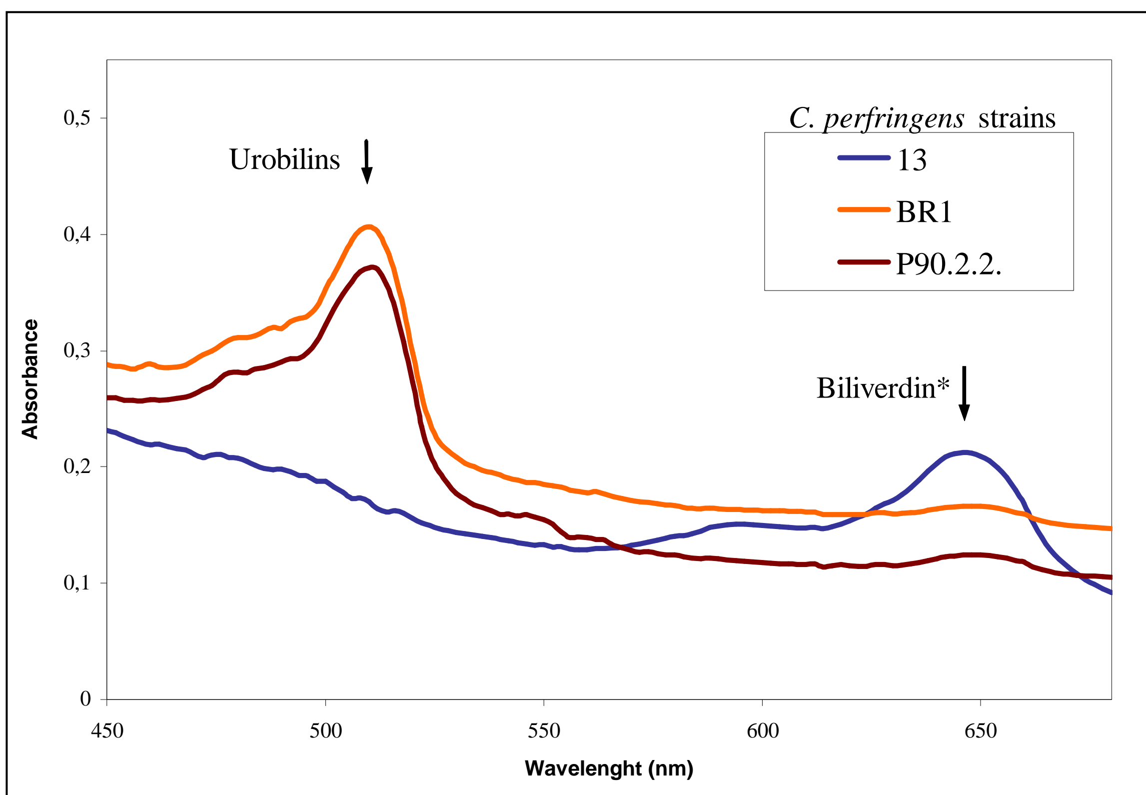

Fig. 5-9. Determination of urobilinoids by direct spectrophotometry. * - biliverdin is oxidative product of unmetabolized bilirubin.

C. perfringens strain	Ratio of bilirubin conversion to urobilinoids (%)	Transformation efficiency
BR1	42	Electroporation was hardly reproducible (This study)
13	0	3×10^5 (Scott and Rood 1989)
P90.2.2.	44	4.4×10^3 (Phillips-Jones 1990)

Tab. 5-11. Bilirubin-reducing properties and transformability of *C. perfringens.*

The only strain that conformed to both prerequisites: (1) the ability to reduce bilirubin and (2) the transformability was *C. perfringens* strain P90.2.2.. This strain was chosen as a suitable candidate for transposon mutagenesis.

Electroporation of *C. perfringens* P90.2.2.

Electroporation of late-stationary phase P90.2.2. cells resulted in transformation efficiency of 4.4 x 10^3 transformants per µg of DNA (Phillips-Jones 1990). This value is relatively low compared to that achieved by electroporation of lysostaphin-treated cells of strain 13 (3.0 x 10^5 transformants per µg of DNA, Scott and Rood 1989). Consequently we tried to increase transformation efficiency of strain P90.2.2. at first by lysostaphin treatment. Early-exponential phase *C. perfringes* cells were treated with varying amounts of lysostaphin (0.5, 1, 10 and 20 µg/ml), but no transformants were obtained. In further experiment we completely omitted lysostaphin treatment, reduced the number of washes in the electroporation buffer and tested whether post-electroporation incubation on ice affects transformation efficiency. Electroporation was carried out at 200 Ω and using pulses of 1.8 - 2.5 kV (9 - 12.5 kV/cm). All manipulations were carried out at room temperature similarly as in the original protocol (RT protocol). Alternatively, manipulations were performed at 4° C using cold electroporation buffer (Cold protocol). Transformants were selected on BHI-agar plates supplemented with Cm (5 µg/ml).

Tables 5-12 and 5-13 show the effect of temperature (during manipulations) and voltage applied on transformation efficiency of *C. perfringens* P90.2.2. transformed with pJIR750 DNA. The highest transformation efficiency (1.37 x 10^4) we obtained when cells at room temperature were prepared, shocked at 9 kV/cm and post-shock incubation on ice was omitted (protocol 4.2.4.4). The ratio of cell killing was 91.8% at 9 kV/cm, 96% at 10 kV/cm and 99% at 12.5 kV/cm for RT protocol. In case of Cold protocol (Tab. 5-13) transformation efficiency neither reached values achieved by Phillips-Jones (1990) (20 transformants per µg of DNA vs. 4.4 x 10^3).

We developed rapid and simple method (4.2.4.4) suitable for electroporation of *C. perfringens* P90.2.2., bilirubin-reducing strain, providing transformation efficiency up to 1.37 x 10^4.

Electric field strength (kV/cm)	Time constant (ms)	Transformation efficiency	
		RT[a]	Ice[b]
9	3.2	1.37×10^4	5.31×10^3
10	3.3	9.03×10^3	4.52×10^3
12.5	3.2	1.19×10^3	0

Tab. 5-12. Electroporation of *C. perfringens* P90.2.2. Manipulations performed at room temperature (RT protocol). Each value represents the average of three experiments. [a] - absence of post-shock incubation on ice. [b] - 10 min post-shock incubation on ice.

Electric field strength (kV/cm)	Time constant (ms)	Transformation efficiency	
		RT[a]	Ice[b]
9	3.4	20	0
10	3.3	20	0
12.5	3.2	10	0

Tab. 5-13. Electroporation of *C. perfringens* P90.2.2.. Manipulations performed at 4 °C (Cold protocol). Each value represents the average of three experiments. [a] - absence of post-shock incubation on ice. [b] - 10 min post-shock incubation on ice.

5.2.2 Transposon mutagenesis of C. perfringens P90.2.2.

5.2.2.1 Tn*916* mutagenesis of *C. perfringens* P90.2.2.

pAM120 electroporation

Initially, we tried to introduce Tn*916* carried by a non-replicative plasmid pAM120 into *C. perfringens* P.90.2.2. by electroporation (4.2.4.4). We used saturating concentration of pAM120 DNA (≥ 6 µg per cuvette) as described by Briolat and Reysset (2002). Plasmid pJIR750 was electroporated as a positive control. Cells transformed with pMA120 were selected on BHI-agar plates containing Tc (10 µg/ml), cells transformed with pJIR750 were selected on BHI-agar plates containing Cm (5 µg/ml). Of the three experiments carried out we obtained only pJIR750 transformants (Tab. 5-14).

Plasmid	Conc. of plasmid DNA (µg)/cuvette	Time constant (ms)	Transformation efficiency
pAM120	6	2.5	0
pAM120	10	2.45	0
pJIR750	2	2.5	7130

Tab. 5-14. Electroporation of *C. perfringens* P90.2.2. with pAM120 and pJIR750. Each value represents the average of three experiments.

In consequence we tried to extend duration of the pulse (time constant), because prolongation of the period during which pores in cell membrane are created may facilitate penetration of such a large plasmid. The time constant was changed using parallel resistors set at 200 Ω, 400 Ω, and 600 Ω. Neither in this case *C. perfringens* was transformed with plasmid pAM120 (Tab. 5-15.). We also performed restriction analysis of pAM120 with *Eco*RI, *Bam*HI and *Nde*I enzymes, to check whether any alterations in the length or rearrangements of pAM120 occurred. This analysis showed that restriction profile of pAM120 remained unaltered (Fig. 5-10).

Vector	Conc. of vector (µg)/cuvette	Resistance (Ω)	Time constant (ms)	Transformation efficiency
pAM120	6	200	2.5	0
pJIR750	2	200	2.4	11760
pAM120	6	400	3.3	0
pJIR750	2	400	3.1	484
pAM120	6	600	3.6	0
pJIR750	2	600	3.5	40

Tab. 5-15. Time constant effect on transformability of *C. perfringens* P90.2.2. with plasmid pAM120. Each value represents the average of three experiments.

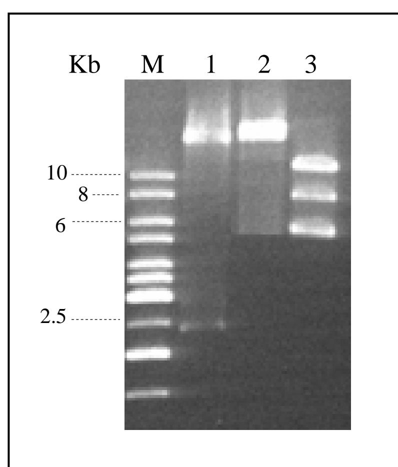

Fig. 5-10. Restriction analysis of pAM120. M - 1 kb ladder (MBI Fermentas), 1 - diggestion of pAM120 with *Eco*RI resulting in bands of 2.4 and 21 kb, 2 - linearization of pAM120 with *Bam*HI resulting in a single band of 23.4 kb, 3 - diggestion of pAM120 with *Nde*I resulting in bands of 5.2, 7.6 and 10.6 kb.

Conjugative transfer of pAM120 from *E. coli* S17-1 to *C. perfringens* P90.2.2.

Tn*916* belongs to a group of conjugative transposons, therefore it might be introduced into *C. perfringens* from *E. coli* by filter-mating procedure (4.2.7). As a donor *E. coli* S17-1 transformed with pAM120 was used. This *E. coli* strain carries an integrated RP4-derivative whose transfer functions are known to be necessary for mating between

bacteria. Mating between *E. coli* S17-1 transformed with pAM120 and *C. perfringens* 13 was performed as a positive control, because conjugative transfer of pAM120 between these two bacteria has been already demonstrated (Cole and Rood 1991). Transconjugants were selected on BHI-agar plates containing Tc (10 µg/ml), and sodium azide (0.2 mg/ml). However, intergeneric transfer of Tn*916* from *E. coli* to *C. perfringens* P90.2.2. did not proceed as shown in table 5-16.

Strain of *C. perfringens*	P90.2.2.	13
No. of transconjugants	0	24

Tab. 5-16. Conjugative transfer of pAM120 from *E. coli* S17-1 to *C. perfringens.* No. of transconjugants was calculated from two experiments.

5.2.2.2 Tn*917* mutagenesis of *C. perfringens* P90.2.2.

Tn*917* mutagenesis was performed by using a vector pTV408, originally developed for mutagenesis of streptococci (Slater *et al*. 2003). pTV408 is a hybrid vector containing the temperature-sensitive broad-host-range replicon from the *Lactococcus lactis* plasmid pWV01 and modified Tn*917* transposon. *L. lactis* replicon allows replication between permissive temperatures of 28 and 30 °C but not between 37 and 42 °C, when plasmid curing and transposition of Tn*917* can occur. However, we couldn't use advantage of temperature-sensitive replicon due to intrinsic resistance of *C. perfringens* to Km, the antibiotic used for selection during incubation at permissive temperatures. pTV408 was electroporated (4.2.4.4) as a non-replicative plasmid, and *C. perfringens* transformants were selected on BHI-agar plates containing Em (50 µg/ml) at 37°C. Alternatively, transformants were regenerated and selected at non-permissive temperature (42 °C), when the transposition is preferred. pJIR750 was used as a positive control like before. *C. perfringens* cells were transformed neither at 37 nor 40 °C (Tab. 5-17). Finally, we performed restriction analysis of pTV408 using *Eco*RI and *Hind*III enzymes, to check whether any alterations in the length or rearrangements of the plasmid occurred. Restriction profile of pTV408 remained unaltered (Fig. 5-11).

Plasmid	Conc. of plasmid DNA (µg)/cuvette	Time constant (ms)	Transformation efficiency, 37 °C	Transformation efficiency, 42 °C
pTV408	3	2.6	0	0
pTV408	6	2.5	0	0
pJIR750	2	2.5	11760	8200

Tab. 5-17. Electroporation of *C. perfringens* P90.2.2. with pTV408 and pJIR750. Each value represents the average of three experiments.

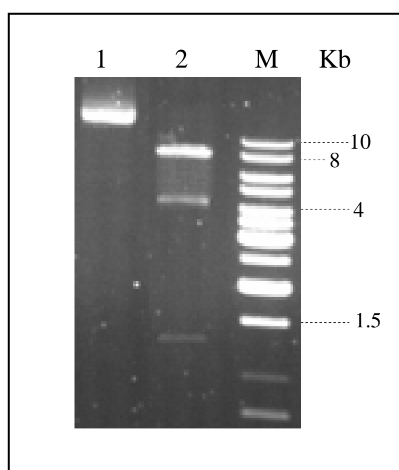

Fig. 5-11. Restriction analysis of pTV408. M - 1 kb ladder (MBI Fermentas), 1 - linearization of pTV408 with *Eco*RI resulting a single band of 13,9 kb, 2 - digestion of pTV408 with *Hind*III resulted in bands of 1.3, 4.2, and 8.4 kb as expected.

Unfortunately, both transposon-based mutagenic approaches failed. We were able neither to introduce transposons into *C. perfringens* P90.2.2. nor to isolate clones incapable to reduce bilirubin.

5.2.3 Construction of C. perfringes P90.2.2. genomic library in E. coli and its screening for bilirubin reducing activity

The last attempt to isolate gene(s) involved in the metabolism of bilirubin was based on the construction of *C. perfringens* genomic library in *E. coli* and its screening for the presence of clones able to reduce bilirubin. Similar approach has been successfully applied in several studies: The method was used for the identification of genes involved in mannose biosynthesis in *C. perfringens* (Walters *et al.* 1999), α-galactosidase gene in *C. stercorarium* (Suryani *et al.* 2003), or genes controlling sucrose utilization in *C. beijerinckii* (Reid *et al.* 1999).

5.2.3.1 Construction of *C. perfringens* P90.2.2. genomic library

Prior to the library construction we tested whether the host strain of *E. coli* DH5α transformed with pK19, the plasmid selected for construction of the library, does not reduce bilirubin (4.2.8.5, Fig. 5-12). Since bilirubin-reductase assay was negative, therefore the DH5α strain of *E. coli* could be used for the maintenance and expression of *C. perfringens* genomic library.

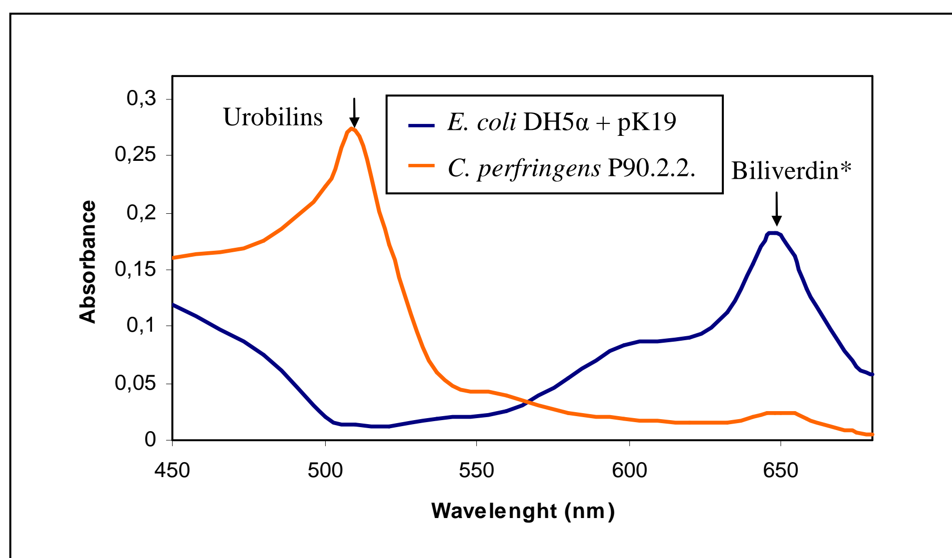

Fig. 5-12. Bilirubin-reducing activity of *E. coli* DH5α. * - biliverdin is oxidative product of unmetabolized bilirubin. *C. perfringens* P90.2.2. served as positive control.

Construction of *C. perfringens* genomic library is described in detail in the section 4.2.8. In total we obtained 6319 *E. coli* clones from two independent experiments. The size of *C. perfringens* chromosomal fragments inserted in pK19 was determined by double digestion with *Bam*HI and *Pvu*I (Fig. 5-13). We expected to obtain the following restriction pattern: one band of about 2.5 kb corresponding to the pK19 and one or more bands of chromosomal DNA inserts giving together 8-12 kb. However, only 30-40 % of putative recombinant clones exhibited such restriction profile. The rest of recombinant plasmids exhibited incorrect, but uniform restriction pattern: one band of 1.2 kb and second band above 5 kb.

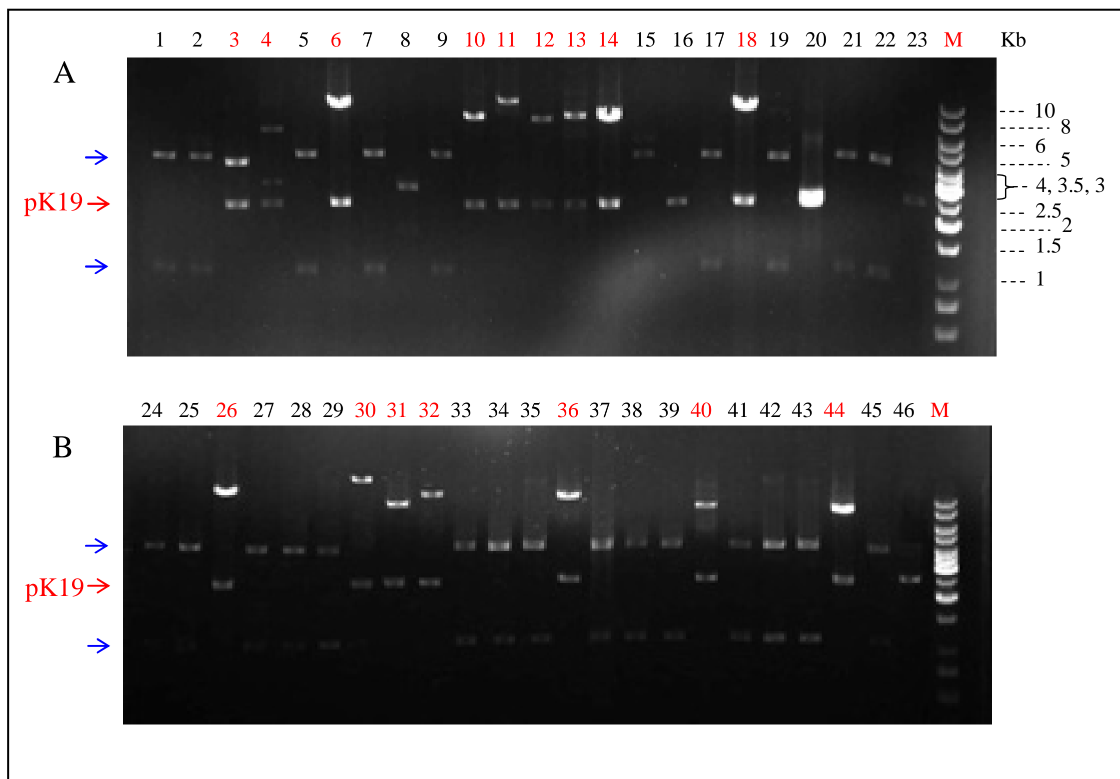

Fig. 5-13. Double digestion (*Bam*HI+*Pvu*I) of vectors from putative positive clones of *E. coli* (lines 1-46) obtained in two ligation experiments (A, B). M - 1 kb ladder (MBI Fermentas). Lines with recombinant pK19 containing DNA inserts are red marked, red arrow indicates position of pK19. Blue arrows indicate incorrect restriction pattern.

Subsequently we checked whether the isolated vectors contain DNA of clostridial origin. We analyzed 12 vectors: 6 with correct restriction pattern (lines 4, 6, 13,

30, 32, 40; Fig. 5-6) and 6 with incorrect restriction pattern (2, 9, 21, 25, 34, 42; Fig. 5-6). All plasmids were sequenced, but sequences only from vectors exhibiting the correct restriction pattern were obtained. However, we did not manage to resequence remaining vectors (2, 9, 21, 25, 34, 42) and to prove the presence of inserts of clostridial origin. Therefore all these plasmids with altered structure were excluded from the library. Sequences were subjected to a nucleotide-nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). All of them manifested high similarity with a genomic sequence of *C. perfringens* 13 (Fig. 5-14). The characteristics of the constructed library are summarized in Table 5-18.

SAMPLE 4:

```
16      AGGTTCTTTTGTATCTCCTAAGAAGAAGACAGCTAATGTTCCTATACCTGTGTGAGAACC  75
        ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
38736   AGGTTCTTTTGTATCTCCTAAAAAGAAGACAGCTAATGTTCCTATACCTGTGTGAGAACC  38795

76      TATAACTATTCCTATATAATCTATAAGTATGTCTTTTACAGTATATTTTTCTCTAATAAT  135
        ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||| ||
38796   TATAACTATTCCTATATAATCTATAAGTATGTCTTTTACAGGATATTTTTCTCTAATCAT  38855

136     TTCTGCTAAAGTCTCTGCATCTTCAATACAATCACTATGAGCAATAAAAATTGTTTGTTC  195
        |||||||||||||||||||||||||| ||||||||| ||||| |||||||||||||||||
38856   TTCTGCTAAAGTCTCTGCATCTTCAACACAATCACTGTGAGCTATAAAAATTGTTTGTTC  38915
```

SAMPLE 6:

```
301     ACCTTTAATGCTCTACATGAAAAGAATAAACAAATTATACTATCATCAGATAGACCGCCT  360
        ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
1130    ACCTTTAATGCTCTACATGAAGAGAATAAACAAATTATACTATCATCAGATAGACCGCCT  1189

361     AAGGAAATTCCTACATTAGAAGACAGGTTAAGATCTAGATTTGAATGGGGTTTAATAGCG  420
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1190    AAGGAAATTCCTACATTAGAAGACAGGTTAAGATCTAGATTTGAATGGGGTTTAATAGCG  1249

421     GATATTCAACCACCTGATTTCGAAACTAGAATGGCAATCCTAAAGAAGAAAGCTGATGTT  480
        ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
1250    GATATTCAACCACCTGATTTCGAAACTAGAATGGCAATCCTAAAGAAAAAAGCTGATGTT  1309
```

SAMPLE 13:

```
223     ATAATTACTATTCATTATCAACAAAGTGGAAGAGAGATATTCATATGTATAAAATTTAAA  282
        ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
345174  ATAATTACTATTCATTATCAACAAAGTTGAAGAGAGATATTCATATGTATAAAATTTAAA  345233

283     TATAAAAATTTAAATTTTAGAGGAGGAATTTATTGTCTTTTAGTAATTAACATGAACTTT  342
        |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
345234  TATAAAAATTTAAATTTTAGAGGAAGAATTTATTGTCTTTTAGTAATTAACATGAACTTT  345293

343     AGGGAGAAAATTATAGAGATAAGAATTATAACTTTTTATTAGTTGATAAAAACTTTAGAG  402
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 
345294  AGGGAGAAAATTATAGAGATAAGAATTATAACTTTTTATTAGTTGATAAAAACTTTAGAA  345353

403     TATAAACTAAAGTTTTTAATAATTAAATTTAAAGTGTGAATTGTAAGTTTTTTAGTGATT  462
        ||||  ||||||||||||||||||||||||||||||||||||||||| ||||||||||||
345354  TATATTCTAAAGTTTTTAATAATTAAATTTAAAGTGTGAATTGTAAG-TTTTTAGTGATT  345412
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SAMPLE 30:

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120       CTGAATCTAAAGGAATTCTTAATTTACTATCAAGTACTATCCTAAAATGCTTTCTTTCTA  179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
705268    CTGAATCTAAAGGAATTCTTAATTTACTATCAAGTACTATCCTAAAATGCTTTCTTTCTA  705209

180       NCGCTTTATTTTTATTANGGCTACATCTTAAATCTGGATTATCCTTTAAAACAGTGTTTA  239
           | |||||||||||||| | ||||||||||||||||||||||||||||||||||||||||
705208    ACTCTTTATTTTTATTAAGTCTACATCTTAAATCTGGATTATCCTTTAAAACAGTGTTTA  705149

240       TTCCAACCATTATTGAGCTATATTTATTTCTTAAGGAATGAACAAACTCTCTTGATTTTC  299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
705148    TTCCAACCATTATTGAGCTATATTTATTTCTTAAGGAATGAACAAACTCTCTTGATTTTC  705089
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SAMPLE 32:

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13        AAAAGAGTAAATGAATTAATTGAAAAGACTAAATTACAAGAAGTTGAGAAAAGGGATTTA  72
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
714207    AAAAGAGTAAATGAATTAATTGAAAAGACTAAATTACAAGAAGTTGAGAAAAGGGATTTA  714266

73        GAGATAAGGATGTTACAAGCACAAATTAATCCTCATTTTCTCTTTAATACATTGAACTCT  132
          ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
714267    GAGATAAGGATGTTACAGGCACAAATTAATCCTCATTTTCTCTTTAATACATTGAACTCT  714326

133       TTAAAGTGGACAGCCTTAATGAATCAAGATTATACAGTAAGCGAGGGGCTTAGTTCCCTA  192
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
714327    TTAAAGTGGACAGCCTTAATGAATCAAGATTATACAGTAAGCGAGGGGCTTAGTTCCCTA  714386
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SAMPLE 40:

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55        AATTTTATTTCTTCCCTTTTTCCAAGGTATAGTGAATTCATCAACATTACTATTTACTGA  114
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
352562    AATTTTATTTCTTCCCTTTTTCCAAGGTATAGTGAATTCATCAACATTACTATTTACTGA  352503

115       GAATATAAACTGATCTGCATTTCTATACTCACAAGGCTCTCTGTATGCCTTTCTCATTTC  174
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
352502    GAATATAAACTGATCTGCATTTCTATACTCACAAGGCTCTCTGTATGCCTTTCTCATTTC  352443

175       AATAGTAATACCAGCATTTTTAACTGATGGATTAAACACCTCATATTCAACTAAAAATTC  234
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
352442    AATAGTAATACCAGCATTTTTAACTGATGGATTAAACACCTCATATTCAACTAAAAATTC  352383
```

Fig. 5-14. Alignment of sequences of DNA fragments inserted in pK19 with the genomic sequence of *C. perfringens* 13. Sequences of DNA inserts are red marked, genomic sequence of strain 13 is black marked.

Experiment	A	B	A+B
No. of Km-resistant clones	3524	2795	6319
No. of recombinant clones (white)	2573	2089	4662
Recombinants with pK19 + chromosomal fragments (%)	39.1	30.4	34.8[a]
Average size of inserted fragment (kb)	9	9	9
∑ Sizes of inserted fragments (kb)	9054	5715	14769

Tab. 5-18. Characteristics of genomic library of *C. perfringens* P90.2.2.. [a] – average of A+B.

5.2.3.2 Screening *C. perfringens* P90.2.2. genomic library in *E. coli* for clones able to reduce bilirubin

Even though the library contained only 30-40 % of clones with iserts of clostridial origin, we performed screening of all recombinant clones (4662) in nearly 100 individual experiments (for details see 4.2.8.5). Urobilinoids, reduction products of bilirubin, were determined by direct spectrophotometry of zinc complex of urobilin in DMSO (4.2.1). Bilirubin-reducing activity was not proved in any of the analyzed samples. Absorption spectra from 4 experiments are shown in Figure 5-15.

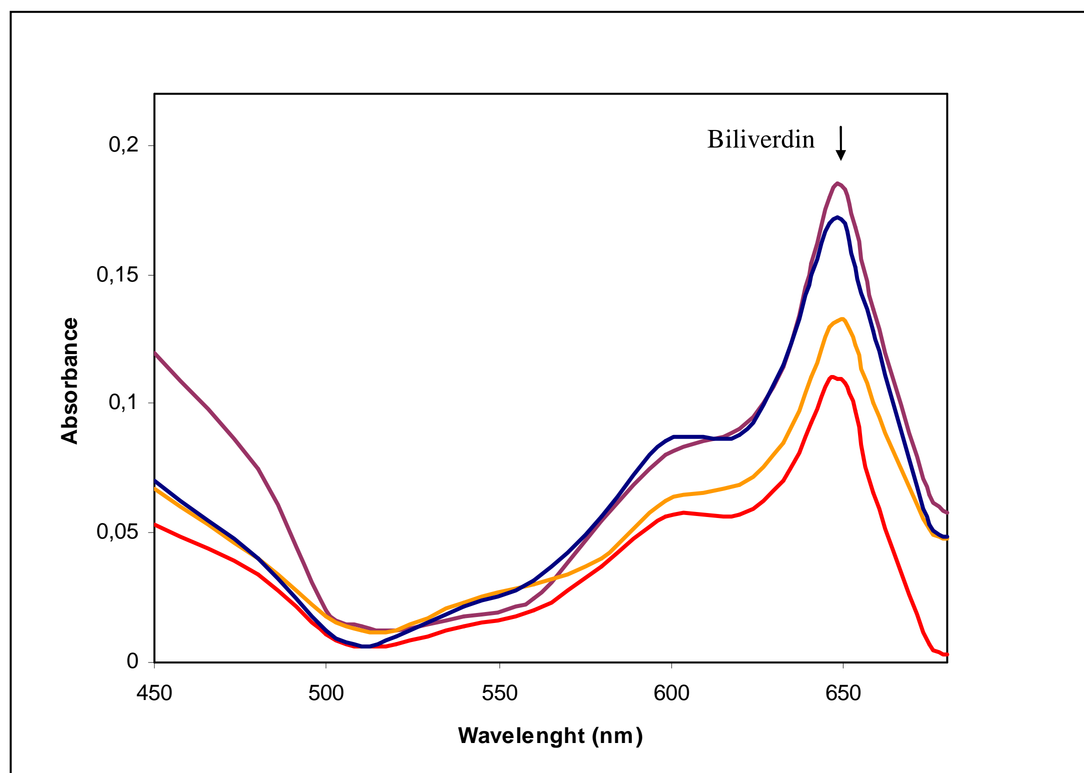

Fig. 5-15. Screening *C. perfringens* P90.2.2. genomic library in *E. coli* for clones able to reduce bilirubin. Absorption spectra from 4 experiments. In all cases only biliverdin, the reduction product of bilirubin was formed, whereas urobilinoids were not produced.

6 DISCUSSION

6.1 Analysis of bile pigments metabolized by *C. perfringens* and their respective reduction products

6.1.1 Analysis of bile pigments metabolized by C. perfringens BR1

In the first part of this study we focused on the process of bilirubin reduction catalyzed by an unique intestinal strain of *C. perfringens*. Surprisingly, the strain BR1 could reduce a wide variety of bile pigments that differ substantially in their polarity and secondary structure, including UCB, MB, bilirubin glucuronides, amides, alkyl esters, and taurates. Since bacteria are not altruistic, it seems that this broad substrate specificity of bilirubin reducing enzyme(s) could be an effective tool for disposal of electrons produced by fermentolytic processes within these anaerobic bacteria. This has been described also for substrates other than bilirubin, which are present physiologically in the gut lumen, such as bile salts (Wells and Hylemon 2000) and their sulfate esters (Eyssen *et al.* 1985).

As we confirmed, bilirubin reductase is not secreted by this bacterium, so reduction of bilirubin must occur intracellularly. Although the weakly polar UCB, MB and its alkyl esters should spontaneously diffuse through phospholipid bilayers, the polar amides, glucuronide and especially taurine conjugates would not (Zucker and Goessling 2000). This fact suggests that there must be carrier-mediated uptake of the bile pigments in *C. perfringens*, as it was demonstrated for bile salts uptake in *Clostridium* sp. Strain TO931 (Wells and Hylemon 2000). Indeed, despite its ready diffusibility, carrier-mediated uptake of UCB is present not only in mammalian cells (Cai *et al.* 2002), but also in simpler organisms such as *Caenorhabditis elegans* (George *et al*. 1999). Even more interestingly, it was demonstrated that the beta-subunit of the inner mitochondrial membrane protein of the rat liver, F1-ATPase, is identical to an organic anion binding protein, a putative bilirubin transporter at the sinusoidal hepatocyte membrane (Goeser *et al*. 1990). However, this β-subunit of F1-ATPase is prevalent in many prokaryotes (Nelson 1989) suggesting possible role in active transport of bile pigments of this or related ABC proteins (Saier and Reizer 1991).

6.1.2 Analysis of bilirubin/mesobilirubin reduction products

C. perfringens BR1 was found to reduce UCB only to the level of urobilinogen. The three urobilin bands found upon reduction of commercial UCB were proven by UV-visible and fluorescence spectroscopy, ferric chloride oxidation (mesobiliviolin reaction), mass spectrometry, and elemental composition to be the oxidation products of different isoforms of urobilinogens III, IX and XIII, formed from the respective isomers of UCB. When pure MB IXα was used as a substrate, only one urobilinogen species was produced. Similarly, TLC of the reduction products of [^{14}C]-labeled bilirubin (Fig. 5-7) demonstrated production of three urobilin species, whereas in case of MB (Fig. 5-1) only one urobilin could be detected. Thus, under *in vitro* conditions molecular scrambling (i.e. dipyrrolic exchange) did not occur and the initial configuration was maintained. The commercial MB was, therefore, apparently much purer than the UCB supplied by a different vendor. This is in accord with results by McDonagh and Assisi (1971), who observed marked differences in the content of these isomers in bile pigments supplied by various vendors.

It is interesting that bile pigments are converted only to the level of urobilinogens and reduction does not proceed to stercobilinogens. This is in agreement with previous suggestions indicating that reduction to levorotary stercobilinogens may require an additional specific enzyme(s) (Watson *et al*. 1969). It should also be stressed that, although our clostridial strain was isolated from an urobilinoid-positive neonatal stool, these cases are rare since colonization of neonatal gastrointestinal tract with bacteria reducing bilirubin is very slow and accounts for the low levels of urobilinoids in neonatal feces (Vítek *et al*. 2000).

Our study was carried out with only one microbial isolate, which might oversimplify the situation within the intestinal lumen. Further studies are needed to fully elucidate details of bilirubin catabolism in the intestinal lumen, in particular by indigenous intestinal microflora.

6.2 Identification of gene(s) involved in bilirubin metabolism

6.2.1 Electroporation of the C. perfringens BR1

Electroporation is a versatile method for introduction of exogenous DNA into the bacteria. The technique involves the application of a brief high-voltage pulse to the sample of cells and DNA. The result is a transient membrane permeability and DNA uptake by the subpopulation of the surviving bacteria (Miller 1994). Development of stable and reproducible electroporation protocol was necessary for introduction of transposons Tn*916* and Tn*917* into the strain BR1.

We electrotransformed late-stationary phase cells of *C. perfringens* with plasmid pJIR750 according to Kim and Blaschek (1989). Some strains of *C. perfringens* undergo autolysis at late-stationary phase (Heefner *et al*. 1984, Rogers 1984), which may destabilize cell wall. Because of the autolysin activity, the cell wall of late-stationary phase *C. perfringens* cells may be more vulnerable to electroporation-induced pore formation and thereby allow for plasmid DNA penetration (Kim and Blaschek 1989). There is no evidence whether the *C. perfringens* BR1 produces autolysins, but we were able to transform late-stationary phase cells of this strain. Transformation efficiency of 9.9×10^3 was comparable to those of other strains: 1.0×10^4 of strain 3624A (Kim and Blaschek 1989), 4.4×10^3 of strain P90.2.2. (Phillips-Jones 1990) and 9.8×10^3 of strain 3626B (Chen *et al*. 1996). The only strain giving much higher efficiency (8.1×10^8) was the strain 13 (Chen *et al*. 1996). On the other hand, the transformation experiments that we performed were not reproducible. *C. perfringens* transformants were obtained in only two out of six experiments.

One factor which may have negative influence on electroporation of *C. perfringens* is a presence of cell associated nucleases (DNases). Chen *at al*. (1996) demonstrated that DNases of *C. perfringens* type 3626B digest plasmid DNA during the pre-shock incubation period. After 3 min plasmid incubation with *C. perfringens* type 3626B competent cells, almost all DNA was digested. Compared to a reference there was only a weak signal on a gel after electrophoretic analysis. In our case no losses of plasmid DNA were observed during prolonging incubation (from 1 min to 10 min), because the density of DNA bands remained the same as a reference (Fig. 5-8). Therefore negative effect of DNases on the transformability of *C. perfringens* BR1 strain could be excluded.

We also examined the effect of the electric pulse parameters on the cell survival and transformability of strain BR1, and changed the regeneration conditions after electroshocking. Regeneration in BHI medium with sucrose probably osmotically stabilized the shocked cells and prevented their lysis during the period necessary for the expression of Cm-resistance genes. The ratio of cell killing was 65 % following electroporation at 6.25 kV/cm and 200 Ω, and up to 98 % at 12.5 kV/cm and 600 Ω. The percentage of cell killing was lower in comparison with transformable *C. perfringens* strain P90.2.2. (64 - 83 % vs. 89 - 91 % at 6.25 kV/cm), what also increased the chance of transforming strain BR1. Neither application of pulse with the highest electric field strength of 12.5 kV/cm and resistance of 600 Ω resulted in the transformation of strain BR1, whereas transformants of strains 3624A and P90.2.2. at electric field strength of 6.25 kV/cm were obtained (Kim and Blaschek 1989, Phillips-Jones 1990). Kim and Blaschek (1989) transformed *C. perfringens* when the pulse duration time (time constant) was 36.0 - 45 ms, while Phillips-Jones (1990) transformed *C. perfringens* when pulses of 5.7 - 8.1 ms were applied. Time constant affects the transformation efficiency of *C. perfringens* rather than the transformability. Therefore, we suppose that time constants of 6 - 15 ms should be sufficient for the electroporation of strain BR1 and the poor transformability is not caused by shortness of the time constant.

Because of the irreproducibility of the transformation of the late-stationary phase cells, we examined the effect of growth phase on the transformability of the *C. perfringens* BR1. Cells harvested at the different points in the growth curve, were not transformed at all (Tab. 5-5). These negative results supported the hypothesis that the cell wall of Gram-positive bacteria interfere with the electrotransformation and must be removed or compromised to allow DNA uptake.

Electroporation of lactic streptococci has been achieved after the pretreatment of the cells with lysozyme (Powell *at al.* 1988). However, pretreatment of *C. perfringens* cells with lysozyme failed to yield any transformants (Scott and Rood 1989). In the same study *C. perfringens* transformants were reproducibly obtained when the cells were pretreated with lysostaphin (2-20 μg/ml, 1h at 37 °C). It was assumed that lysostaphin partially digests the *C. perfringens* cell wall so that the plasmid DNA has access to the pores subsequently created in the cell membrane by electroporation (Scott and Rood 1989). This conclusion is in the agreement with the suggestion made by Kim and Blaschek (1989) regarding the potential role of autolysins. When we carried out the transformation experiments with lysostaphin pretreated cells, we obtained up to 6.4×10^4 transformants per

μg DNA. The transformation efficiency that we achieved was comparable to that of Scott and Rood (1991), but we had difficulties to repeat experiments (Tab. 5-6) similar as in case of late-stationary phase cells.

Therefore we tried to find out the point in the process of competent cells preparation, when the cells die, and examine whether the lysostaphin concentration did not affect *C. perfringens* viability. We found out that cells lysed during washing in electroporation buffer (Tab. 5-7) or during pre-shock incubation on ice (Tab. 5-8). In addition, untreated *C. perfringens* cells also died under the same conditions (Tab. 5-7, 5-8). We supposed that the extensive washing in electroporation buffer together with a relatively high treatment temperature (20 °C) caused the lysis of competent cells independently of the lysostaphin treatment. Consequently, we reduced a number of the washes, increased the volume of electroporation buffer, and performed part of the manipulations at 4 °C. Cm-resistant transformants were obtained when cells were washed in cold (4 °C) electroporation buffer after lysostaphin treatement. When we prepared competent cells at 20 °C, the cells were transformed only when lysostaphin at concentration of 5 μg/ml was used. Furthermore, when the post-electroporation incubation on ice was omitted, the transformants were obtained at all three concentrations of lysostaphin. The lysostaphin concentration of 0.5 μg/ml gave the highest transformation efficiency of 2 x 10^4 (Tab. 5-9). However, these preliminary results were not seen in the repeated experiments (Tab. 5-10) and it was impossible to define the relation between lysostaphin concentration and the transformation efficiency and design the optimal concentration of lysostaphin. As well, on account of these inconsistent data we did not continue in optimization of the lysostaphin method.

We found that strain BR1 is hardly transformable, and it was only transformed in agreement with the hypothesis that cell wall must be destabilized prior electroshocking. Even thought we were able to transform late-stationary phase cells and lysostaphin treated cells. However, none of performed optimizations brought a stable protocol for electroporation of this recalcitrance strain. One explanation of the poor transformability could be that all manipulations we performed aerobically, whereas *C. perfringens* is anaerobic bacterium, and viability of cells could be decreased by O_2 damage already before electroshocking. On the other hand *C. perfringens* is an aerotolerant anaerobe and the electrocompetent cells of strains P90.2.2. and 13 were also prepared aerobically (J.I. Rood, personal communication; M.K. Phillips-Jones, personal communication). Another reason for variations in transformability could be the usage of different commercial batches of the

culture media and their compounds or even small differences in their preparation resulting in alterations in either the thickness or composition of the cell wall. Finally, it is interesting to note that strain BR1 is a non-pathogenic and it seems that there is a relationship between type of the strain and transformability by electroporation in pathogenic *C. perfringens* strains (Scott and Rood 1989, Allen and Blaschek 1990, Chen *at al*. 1996). It was demonstrated that four type A strains and one type C strain were transformable when the mid-exponential-phase cells were used, while the three type B strains tested were not (Allen and Blaschek 1990). Chen *et al*. (1996) demonstrated that the cell associated nucleases disabled plasmid transformation of *C. perfringens* strain 3626B. The authors proposed a modified protocol for the transformation of strains with similar phenotype. As it was shown, not all *C. perfringens* strains are transformable (Scott and Rood 1989, Allen and Blaschek 1990) and the strain BR1 probably belongs to this group of nontransformable strains.

6.2.2 Electroporation of C. perfringens P90.2.2.

C. perfringens P90.2.2. exhibits two features essential for the isolation of mutants defective in bilirubin metabolism by transposon mutagenesis: (1) the ability to reduce bilirubin and (2) the transformability. Another potential candidate, the transformable strain 13, does not reduce bilirubin, and this example demonstrates that the ability to reduce bilirubin is not incident to all *C. perfringens* strains.

We carried out electroporation of *C. perfringens* P90.2.2. to establish electroporation protocol providing higher transformation efficiency than the previous one (4.4×10^3, Phillips-Jones 1990). We attempted to increase the transformation efficiency of the strain P90.2.2. by the lysostaphin treatment. We failed to obtain any transformants using varying amounts of lysostaphin (0.5, 1, 10, and 20 μg/ml). Similar result was obtained by Scott and Rood (1989) when they used the lysostaphin protocol for electroporation of the other *C. perfringens* strains (CW504, JIR81 and 3626B) than the strain 13. Until now, the lysostaphin protocol has been successfully applied only on the strain 13 and its derivatives (Scott and Rood 1989, Awad and Rood 1997, Briolat and Reysset 2002).

The new protocol was derived from lysostaphin method: We transformed early-exponential phase cells, reduced number of washes in electroporation buffer and omitted

the lysostaphin treatment. The highest transformation efficiency (1.37 x 10^4) was obtained when cells were prepared at room temperature and the post-shock incubation on ice was omitted. Higher transformation efficiency than that achieved in the previous study (Phillips-Jones, 1990) might also have been influenced by usage of different plasmid DNA (pJIR750 vs. pSB92A2). Transformation efficiency decreased significantly (100 - 700 times), when competent cells were prepared at 4° C, using cold electroporation buffer. In the case of the RT protocol, the elimination of post-shock incubation on ice resulted in up to 2.5 fold increase of the number of transformed cells, except for shocking at 2.5 kV, when cells were not transformed (Tab. 5-12). Using the Cold protocol, transformants were obtained only in the absence of post-shock incubation on ice (Tab. 5-13). In case of RT protocol the highest voltage of 2.5 kV gave the lowest transformation efficiency. It seems that such high field strength (12.5 kV/cm) produces unacceptable levels of cell killing (99%) and is unsuitable for the electroporation of the early-exponential phase cells of *C. perfringens* P90.2.2.. As the voltage decreased to 1.8 kV (9 kV/cm), the transformation efficiency increased by approximately 10-fold (Tab. 5-11). The ratio of cell killing (91.8% at 9 kV/cm and 96% at 10 kV/cm) was similar to the values, when late-stationary phase cells were transformed (89 - 91 % at 6.25 kV/cm, Philips-Jones 1990). We concluded that manipulation at low temperatures in the case of the Cold protocol was the main factor affecting transformation efficiency and compromised the impact of the voltage applied.

The new protocol is time-saving, because early-exponential phase cells are transformed. The complete procedure takes approximately 6 - 7 h. Furthermore, no enzymatic manipulations are required. Keeping the cells at room temperature and the absence of post-shock incubation on ice significantly increased the transformation efficiency, and might be applied for electroporation of previously non-transformable *C. perfringens* strains.

The complex nature of the factors involved in the transformation by electroporation indicates that it may be difficult to develop a universal electroporation-induced transformation protocol for whatever strain of *C. perfringens*. Our data, consistent with published results (Scott and Rood 1989, Allen and Blaschek 1990, Phillips-Jones 1990, Chen *et al*. 1996), show that selection of a convenient electroporation technique and the optimization of the transformation conditions is necessary.

6.3 Transposon mutagenesis of *C. perfringens*

Transposon mutagenesis is a versatile method useful for the identification of essential and pathogenicity-related genes in both pathogenic and nonpathogenic bacteria. It is based on random integration of transposon into a genomic DNA, and searching for the loss of desired activity among bacterial clones bearing the transposon.

6.3.1 Tn916 mutagenesis of C. perfringens P90.2.2.

Initially, we performed the transposon mutagenesis with Tn*916*, the element, whose usage has been reported in *C. perfringens* (Awad and Rood 1997, Briolat and Reysset, 2002). We electroporated the non-replicative plasmid pAM120 bearing Tn*916* into the bilirubin-reducing strain P90.2.2.. No Tc-resistant clones were obtained either when recommended saturating concentration of pAM120 was used (Briolat and Reysset, 2002), or when the time constant was prolonged. We supposed that extension of the time constant might facilitate the penetration of such a large plasmid (23.4 kb).The disability of *C. perfringens* cells to accept plasmid DNA was excluded by a parallel transformation with replicative plasmid pJIR750 (Tab 5-13, 5-14). We also excluded the possible excision of Tn*916* from pAM120, because the antibiotic resistance gen *tet(M)* related with the transposon conferred Tc-resistance to the host of *E. coli*. Moreover the restriction analysis did not reveal any rearrangements in the structure of pAM120 (Fig. 5-10). The integrity and the ability of Tn*916* to be transferred into *C. perfringens*, was demonstrated by the conjugative transfer of pAM120 from *E. coli* S17-1 into *C. perfringens* strain 13. We obtained Tc-resistant transconjugants of strain 13, whereas of strain P90.2.2. not (Tab. 5-16). To exclude the possibility that pAM120 had mutated during its passaging in the source laboratory (J.I. Rood) we used pAM120 obtained from a different laboratory (B. Depuy) nevertheless without success (data not shown).

We concluded that transformation efficiency of 10^4 (Tab. 5-15) is too low for the successful introduction of pAM120 into *C. perfringens* P90.2.2., because the Tn*916* mutants only of high electrocompetent strain 13 and its derivatives could be obtained (Awad and Rood 1997, Briolat and Reysset 2002). It also seems that strain P90.2.2. is not a suitable acceptor strain in mating experiments.

6.3.2 Tn917 mutagenesis of C. perfringens P90.2.2.

The second transposon that has been reported to be used in *Clostridium* genus, is Tn*917*. pTV408 bearing Tn*917* was electropotated as a nonreplicative plasmid. Due to intrinsic Km resistance of *C. perfringens* the advantage of temperature-sensitive replicon presented in pTV408 (Slater *et al*. 2003) could not be used. Em-transformants we selected neither at 37 °C, nor at nonpermisive temperature of 42°C when curing and transposition of Tn*91*7 is preferred. Restriction analysis of pTV408 did not reveal any rearrangements in its structure (Fig. 5-11). Also excision of Tn*917* from pTV408 was excluded, since *E. coli* cells carrying pTV408 were always selected on Em indicating that the antibiotic resistance gen *erm* related with the transposon was expressed.

The possible explanation of these negative results could be the inability of Tn*917* to integrate into the genome of *C. perfringens* P90.2.2.. In addition, there is no evidence whether promotor of *erm* gene of Tn*917* may induce its expression in *C. perfringens*. Negative results were also obtained for the high electrocompetent strain 13 (J.I. Rood, personal communication), and therefore it seems that *C. perfringens* is not a suitable candidate for Tn*917* mutagenesis.

6.4 Construction *of C. perfringes* P90.2.2. genomic library in *E. coli* and its screening for bilirubin reducing activity

The last attempt to isolate gene(s) involved in the metabolism of bilirubin was based on the construction of *C. perfringens* genomic library and its screening in *E. coli* for the clones expressing bilirubin reducing activity.

The construction of a genomic library is a relatively simple process involving ligation of vector DNA and fragmented chromosomal DNA. Sufficiently random fragmentation of chromosomal DNA can be obtained by partial digestion using frequently cutting restriction enzymes. We considered of digesting the chromosomal DNA with either *Tsp*509I or *Sau*IIIA, the enzymes often used for generating partial digestion libraries. The sequence analysis of chromosomal DNA of strain 13, revealed above 40 000 target sites of *Tsp*509I, while only 4000 sites of *Sau*IIIA were detected. *C. perfringens* strain 13 has low G+C content (28.6%) (Shimizu *et al.* 2002), and therefore the target site of *Sau*IIIA (GATC), is in the genome less often represented than the target site of *Tsp*509I (AATT). As well, when we digested chromosomal DNA of strain P90.2.2. with these enzymes, *Sau*IIIA cut the DNA less frequently than *Tsp*509I (data not shown). Consequently, the partial digestion was performed using *Tsp*509I and it was set up to compromise cloning capacity of vector pK19 with the maximal desired length of cloned chromosomal fragments (8-12 kb).

In total we obtained 4662 recombinant *E. coli* clones from two independent experiments (Tab. 5-18). Nevertheless, only 30-40 % of the putative recombinant clones carried recombinant pK19 containing inserts of 8-12 kb in large. The majority of clones carried 6 kb vectors. Digestion of these vectors with *Bam*HI and *Pvu*I did not result in fragments corresponding to the pK19 (2.5 kb) and inserts of chromosomal DNA. Plasmids were cleaved into two fragments of 1.2 and 5 kb (Fig. 5-13). In addition, we failed to sequence these aberrant plasmids and to prove DNA inserts of clostridial origin. It seems that these DNA molecules were derived from pK19, because they replicated independently of chromosomal DNA and provided Km-resistance to the host of *E. coli*. However, it remains unclear why this phenomenon occured. One possible explanation of the aberrant plasmids could be the occurance of DNA rearrangements in the major fraction of recombinant clones.

Since we obtained aberrant plasmids in both library construction experiments, we supposed that repeating ligations would be unreasonable and we performed screening of

the existing library for the presence of clones able to reduce bilirubin. The screening was accompanied by few complications: Firstly, there is no evidence whether bilirubin could be transported or enters into *E. coli*. This ability is associated with bilirubin-reducing strains of *C. perfringens* (L. Vítek, unpublished results) and we suppose that the pigment probably does not enter into *E. coli* as it does not reduce bilirubin (Fig. 5-12). So, the *E. coli* cells must be disintegrated prior incubation with bilirubin, and the only suitable technique that does not destroy the bilirubin-reducing activity in *C. perfringes* is a French press disintegration (L. Vítek, unpublished results). Secondly, determination of urobilinoids, reduction products of bilirubin, is rather tedious method, because it involves measurement of absorption spectra and it could not be replaced by any simpler technique, e.g. color reaction. So, the whole screening process involves culturing particular *E. coli* clone, disintegration of cells using French pressure cell, incubation of the protein extract with bilirubin and determination of urobilinods spectrofotometrically. It is evident that subjecting every particular clone (from the count of 4662) to such procedure is impossible. As consequence we pooled clones from a half of 96-well plate and screened them in one experiment, what decreased total number of quantifications to nearly 100. Nevertheless, this experimental layout did not lead to isolation of any clone of *E. coli* with bilirubin-reducing activity.

There are several possible explanations of this negative result: Gene coding putative bilirubin-reductase is not present in the library. This assumption could be true if the chromosomal DNA was not cleaved completely randomly, or the fragment carrying gene coding putative bilirubin-reductase was larger than the cloning capacity of pK19. On the other hand, the probability of isolating a fragment of interest reached almost 99 %, because the total number of base pairs present in clones screened (14769 kb, Tab. 5-18) represented 4.6 fold excess over the total number of base pairs in the genome (3.031 kb of strain 13), as it was calculated by Seed *et al*. 1982. Other possible explanation could be that expression of genes of *C. perfringens* P90.2.2. was altered, or even more they were not expressed in *E. coli*, although expression of clostridial genes in *E. coli* under either aerobic (Walters *et al.* 1999) or anaerobic (Rafii and Coleman 1999) conditions was demonstrated. As well nascent proteins might require special post translation modification for their function that could not be provided by the host bacterium. Finally, the bilirubin-reducing activity might have been too weak to be detected spectrofotometrically, since we screened 48 clones in one experiment. However, reduction of clones screened to a half would result in increase of screening experiments to nearly 200, which is technically impracticable.

Despite the all negative attempts, there are still a few possibilities how to identify genes involved in the bilirubin metabolism in *C. perfringens*. First, to finalize purification of bilirubin-reducing protein and to determinate its sequence. Then the relevant gene might be identified by screening the genomic library of *C. perfringens* P90.2.2. by hybridization with an oligonucleotide probe designed according to the amino acid sequence of the purified protein. Secondly, the complete genome sequences of two additional *C. perfringens* type A strains, ATCC 13124 and SM101, have been determined and compared with the published *C. perfringens* strain 13 genome recently (Myers *et al*. 2006). ATCC 13124 produces large quantities of gangrene-associated toxins and the strain SMS101 is a transformable derivative of the *C. perfringens* food poisoning isolate NCTC 8798 producing enterotoxin (CPE). The comparison of these three genomes revealed considerable genomic diversity with >300 unique “genomic islands” identified. These islands encode genes that correlate to differences in virulence and phenotypic characteristic of these strains. Assuming that one of these novel sequenced strains would reduce bilirubin, and then based on sequence comparison of the active strain with the non-active strain candidate genes coding putative bilirubin reductase might be proposed. In subsequent studies findings obtained in this work might be applied, especially the aspects regarding genetic manipulation with *C. perfringens*.

7 CONCLUSIONS

In this study we focused on the process of bilirubin reduction catalyzed by an anaerobic intestinal bacterium *C. perfringens*. We aimed to undertake analysis of bile pigments metabolized by *C. perfringens* and their respective reduction products and to identify gene(s) encoding protein(s) involved in metabolism of bilirubin.

7.1 Analysis of bile pigments metabolized by *C. perfringens* and their respective reduction products

(1) The *C. perfringens* strain BR1 isolated from neonatal stools reduces a variety of different bile pigments indicating that this broad substrate specifity could be an effective tool for disposal of electrons produced in catabolic processes within these bacteria.

(2) The examined strain reduces UCB only to the level of urobilinogen. Other bacterial strains and species, absent in neonates, are presumed to be essential for catabolism to the level of stercobilinogen.

7.2 Identification of gene(s) involved in bilirubin metabolism

(1) The *C. perfringens* strain BR1 is resistant to the transformation of plasmid DNA mediated by electoporation and therefore it is not a candidate suitable for transposon mutagenesis.

(2) A transformable *C. perfringens* P90.2.2. strain was found to reduce bilirubin. Rapid and simple method suitable for electroporation of this strain was developed providing transformation efficiency up to 1.37×10^4.

(3) Transposon mutagnenesis of the strain P90.2.2. failed to identify gene(s) involved in metabolism of bilirubin. We were not able either to introduce transposons Tn*916* and Tn*917* into this strain or to isolate clones incapable to reduce bilirubin.

(4) The attempt, based on construction of *C. perfringens* P90.2.2. genomic library and its screening in *E. coli* for clones able to reduce bilirubin, did not result in isolation of gene(s) responsible for the reduction of bilirubin as well.

(5) Even thought we did not manage to identify gene(s) involved in metabolism of bilirubin, this study brought new findings regarding genetic manipulatios with *C. perfringens.*

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