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Adding Upstream Sequence and a Downstream Reporter to the Bile Acid Inducible Promoter of *CLOSTRIDIUM scindens* VPI 12708

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ADDING UPSTREAM SEQUENCE AND A DOWNSTREAM REPORTER TO THE BILE ACID INDUCIBLE PROMOTER OF *CLOSTRIDIUM SCINDENS* VPI 12708

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Bile acids in the small intestines of animals serve to breakdown fats and fat-soluble vitamins. Most of the bile acids are reabsorbed into the enterohepatic circulation, but approximately five percent of these bile acids pass into the large intestine. These bile acids are swiftly deconjugated by the bacterial population, and then subjected to further intestinal bacterial chemical modifications. The most significant of these modifications are 7α-dehydroxylations which form secondary bile acids (deoxycholate and lithocholate). Much research has illuminated the 7α-dehydroxylation pathway: of particular interest is the bile acid inducible operon, for which *Clostridium scindens* VPI 12708 serves as the model organism. There is a lack of knowledge on how this operon is regulated, so the goal of this project was to create a genetic construct consisting of upstream regulatory elements, a bile acid inducible promoter, and a *β*- glucuronidase reporter. Cloning strategies utilized PCR to amplify desired DNA fragments and sewing methodology to combine DNA fragments. DNA fragments were ligated into plasmids and transformed into competent *E. coli*. Transformants were evaluated for the desired reporter gene fusion by blue/white screening, additional PCR, and/or restriction digestion. The bile acid inducible promoter was successfully amplified, and the upstream sequence and *uid*A (*β*- glucuronidase) reporter was demonstrated. However, no *E. coli*

transformants were demonstrated to possess the *bai*P-*uidA* gene fusion. The project strategy is plausible and data regarding the bile acid inducible promoter are greatly needed.

Introduction

An important function of the liver is the synthesis of bile. Bile composition is composed of organic and inorganic electrolytes, calcium, magnesium, sulfate, phospholipids, proteins, cholesterol, and bile acids. Bile secretion by hepatocytes serves as an excretion route for organic solutes (e.g. bilirubin) and assists in lipid and fat-soluble vitamin absorption (Scharschmidt 1990). Lipids and fat-soluble vitamins are hydrophobic and will form larger insoluble aggregates in aqueous solutions. Bile acids prevent aggregation of these compounds and promote lipid emulsification so that these can be broken down into ever smaller sizes (Monte et al. 2009).

Bile Acid Biochemistry

In vertebrates, hepatic biosynthesis of primary bile acids provides an elimination route for cholesterol. Cholesterol (Fig. 1) comprises the basic 'chemical chassis,' a 19-carbon sterol nucleus of bile acid compounds (Vlahcevic et al. 1990). Key chemical groups attached to the sterol nucleus include a carbon side chain (bonded at carbon 17) that includes a branched 5 carbon side chain ending in a carboxylic acid, 5β hydrogen, and a 3α hydroxyl (Samuelsson 1960; Vlahcevic et al. 1990). Therefore, the primary bile acids are 24-carbon cyclopentanephenanthrene sterols and include cholic acid (3α, 7α, 12α-trihydroxy-5β-cholanic acid) and chenodeoxycholic acid (3α, 7α-dihydroxy-5β-cholanic acid) (Vlahcevic et al. 1990; Monte et al. 2009).

The primary bile acids (Fig. 1) function as ionic detergents to aid the solubilization of cholesterol and other organic compounds such as bilirubin, and the absorption of dietary lipids and lipid soluble vitamins (A, E, D, and K). In humans, after

synthesis within the liver, primary bile acids are stored in the gall bladder, awaiting a postprandial cholecystokinin cue. Cholecystokinin is a polypeptide that stimulates the gall bladder to contract and to empty accumulated bile acids into the duodenum. Research suggests that the bile acid pool cycles from hepatic origin, to the gall bladder, to the small intestine, and back to the liver via the portal vein. This enterohepatic circulation occurs twice per meal. Predictably, bile acid re-absorption is most efficient in the ileum, allowing the detergent action of these compounds to be effective throughout the length of the small intestine. Re-absorption is efficient; 95% of the bile acid pool is returned to the liver via the portal vein (Hill and Drasar 1968; Vlahcevic et al. 1990). Any deconjugated bile acids are reconjugated before the liver resecretes these into the bile and completes the enterohepatic circulation (Prabha and Ohri 2006). The remaining 5% (~400 – 600 mg/day) enter the large intestine and are modified by intestinal microbes (Hill and Drasar 1968; Vlahcevic et al. 1990).

Within the liver, >99% of primary bile acids are conjugated to different modifying groups (amino acids, sulfates, glucuronides, etc.). Conjugation with either glycine or taurine occurs most frequently at C24 (Vlahcevic et al. 1990; Vlahcevic et al. 1992). Modifying groups are linked via ester, ether, or amide bonding to either side chain carboxyls or one of the hydroxyl groups of the steroid rings. These chemical modifications prevent precipitation of the primary bile acids under neutral aqueous conditions (Hill and Drasar 1968; Vlahcevic et al. 1990; Russell and Setchell 1992; Vlahcevic et al. 1992; Ridlon et al. 2006). The average concentration of bile acids is 12 g/L in human hepatic ductal bile. Cholates, deoxycholates, and chenodeoxycholates

represent 35%, 25%, and 35%, respectively, of primary bile acids in human bile (Vlahcevic et al. 1990).

The intestinal microflora consist of a diverse group of bacteria estimated at a density of 1 x 10^{12} colony forming units (CFU)/g and consisting of over 500 species (Lievin-Le Moal and Servin 2006; Canny and McCormick 2008). Notable genera include *Bifidobacterium, Escherichia, Clostridium, Bacteroides, Streptococcus, Fusobacteria, Peptostreptococcus, Lactobacillus, Methanobrevibacter, Helicobacter,* and *Enterococcus* (Lievin-Le Moal and Servin 2006; Canny and McCormick 2008; Huys et al. 2008). Microbial populations become increasingly numerous and anaerobic toward the distal end of the intestinal tract. These microorganisms perform useful functions for the host including vitamin synthesis, protection from exogenous pathogens, and fermentation of non-digestible carbohydrates (Canny and McCormick 2008). Additionally, these microbes are known to modify bile acids that escape reabsorption by the ileum.

There are several potential microbial modifications that can occur to the bile acids that enter the large intestine. Intestinal microorganisms that possess bile salt hydrolases perform deconjugation (the removal of amino acids from primary bile acids) which provides an available source of carbon, nitrogen and sulfur (Ridlon et al. 2006). For example, taurine is known as both an aerobic and anaerobic energy source, and glycine is used in an amino acid fermentation reaction in which one amino acid donates electrons to a different amino acid. Other post-deconjugation biotransformations result from 7α-hydroxysteroid oxidoreductases, 7β- dehydroxylases, 12α/β hydroxysteroid dehydrogenases, and 3α-hydroxysteroid oxidoreductases (Vlahcevic et al. 1990; Ridlon et al. 2006). However, it is the irreversible action of 7α-dehydroxylases, which is deemed

the most important quantitative microbial modification, that require careful consideration (White et al. 1980).

The 7α-dehydroxylation activity is notable because of the prevalence of secondary bile acids (Fig. 1) in human feces. Deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) (DCA) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) (LCA) (produced via dehydroxylation of cholic acid (CA) and chenodeoxycholic acid (CDCA), respectively), can only be transformed after deconjugation (Ridlon et al. 2006). Secondary bile acids differ functionally from the compounds from which they are derived because these do not solubilize lipids on par with CA/CDCA. In addition, secondary bile acids are more cytotoxic and carcinogenic than primary bile acids, and these alter biosynthesis of primary bile acids (Franklund et al. 1993). Biochemically, secondary bile acids are significantly more hydrophobic than primary bile acids after microbial modifications (Nihira et al. 1988; Lamcharfi et al. 1997).

Primary bile acid 7α-dehydroxylation results in a net reduction of two electrons. This large, energetically expensive pathway is thought to benefit 7α-dehydroxylating organisms by giving these a competitive edge against other organisms that are susceptible to the cytotoxicity of secondary bile acids and/or by providing an energetic advantage in using bile acids as electron acceptors to dispose of electrons from other metabolic processes (Ridlon et al. 2006).

It is noteworthy that only a tiny percentage of the colonic microbiota (~0.0001%) possess the 7α-dehydroxylation pathway, yet the conversion rate from primary to secondary bile acids is relatively rapid (Ridlon et al. 2006). Equally noteworthy is that the genus that is most often implicated in 7α-dehydroxylations within the intestine is

Clostridium (Midtvedt and Norman 1967; Ferrari et al. 1977; Stellwag and Hylemon 1979; Hirano et al. 1981; Takamine and Imamura 1995; Doerner et al. 1997). The presence of *Clostridium* species was confirmed by 16S rDNA analysis of fecal 7α-DH bacterial isolates (Wells et al. 2000; Ridlon et al. 2006).

Elevated levels of secondary bile acids have been found in patients with gallstones and in colorectal patients (Hill et al. 1975; Nagengast et al. 1988; Berr et al. 1996). One study focused on cholesterol gallstone patients with elevated biliary cholesterol and DCA implicated 7α-dehydroxylating bacteria in the formation of gallstones (Berr et al. 1996). Additionally, 70% of large bowel cancer patients exhibited high levels of fecal bile acids and Clostridia capable of modifying primary bile acids as compared to 9% of the non-cancer patients (Hill et al. 1975).

Secondary bile acids re-enter enterohepatic circulation after passive reabsorption in the colonic mucosa. The liver is unable to convert LCA and DCA back into CDCA and CA. The process of 7α-hydroxylation is not performed on returning bile acids. Instead, upon return to the liver, LCA and DCA are conjugated with either glycine or taurine. LCA is also often sulfated on the third carbon of its sterol rings (Hofmann 1984; Ridlon et al. 2006).

Clostridium and Bile Acid Metabolism

Clostridum scindens VPI 12708 is the model organism for studying the bile acid inducible operon and elucidating the genes and proteins involved in the transformation of primary bile acids into secondary bile acids (Coleman et al. 1988; White et al. 1988a; White et al. 1988b; Krafft and Hylemon 1989; Mallonee et al. 1990; Mallonee et al. 1992; Franklund et al. 1993; Mallonee et al. 1995; Mallonee and Hylemon 1996; Ye et al. 1999; Kang et al. 2008). The organism of interest for this project was renamed

Clostridium scindens VPI 12708 (previously *Eubacterium* VPI 12708) based on phylogenetic comparison (Kitahara et al. 2000).

Clostridium scindens VPI 12708 initiates the bile acid 7α-dehydroxylation pathway by importing primary bile acids intracellularly via a bile acid transporter (Mallonee and Hylemon 1996). After importation, bile acid-CoA ligase adds co-enzyme A on to the bile acid carboxylic acid group (Mallonee et al. 1992). If cholic acid is present, modification by a 3α-hydroxysteroid dehydrogenase produces 3-oxo-cholyl-CoA (Mallonee et al. 1995). Modification by a 3-dehydro-4-CA steroid reductase produces 3-oxo-Δ^4-cholyl-CoA (Franklund et al. 1993), followed by dehydration by a bile acid CoA hydrolase (Ye et al. 1999). The resulting 3-oxo-Δ^4-cholic acid is further dehydrated by 7α-dehydratase (Wells and Hylemon 2000). This 3-oxo-$\Delta^{4,6}$-deoxycholic acid intermediate is converted into 3-oxo-Δ^4-deoxycholic acid, into 3-oxo-deoxycholic acid, and ultimately into deoxycholic acid after accepting electrons from NAD(P)H. The specific enzymes involved in each of these steps have not been yet. Lastly, the resulting secondary bile acid is hypothesized to be removed from the cell via a bile acid exporter (Ridlon et al. 2006).

In earlier scientific work, the first reports of 7α-DH induction among *Clostridium* species VPI 12708 stated that amending growth media with [C^{14}] – CA or [C^{14}] – CDCA induced 7α-DH activity. Induction was measured radiochromatographically by rate of formation of [C^{14}] – DCA or [C^{14}] – LCA. Notably, this induction was specific in that both a free C-24 carboxyl group and a free 7α-hydroxyl group were required (White et al. 1980).

The bile acid inducible operon (Fig. 3) is a 12 kb gene regulation system that encodes at least 8 structural genes, a bile acid inducible promoter, and two upstream *ara*C homologous regions which may serve to regulate transcription (Wells and Hylemon 2000; Ramasubbaiah 2004; Ridlon et al. 2006). The *bai* genes are ordered after the promoter as follows: *bai*B, *bai*CD, *bai*E, *bai*A2, *bai*F, *baiG*, *bai*H, and *bai*I. Additional *bai* genes are still expected to be discovered. The mechanism of the *bai* operon regulation is unknown (Ridlon et al. 2006).

An attempt to understand the genetic regulation of 7α-DH expression began with the discovery of two cholic acid induced polypeptides (45 kDa and 27 kDa) which led to the cloning of the gene encoding the 45 kDa protein. Additionally, a Northern blot revealed a cholic acid induced >6,000 nucleotide mRNA which suggested the possibility of a large bile acid-inducible (*bai*) operon (White et al. 1988a). The discovery of the previously mentioned 27 kDa protein led to the cloning of a second gene implicated in the 7α-DH pathway (Coleman et al. 1988). DNA sequence analysis revealed an open reading frame upstream of the 45kDa gene that could encode a second protein. An additional 27kDa polypeptide was discovered and a comparison of these two 27kDa genes (temporarily designated as 27K-1 and 27K-2) revealed homology within nucleotide sequences; the deduced amino acid sequences were also homologous. The discovery of these polypeptides suggested a multigene family, the existence of and the need to clone an entire operon, and the need to elucidate the function of all the implicated polypeptides (White et al. 1988b). Genes 27K-1 and 27K-2 were renamed to *bai*A1 and *bai*A2, respectively. A third gene (*bai*A3), which is not part of the operon and exists on a separate chromosomal fragment, was found to be 100% identical to *bai*A1. This

determination was followed by the discovery of six bile acid-inducible polypeptides and their respective DNA fragments that constitute an operon (Mallonee et al. 1990). One of these six DNA fragments was subcloned into *Escherichia coli* and shown to encode an ATP-dependent bile acid-coenzyme A ligase by activity; this gene's designation is *bai*B (Mallonee et al. 1992). A second fragment, *Bai*H, encodes a NADH:Flavin oxidoreductase (Franklund et al. 1993). Four additional fragments were designated *bai*A1, *bai*A3, *bai*E, and *bai*G. Further study of *bai*A1 and *bai*A3 revealed 3α-hydroxysteroid dehydrogenases with specific activity toward coenzyme A-containing moieties, especially cholyl-CoA and deoxycholyl-CoA (Mallonee et al. 1995). *Bai*E codes for a bile acid 7α-dehydratase (Dawson et al. 1996), and *Bai*G codes for a bile acid transporter, presumably driven by the proton motive force, which specifically recognizes CA and CDCA as substrates (Mallonee and Hylemon 1996). The *bai*F gene encodes a bile acid co-enzyme A hydrolase (Ye et al. 1999). Most recently, *bai*CD was found to have a stereo-specific NADH-dependent 7β-hydroxy-3-oxo-Δ^4-cholenoic acid oxidoreductase activity. *Bai*I is predicted to encode a 7β-dehydratase based upon comparison with the *bai*E gene (Kang et al. 2008). Figure 2 reviews the structural transformations involved in 7α-DH.

The homodimeric AraC protein functions as both a positive and negative regulator for the uptake and metabolism of L-arabinose. In *E. coli*, dimeric AraC binds near the *ara* promoter site and forms a loop in the DNA that represses transcription when L-arabinose is absent. If the sugar is present, AraC changes its binding at the promoter to directly interact with RNA polymerase and activates genes under the control of the *ara* promoters which converts L-arabinose to D-xylulose phosphate (Schleif 2003). AraC

regulation is relevant regarding the DNA sequence (~1200 bp) that precedes the *bai* promoter because it is homologous to *ara*C. Figure 4 shows a clustal W alignment of *araC* homologous sequences within several bacterial species. If it regulates in a similar manner, then it will be necessary to include this DNA upstream of the *bai* promoter for functionality.

The specific aims of this project were as follows: 1. To create a reporter gene fusion containing the *bai* promoter, potential regulatory sequence, and the *uid*A reporter gene, and 2. To test for the regulation of expression of the reporter gene fusion by primary bile acids. We hypothesized that the addition of *araC* homologous sequence upstream of the *bai* promoter would make no difference in governing response to bile acids and that there would be no difference in *bai* operon activity in the presence or absence of primary bile acids.

Materials and Methods

Bacterial Strains

The model organism, *Eubacterium* VPI 12708 (White et al. 1980), was renamed *Clostridium scindens* VPI 12708 by Kitahara et al. in 2000. The organism was originally obtained from Dr. Philip Hylemon from Virginia Commonwealth University.

Media

E. coli strains were grown aerobically in Luria-Bertani (LB) media consisting of 10 g tryptone (Fisher Scientific; Pittsburg, PA), 5 g sodium chloride (Fisher), and 5 g yeast extract (Becton Dickinson; Franklin Lakes, NJ) per liter. LB media were autoclaved and stored at ambient temperature. For plates, 15 grams of granulated agar (Fisher Sci) was added to LB media prior to autoclaving (Ausubel et al. 2002).

Clostridium scindens VPI 12708 was cultured anaerobically in Brain Heart Infusion (BHI) (Becton Dickinson) with modification (Holdeman et al. 1977). Amendments to 37g BHI included (per liter): 40 mL VPI salt solution, 1 mL hemin , 4 mL resazurin solution, 10 g yeast extract (Becton Dickinson), 2 g fructose (Sigma Aldrich; St. Louis, MO), 1 g cysteine-HCl (Sigma Aldrich), 2.69 g sodium bicarbonate (Sigma Aldrich), and q.s. NANOpure® (np) water (Barnstead; Dubuque, IA). Hemin solution consisted of 0.2 mg hemin chloride (ICN Biomedicals), 10 mL 1 M sodium hydroxide, and 90 mL np water. This solution was autoclaved and stored at 4°C. The resazurin solution consisted of 25mg resazurin (Sigma Aldrich) in 100 mL np water. All contents except hemin, cysteine-HCl, and sodium bicarbonate were added to a 2 L round

bottom flask and boiled while sparging with nitrogen gas. Boiling was ceased after the resazurin indicator became transparent and the solution cooled to ambient temperature. Hemin, cysteine-HCl, and sodium bicarbonate were added and mixed; pH was adjusted to 7.0 drop-wise with HCl. Contents were transferred to 18 x 150 mm anaerobic culture tubes (Bellco Glass; Vineland, NJ), sealed with serum stoppers with a nitrogen head space, secured with aluminum seals, crimped, autoclaved and stored at ambient temperature.

VPI salt solution contained (per liter): 0.26 g calcium chloride dihydrate, 0.48 g magnesium sulfate heptahydrate, 1 g dipotassium phosphate, 1 g potassium dihydrogen phosphate, 10 g sodium bicarbonate, 2 g sodium chloride. This solution was adjusted to pH 7.5 with NaOH and stored at 4°C (Holdeman et al. 1977).

Antibiotics

Ampicillin (Sigma) stocks were made by adding 100 mg ampicillin per milliliter to a 1:1 np water/ 100% ethanol solution. Tetracycline (Sigma) stocks consisted of 10 mg tetracycline per milliliter of ethanol. Chloramphenicol (ICN Biomedicals) stocks consisted of 20 mg chloramphenicol per milliliter of ethanol. Working concentrations in media are stated in Table 1. All stocks were stored unfiltered.

Plasmids

Bannam and Rood (1993) constructed versatile shuttle vectors to transfer genetic material in between *E. coli* and *C. perfringens*. This (Doerner) laboratory has demonstrated conjugal gene transfer of pECU-001 (modification of pJIR750) from *E. coli*

S17-1 to *C. scindens* VPI 12708 of pECU-001 (which is a modified pJIR750 (Fig. 5)) (Ramasubbaiah 2004). Plasmid pWKU-001, derived from the pECU-001 plasmid, was also conjugally transferred. Additions to pECU-001 include a 195 bp cloned bile acid inducible promoter region from *C. scindens* VPI 12708 which is upstream of the 1811 bp *uid*A gene (Ramasubbaiah 2004).

Plasmid recovery was accomplished using either the UltraClean™ Standard Mini Plasmid Prep Kit (Mo-Bio; Carlsbad, CA) or a Plasmid Midi kit (Qiagen; Valencia, CA). When using the Midi preps, purified plasmid DNA was concentrated using centricon devices (Millipore). Briefly, each sample was diluted in 2 mL np water, placed into a centricon spin filter, and subjected to centrifugation (1.0 x g, ambient temperature, ≥ 15 minutes). The resulting DNA concentration was determined with a NanoDrop ND-1000 (Thermo Scientific) spectrophotometer measuring absorbance at 280nm.

Electrophoresis and Visualization of Agarose Gels

Agarose gels were prepared at 1% (grams per 100 mL) agarose (Fisher) in 1x tris acetate disodium ethylenediaminetetraacetate (TAE) buffer (Ausubel et al. 2002). TAE was prepared in a 50x concentration as follows (per liter): 242 g Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol (Fisher)), 57.1 ml concentrated glacial acetic acid (Fisher), and 100 ml 0.5 M disodium ethylenediaminetetraacetate (Sigma Chemical), pH 8.0 (Ausubel et al. 2002).

DNA was visualized using Sybr Green I stain (Cambrex Bio Science; East Rutherford, NJ). Briefly, 10,000x Sybr Green was diluted 1:100 in dimethyl sulfoxide (DMSO) and 1 µL was directly added to every 10 µL of loading buffer containing DNA.

DNA bands were observed using a Kodak EDAS transilluminator and processed with Kodak's Scientific Imaging System.

Ligation and Blunt Ending Reactions

Removal of DNA overhangs was accomplished using an End-It™ DNA Repair kit according to the manufacturer's instructions (Epicentre; Madison, WI). Approximately 1 μg of DNA was used for reactions; reactants were mixed and incubated at room temperature. Reactions were deactivated by heating to 70°C for 10 minutes.

All ligation reactions were performed using a Fast-link™ DNA ligation kit (Epicentre). DNA concentrations near 1 μg were used in reaction vessels; reactants were combined and reactions were conducted at room temperature. Reactions were stopped by heating to 70°C for 15 minutes.

Gel Purification

After gel electrophoresis, DNA fragments were visualized on an ultraviolet illuminator (Fisher), excised, and transferred to 1.5 mL snap cap tubes. Gel purifications were performed using the Wizard® SV Gel and PCR Clean-Up System (Promega; Madison, WI).

Primers

All primers were designed using Vector NTI software (Invitrogen; Carlsbad, CA) and synthesized by Integrated DNA Technologies. Primer sequences were as given in Table 2.

Enzymes

All restriction exonuclease enzymes used in this project were from Promega (Madison, WI) and were used as recommended by the manufacturer. Prior to ligation, restriction enzymes were inactivated by required heating to 65-75°C.

An APex™ heat-labile alkaline phosphatase (Epicentre) was used for cloning procedures to prevent self-ligation of DNA fragments.

Blue-White Screening

Blue-white screening allows for a qualitative detection of a disrupted *lacZ* gene-containing plasmid within *E. coli*. The *lacZ* gene codes for beta-galactosidase which can hydrolyze X-gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside) into a visible blue product. This reaction includes the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) which acts as a synthetic analog of lactose that functions to inactivate the *lac* repressor (Yanisch-Perron et al. 1985).

Competent XL-1 Blue *E. coli* strains are engineered to produce the C-terminal beta galactosidase fragment. Transforming these *E. coli* with pUC18 (Fig. 6) plasmids allows the organism to make the N-terminal beta galactosidase fragment. The N-terminal portion of the *lacZ* gene contains a multiple cloning site in the pUC18 plasmid. If this gene is disrupted by a DNA insertion, the gene will no longer function. For this method, LB plates were supplemented with 100µg/mL ampicillin, 40 µL of 20 mg/mL X-gal, and 40 µL of 100 mM IPTG. These chemicals were spread using a sterile glass spreader over the surface of the plate. *E.coli* transformants were plated and incubated at 37°C for 24 hours. Blue colonies were disregarded, but white colonies were sub-cultured and checked

for recombinant plasmids by plasmid recovery, endonuclease digestion, and PCR (Yanisch-Perron et al. 1985).

Sewing Reactions

The technique referred to as "sewing" denotes the joining of two separate DNA fragments resulting from 15 to 20 base-pair complementation between the 3' end of one DNA fragment and the 5' end of another (In this case, fragments that contain the *ara*C homolog, *bai* promoter, and *uid*A reporter sequence). The *bar*A -*bai*P fragment has a 3' DNA overhang possessing complementary base pairs to that of the joining 5' *uid*A DNA. PCR cycler settings: 1. 95°C, 2" 2. 95°C, 30 s 3. Cool 1°C /s until 40°C, hold 1" 4. 74°C, 2" 5. Repeat steps #2 – #4 thirty times 6. 74°C, 10" 7. Cool to 4°C. The newly combined DNA was amplified using outside primers and *Taq* polymerase.

Results and Discussion

The ultimate goal of this project was to create a reporter gene construct under the regulatory control of the *Clostridium scindens* VPI 12708 bile acid inducible promoter. This construct was designated pWKU-003. The synthesis of a functioning construct will be an excellent tool for investigating the *bai* operon and for achieving a better understanding of the process of 7α-dehydroxylation.

Though several publications address the bile acid inducible operon, *bai* genes, and bile acid operon inducibility (White et al. 1988b; Gopal-Srivastava et al. 1990; Mallonee et al. 1990), none address regulation of the promoter. The bile acid inducible operon is activated in the presence of cholic acid or chenodeoxycholic acid, but the complete regulatory mechanism is unknown. The *bai* promoter is approximately 254 nucleotides in length (Ridlon et al. 2006). We chose to include an additional ~1200 nucleotides upstream of the *bai* promoter due to the possibility that this region may contain regulatory elements necessary for the function of the promoter. Ridlon *et al.* (2006) reported two regulatory elements, *barA* and *barB,* preceding the *bai* promoter that are homologous to *araC.* A *bai*P/*uid*A construct that omitted upstream regulatory elements previously assembled in this (Doerner) laboratory did not provide evidence of gene activity as measured by a GUS spectrophotometric assay. We hypothesize that this construct failed due to the absence of upstream regulatory elements since this construct consisted of only the promoter and reporter (Ramasubbaiah 2004).

The *uid*A reporter gene has been widely used in genetics systems to assess promoter activity (Jefferson et al. 1987; Klijn et al. 2006). The *uidA* gene codes for β-

glucuronidase, a hydrolase that breaks down β-glucuronides. This stable hydrolase enzyme cleaves a substrate (p-nitrophenyl β-D-glucuronide); the end product is quantified with a spectrophotometer (Jefferson et al. 1987). Our project's special consideration is the fact that we are using an obligate anaerobe. The *uidA* gene is a viable reporter for this project because Feldhaus et al. (1991) successfully employed the *uid*A reporter in *Bacteroides*, an obligate anaerobe.

The bile acid inducible promoter and upstream sequence were amplified from *C. scindens* VPI 12708 chromosomal DNA (5' primer = Up AraC/bai (1), 3' primer = Dn AraC-bai or Downstream AraC-hol). The restriction sites on the *bar*A 5' end (*Bgl*II) and the *uid*A 3' end (*Bam*H1) allow for future excision from the pWKU-001 plasmid (Fig. 5, 7). Fifteen *uid*A-complementary bases were added to the *bar*A-*bai*P 3' end to allow these fragments to be sewn together. A high fidelity *Tli* DNA polymerase (Promega) was used to amplify these sequences from *C. scindens* VPI 12708 (*bar*A -*bai*P) and pWKU-001 (*uid*A). The data (Fig 8A) show that the *bar*A -*bai*P (lane 5, ~1,500 bp) and *uid*A (lane 1, ~1800 bp) fragments were amplified. A 16s RNA gene was amplified as a template control to confirm the presence of bacterial DNA (Primers = 27f, 1492r; Fig 8A, lane 2). The sewing of these amplicons together was unsuccessful (Fig 8B, lane 1) since a ~2,000 bp fragment was visualized, but the expected ~3,300 bp DNA fragment was not detected.

A plausible explanation for this failure could be that the annealing reaction was inefficient. The current protocol adjusted temperature from 95°C down to ~40°C in a matter of seconds. A gradual temperature decrease over two minutes following denaturation might enhance the joining of the two DNA fragments by promoting greater base pair complementarity. A second possibility is that both *Taq* and *Tli* have

exonuclease activity which might degrade the 3' overhang on the *bar*A - *bai*P DNA. This partial success, the amplification of the *barA-baiP* and the *uidA* fragments, led to the next protocol variation which used a different sewing polymerase but preserved the protocol strategy.

Since the amplicons that were to be joined were successfully amplified, a modified cloning strategy was developed. The second cloning protocol (Fig 7) also produced the previously mentioned DNA amplicons as seen in Fig 9A, lanes 1 and 2. Most steps were identical to the previous protocol, but Klenow polymerase was used instead of *Taq* or *Tli*. Klenow polymerase lacks 5' to 3' exonuclease activity and therefore better preserves the 3' overhang in the AraC/*bai*P fragment. For gradual cooling following deannealing (the separation of double stranded DNA), the temperature was reduced by five degrees Celsius every 30 seconds until holding at 37°C. Klenow polymerase, nucleotides, and buffer were added; reactions were incubated at 37°C for 30 minutes and were then amplified by flanking primers. Fig 9B shows the final amplification result of four different reaction mixtures in lanes 2 – 5. Even with this modification, a ~3,300 bp fragment was not recovered. Repeated attempts to amplify these reaction mixtures with flanking primers were unsuccessful (data not shown).

It is not possible to confirm whether the Klenow polymerase successfully filled in nucleotides after the *barA-baiP* and *uidA* fragments were sewn. This approach did not control for the template amplification limit of *Taq* polymerase (0.2 kbp to 2.0 kbp, which was not known until the fourth cloning protocol was devised (Unknown 2007)).

The third cloning protocol (Fig. 10) amplified a 120bp fragment from the *uidA* gene contained within pWKU-001 (Fig. 11A, lanes 1 – 3). The *bar*A-*bai*P fragment was amplified from *C. scindens* VPI 12708 as described previously (Fig 11A, lanes 5 and 7). During the second round of PCR amplification, the *bar*A -*bai*P fragment with its 3' *uid*A complementary bases and the 120 bp *uid*A fragment were sewn, and then amplified using the upstream AraC primer and the *uid*A (2) as the downstream primer (Fig. 11B). Several annealing temperatures (51.5°C to 64.6°C) were employed but this range seemed to have had no effect on the outcome (Fig. 11B; lanes 1 – 7, 9 – 16). We did observe that the polymerase used in the amplification reactions (*Taq* versus *Tli*) affects the amplified products; a clear difference can be seen by comparing the bands seen in Fig. 11B. Two amplicon sizes were observed when *Taq* was used (~1,100 and ~1,600 bp) and several amplicons were observed (~100, 300, 700, 1000, and 1600 bp) when *Tli* was used. The ~1,600 bp band is the expected fragment size; this PCR product was gel purified and cut with *BclI*. The pWKU-001 plasmid was cut with *EcoR1*, blunt ended (5' and 3' DNA ends do not have overhangs), and cut with *Bcl1*. The restriction enzymes remove the existing *bai*P and first 120 bp of the *uidA* gene from pWKU-001. This also allows for a directional ligation of the ~ 1,600 bp fragment insert (also cut by *Bcl1*) into the cut pWKU-001. The vector and the *barA-bai*P-*uidA* fusion were transformed into *E. coli* XL1 Blue and grown on LB agar with ampicillin, ITPG, and X-gal.

Putative transformant *E. coli* were screened using whole colony PCR to confirm the presence of the desired pWKU-003. All of the amplified fragments were smaller than expected (Fig. 11C). Since colony PCR was unsuccessful, two colonies (L4-2, L1-2) were selected for further analysis. Plasmids were recovered from transformants, cut with

*Eco*R1 and analyzed for the presence the proposed 10.6 kbp pWKU-003 (Fig. 12A). Two plasmids seen in Fig. 12A, lanes 1 and 5, exhibited fragments running near the expected size of 10.6 kbp (pWKU-001 after digestion = ~9,000 bp, insert = ~1,600 bp). However, upon a secondary, confirmatory *Hin*dIII digestion of the same plasmids (Fig. 12A, lanes 1 and 3), transformants exhibited fragments of ~3 kbp and ~5.5 kbp, respectively, instead of the expected 10.6 kbp band. Further PCR amplification confirmed the presence of the *uidA* gene, but could not confirm the presence of *bai*P or *barA* sequence (data not shown).

A previously unknown *Bcl*I site was discovered within the AraC-*bai*P insert after analysis with Vector NTI software in assessing the failure to detect the desired full *barA-baiP-uidA* fusion. Vector NTI predicts that digestion of the insert with the *Bcl*I restriction enzyme should result in two fragments 336 bp and 1151 bp (confirmatory insert digest not performed). Since the gene fusion was prepared for insertion by *Bcl1* digestion, this would diminish the likelihood that all ~1500 bp of the *barA-bai*P nucleotides would be included in the plasmid. In the next method, we also addressed the template size limitations of *Taq* polymerase by switching this enzyme in favor of one that can amplify longer templates with high fidelity.

To overcome the template size limitations of *Taq* polymerase (which amplifies templates 0.2 kbp to 2 kbp), a polymerase that can amplify >2 kbp with high fidelity was obtained, and this led to a fourth cloning strategy (Fig. 13). *BarA-bai*P (~1500 bp) and *uid*A (~1820 bp) were amplified as in previous protocols, but these fragments were joined using a combination of controlled temperature reduction (-1°C/30s) to 40°C and a

high fidelity, long amplifying DNA polymerase (LA Taq; Takara/ClonTech Labs). As the thermocycler holds 40°C, dNTPs were added before an elongated extension period at 74°C. Primers were added that annealed to the 5' end of the *bar-baiP* fragment and the 3' end of the *uidA* fragment and results suggest (Fig 14A, lanes 1 and 3) that the sewing reaction was successful because a 3,300 bp fragment was observed. The blue/white screening produced twelve candidates that were analyzed by PCR. Unfortunately, none of these candidates were proven to contain the desired construct. Amplifications using flanking, *uid*A, and AraC primers on a prospective colony identified by restriction enzyme digestion (data not shown) were inconclusive. DNA amplicons of 3,300; 1,800; and 1,500 bp should have appeared in lanes 1, 2, and 3of the gel (Fig 14B), respectively. Fig 14B shows that only the *uidA* amplification (lane 2) was successful; the combined *barA-bai*P-*uidA* (lane 1) and the *barA-bai*P fragment (lane 3) did not amplify from the plasmid. No inconsistencies were noted among negative controls. Further attempts to perform the reaction protocol using an alternate high fidelity DNA polymerase (ABgene Extensor) instead of LA Taq were unproductive (data not shown).

It is unknown if the failure to clone the complete *barA-bai*P-*uidA* fusion was due to the lack of complete assembly of the construct or if it was due to an incompatibility issue of introducing a high-copy plasmid containing a foreign set of instructions into *E. coli*. High copy plasmids can impose metabolic burdens upon cells and foreign DNA can impose toxic effects on the host cell (Carnes and Williams 2009). We did address this possibility by considering a low-copy plasmid for use in future methods.

One alternative cloning possibility would be to amplify the *bai*P-*uidA* regions within the existing pWKU-001 and to design an upstream primer that adds an extra ~20

bp (complementary to the 3' *barA* nucleotides) to the 5' *bai*P end. Primers could then be designed to amplify the *barA* sequence from *C. scindens* VPI 12708, and a sewing reaction can be performed to unite the two fragments. If restriction site locations allow, ligating the fragment directly into the pWKU-001would be preferable. If not, then the fragment can be ligated into pBR322.

Ultimately, we succeeded in amplifying the *bai*P, upstream sequences, and the *uid*A reporter. The desired *bar*A-*bai*P-*uidA* composite fragment was only produced with the fourth cloning strategy, but this result was not consistently reproducible. Nonetheless, this result suggests that the use of a high fidelity, large template amplifying polymerase and a slow cooling step within the PCR method may be a useful strategy in the future for constructing a *barA*-*bai*P-*uidA* composite fragment. The use of a simple *Taq* polymerase to perform larger amplifications than it was designed to perform was a major flaw in the experimental design of this study. Since the proposed *barA*-*bai*P-*uidA* construct was not successfully transformed into *E. coli* and then mated with *C. scindens* VPI 12708, no further information could be ascertained about the *bai* promoter or operon from this project. Future studies will be necessary to develop successful cloning protocols that create *bai* promoter-reporter gene fusions and allow measurement of *bai* operon activity.

Table 1: Stock and working antibiotic concentrations

Antibiotic Stock	Working Concentration
Ampicillin 100 mg/mL	100 μg/mL
Tetracycline 10 mg/mL	10 μg/mL
Chloramphenicol 10 mg/mL	20 μg/mL

Table 2: Primer labels, length, and nucleotide sequence

Primer Pair ID	Length (BPs)	Primer Seq 5' to 3'
Up AraC	35	ATAGATCTTCATTGGTTTTCACCTCCCCGGTAGAG
Up AraC(2)	36	GGAAGATCTTCATTGGTTTTCACCTCCCCGGTAGAG
Dn AraC	48	GTTTCTACAGGACGTAACATTTTCTTAATGCTTAATATCTTTTCTTCG
Dn AraC(2)	55	GGTTGGGGTTTCTACAGGACGTAACATTTTCTTAATGCTTAATATCTTTTCTTCG
Up uidA	30	ATGTTACGTCCTGTAGAAACCCCAACCCGT
Dn uida	32	ATGGATCCTCATTGTTTGCCTCCCTGCTGCGG
Dn uida-Kpn1(2)	25	TTGTAACGCGCTTTCCCACCAACGC
27f (16S rDNA)	21	AGAGTTTGATC(AC)TGGCTCAG
1492r (16S rDNA)	22	ACGG(CT)TACCTTGTTACGACTT

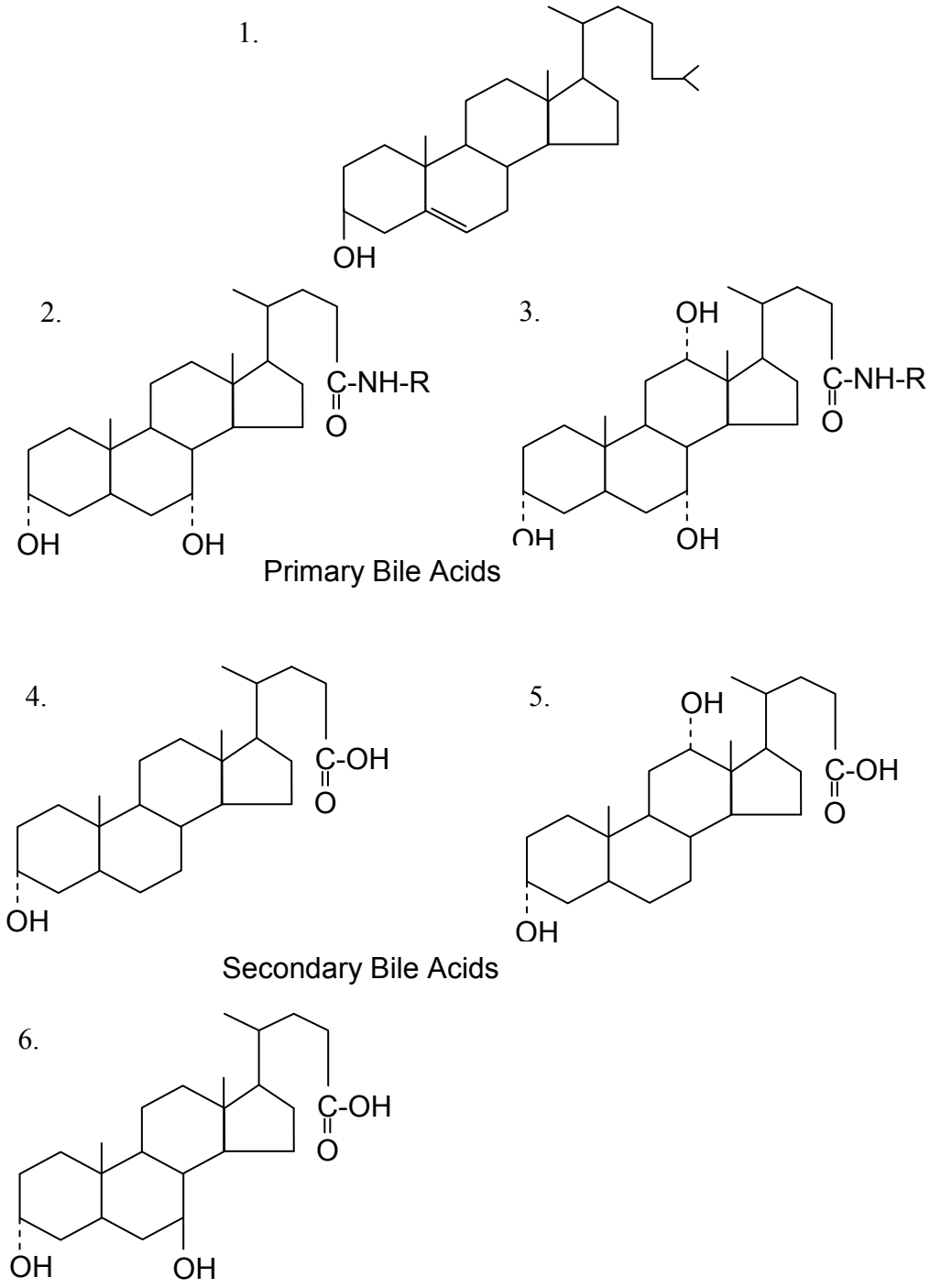


Figure 1: Structures of cholesterol, primary and secondary bile acids
Cholesterol (1), conjugated chenodeoxycholic acid (2), conjugated cholic acid (3), lithocholic acid (4), deoxycholic acid (5), and ursodeoxycholic acid (6).

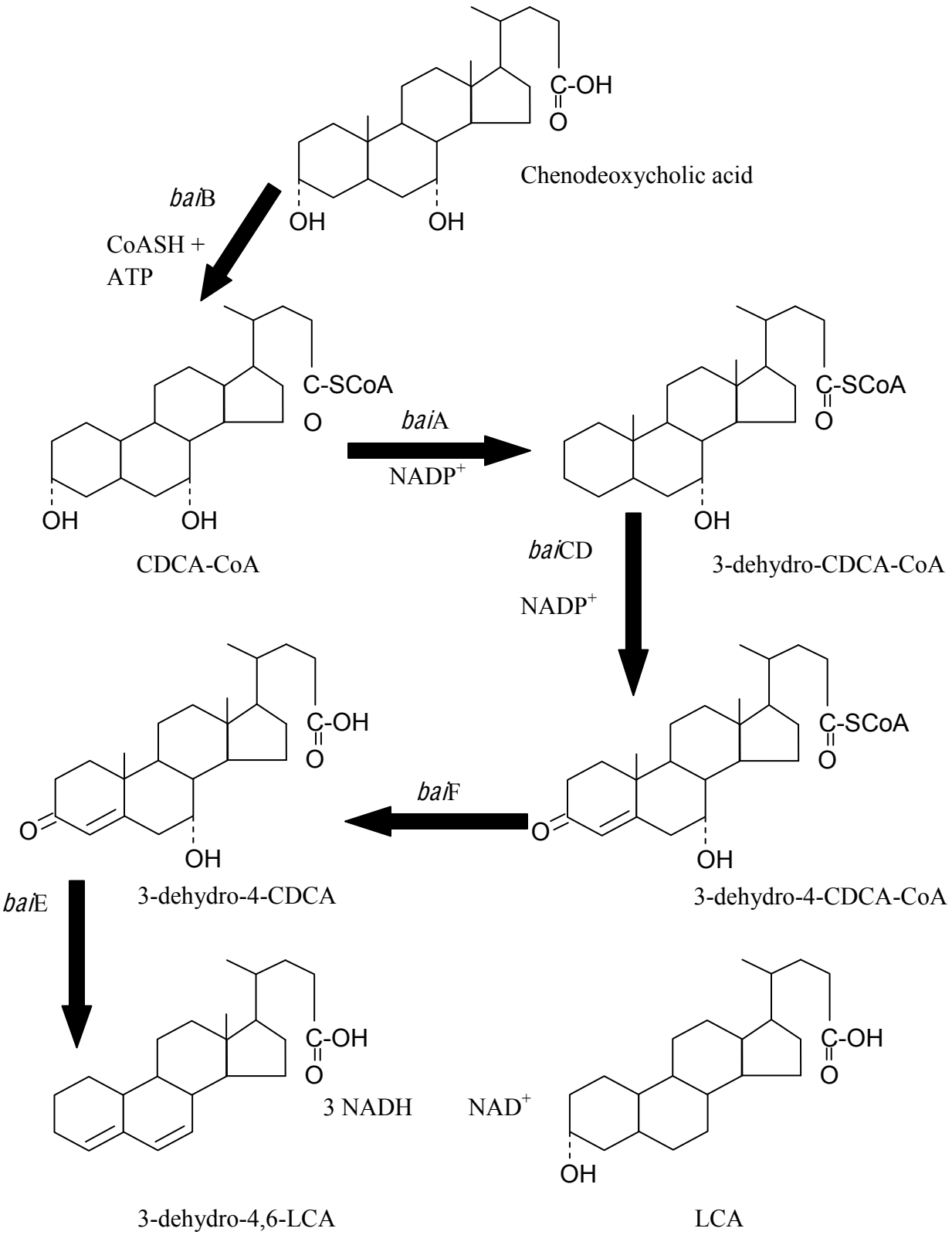
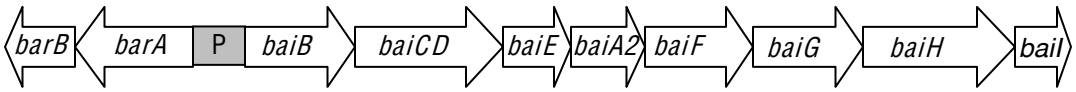


Figure 2: Proposed chenodeoxycholic acid bile acid 7α-dehydroxylation pathway in *Clostridium scindens* VPI 12708



bai Operon

Figure 3: Proposed map of the bile acid inducible operon
P designates the bile acid inducible promoter. Genes and functions: *bar*A &B encode putative transcriptional regulators, *baiB* encodes bile acid CoA ligase, *bai*CD encodes 3-dehydro-4 chenodeoxy/cholic acid oxidoreductase, *bai*E encodes7α-dehydratase, *bai*A2 encodes 3α-hydroxysteroid dehydratase, *bai*F encodes putative bile acid CoA transferase, *bai*G encodes H^+ dependent bile acid transporter, *bai*H encodes 3-dehydro-4-ursodeoxycholic/7-epi cholic acid oxidoreductase, and *bai*I encodes 7β-dehydratase.

```
E._coli_E24377A_araC              --------------------------------------------------
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               --------------------------------------------------
C._botulinum_A_str._ATCC_3502_    --------------------------------------------------
B._thuringiensis_konkukian_str    --------------------------------------------------
C._scindens_12708_araC_hom        TCATTGGTTTTCACCTCCCCGGTAGAGACTCCGGTATTCGCCAGGCGTCA 50


E._coli_E24377A_araC              --------------------------------------------------
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               --------------------------------------------------
C._botulinum_A_str._ATCC_3502_    ----------------------------------------------TTGGAAA 7
B._thuringiensis_konkukian_str    --------------------------------------------------
C._scindens_12708_araC_hom        TCTTATAGAGTTTGTTAAAGGCCCTGGAAAAGGAGCGGCTGCAGTTGAAC 100


E._coli_E24377A_araC              --------------------ATGGCT---GAAGCGCAAAATGATCCCCTG 27
S._enterica_CT18_araC             --------------------ATGGCT---GAAACGCAAAATGATCCGCTA 27
Y._pestis_CO92_araC               --------ATGTATCAACGCATGGTTCAGGAACCGCAACCCAATCCTTTG 42
C._botulinum_A_str._ATCC_3502_    CAATCATGTTACTTAACAATACACTTGAATATATTGAAAGAAATTTGCAT 57
B._thuringiensis_konkukian_str    --ATGGAGAGTAAACAAAGTAGAAATGAGTATTTACAGTGCATATACAAA 48
C._scindens_12708_araC_hom        CCTACATTTACCGCCACATCCAGCACCGGAAGATTCGTCGTAAGCAGAAG 150
                                                      *

E._coli_E24377A_araC              CTGCCGGGATACTCGTT-TAATGCCCATCTGGTGGCGGGTTTAACGCCGA 76
S._enterica_CT18_araC             TTGCCGGGATATTCATT-TAATGCCCATCTGGTCGCCGGGCTGACGCCAA 76
Y._pestis_CO92_araC               CTGCCAGGCTACACGTT-CAATGCTTATCTGGTCGCAGGATTAACCCCGA 91
C._botulinum_A_str._ATCC_3502_    ACGGCAATTAATATAGA-TGATATATCAAAAGTTGCATGTTCATCACGAT 106
B._thuringiensis_konkukian_str    GTACAAAATTATATAGAATTACACATAAATGATT-CACTTTCAATTGAAG 97
C._scindens_12708_araC_hom        GGCTGCAGCATGACGCAGCCTTGTAAGGACGGTGATGTCCTTGTAGGACG 200
                                                        *

E._coli_E24377A_araC              TTGAGGCCAACGGTTATCTCGATTTTTTTATCGACCGACCGCTGGGAATG 126
S._enterica_CT18_araC             TTGAAGCGAATGGATATCTGGATTTTTTTATCGATCGTCCGTTGGGCATG 126
Y._pestis_CO92_araC               TTCTGGCTGACGGCCCACTCGATTTCTTCATTGATCGGCCTGGCGGCATG 141
C._botulinum_A_str._ATCC_3502_    ATCACTTTCAGCGTGTTTTTCATTCATTAACAGGATTTACAGTAACTCAG 156
B._thuringiensis_konkukian_str    AGTTAGCGGAT-ATAGCAGGATTTTCAAAGTATCATTTTCATAGAATTTT 146
C._scindens_12708_araC_hom        CGCCGAATTTCTCCTGGACCAGTTCCCGGATAACGCTCTCGGACAGATAG 250
                                            **            *

E._coli_E24377A_araC              AAAGGTTATATTCTCAATCTCACCATTCGCGGTCAGGGGGTGGTGAAAAA 176
S._enterica_CT18_araC             AAGGGATATATTCTTAACCTGACCATCCGCGGAGAGGGCGTCATTAATAA 176
Y._pestis_CO92_araC               AAGGGCTATATTCTGAATTTGACCATTAAGGGGCAGGGGAAGATTTTCGA 191
C._botulinum_A_str._ATCC_3502_    TATATAAAAAATCGCAGACTTACCCTTGCCGCAGAGGAGTTGGTTTCAAC 206
B._thuringiensis_konkukian_str    TAAAGGGATAGTAGATGAACCATTATCTCGGTATGTAAACCGATTGAAAC 196
C._scindens_12708_araC_hom        AGGGATTTGGAGGTATCCTTGATGTCGATGGGAGATGAAAAACTTGCATA 300
                                            *       *           *

E._coli_E24377A_araC              TCAGGGACGAGA--ATTTGTTTGCCGACCGGGTGATATTTTGCTGTTCCC 224
S._enterica_CT18_araC             TAATGGCGAGCA--GTTTGTCTGTCGGCCTGGCGATATATTATTGTTTCC 224
Y._pestis_CO92_araC               TGGTGAAAATAC--CATCTACAGTAATCCTGGTGATTTACTGTTGTTCTC 239
C._botulinum_A_str._ATCC_3502_    AGACAGAAGAAT--AATTGAT----ATAGCATTGAAATATGGTTATGAAA 250
B._thuringiensis_konkukian_str    TGGAAAGGGCAACAAACCTTCTTACATACCGTTCGGATATGACGATTA-- 244
C._scindens_12708_araC_hom        GGAGTGATTCAGCACATCCATCAGCATCTGGCTCATGATCA--TGCCGCG 348


E._coli_E24377A_araC              GCCAGGAGAGATTCATCACTACGGTCGTCATCCGGAGGCTCGCGAATG-G 273
S._enterica_CT18_araC             GCCGGGCGAGATTCATCACTATGGACGGCATCCGGATGCCAGCGGGTG-G 273
Y._pestis_CO92_araC               ACCTAAGTCGCCTCACCATTATGGTCGTTCACCGAATAGTGATTGTTG-G 288
C._botulinum_A_str._ATCC_3502_    GTCCAGAAGCATTTACTAA-------AGCATTTAAAAGATTACATGGA-A 292
B._thuringiensis_konkukian_str    --CTAATATCGCTTACCATT------TTGGTTTCACAGATT-CAGCAG-T 284
C._scindens_12708_araC_hom        GTATACCTTCTCCCCGCTCTCCAGCATCTGCTTGCGATATTCCAGAGGTG 398


E._coli_E24377A_araC              TATCACCAGTGGGTTTACTTTCGTC----CGCGCGCCTACTGGCATGAAT 319
S._enterica_CT18_araC             TATCACCAGTGGGTTTATTTCCGGC----CTCGCGCCTACTGGCAGGAGT 319
Y._pestis_CO92_araC               TATCACCGCTGGGTCTATTTCCGAC----CACGGGCTTATTGGGCCGATT 334
C._botulinum_A_str._ATCC_3502_    TATCACCATCAGCTTTAAAAAAGCT----TAATGGAAAAATTAAAGCTTT 338
B._thuringiensis_konkukian_str    TTTCTCCCGTACATTTAAAAATTAT----TATGGAGTAAGTCCGTCTCAA 330
C._scindens_12708_araC_hom        TCGTCCCGGTATATTTCTTAAAGATGCGGTAGAAGGCTACTTCCGAGGAA 448
                                  *    **      * *                    * *
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E._coli_E24377A_araC              GGCTTAACTGGC-CGTCAA-TATTTGCCAATACGGG----TTTCTT--TC 361
S._enterica_CT18_araC             GGCTGACCTGGC-CGACAA-TCTTTGCTCAGACAGG----ATTTTT--CC 361
Y._pestis_CO92_araC               GGTTGGAATGGCACAGTAA-AACCCATGAGGTAGGG----CGTTTA--TC 377
C._botulinum_A_str._ATCC_3502_    TCCCAAAATCTCTTTTCAAATATCTATAAAAGGAGA----GTGCGAAATC 384
B._thuringiensis_konkukian_str    TATCGAAACGACAATAGCAAGAATTGCAAAGACGTA----AGAAGAATTT 376
C._scindens_12708_araC_hom        AATCCGGAATGCTCGGCCACGTATGACAGAGACATGTCTTCGTAGAGAAG 498
                                             *     *

E._coli_E24377A_araC              GC--CCGGATGAAGCGCACCA------GCCG--CATTTCA-----GCGAC 396
S._enterica_CT18_araC             GC--CCGGACGAGGCGCGCCA------GCCG--CATTTCA-----GCGAA 396
Y._pestis_CO92_araC               TTTACCTAACAATAACTTAAT------GCTG--GAGTTTG-----ACCGC 414
C._botulinum_A_str._ATCC_3502_    ATTTATAGAATAGTTGAAAAA------GAAG--CATTTAA-----GGTAT 421
B._thuringiensis_konkukian_str    CTCAATACAATGAATGTAAGAAGGTTCGAGG--GAATGTT-----GAAAT 419
C._scindens_12708_araC_hom        CGCGCCGGAAGCGATATTCACCCGTACGCGGTTCAGTGTGTGGGCAAAGT 548
                                         *                 *  *   * *

E._coli_E24377A_araC              C-TGTTTGGGCAAATCATTAACGCCGGGCAAGGGGAAGGG--CGCTATT- 442
S._enterica_CT18_araC             C-TGTTCGGGCAGATCATCAGCGCCGGGCAAGGGGAAGGT--CGCTATT- 442
Y._pestis_CO92_araC               T-TATTTGCCAATATTGAGCAGACACAGAAATCGGGGCGG--CGCTTCG- 460
C._botulinum_A_str._ATCC_3502_    T-TGGTACAGGATTTGAAACAACTAGAATTAATTATGCGG--C-TTATA- 466
B._thuringiensis_konkukian_str    TGTAACAGTAGACAATATAAACGTCGCATACATAAGGCATATCGGTACAT 469
C._scindens_12708_araC_hom        TGTAGCCGCTGATATTGCGCAGTTCCCGGTTCAGCGTAGAAGCGTTGGTA 598
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E._coli_E24377A_araC              CGGAGCTGCT-GGCGATAAATCTGCTTGAGCAATTGTTACTGCGGCGCAT 491
S._enterica_CT18_araC             CTGAGCTACT-GGCGATCAATCTGCTGGAGCAGTTGTTGCTCAGACGTAT 491
Y._pestis_CO92_araC               GTGAAGAACT-GGGTATGAATTTGCTCGAGCGGTTACTGCTACGAGCAAT 509
C._botulinum_A_str._ATCC_3502_    AAGAAATACC-AGAATTTA--TTAATAAGATTTTTAAAGATGGAACACAT 513
B._thuringiensis_konkukian_str    ATGAAGAGTT-AACTATAGCTTTTCCGAAAATGATAGAGAAACTATTTCA 518
C._scindens_12708_araC_hom        GAAAAACGCTCAGCCACGCTCTCGGCCGTAATGTCCTCGGCAAAATGGGA 648
                                     *

E._coli_E24377A_araC              GGAAGCGA--TTAACGAGTCGCTCCATCCACCGATGGATAATCGGGTACG 539
S._enterica_CT18_araC             GGCGGTAA--TTAATGAGTCGTTGCATCCGCCGATGGATAGCCGTGTGCG 539
Y._pestis_CO92_araC               GGAAGAAGACCCACTTAGTC--CACAACGTATTATGGACCCACGTATTAT 557
C._botulinum_A_str._ATCC_3502_    GATAGAA---TTAATGAAGCATTAGGTAATTCTAAAGGTAGTTTATTAGA 560
B._thuringiensis_konkukian_str    ATACGCAA--CCAAGCAAAACTATCATGTATTTGAGGATACGAAAGTATT 566
C._scindens_12708_araC_hom        GCCGATA----TAAAGGGTTGCCTGCCATCCAAGAGGCCAGTCCTTGTCC 694
                                           *                   *

E._coli_E24377A_araC              CGAGGCTTGTCAGTACATCAGCGATCACCTGGCAGACAGCAATTTTGATA 589
S._enterica_CT18_araC             CGATGCCTGCCAGTATATCAG------------------------------ 560
Y._pestis_CO92_araC               TGAGGCCTGTCAATTTATTACCGGAAATCTGGCAG---GGGAATTACGTA 604
C._botulinum_A_str._ATCC_3502_    TGGATTTCATTATGATTTTAAAGAAAATGGTACAA---GAAAATATATGA 607
B._thuringiensis_konkukian_str    AACCATTTACCACGATCATCATGAATTTACGGAGGACAATCATTTAAGAA 616
C._scindens_12708_araC_hom        TGGTCTTTCTGGCTTTGATGGATGCCATGCTTGATGAAAAAGTACGCAAA 744

E._coli_E24377A_araC              TCGC---CAGCGTCGCA-CAGCAT-GTTTGCTTGTCACCGTCGCGTCTGT 634
S._enterica_CT18_araC             ------------------------------------------------- 
Y._pestis_CO92_araC               TTGA---CGAAGTTGCC-CGCCAT-GTTTGCCTATCACCATCACGCTTAG 649
C._botulinum_A_str._ATCC_3502_    TGGG---ATATGAAATA-CCTAGA-ACAGAGATATCAGATGAATTTACAA 652
B._thuringiensis_konkukian_str    CAAGTTTATGTATAACAATTCCAG-GTGAATCTGCAATAGAAACGAACGA 665
C._scindens_12708_araC_hom        CTGCCCAAGGATGGAGACCTTTATCAGTGAACTGCCAGGATCGAAGCAGG 794

E._coli_E24377A_araC              CACATCTTTTCCGCCA---GCAGTTAGGGATTAGCGTCTTAAGCTGGCGC 681
S._enterica_CT18_araC             ------------------------------------------------- 
Y._pestis_CO92_araC               CGCATTTATTCCGTGA---ACAGGTTGGCATTAATATATTACGTTGGCGT 696
C._botulinum_A_str._ATCC_3502_    TACTTCAAGTACCTAA---GCTTACATGGGC-AGTATTTGAAG--GATAT 696
B._thuringiensis_konkukian_str    CATTGGAATAATGGTA---ATACCTTCAGGCAAGTATGCAGTAG-GACAT 711
C._scindens_12708_araC_hom        AATCTTCCTTTTCAAATTCATCGAATAAATCCCGGATCTTTTGCTGATAT 844

E._coli_E24377A_araC              GAGGAC--CAACGTATCAGCCAGGCGAAGCT--GCTTTTGAGCACCACCC 727
S._enterica_CT18_araC             ------------------------------------------------- 
Y._pestis_CO92_araC               GAGGAT--CAGCGGGTGATCCGGGCGAAATT--ATTACTGCAAACGACCC 742
C._botulinum_A_str._ATCC_3502_    GGGGCT--ATGCCAGATAACTTGATTATACAGGATATATGGAGACGTATA 744
B._thuringiensis_konkukian_str    TTTGAA--ATATT--CCAAGATGAATATAAAA-GAGCATGGGACTTTATA 756
C._scindens_12708_araC_hom        ACTCCTTCCAGCCGGATAACGGGGAGG-GCGACCATAATGGCGTCCACCG 893
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Y._pestis_CO92_araC               AAGAGCCAATTGCGACCATTGGCCGGGTGGTAGGCTATGATGACCAACTC 792
C._botulinum_A_str._ATCC_3502_    TATTCTGAATGGTTTCCATCCTCTGGAT--TTGAGCAAGTGGAAGGTCCA 792
B._thuringiensis_konkukian_str    TATGGTGAGTGGCTGCCAAATAGCGGAT-----ATAAACCGAGAGACTCA 801
C._scindens_12708_araC_hom        TATCAGGGCTTGGTTCCCTGAGCGCCAGAAAGGAAGAGAGCGGATAGTCA 943

E._coli_E24377A_araC              TATTTCTCGCGAGT--ATTTAAAAAATGCACCGGGGCCAGCCCGAG---C 822
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               TATTTTTCCCGTGT--TTTCCGTAAACGAGTGGGGGTCAGCCCCAG---C 837
C._botulinum_A_str._ATCC_3502_    TGTATTGAAAAAAA--TTTTTGGAATAATAAAAAAACATGATGAATA---C 837
B._thuringiensis_konkukian_str    TATCCTTTTGAAGT--ATATAGAAATGATCCAAAGCAGCATCCAAAGCAT 849
C._scindens_12708_araC_hom        TACACGCAGATAGACAACTCAAGAGGATCGTCGGAATCCGCTTTGATCGT 993

E._coli_E24377A_araC              GAGTTCCGTGCCGGTTGTGAAG-AAAAAGTGAATGATGTAGCCGTCAAGT 871
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               GATTTCAGGCGGCGCAGTATCG-AAACTAACTATCCACAACGTACTTTTC 886
C._botulinum_A_str._ATCC_3502_    AAATGCGAAGTATGGATTCCCGTAAAAACAAAGTCAATCAATTGGTGA-- 885
B._thuringiensis_konkukian_str    AAACATATAGTTGATATATATGTACCTATCGAACCTTTTTAA-------- 891
C._scindens_12708_araC_hom        GAACGTGTGGTACG-ACTGAAGCCAGACGAAGGTACTCTGTTCAAATGGG 1042

E._coli_E24377A_araC              TGTCATAA----------------------------------------- 879
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               GTCCAGCGGACTGGCAGGATAATCAGGGGGCATTGGTGCGGGTTTAA--- 933
C._botulinum_A_str._ATCC_3502_    --------------------------------------------------
B._thuringiensis_konkukian_str    --------------------------------------------------
C._scindens_12708_araC_hom        AACTCTACGCCATTGATCGTAATTGTGCCGCATCCGGAATTCACCAGTAT 1092

E._coli_E24377A_araC              --------------------------------------------------
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               --------------------------------------------------
C._botulinum_A_str._ATCC_3502_    --------------------------------------------------
B._thuringiensis_konkukian_str    --------------------------------------------------
C._scindens_12708_araC_hom        GAAAAGGGACTCATTTTCACAGATGGGCGGGGTGATGTCGCTGATGGTAT 1142

E._coli_E24377A_araC              --------------------------------------------------
S._enterica_CT18_araC             --------------------------------------------------
Y._pestis_CO92_araC               --------------------------------------------------
C._botulinum_A_str._ATCC_3502_    --------------------------------------------------
B._thuringiensis_konkukian_str    --------------------------------------------------
C._scindens_12708_araC_hom        GCTTCTGCAGGCGCAGATATTTGTCCTTTCCGGTAAAGACAGAATGGACA 1192

E._coli_E24377A_araC              ------------------------------------------
S._enterica_CT18_araC             ------------------------------------------
Y._pestis_CO92_araC               ------------------------------------------
C._botulinum_A_str._ATCC_3502_    ------------------------------------------
B._thuringiensis_konkukian_str    ------------------------------------------
C._scindens_12708_araC_hom        GGGCTGCCACAGATGGGCTTGGATTTTATTGTGTTTGGCAT 1233
```
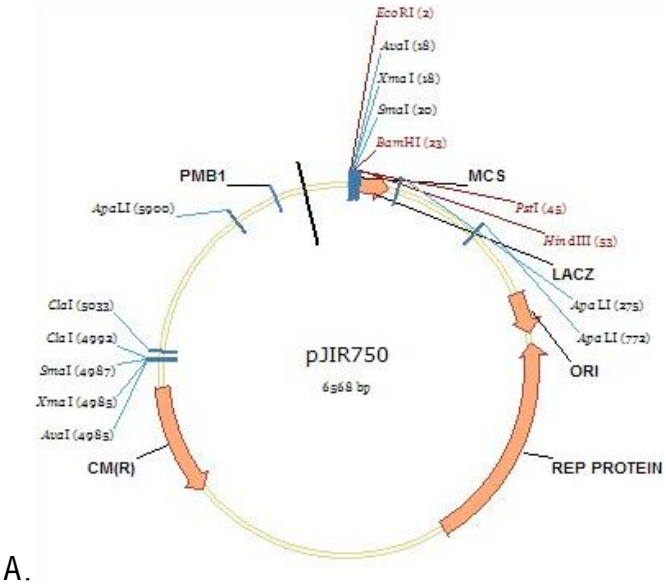
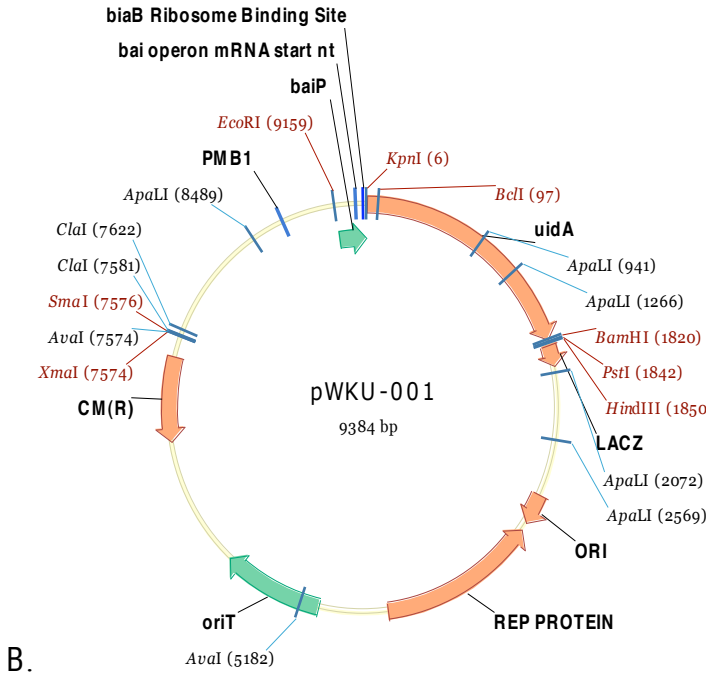

Figure 4: ClustalW sequence alignment

Six bacteria were aligned using clustalW software to compare the putative regulatory upstream sequence of *C. scindens* VPI 12708 with other known *ara*C gene types. Identity positions are denoted with a "*".



A.



B.

Figure 5: Plasmids pJIR750 and pWKU-001
(A) pWKU-001 was constructed from pJIR750 which possesses a chloramphenicol resistance marker and a partial *lacZ* gene.
(B)Existing vector pWKU-001 contains the *uid*A gene and a chloramphenicol resistance marker (Ramasubbaiah 2004).

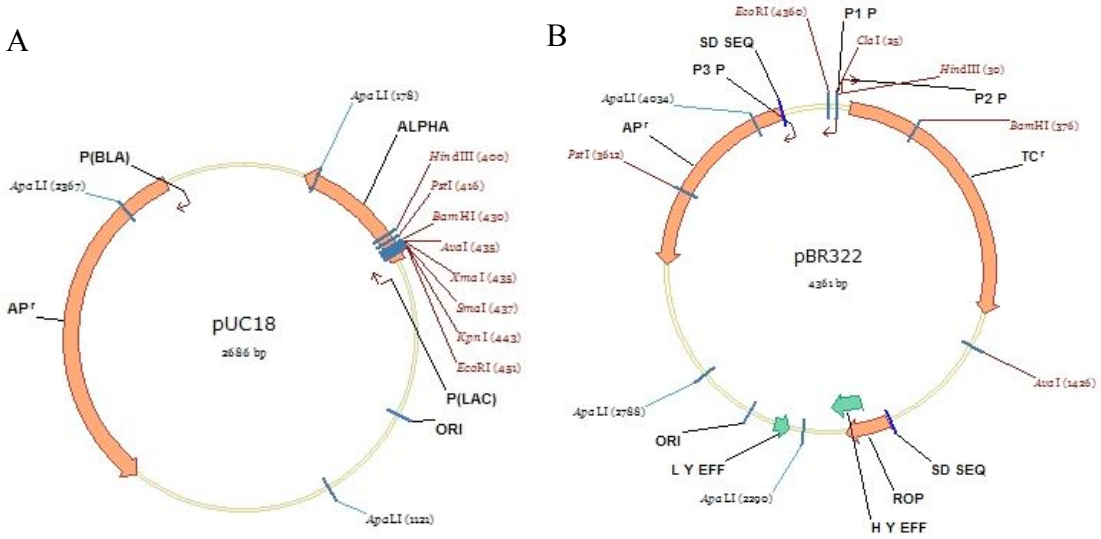


Figure 6: Vectors for transforming bacteria

Physical and genetic maps of pUC18 (A) and pBR322 (B) cloning vectors. The fully assembled *bar*A -*bai*P-*uid*A construct is intended for blunt end ligation into either pUC18 at the Sma I (blunt) site or into pBR322 at the BamH1 site [cut and blunt ended by End-It® kit (Epicentre)].

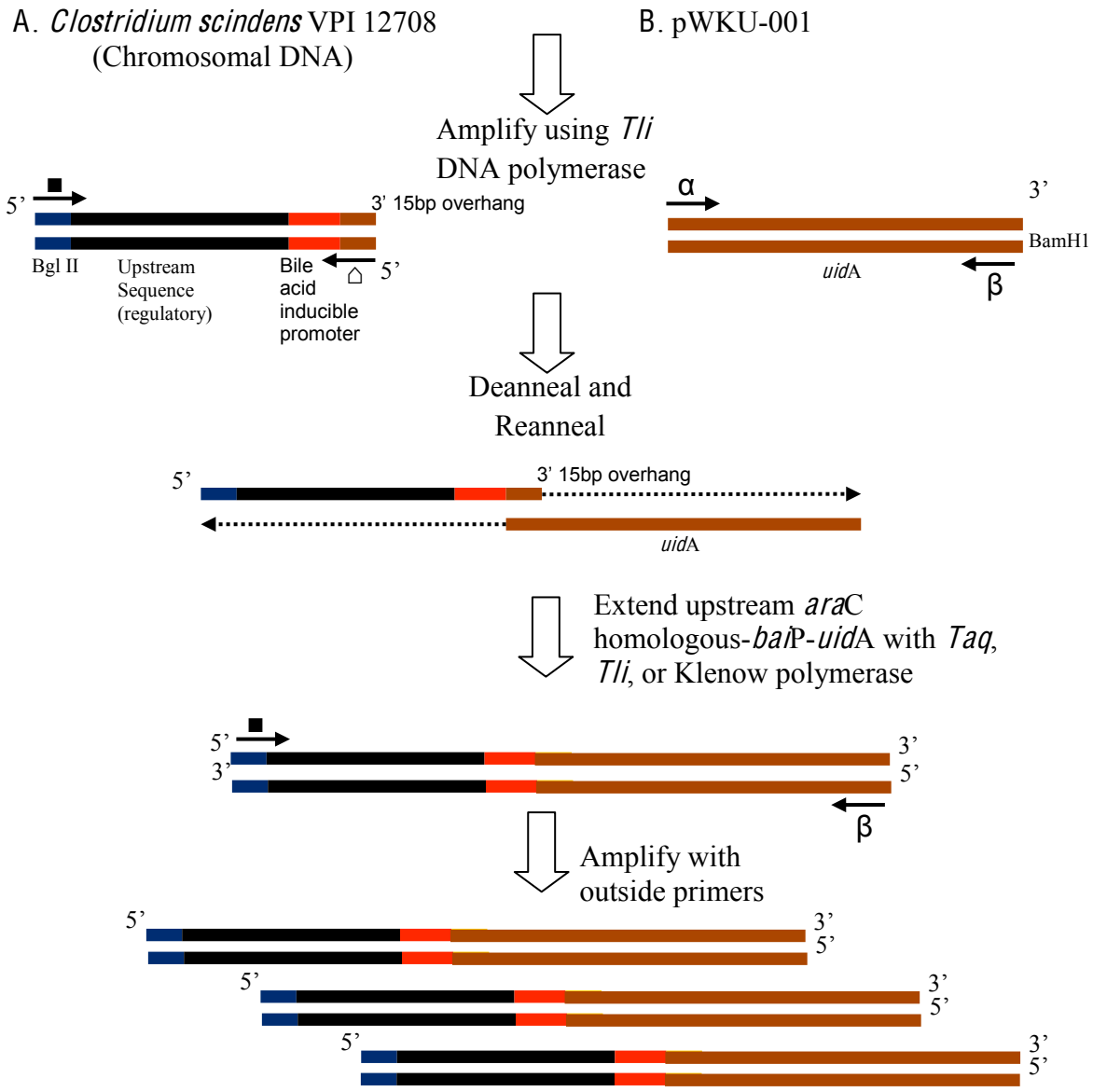


Figure 7: Cloning Strategy 1 and 2

Chromosomal DNA from *Clostridium scindens* VPI 12708 (A) was amplified (5' primer (■) = Up AraC/bai (1), 3' primer (⌂) = Dn AraC or Downstream AraC(2)) for the bile acid inducible promoter including upstream sequence. A 3' 15 base pair overhang complementary to the 5' *uid*A sequence was added by the downstream primer to facilitate the annealing of these two fragments. An existing plasmid, pWKU-001(B), contained the reporter sequence for *uid*A. This was amplified (5' (α) = Up uidA, 3' (β) = Downstream uidA Amplication) and purified. Both DNA fragments were combined and an attempt was made to make them contiguous using outside primers and either *Taq*, *Tli*, or Klenow DNA polymerase. Both strategies use similar methodology, but the second uses Klenow DNA polymerase for extension.

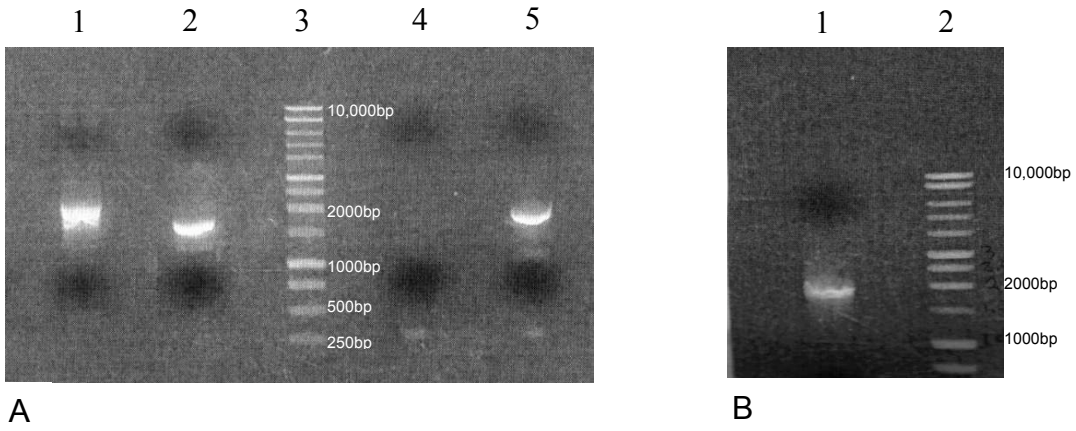


Figure 8: Results from first cloning strategy
(A) Lane 1 shows amplification of the *uid*A reporter sequence from pWKU-001 using Up uidA and Dn uidA primers (expected size = ~1,800 bp). Lane 2 shows amplification of *C. scindens* VPI 12708 for a nearly full-length 16S rDNA product considered universal for the domain *Bacteria* using 5' 27f and 3' 1492r primers (expected size = ~1,500 bp). Lane 3 contains a 1 kb DNA standard. Lane 4 contains a negative control from a PCR amplification consisting of Up AraC and Dn AraC primers, *Taq* polymerase and nuclease-free water. Lane 5 shows amplification of upstream AraC homologous sequence and the *bai* promoter from *C. scindens* VPI 12708 chromosomal DNA (Primers = 5' Up AraC and 3' Dn AraC; expected size = ~1,500). (B) Attempt to sew the upstream AraC sequence and *bai* (bile acid inducible) promoter with the *uid*A reporter gene. Lane 1 contains a PCR amplification of AraC-*bai*P and *uid*A DNA fragments using flanking primers (Up AraC and Dn uidA). Lane 2 contains a 1 kb DNA standard.

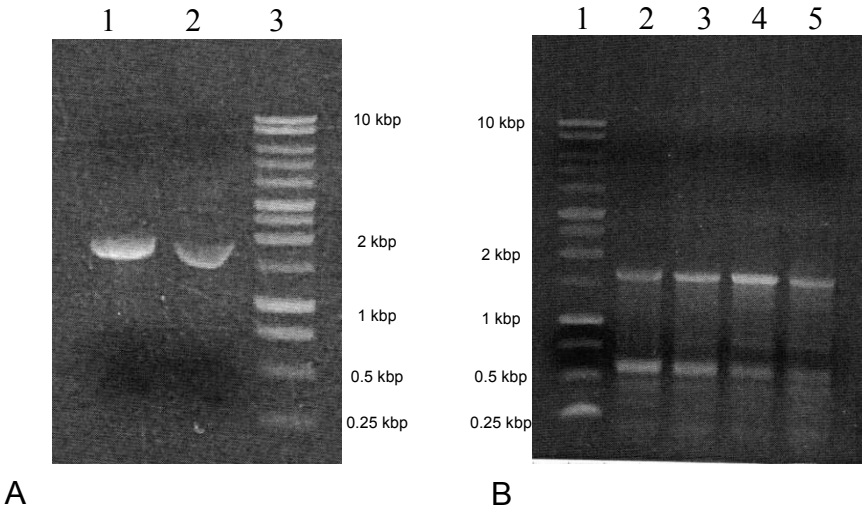


Figure 9: Results from second cloning strategy
(A) Shows amplification of the *bar*A -*bai* promoter and *uid*A reporter using Tli high fidelity DNA polymerase. Lane 1 shows *uid*A (pWKU-001 template) amplified by 5' Up uidA and 3' Dn uidA primers (expected size = ~1,800 bp). Lane 2 shows upstream *bar*A sequence and the bile acid inducible promoter amplified by 5' Up AraC and 3' Dn AraC primers (expected size = ~1,500 bp). Lane 3 contains a 1 kb DNA standard. (B) Shows result of the amplified sewing reaction of *bar*A -*bai*P with *uid*A by varying the constituents in the reaction mixture. The sewing reaction in each lane was set up with an initial reaction volume of 10 µL comprised of the two DNA amplicons and the remainder in nuclease-free water (Lane 2 = 0.5 µL *bar*A -*bai*P, 0.5 µL *uid*A; Lane 3 = 1 µL *bar*A -*bai*P, 1 µL *uid*A; Lane 4 = 2.5 µL *bar*A -*bai*P, 2.5 µL *uid*A; Lane 5 = 5 µL *bar*A -*bai*P, 5 µL *uid*A). These reaction tubes were subjected to 95°C, then temperature was decreased 5°C every 30 sec until reaching 37°C. Buffer, nucleotides, and Klenow polymerase were added for 30 minute incubation at 37°C. Amplification reactions were set up using flanking primers (Up AraC and Dn uidA); a 3,300 bp fragment was expected. Lane 1 contains a 1 kb DNA standard.

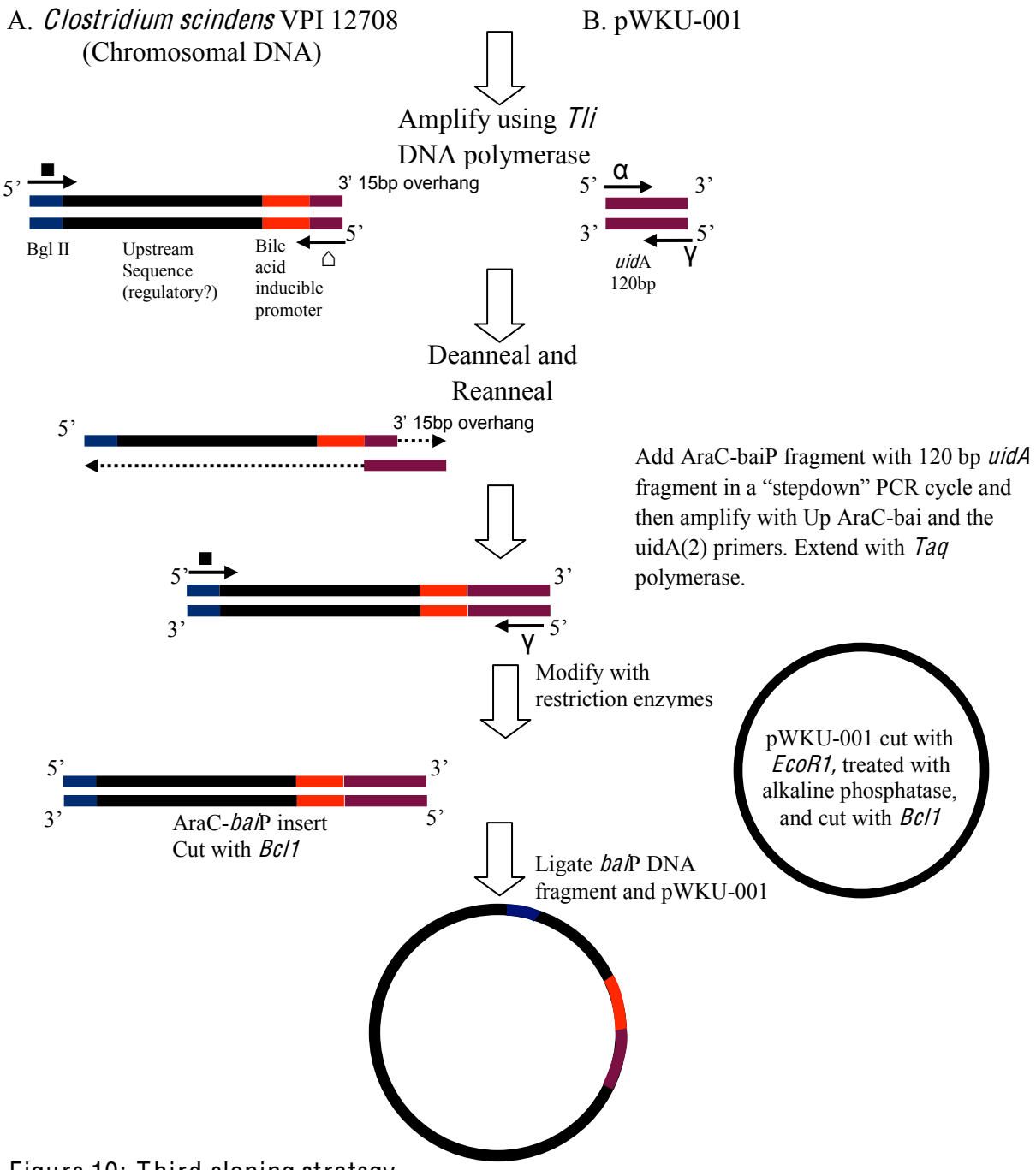


Figure 10: Third cloning strategy

Chromosomal DNA from *Clostridium scindens* VPI 12708 (A) was amplified [5' primer (■) = Up AraC/bai (1), 3' (⌂) = Dn AraC or Dn AraC(2)] for the bile acid inducible promoter including upstream sequence. An existing plasmid, pWKU-001 was used as a template (5' (α) = Up uidA, 3' (γ) = Dn uidA (2)) and produced a 120bp *uidA* fragment that was gel purified. A second reaction combined this DNA fragment with the *bar*A -*bai*P DNA fragment using a PCR cycler program that lowered temperature from 95°C to 40°C by 1°C/ 30s. The *bar*A -*bai*P DNA insert fragment was treated with an End-It kit (Epicentre). Plasmid pWKU-001 was cut with *EcoR*I, treated with alkaline phosphatase, and cut with *Bcl*I. The AraC-*bai*P insert was cut with *Bcl*I. Insert and vector were ligated together.

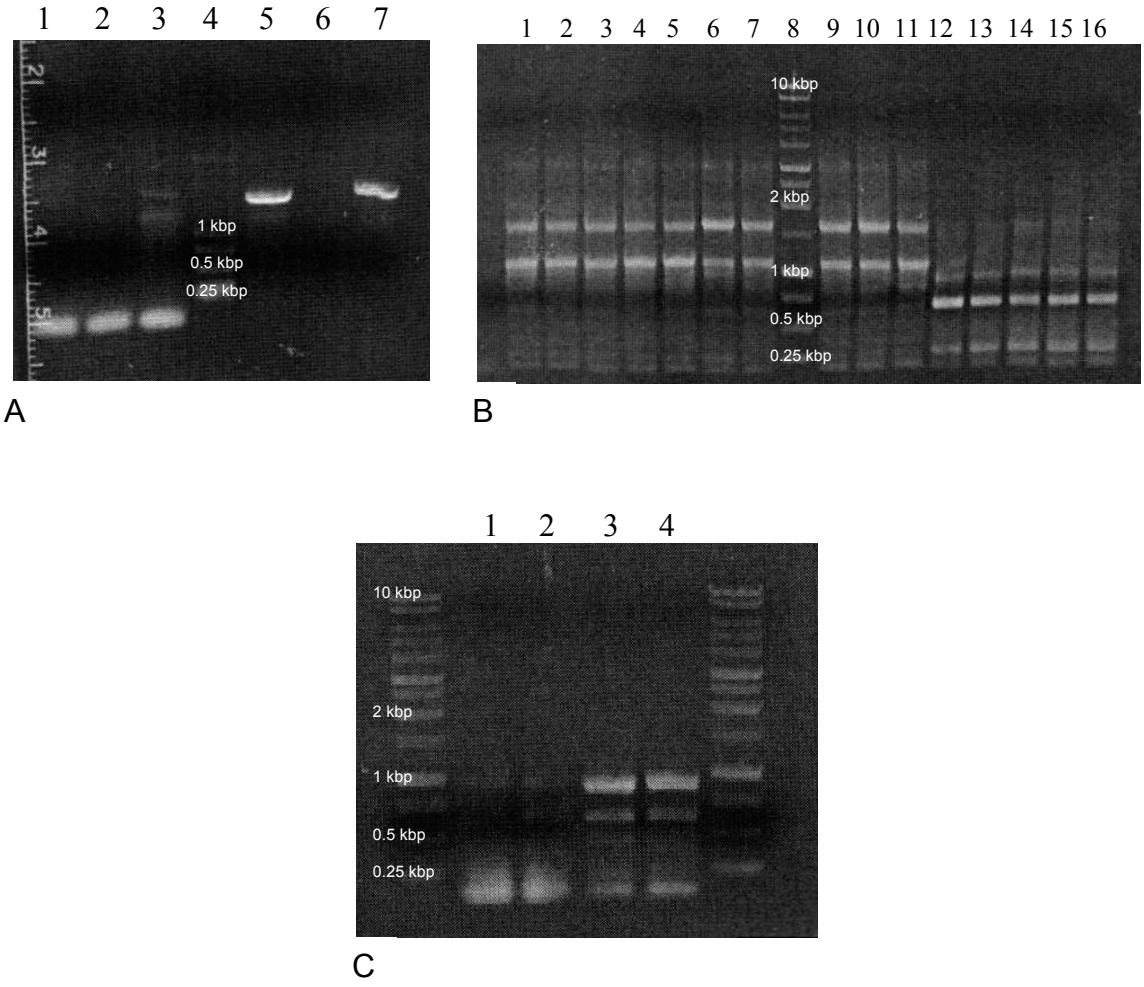


Figure 11: Results of the third cloning strategy

(A) The *bar*A -*bai* promoter and *uid*A fragment sequences were amplified using *Tli* high fidelity polymerase. Lanes 1-3 contains amplifications of the a 120 bp *uid*A fragment using 5' Up uidA and 3' Dn uidA(2) primers. Lane 4 contains a 1 kb DNA standard. Lanes 5 and 7 contain amplifications of upstream *bar*A homologous sequence and the *bai*P using Up AraC and Dn AraC primers. Lane 6 shows a negative control from a PCR reaction that contained Up AraC and Dn AraC primers, *Taq* polymerase, and nuclease free H_2O. (B) Amplifications of the combined AraC-bai promoter and *uid*A fragment (~1,600 bp). Lanes 1-7, 9-11 amplified *bar*A -*bai*P/*uid*A using *Taq* DNA polymerase and Up AraC and Dn uidA(2) primers. Lanes 12-16 amplified *bar*A -*bai*P/*uid*A using *Tli* DNA polymerase. Lane 8 contained a 1 KB molecular standard. Annealing temperature varied as follows: lanes 1, 6, and 12, 51.7°C; lanes 2, 7, and 13, 55.5°C; lanes 3, 9, and 14, 58.4°C; lanes 4, 10, and 15, 61.8°C; lanes 5, 11, and 16, 64.6°C. (C) Direct PCR whole colony screen of transformant *E.coli*: Lanes 1 and 2 were amplified by Upstream AraC and Downstream *uid*A primers (~3,300 bp expected) without PCR clean-up. Lanes 3 and 4 were amplified identically, though the template was subjected to clean-up (Mo-Bio).

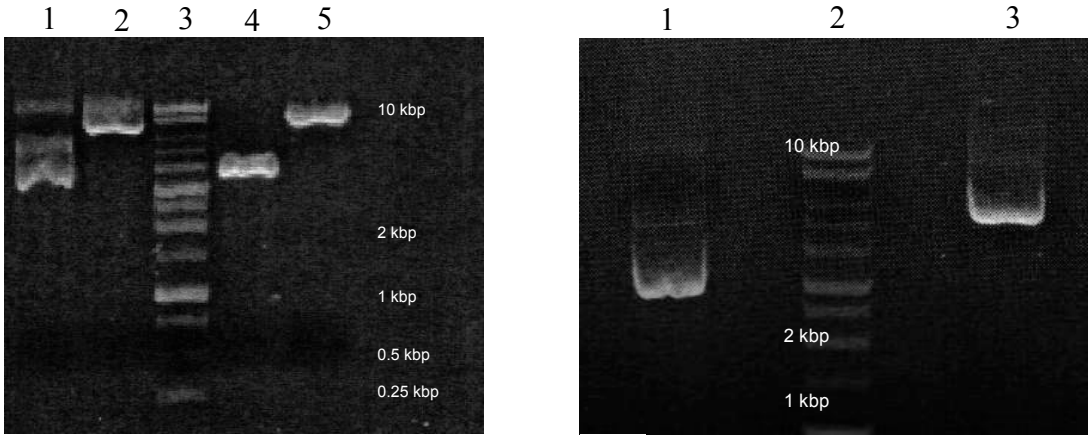


Figure 12: Results of the third cloning strategy (continued)
(A) *Eco*R1 digests of plasmids recovered from putative transformants (desired size = ~10.6 kbp). Lanes 1, 2, 4, and 5 represent recovered plasmids from isolated colonies. Lane 3 contains a 1 kb DNA standard. (B) *Hin*DIII digests of two putative transformants for further confirmation of the attempted ligation of pWKU-001, *bar*A -baiP, and *uid*A into the proposed pWKU-003 (expected size = ~10.6 kbp). Lane 1 is a *Hin*DIII plasmid digest of the same colony as Fig 12A, lane 1. Lane 2 holds a 1 kb DNA standard. Lane 3 is a *Hin*DIII plasmid digest of the same colony as Fig 12A, lane 5.

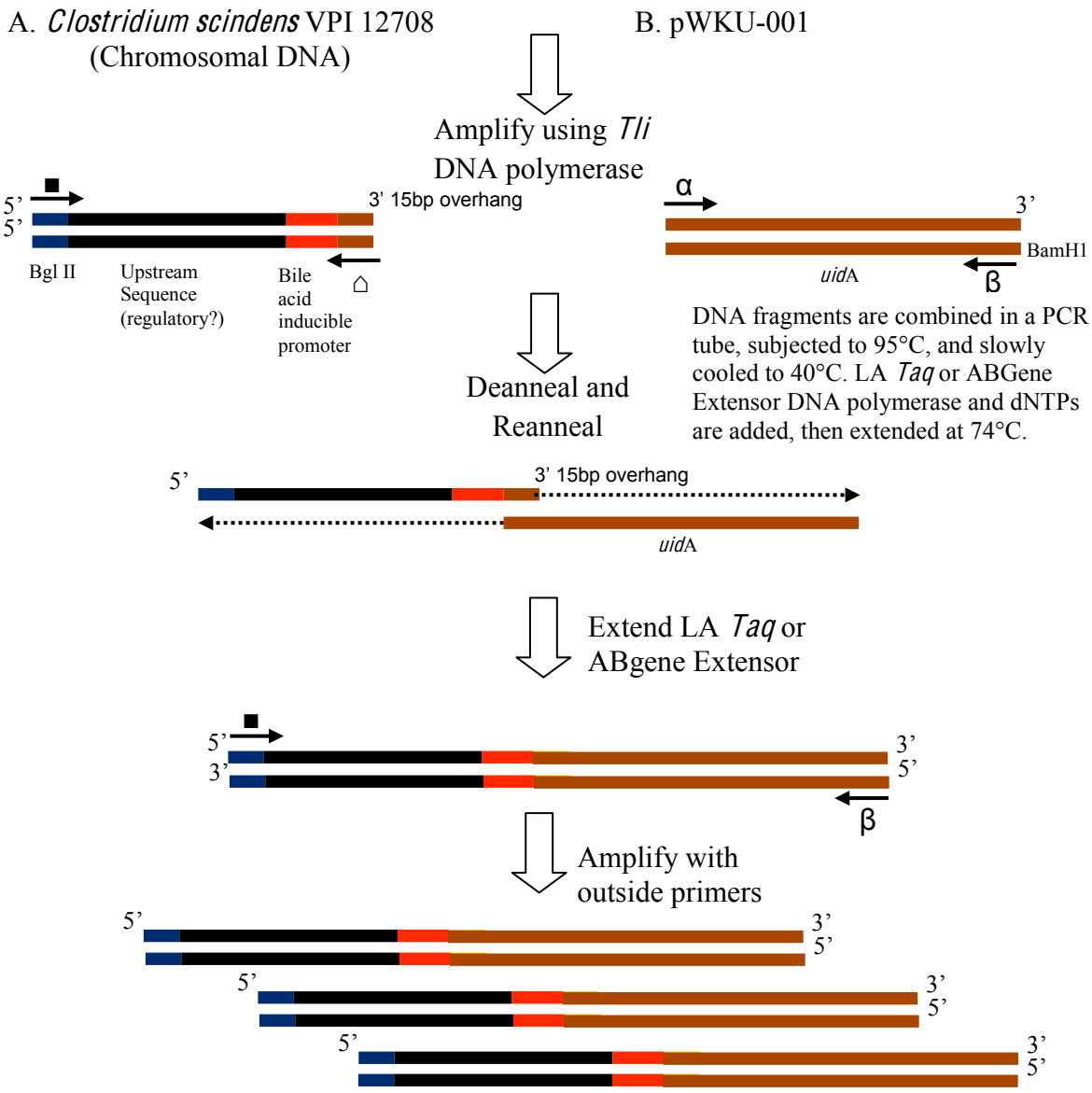


Figure 13: Fourth cloning strategy

Chromosomal DNA from *Clostridium scindens* VPI 12708 (A) was amplified (5' primer (■) = Up AraC/bai (1), 3' (⌂)= Dn AraC or Dn AraC(2)) for the bile acid inducible promoter including upstream sequence. An existing plasmid, pWKU-001(B), contained the reporter sequence for *uid*A. This was amplified (5' (α) = Up uidA, 3' (β) = Downstream uidA Amplication) and purified. Both DNA fragments were combined and an attempt was made to make them continuous using outside primers and either LA TAQ or ABGene Extensor (long amplifying, hi-fidelity) DNA polymerase. Amplicons were further amplified by adding outside primers (5' primer (Δ) = Up AraC(2), 3' (β) = Dn uidA). A pUC18 vector was cut with *Sma*I and treated with alkaline phosphatase. The *bai*P insert was treated with an End-It kit (Epicentre) to ensure a necessary 5' phosphate group is present. The vector and insert were ligated using a Fast-link ligation kit (Epicentre).

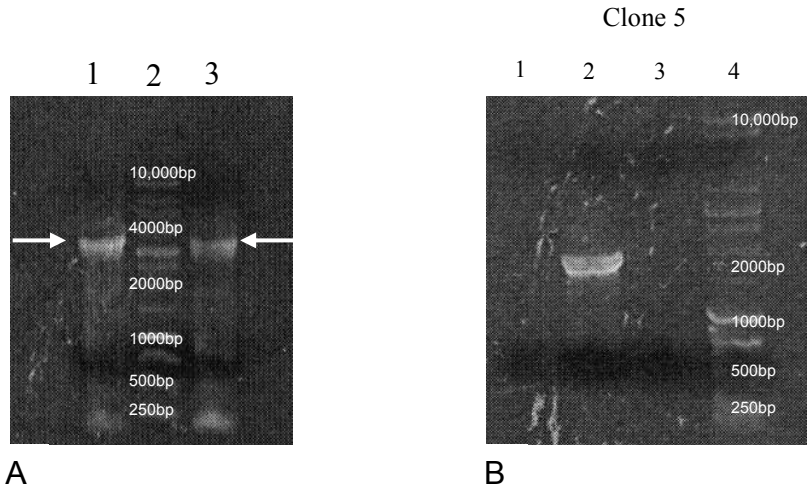


Figure 14: Results from the fourth cloning strategy
(A) Ligation of AraC-*bai*P-*uid*A using LA Taq DNA polymerase. *Bar*A -*bai*P and *uid*A DNA were mixed in equal volumes in a PCR tube and deannealed at 95°C. The tubes were slowly cooled 1°C every 30 seconds until they reached 40°C to bind AraC-*bai*P 3' *uid*A complementary base pairs to the 5' end of the *uid*A DNA. LA *Taq*, buffer, and dNTPs were added before adjustment to 74°C for 10 minutes. Flanking primers were added (5' = Up AraC, 3' = Dn uidA) to the same vessel to continue an amplification reaction with multiple deanneal, reanneal, and extension cycles. Lanes 1 and 3 show ligations of *bar*A-*bai*P-*uid*A (~3,300 bp). Lane 2 contains a 1 kb DNA standard. (B) The *bar*A-*bai*P-*uid*A insert generated in (A) was ligated into pUC18. The modified plasmid was transformed into electrocompetent XL1 Blue *E. coli*. Transformants were isolated by blue/white screening. Plasmids were recovered from five different isolated transformants and cut with *Bam*H1 or *Hin*DIII (data not shown). No clones that were digested showed the desired ~6,000 bp. Three representatives of the twelve isolates were then selected for further digestion with *Bcl*1 and *Bgl*2. Clone 5 was selected after cutting because its bands were near the approximated size (data not shown). The gel in (B) shows the attempted amplification of the recovered plasmid from clone 5 for *bar*A-*bai*P-*uid*A (Lane 1), *uid*A (Lane 2), and *bar*A-*bai*P (Lane 3). Lane 4 contains a 1 kb DNA standard.

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