Construction of recombinant *E. coli* Nissle 1917 (EcN) strains for the expression and secretion of defensins

Konstruktion von rekombinanten *E. coli* Nissle 1917 (EcN) Stämmen, die Defensine exprimieren bzw. sekretieren

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Summary

The probiotic *Escherichia coli* strain Nissle 1917 (EcN) is one of the few probiotics licensed as a medication in several countries. Best documented is its effectiveness in keeping patients suffering from ulcerative colitis (UC) in remission. This might be due to its ability to induce the production of human beta defensin 2 (HBD2) in a flagellin-dependent way in intestinal epithelial cells. In contrast to ulcerative colitis, for Crohn´s disease (CD) convincing evidence is lacking that EcN might be clinically effective, most likely due to the genetically based inability of sufficient defensin production in CD patients.

As a first step in the development of an alternative approach for the treatment of CD patients, EcN strains were constructed which were able to produce human alpha-defensin 5 (HD5) or beta-defensin 2 (HBD2). For that purpose codon-optimized defensin genes encoding either the proform with the signal sequence or the mature form of human alpha defensin 5 (HD5) or the gene encoding HBD2 with or without the signal sequence were cloned in an expression vector plasmid under the control of the T7 promoter. Synthesis of the encoded defensins was shown by Western blots after induction of expression and lysis of the recombinant EcN strains. Recombinant mature HBD2 with an N-terminal His-tag could be purified by Ni-column chromatography and showed antimicrobial activity against *E. coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*. In a second approach, that part of the *HBD2*-gene which encodes mature HBD2 was fused with *yebF* gene. The resulting fusion protein YebFMHBD2 was secreted from the encoding EcN mutant strain after induction of expression. Presence of YebFMHBD2 in the medium was not the result of leakage from the bacterial cells, as demonstrated in the spent culture supernatant by Western blots specific for ß-galactosidase and maltose-binding protein. The dialyzed and concentrated culture supernatant inhibited the growth of *E. coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* in radial diffusion assays as well as in liquid coculture. This demonstrates EcN to be a suitable probiotic *E. coli* strain for the production of certain defensins.

Zusammenfassung

Der probiotische *Escherichia coli* Stamm Nissle 1917 (EcN) ist eines der wenigen Probiotika, die als aktive Komponente eines Medikaments in mehreren Ländern zugelassen sind. Am besten ist die Wirksamkeit des EcN für die Remissionserhaltung von an Colitis Ulcerosa leidenden Patienten dokumentiert. Diese Fähigkeit ist vermutlich darauf zurückzuführen, dass EcN in der Lage ist die Produktion des humanen beta-Defensins 2 (HBD2) mittels seiner Flagelle zu Induzieren. In dieser Studie wurden rekombinante EcN Stämme konstruiert, die ein Defensin zu produzieren vermögen. Zu diesem Zweck wurden Kodon-optimierte Defensingene in Expressionsplasmidvektoren kloniert, die entweder die Proform mit der Signalsequenz oder die reife Defensinform des humanen α-Defensins 5 (HD5) oder des humanen β-Defensins 2 (HBD2) unter der Kontrolle des T7-Promotors kodieren. Die Synthese dieser Defensine wurde mittels Western-Blot nach der Induktion der Expression und der Lyse der rekombinanten EcN Stämme demonstriert. Das rekombinante reife HBD2 mit einem N-terminalen His-Tag konnte mittels Ni-Säulen-Chromatographie aufgereinigt werden. Das so gewonnene HBD2 zeigte antimikrobielle Aktivität gegen *E. coli*, *Salmonella enterica* Serovar Typhimurium und *Listeria monocytogenes*. In einem zweiten Ansatz wurde der Teil des HBD2-Gens mit dem *yebF*-Gen fusioniert, der das reife HBD2 kodiert. Das resultierende Fusionsprotein YebFMHBD2 wurde von dem entsprechenden EcN Stamm nach Induktion der Expression sekretiert. Die Präsenz von YebFMHBD2 im Medium war nicht das Ergebnis von Zellyse wie Western-Blots spezifisch für die β-Galaktosidase und das Maltose-Bindeprotein mit dem Kulturüberstand zeigten. Dieser Kulturüberstand inhibierte das Wachstum von *E. coli*, *Salmonella enterica* Serovar Typhimurium und *Listeria monocytogenes* nach Dialyse und Aufkonzentration sowohl in Agardiffusionsassays als auch in Flüssigcokultur. Damit konnte gezeigt werden, dass EcN ein für die Produktion von bestimmten humanen Defensinen geeignetes Probiotikum darstellt.

EcN ist bei der Behandlung von Morbus Crohn Patienten nicht aktiv. Dies ist vermutlich in der genetisch bedingten Unfähigkeit zur ausreichenden Defensinproduktion solcher Individuen begründet. Als ein erster Schritt in der

Entwicklung von alternativen Ansätzen zur Behandlung Morbus Crohn Patienten wurden in dieser Arbeit EcN Stämme konstruiert, die in der Lage sind HD5 oder HBD2 zu produzieren.

1. Introduction

1.1. Probiotic *Escherichia coli* strain Nissle 1917 (EcN)

1.1.1. Probiotics

Probiotics are by definition living non-pathogenic micro-organisms, which exert a positive effect on the host organisms when they get into the gastrointestinal tract in a viable condition in sufficiently large numbers (Food and Agriculture Organization of the United Nations. and World Health Organization. 2006). Probiotic microorganisms are often found as food ingredients, e.g. in fermented milk products and food supplements, but are also employed as feed additives in livestock breeding and as active components of medical remedies in human and veterinary medicine (Bansal and Garg 2008, Bengmark 2005, de Vrese and Schrezenmeir 2008, Marteau 2006). Usually in the food industry, probiotics mostly are lactic acid bacteria (LAB), such as lactobacilli, lactococci, bifidobacteria and streptococci (enterococci) (Gill and Prasad 2008). However, other non-pathogenic microorganisms, e.g. certain yeast strains (*Saccharomyces boulardii*) and *Escherichia coli* strains, like *E. coli* Nissle 1917 (EcN) are also used in human and veterinary medicine (Fric 2002, Hart et al. 2003, Heselmans et al. 2005, Reid and Devillard 2004, Schultz 2008).

1.1.2. *E. coli* Nissle 1917 (EcN)

The requirements to be met by a probiotic intended for medical application are demanding: proof is required of clinical efficacy in defined medical indications, evidence of effects and mechanisms of action, and compliance with specific criteria relating to product quality including non-pathogenicity and genetic stability of the micro-organism used therapeutically.

All the criteria for a probiotic medicine are fulfilled completely by *E. coli* Nissle 1917 (EcN), the active substance of Mutaflor® (Ardeypharm GmbH, Herdecke, Germany), which is used for treatment of various intestinal disorders and is known to be a successful colonizer of the human gut (Boudeau et al. 2003, Lodinova-Zadnikova and Sonnenborn 1997, Rembacken et al. 1999, Sonnenborn and Schulze

2009). This strain was originally isolated by Alfred Nissle in 1917 from a soldier who was not affected, in contrast to his comrades, by a severe outbreak of diarrhea (Blum-Oehler et al. 2003).

1.1.2.1. General properties of EcN

E. coli Nissle 1917 has been thoroughly analyzed by means of microbiological, biochemical and molecular genetic methods (Blum-Oehler et al. 2003, Blum et al. 1995, Grozdanov et al. 2004, Grozdanov et al. 2002). The strain does not exhibit any virulence factors, but has gene clusters located on genomic islands (GEIs) responsible for the synthesis of several so-called `fitness factors´, which contribute to the strain's probiotic nature. Serologically, EcN belongs to the *E. coli* O6 group and is of serotype O6:K5:H1 (Blum et al. 1995, Grozdanov et al. 2002). EcN is a typical gram-negative enterobacterium containing lipopolysaccharide (LPS) as a structural component of its outer cell membrane. The O6 surface antigen represents the outer part of the strain's LPS and exhibits some peculiar features. The O6 polysaccharide side-chain is very short, consisting of only one single `repeating unit´ of the oligosaccharide building block typical of the O6 antigen, giving the strain a so-called `semi-rough´ phenotypic appearance. EcN is able to form an extracellular capsule of the K5 serotype. This kind of capsule is present in only about 1 % of *E. coli* isolates. The chromosomal gene locus responsible for the synthesis of the K5 capsule has been detected by using a gene probe specific for the K5 capsule gene cluster (Ott et al. 1991). EcN possesses flagella of serotype H1 and is thus quite mobile. The possession of flagella enables the microbe to actively move within the gut lumen and the outer layer of the two layered mucus, which serves as an oxygen source, useful for aerobic catabolism of substrates by *E. coli*. Besides their function as driving apparatus, the flagella are important in bacterial crosstalk with the epithelium (Cario 2005, Wang et al. 2005). EcN possesses three different types of fimbriae: F1A (type 1) and F1C fimbriae as well as so-called `curli´ fimbriae, which mediate adhesion to intestinal epithelial cells in cell culture experiments or to the mucus layer of the intestinal wall *in vivo*, facilitating colonization of the gut (Blum et al. 1995, Grozdanov et al. 2004, Hammar et al. 1995, Lasaro et al. 2009, Stentebjerg-Olesen et al. 1999).

1.1.2.2. Fitness factors of EcN

Non-pathogenic (avirulent) commensal bacteria may also produce specific factors that enable them to compete with other strains in the ecological system of the gut and to effectively communicate with the host organism. These factors are designated fitness factors, which are also often encoded by special DNA sequences (genomic islands, GEIs) on the bacterial chromosome (Hacker and Carniel 2001, Hacker et al. 2003). Different fitness factors have been detected in EcN strain.

Siderophores are iron-chelating substances needed for bacterial iron uptake. EcN produces an unexpectedly wide array of siderophores: aerobactin, enterobactin, salmochelin, yersiniabactin as well as a hemin- and a citrate-dependent iron acquisition system. Besides the siderophore ferric iron uptake systems, EcN also possesses an elemental ferrous iron uptake system (EfeU) (Grosse et al. 2006).

The antagonistic actions of EcN are due, at least in part, to the formation of microcins against which the producer strain itself is immune. One of these microcins (microcin H47) had already been described before for another *E. coli* strain, whereas the second microcin was unknown so far. The latter has been termed `microcin M´ (M from Mutaflor®) by Klaus Hanke and colleagues (Azpiroz et al. 2001, Gaggero et al. 1993, Patzer et al. 2003, Rodriguez et al. 1999) (Fig. 1).

In addition to the production of microcins, EcN belongs to the phylogenetic subgroup of *E. coli* bacteria (group B2) that contains strains able to synthesize peptide/polyketide hybrids (Homburg et al. 2007, Nougayrede et al. 2006). These substances belong to a heterogeneous group of molecules with antimicrobial and antitumor activities.

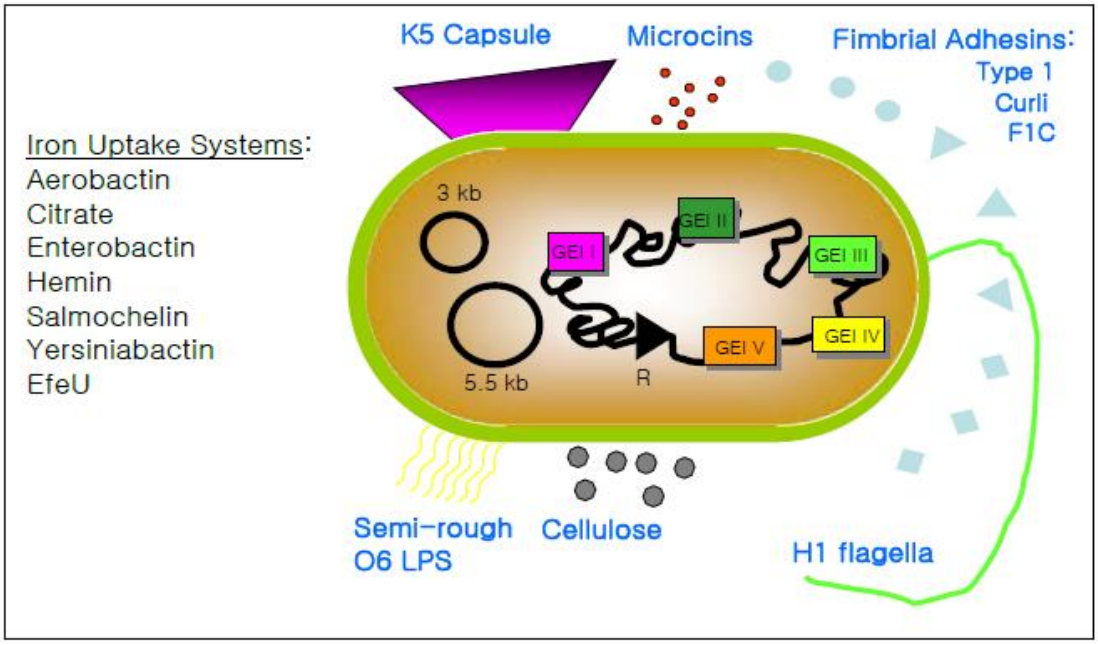


Fig. 1. Fitness factors of EcN, Schematic representation of the major surface structures and other factors that contribute to the fitness of the strain (modified from Blum-Oehler et al. 1995).

This probiotic drug, Mutaflor®, is successfully used in several countries for the treatment of various diseases of the digestive tract, including acute and prolonged diarrhea (Henker et al., 2007; 2008), uncomplicated diverticular disease (Fric and Zavoral, 2003), and inflammatory bowel disease (IBD) (Kruis et al. 1997, 2004, Matthes et al., 2010, Rembacken et al., 1999). It is particularly used for maintenance therapy of remission in patients with ulcerative colitis (UC) (Schultz, 2008). In ulcerative colitis, it is thought that one of the main mechanisms of action of EcN is the induction of defensin synthesis in intestinal epithelial cells (Wehkamp et al., 2004). The molecular mechanism behind the clinical effectiveness of EcN in UC-treatment might be the flagellin-mediated enhancement of human beta-defensin 2 (HBD2) production which is due to induction of a NF-kB- and AP-1-dependent signaling pathway (Schlee et al., 2007).

Wehkamp and his colleagues showed that the induction of HBD2 by EcN was dose-dependent with a maximum of 362-fold at the highest bacterial concentration tested in contrast to HBD-1 and HD-6 (Wehkamp et al. 2004a).

1.2. Expression of antimicrobial peptides in the gastrointestinal (GI) tract

1.2.1. Microbial/Host interactions

The gastrointestinal (GI) tract is unique, as it represents the largest area of the body that is constantly exposed to microorganisms. This exposure occurs in association with oral intake (which may be contaminated with microorganisms) and the resident microbial flora, varying in distinct proximal to distal regions of the GI tract. Of the resident flora in the oral cavity, *Streptococci* predominate. In the stomach, although the secreted gastric acid aims to keep the lumen largely sterile, *Helicobacter pylori* are resident in the mucus layer of many individuals. The proximal small intestine (duodenum, jejunum, proximal ileum), which mediates the important functions of digestion and absorption of nutrients, is colonized by small numbers of bacteria (10^2-10^3). In the distal ileum and the colon, there is an extensive resident bacterial flora (total ~10^{14}) consisting of ~400 different species of anaerobic and aerobic bacteria (Heneghan 1965, Sartor 2008).

Gastric and other secretions, motility, and secretory immunoglobulin A (a composition of mucosal adaptive immunity) have been known for many years to provide protection against microorganisms in the GI tract. In recent years, there has been increasing appreciation of the likely importance of antimicrobial peptides and proteins as components of innate immunity against microorganisms. The antimicrobial peptides/proteins are expressed predominantly by epithelial cells, which have distinct characteristics in different regions of the GI tract.

Numerous factors may regulate the expression of antimicrobial peptides in the GI tract. These include intra- and extra- cellular processing of biologically inactive precursor forms of a peptide, epithelial interactions with pathogenic and resident inflammation (e.g. inflammatory bowel disease). Additional factors include the type and number of antimicrobial peptide expressing epithelial cells and their state of differentiation. In inflamed tissue, there is infiltration from the circulation by antimicrobial peptide/protein expressing polymorphonulcear leukocytes, which contribute to innate mucosal host defense (Cunliffe and Mahida 2004).

1.2.2. Intestinal epithelial cells and the expression and regulation of antimicrobial peptides

In the small intestine, there are four main epithelial cell types: absorptive cells, goblet cells, enteroendocrine cells and Paneth cells, which are derived from multipotent stem cells (Booth and Potten 2000, Cheng and Leblond 1974). Following their origin from stem cells present near the crypt base, the epithelial cells (apart from Paneth cells) differentiate as they migrate up to the villus tip. They are subsequently lost into the lumen via exfoliation and/or apoptosis, which not only facilitates the removal of adherent bacteria but is also associated with the release of antimicrobial activity (Rose et al. 1998). Paneth cells have generated considerable interest as mediators of innate immunity in the GI tract, as they express a number of antimicrobial peptides and proteins (Ouellette 1997). In addition to the antimicrobial proteins lysozyme and secretory phospholipase A_2 (PLA_2), Paneth cells have also been shown to express members of the α-defensin family in humans (Ghosh et al. 2002, Jones and Bevins 1992, 1993, Mason and Taylor 1975, Ouellette 1997, Porter et al. 1998, Selsted et al. 1992). Paneth cells are restricted to the small intestine, where they may play an important role in maintaining the relatively low number of bacteria of the lumen and/or provide protection to stem cells (which are located close to Paneth cells) in the crypt (Ouellette and Bevins 2001). Cells with morphological features of Paneth and goblet cells, designated intermediate cells have been shown to express α-defensins by Cunliffe and his colleagues (Cunliffe et al. 2001).

In contrast to enteric α-defensins, human β-defensin 1 (HBD1), a member of the β-defensin family, appears to be expressed by most epithelial cells of the small and large intestine (O'Neil et al. 1999). Only during inflammation or infection is the synthesis of additional defensins and other antimicrobial peptides by the colonic mucosa induced. The inducible antibacterial peptides including HBD2-4, LL37 and bactericidal/permeability increasing protein may prevent further bacterial entry into an already compromised epithelium and contribute to antimicrobial defense during inflammation at this site. The inducible β-defensins are regulated by NF-kB, a transcription factor also regulating many proinflammatory cytokines (O'Neil et al. 1999). Human cathelicidin LL-37/human cationic antimicrobial protein 18 is expressed by mature enterocytes in the intestine, but its expression is absent in crypts (Hase et al. 2002, Schauber et al. 2003). Bactericidal/permeability increasing

protein, an antibacterial and endotoxin-neutralizing protein known to be produced by neutrophils, has recently been shown to be expressed by colonic epithelial cells, predominantly in the crypt and surface epithelial cells with reduced expression in cells in the intermediate zone (Canny et al. 2002).

1.2.3. Antimicrobial peptides

Antimicrobial peptides are small cationic peptides which protect their hosts against a vast array of microorganisms. These peptides are produced by several species including bacteria, insects, plants, vertebrates and they have been recognized as ancient evolutionary molecules that have been effectively preserved in mammals (Lehrer and Ganz 1999).

Based on their amino acid composition, size and conformational structures, antimicrobial peptides are divided into several categories such as peptides with linear α-helix structures, like human cathelicidin; peptides with β-sheet structures stabilized by disulfide bridges, like human defensins; peptides with extended structures, like indolicidin a bovine antimicrobial peptide; and peptides with loop structures, like cyclic defensins found in rhesus macaques (Lai and Gallo 2009).

Antimicrobial peptides are expressed on the primary barriers of organism such as skin and mucosal epithelia, preventing the colonization of host tissues by pathogens (Bulet et al. 2004). Moreover, these peptides are stored in granules within phagocytes, where they assist in the killing of engulfed microorganisms (Ganz 1999, Zasloff 2002).

1.2.3.1. Defensins

Defensins are antimicrobial peptides secreted by various cells as a component of the innate host defense and they are cationic, arginine-rich, small peptides between 3.5 and 4 kDa in size with six cysteines that form three disulfide bridges (Ganz and Lehrer 1994, Lehrer et al. 1993, Martin et al. 1995, Wehkamp et al. 2007a). They exhibit a broad spectrum antimicrobial activity against bacteria, fungi

and some enveloped viruses and can also act as chemokines (Chertov et al. 1996, Yang et al. 1999).

1.2.3.1.1. The mechanism of defensins

Permeabilization of target membranes is the crucial step in defensin mediated antimicrobial activity and cytotoxicity. Lehrer and his colleagues observed that *Escherichia coli* ML-35 that were treated by defensins became permeable to small molecules (Lehrer et al. 1989). The mechanism of action was found as attachment of the cationic defensins to the negatively charged bacterial cell surface resulting in a membranolytic disruption of the plasma membrane (Ganz 2003, Harder et al. 2001, Kagan et al. 1990, Shimoda et al. 1995). Defensins were more active against vesicles that included negatively charged phospholipid. In general, the activity of defensins against vesicles was diminished in the presence of increased salt concentrations, supporting the importance of electrostatic forces between the anionic phospholipid headgroups and cationic defensins. This antimicrobial quality allows defensins to protect the host epithelium and stem cells from virulent pathogens and also help to regulate the number and composition of commensal microbiota (Ramasundara et al. 2009, Salzman et al. 2007).

1.2.3.1.2. Classes of Defensins

Defensins are classified as α- or ß-defensins based on the position of 3 intramolecular disulfide bridges (Nissen-Meyer and Nes 1997). 6 α-defensins (HNP1-4, HD5 and HD6) and 6 ß-defensins (HBD1 to 6) have been identified in humans (Table 1) (Bensch et al. 1995, Ganz et al. 1985, Garcia et al. 2001a, Garcia et al. 2001b, Harder et al. 1997, 2001, Ramasundara et al. 2009, Yamaguchi et al. 2002).

Table 1. Defensin in the gut (modified from Ramasundara et al. 2009)

	Precursor size	Mature peptide size	Cystein linkages	Defensin	Tissue distribution	Stimuli
α-defensins	~90-105 aa	~30-34 aa	1-6, 2-4, 3-5	HNP 1-4	Sparse lamina propria neutrophils. In active inflammation seen in scattered intestinal epithelial cells.	Increased in active inflammation but possibly a result of increased neutrophil influx.
				HD5-6	Paneth cells and some villous epithelial cells in normal duodenum, jejunum and ileum	Constitutively expressed, however processing is required for biological activity.
β-defensins	~60-70 aa	~35-44 aa	1-5, 2-4, 3-6	HBD1	Colonic epithelia (and some other mucosal epithelia)	Constitutively expressed
				HBD2-4	Colonic epithelia (and some other mucosal epithelia)	IL-1α and entero-invasive bacteria
					Colonic plasma cells	

HBD, human β-defensin; HD, human α-defensin; HNP, human neutrophil peptides; IL, interleukin.

1.2.3.1.2.1. Human α-defensins

Human α-defensins provide antimicrobial host defence throughout the periphery and small intestine through their expression and release from granulocytic neutrophil and Paneth cell populations (Ganz et al. 1985). Human α-defensins are synthesized as larger, inactive pro-peptide molecules and the mature, active proteins result from post-translational processes (Porter et al. 1998, Valore et al. 1996). In the small bowel, human α-defensins (HD) 5 and 6 are most commonly expressed at the base of the crypts of Lieberkühn. Human defensin 5 and 6 (HD5, HD6) is primarily expressed in the epithelial intestinal Paneth cells (Ghosh et al. 2002) as inactive pro-peptide. They are cleaved into active mature forms by trypsin and trypsinogen stored in Paneth cell granules (Dann and Eckmann 2007, Salzman et al. 2003). Human α-defensins 1-4, known also as human neutrophil peptides (HNP), are present in azurophil granules of polymorphonuclear cells-forming part of their armory and hence contributing to innate immunity at a systemic level (Cunliffe 2003). Their expression in the gut coincides with the presence of lamina propria neutrophils (Cunliffe 2003).

1.2.3.1.2.2. Human ß-defensins

Human ß-defensins are produced by epithelial cells that are part of both the innate and adaptive immune responses (Harder et al. 1997, 2001, Singh et al. 1998, Yang et al. 1999). In contrast to human α-defensins, human ß-defensins are not expressed as pro-forms. Epithelial cells constitutively express HBD1. HBD2, 3 and 4 are induced in the skin as well as in urinary, gastrointestinal and respiratory epithelia by stimulation of epithelial cells by contact with microorganisms or cytokines such as TNF-α and IL-1β (Harder et al. 1997, Raj and Dentino 2002).

Since innate immune responses in the gut are directed against luminal bacteria, a defect in the expression and/or function of defensins could give rise to an increase in frequency and severity of intestinal infection (Fellermann et al. 2003). Many fields of investigation agree that such a deficiency could lead to gradual bacterial invasion, inflammation and a loss of tolerance to gut bacteria (Ramasundara et al. 2009).

1.3. Inflammatory Bowel Diseases (IBD)

Inflammatory Bowel Diseases (IBD) are chronic, relapsing and debilitating conditions that have significant impact on quality of life. Inflammatory bowel disease includes two main conditions, ulcerative colitis (UC) and Crohn´s disease (CD) which are defined based on characteristic endoscopic and histological finding. UC is characterized by superficial inflammation limited to the mucosa of the colon. In contrast, CD is characterized by discontinuous skip lesions that can occur anywhere in the gastrointestinal (GI) tract with transmural inflammation and non-caseating granulomas.

The pathogenesis of IBD is not clearly understood and its presentation regarding disease localization, progression and response to therapies is still under investigation. The high concordance rates of CD among monozygotic twins suggest the role of genetics in IBD pathogenesis in Jess's study (Jess et al. 2005). The evidence of this is seen in the identification on NOD2/CARD 15 allele variants and more recently, single nucleotide polymorphism (SNPs) in the autophagy related 16-like 1 (ATG16L1) gene which are associated with increased risk of developing CD (Hampe et al. 2007). On the other hands, the increased incidence of CD over the last 50 years, especially in developed nation, has linked environmental influences such as increased hygiene during infancy and provision of better house-hold amenities to increased susceptibility of an inappropriate response to GI infection later in life (Gent et al. 1994). Therefore, IBD is known to involve a combination of genetic susceptibility and environmental influences.

The most commonly accepted aetiology of IBD is a loss of tolerance to commensal microbiota. The normal intestine maintains a balance between factors that activate the host immunity (gut bacteria, dietary antigens, endogenous inflammatory stimuli) and the host defences that maintain the integrity of the mucosa and down-regulate the inflammatory response (Marks and Segal 2008). Alterations in the composition of the gut microbiota may give way to an increase in virulent species and a lack of protective species (Sartor 2008). Hence the host immune system is overwhelmed by bacterial antigens in the gut lumen and an ineffective down regulation shifts the balance toward immune-mediated intestinal injury. Also

alteration of the bowel flora by either antibiotics or the use of specific dietary changes can ameliorate the symptoms of IBD and help maintain remission (Farrell and LaMont 2002, Marks and Segal 2008). In contrast to normal mucosa containing relatively small numbers of bacteria, some studies demonstrate adherent and invading bacteria in the mucosa of patient with IBD (Wehkamp et al. 2005). Overall, the evidence suggests that alteration of commensal bacteria and the cellular response of inflammation are intrinsic to IBD pathogenesis.

A detailed understanding of the host responses and molecular events consequent to changes in the intestinal flora is not yet available. The expression and regulation of defensins produced in the intestinal mucosa are altered in IBD. Therefore, these host defense proteins may play a role in perpetuating or even inciting changes in gut microbiota and hence contribute to IBD (Ramasundara et al. 2009).

1.3.1. Defensin deficiency in inflammatory bowel diseases

1.3.1.1. The alpha defensin deficiency in Crohn´s disease

In ileal Crohn´s disease, the decreased expression of the α-defensins HD5 and HD6 was demonstrated by Wehkamp and his colleagues and it results in a defective antimicrobial shield in humans (Wehkamp et al. 2004b, Wehkamp et al. 2005). Mutations in the NOD2 (nucleotide-binding oligomerization domain 2) gene, an intracellular receptor for bacterial muramyl dipeptide, are clearly associated with ileal Crohn´s disease (Hugot et al. 2001, Ogura et al. 2001). It is noteworthy that Paneth cells, which are responsible for an effective antibacterial shield in the small intestine, also express NOD2 (Lala et al. 2003). Wehkamp and his colleagues also found a pronounced decrease of alpha-defensin transcripts in patients of Crohn´s disease with a NOD2 mutation as compared to the wild type (Wehkamp et al. 2004b, Wehkamp et al. 2005). These studies implicate that the specific deficiency of Paneth cell defenses in ileal Crohn´s disease, which is even more pronounced in case of NOD2 mutations, results in a dysfunction of the mucosal barrier. The defect in innate immune defense of the ileal mucosa appears to be primary and could cause and/or maintain this inflammatory disease.

Wnt signaling is one of the crucial pathways in the differentiation from the intestinal stem cell towards the Paneth cell. In particular the Wnt transcription factor TCF4 is a known regulator of Paneth cell differentiation and also alpha-defensin expression (van Es et al. 2005). The Wehkamp´s research group was able to show a reduced expression of TCF4 in patients with ileal Crohn´s disease as compared to colonic Crohn´s disease and ulcerative colitis (Wehkamp et al. 2007b). Remarkably, this decrease of TCF4 in ileal Crohn´s disease was independent of the degree of inflammation and independent of the NOD2 genotype and TCF4 expression correlated with HD5 and HD6 (Wehkamp et al. 2007b). Overall, a defect in intestinal stem cell differentiation towards Paneth cells mediated via the Wnt signaling transcription factor TCF4 could result in a defective antibacterial shield in ileal Crohn´s diseases by the absence of a sufficient expression of the protective Paneth cell defensins HD5 and HD6.

1.3.1.2. The beta-defensin deficiency in Crohn´s disease

The beta-defensins HBD1-3 are preferentially expressed in the colon. HBD1 is constitutively expressed in Crohn´s disease independent of the grade of inflammation, whereas HBD2 and HBD3 are inducible in intestinal bowel disease (Fahlgren et al. 2003, O'Neil et al. 1999, Wehkamp et al. 2003, Wehkamp et al. 2002). This deficiency of HBD2 expression in Crohn´s colitis was also confirmed on the protein level by immunohistochemistry (Wehkamp et al. 2002). HBD2 and HBD3 are strongly correlated to each other, especially in case of inflammation (Fahlgren et al. 2003, Wehkamp et al. 2003). The observed colonic defect of the antimicrobial barrier caused by a diminished expression of beta-defensins was supported by a recent study which showed a significantly lower bacterial killing using biopsy extracts from Crohn´s disease patients compared to ulcerative colitis and healthy controls (Nuding et al. 2007).

In humans, the beta-defensins HBD2 and HBD3 are neighboring genes on chromosome 8p23.1. The DNA copy number of this beta-defensin gene cluster is highly polymorphic within the healthy population (Hollox et al. 2003). A genome wide copy number profiling using a DNA microarray showed that the defective induction of

HBD2 and HBD3 in colonic CD may be due to a lower gene copy number in this gene area (Fellermann et al. 2006). The number of HBD2 gene copies was shifted to lower numbers in colonic CD as compared to controls (3 vs 4) (Fellermann et al. 2006). Since patients with 3 or less HBD2 gene copies were shown to have a significantly higher risk of developing colonic CD as compared to individuals with four or more copies, the number of HBD2 gene copies seems to be functionally relevant (Fellermann et al. 2006).

Therefore, a lower HBD2 gene copy number in the beta-defensin locus predisposes to colonic CD, which is most likely mediated through a diminished beta-defensin expression.

1.3.1.3. Beta defensins deficiency in ulcerative colitis

Expression of the inducible β-defensins is significantly increased in areas of active inflammation in UC. Human β-defensin 2 expression is significantly greater in UC compared to controls and also to colonic CD patients (Wehkamp et al. 2002). Similarly, the expression of HBD3 is strongly correlated with HBD2 and is also greater in UC than in controls (Wehkamp et al. 2003). In contrast, non-inflamed UC samples showed no significant difference in expression of HBD2 compared to control, indicating that the increased expression of these β-defensins occurs only in active inflammation (Wehkamp et al. 2003). Nuding and colleagues found that colonic samples from patients with UC contained significantly more plasma lineage cells than colonic samples from patients with CD and control patients as defined by CD138 staining (Nuding et al. 2007). Ulcerative colitis tissue contained on average 16.4 % CD138 positive cells compared to 7.8 % for CD and 8.3 % for controls. The high concentration of β-defensin expressing colonic plasma cells in the mucosa seen in UC could account for the higher levels of β-defensin detected. In addition, the high concentration of pro-inflammatory cytokines such as IL-1α and entero-invasive bacteria present in the inflamed tissue of UC, may also contribute. The antimicrobial peptides are appropriately upregulated consequent to the inflammatory events and not likely have a role in the pathogenesis of the disease in UC.

Table 2. Antimicrobial peptide expression in ulcerative colitis, colonic and ileal Crohn´s disease (modified from Wehkamp et al. 2009).

UC (colon)	CD (colon)	CD (ileum)
Normal expression of β-defensin HBD1 and regular induction of HBD2, HBD3 and HBD4 Regular induction of cathelicidin LL37 HD5 and HD6 expression due to metaplastic Paneth cells Regular induction of BPI	Attenuated induction of β-defensins HBD2, HBD3 and HBD4 Attenuated induction of cathelicidin LL37 HD5 and HD6 expression due to metaplastic Paneth cells Regular induction of BPI Overall reduced mucosal antibacterial activity towards different bacteria	Specific reduction of ileal Paneth cell defensins (HD5 and HD6), even more pronounced in patients with a NOD2 mutation (in both cases independent of the grade of histological inflammation and not observed in inflammatory controls) Regular induction of BPI Overall reduced mucosal antibacterial activity towards different bacteria

UC: Ulcerative Colitis

CD: Crohn´s disease

BPI: Bactericidal/Permeability Increasing protein

1.4. Aims of this thesis

As mentioned above, the gut of CD patients possesses a low amount of antimicrobial peptides, especially defensins because of a primary defect of defensin synthesis. Therefore, we constructed defensin-producing and -secreting probiotic EcN bacteria for supplementing the lacking endogenous defensin synthesis.

- Expression of defensins in probiotic EcN

Small cationic antimicrobial peptides have already been successfully synthesized in *E. coli* by recombinant DNA methods (Piers et al. 1993). However, there are several pitfalls using *E. coli* as the host cell for cationic antimicrobial peptide expression, such as the host-killing activity of the product and its susceptibility to proteolytic degradation. The fusion expression of the target peptide with a partner alleviates these problems. However, there arises another problem in that most fusion products are inactive or produced in an insoluble form (Lee et al. 2000; Piers et al. 1993). It has been reported that a fusion expression system with thioredoxin (TrxA) resulted in an increased yield of soluble products (LaVallie et al. 2000). This has been observed for HBD2 and HBD3 (Huang et al., 2006; Xu et al., 2006).

However, it has not been attempted to produce antimicrobial peptides i.e. defensins in probiotic bacteria. EcN was selected because this probiotic strain was already licensed as a medication for therapeutic purposes. Therefore, the primary aim of this thesis was to achieve the expression of defensins, HD5 and HBD2 in EcN. HD5 is encoded as an inactive proform and the inactive proform of HD5 should not affect viability of EcN. HBD2 was selected because this defensin is induced in Caco-2 cells by EcN via its flagellin and its induction in Crohn's colitis is typically impaired (Schlee et al. 2007, Gersemann et al. 2008).

- Secretion of defensins by recombinant *E. coli* strains

The second aim of this thesis was the efficient secretion of a defensin protein by the producing bacterial cells. *E. coli* protein YebF (10.8 kDa) is a soluble endogenous protein secreted into the medium and it is used as a carrier for transgenic proteins (Zhang et al., 2006). It was reported that passenger proteins

linked to the carboxyl end of YebF are efficiently secreted by the respective recombinant *E. coli* K-12 strain. Therefore, YebF was applied and used as the fusion partner for construction of recombinant EcN secreting a defensin.

2. Materials

2.1. Bacterial strains

All strains used/constructed in this study are listed in table 3.

Table 3. Strains used in this study.

Strains	Characteristics	References/ Sources
BL21 (DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	(Sambrook and Russell 2001)
BL2171	Km^R, BL21 harbouring plasmid pCON7-71	(Anthony et al. 2004)
BL2174	Km^R, BL21 harbouring plasmid pCON7-74	(Anthony et al. 2004)
BL2186	Km^R, BL21 harbouring plasmid pCON3-86B	(Anthony et al. 2004)
BL2171HD5	Km^R, BL21 harbouring plasmid pCON71HD5	this study
BL2174HD5	Km^R, BL21 harbouring plasmid pCON74HD5	this study
BL2186HD5	Km^R, BL21 harbouring plasmid pCON86HD5	this study
BL21T73	Ap^R, BL21 harbouring plasmid pT7-3	this study
BL21T74	Ap^R, BL21 harbouring plasmid pT7-4	this study
BL21T73HD5	Ap^R, BL21 harbouring plasmid pT73HD5	this study
BL21T74HD5	Ap^R, BL21 harbouring plasmid pT74HD5	this study
DH5α	F´Phi80d*lac*Z DeltaM15 Delta(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1)	(Bethesda Research Laboratories 1986)
EcNnirB5	Tc^R, EcN harbouring plasmid pNIR5	this study

EcNbla5	Tc^R, EcN harbouring plasmid pBLA5	this study
EcNlac5	Tc^R, EcN harbouring plasmid pLAC5	this study
EcNacrB5	Tc^R, EcN harbouring plasmid pACRB5	this study
EcN1219	Ap^R, EcN harbouring plasmid pAR1219	this study
EcNc1219	Ap^R, EcNc (EcN cured from both cryptic plasmids) harbouring plasmid pAR1219	this study
EcN100	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pET-28a(+)	this study
EcNc100	Ap^R, Km^R, EcNc harbouring plasmids pAR1219 and pET-28a(+)	this study
EcN101	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pEAS101	this study
EcN102	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pEAS102	this study
EcN103	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pEAS103	this study
EcNc103	Ap^R, Km^R, EcNc harbouring plasmids pAR1219 and pEAS103	this study
EcN104	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pEAS104	this study
EcN105	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pEAS105	this study
EcN106	Ap^R, Km^R, EcN harbouring plasmids pAR1219 and pEAS106	this study

SK22D105	Ap^R, Km^R, SK22D (EcN´s isogenic microcin-negative mutant) harbouring plasmids pAR1219 and pEAS105	this study
SK22D106	Ap^R, Km^R, SK22D harbouring plasmids pAR1219 and pEAS106	this study

2.2. Plasmids

All plasmids used and generated in this study are listed in table 4.

Table 4. Plasmids used in this study.

Plasmids	Characteristics	Reference/ Sources
pCON7-71	Km^R, low-copy number expression vector, which utilize the lactose promoter/operator system	(Anthony et al. 2004)
pCON7-74	Km^R, low-copy number expression vector, which utilize the lactose promoter/operator system	(Anthony et al. 2004)
pCON3-86B	Km^R, low-copy number expression vector, which utilize the lactose promoter/operator system	(Anthony et al. 2004)
pCON71HD5	Km^R, pCON7-71 containing *nHD5* gene under the control of the lactose promoter	this study
pCON74HD5	Km^R, pCON7-74 containing *nHD5* gene under the control of the lactose promoter	this study
pCON86HD5	Km^R, pCON3-86B containing *nHD5* gene under the control of the lactose promoter	this study

pGEM T-easy vector	Ap^R, Convenient system for the cloning of PCR products with single 3'-T overhangs	Promega, USA
pMW211	Ap^R, Plasmid containing *DsRed* gene under the control of *acrB* promoter	(Sorensen M. et al. 2003)
pMUT1-Tc	Tc^R, pMUT1 with *tetA* cassette	Sibylle Oswald
pNIRB5	Tc^R, pMUT1-Tc containing *nHD5* gene under the control of *nirB* promoter	this study
pBLA5	Tc^R, pMUT1-Tc containing *nHD5* gene under the control of *bla* promoter	this study
pLAC5	Tc^R, pMUT1-Tc containing *nHD5* gene under the control of *lac* promoter	this study
pACRB5	Tc^R, pMUT1-Tc containing *nHD5* gene under the control of *acrB* promoter	this study
pAR1219	Ap^R, pBR322 derived plasmid with *lac* UV5 promoter encoding the T7 promoter	(Davanloo et al. 1984)
pYebF01	Ap^R, Plasmid containing *yebF*-gene under the control of *acrB* promoter	Stephanie Weibel
pET-28a(+)	Km^R, Plasmid vector for cloning genes under the expression control of the T7 promoter and for creating a fusion protein containing either an N- or a C-terminal His-tag.	Novagen, USA
pEAS101	Km^R, pET-28a(+) containing *nHBD2*-gene under the control of the T7 promoter and encoding HisHBD2 fusion protein with an N-terminal His-tag	this study
pEAS102	Km^R, pET-28a(+) containing *nMHBD2*-gene under the control of the T7 promoter and	this study

	encoding HisMHBD2 fusion protein with an N-terminal His-tag	
pEAS103	Km^R, pET-28a(+) containing *nHD5*-gene under the control of the T7 promoter and encoding HisHD5 fusion protein with an N-terminal His-tag	this study
pEAS104	Km^R, pET-28a(+) containing *nMHD5*-gene under the control of the T7 promoter and encoding HisMHD5 fusion protein with an N-terminal His-tag	this study
pEAS105	Km^R, pET-28a(+) containing *yebF* gene under the control of the T7 promoter and encoding YebF protein	this study
pEAS106	Km^R, pET-28a(+) containing *yebF* and *nMHBD2* genes and under the control of the T7 promoter and encoding YebFMHBD2 fusion protein	this study
pT7-3	Ap^R, expression vector into which genes can be cloned under the control of the T7-RNA-polymerase	(Tabor and Richardson 1985)
pT7-4	Ap^R, expression vector into which genes can be cloned under the control of the T7-RNA-polymerase	(Tabor and Richardson 1985)
pT73HD5	Ap^R, pT7-3 containing *nHD5* gene under the control of the T7-RNA-polymerase	this study
pT74HD5	Ap^R, pT7-4 containing *nHD5* gene under the control of the T7-RNA-polymerase	this study

2.3. Oligonucleotides

Oligonucleotides for PCR reaction and sequencing were synthesized by Eurofins MWG Operon. All primers designed for and used in this study are listed in Table 5.

Table 5. Primers used for amplification and subsequent cloning into vector pET-28a(+).

Primer	Sequence	Applications and relevant proterties
pMUTHD5F1	5'- TAGAAA**AGGCCT**CAGGAGGC – 3' StuI	5'- Amplification of *nHD5* gene for cloning in pMUT1
pMUTHD5R1	5'- CCGGGCTGCAG**GAATTC**GAT -3' EcoRI	3'- Amplification of *nHD5* gene for cloning in pMUT1
nirBF1	5'- CCCTCGAGAC**GAATTC**GCC-3' EcoRI	5'- Amplification of *nirB* gene for cloning in pMUT1
blaF1	5'- CATTTATC**AGGCCT**ATTGTCT-3' StuI	5'- Amplification of *bla* promoter gene for cloning in pMUT1
blaR1	5'- GTTTATTTTTCT**GAATTC**ATTCAA - 3' EcoRI	3'- Amplification of *bla* promoter gene for cloning in pMUT1
lacF1	5'- CGTAATCAT**AGGCCT**AGCTG -3' StuI	5'- Amplification of *lac* promoter gene for cloning in pMUT1
lacR1	5'- CCGACTG**GAATTC**GGGCAGT -3' EcoRI	3'- Amplification of *lac* promoter gene for cloning in pMUT1
acrBF1	5'- GACGTGG**GAATTC**CCAGGTT -3' EcoRI	5'- Amplification of *acrB* gene for cloning in pMUT1

acrBR1	5’- ATGATAC**AGGCCT**TCGCAAA -3’ StuI	3’- Amplification of *acrB* gene for cloning in pMUT1
CONHD5F1	5’-TAGGCGGTA**GTCGAC**AAGAA -3’ SalI	5’- Amplification of *nHD5* gene for cloning in plasmids pCON and pT7
CONHD5R1	5’-GCAGGAATTCGATATCAAGCT -3’	3’- Amplification of *nHD5* gene for cloning in plasmids pCON and pT7
PURERBSF1	5’-TAAG**AAGGAG**ATATACCAAT RBS GCGTACCATTGCCATTCT -3’	5’- Generation of RBS for expression by “*In Vitro* Protein Synthesis Kit” (NEB, USA)
PURER1	5’-TATTCATCATTAACGGCAGCACAGAC C -3’	3’- Amplification of *nHD5* gene for expression by “*In Vitro* Protein Synthesis Kit” (NEB, USA)
PURET7F1	5’-GAAAT**TAATACGACTCACTA** T7 promoter **TA**GGGAGACCACAACGGTTTC CCTCTAGAAATAATTTTGTTTAA CTTTAAG**AAGGAG**ATATACCA -3’ RBS	5’- Generation of T7 promoter and RBS for expression by “*In Vitro* Protein Synthesis Kit” (NEB, USA)
HBD2F1	5’- CTTTAA**GAATTC**ATGCGTGTT -3’ EcoRI	5’- Amplification of *nHBD2* gene for cloning in pET-28a(+)
HBD2R1	5’- CTTATT**AAGCTT**TCATTACGGT -3’	3’- Amplification of

	HindIII	*nHBD2* gene for cloning in pET-28a(+)
MHBD2F1	5’- CTGCCGGGT**GAATTC**GGTG -3’ EcoRI	5’- Amplification of *nMHBD2* gene for cloning in pET-28a(+)
HD5F1	5’- TCAGGAGGCC**GGATCC**ATG -3’ BamHI	5’- Amplification of *nHD5* gene for cloning in pET-28a(+)
HD5R1	5’- GCAGGAATTCGATATC**AAGCTT** -3’ HindIII	3’- Amplification of *nHD5* gene for cloning in pET-28a(+)
MHD5F1	5’- GTACCAGC**GGATCC**CAGG -3’ BamHI	5’- Amplification of *nMHD5* gene for cloning in pET-28a(+)
YebFF1	5’- TAGAGAGCTC**CCATGG**AGAA -3’ NcoI	5’- Amplification of *yebF* gene for cloning in pET-28a(+)
YebFR1	5’- T**CTCGAG**GATATCTCATT -3’ XhoI	3’- Amplification of *yebF* gene for cloning in pET-28a(+)
T7F1	5’-TAATACGACTCACTATAGGG -3’	5’- sequencing primer for the insert in pET-28a(+)
T7R1	5’- GCTAGTTATTGCTCAGCGG -3’	3’- sequencing primer for the insert in pET-28a(+)

2.4. Chemicals, enzymes and antibodies

All chemicals used in this study were purchased from the following companies: Roche Diagnostics (Switzerland), Promega (USA), QIAGEN (Germany), Merck (USA), Roth (Germany), Sigma-Aldrich (USA), Difco (USA), Calbiochem (Germany), Peptanova (Germany), Spectrum Laboratories, Inc. (USA).

Antibodies and antiserums were provided by Dianova (Germany), Cell signalling (USA) and Macherey-Nagel, (Germany).

All restriction enzymes and T4 DNA ligase were purchased from NEB (USA).

Kits were used as follows:

• “QIAGEN Plasmid Mini Kit”, QIAGEN (Germany)

• “QIAquick Gel Extraction Kit (PCR purification Kit)”, QIAGEN (Germany)

• “ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit”, Applied Biosystems (USA)

• “PURExpress *In Vitro* Protein Synthesis Kit”, NEB (USA)

• “Protein Refolding Kit”, Novagen (USA)

Antibodies and Antiserums

- Anti-HBD2 rabbit polyclonal antiserum (was provided by Tomas Ganz at UCLA)
- Anti-HD5 rabbit polyclonal antiserum (was offered by Edith Porter at CSLA)
- Anti-HD5 mouse monoclonal antibody (Hycult biotechnology, Germany)
- Anti-ß-galactosidase mouse monoclonal antibody (Cell signaling, USA)
- Anti-Maltose binding protein rabbit polyclonal antiserum (NEB, USA)
- HRP-conjugated polyclonal goat anti-rabbit serum (Dianova, Germany)
- HRP-conjugated polyclonal rabbit anti-mouse serum (Dako, Denmark)

2.5. Technical equipments

Autoclave	Fedegari FOM/B50

	Biomedis Tecnoclav
	H+P Varioclav
Balance	IL-180, Chyo Balance Corp.
Clean bench	Nuaire Class II Type A/B3
Centrifuges (cooling)	Heraeus Megafuge 1.0R
	Heraeus Biofuge 13R
(table top)	Eppendorf Centrifuge 5415C
Electrophoresis chamber	
for agarose gel	Harnischmacher Midi-large 460.000
for protein gel	Bio-Rad MiniProtean 3 Cell
Electroporator Gene Pulser	Bio-Rad GenePulser XCell
Incubators	Heraeus B5050E
Ice machine	Scotsman AF30
Luminescence image analyzer	Image Quant LAS4000
Magnetic stirrer	Model L-81
Microwave	Moulinex Micro-Chef FM 2910
pH-Meter	WTW Multiline P4
Photometer	Amersham Ultrospec 3100 pro
Pipettes (20 µl, 200 µl, 1000 µl)	Abimed Discovery Starter-Kit
Pipette helper	Brand accu-jet pro
Power supply	Consort E455
Refrigerator	

	-20 °C	Privileg
	-80 °C	Thermo Scientific
Sequencer		ABI PRISM 310 Genetic Analyzer
Shaking incubator		Certomat U, Certomat H
		New Brunswick Scientific Innova 4300
Shaking table		GLW L-40
Sonicator		Branso Sonifier B12
Thermocycler		Biometra T3
UV-transilluminator		NTAS
Vortexer		Bender & Hobein AG Vortex Genie 2
Water bath		Memmert Wasserbad

2.6. Media

All media were autoclaved at 121 °C for 20 min. And 1.5 % (w/v) agar (difco) was added for agar plates.

LB- (Luria-Bertani-) Medium (Sambrook and Russell 2001)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
dH_2O	ad 1000 ml, pH 7.5

Turbo Medium

Tryptone	20 g
Yeast extract	10 g

NaCl	5 g
Glycerol	2 g
KH_2PO_4	6.8 g
K_2HPO_4	11.4 g
dH_2O	ad 1000 ml

Tryptic Soy Broth (TSB)

TSB	30 g
dH_2O	ad 1000 ml

SOC medium

Tryptone	2 g
Yeast extract	0.5 g
NaCl	0.5 g
KCl	0.186 g
MgCl	0.952 g
$MgSO_4$	2.408 g
glucose	3.603 g
dH_2O	ad 1,000 ml

2.7. Antibiotics

Antibiotics were sterile filtrated and stored at -20 °C.

Antibiotics	final concnetration	Solvent
Ampicillin (with Kanamycin 10 µg/ml)	20 µg/ml	dH_2O, sterile filtrated
Ampicillin	50 µg/ml	dH_2O, sterile filtrated
Kanamycin (with Ampicillin 20 µg/ml)	10 µg/ml	dH_2O, sterile filtrated
Kanamycin	30 µg/ml	dH_2O, sterile filtrated
Tetracyclin	10 µg/ml	100 % EtOH, sterile filtrated

2.8. Enzymes and solutions

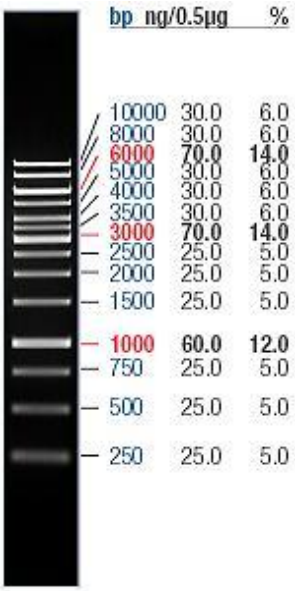
The buffer supplied by the manufacturer and solutions were used for enzyme reactions. The details of buffer solutions were described under the respective methods sections.

2.9. DNA and protein markers

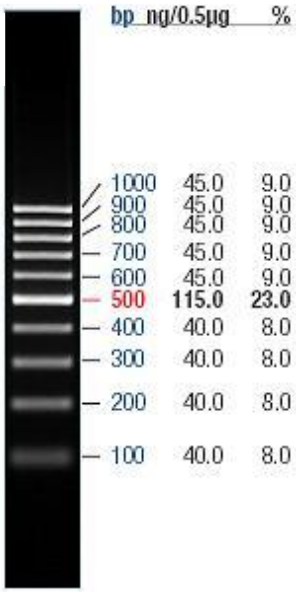
2.9.1. DNA marker

To determine the size of DNA fragments in agarose gels, the Generuler 1 kb DNA ladder and 100 bp DNA ladder were purchased from MBI Fermentas and were used.

A



B



Used DNA standard marker

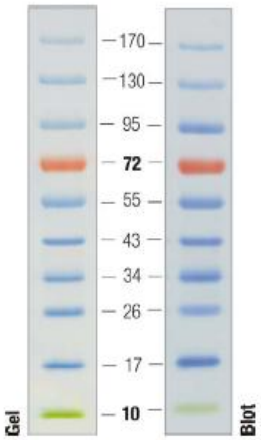
(A) GeneRuler 1 kb DNA Ladder

(B) GeneRuler 100 bp DNA Ladder

Source: http://www.fermentas.com

2.9.2. Protein marker

'Prestained protein ladder' from MBI Fermentas was used for determination of the molecular weight of proteins separated by polyacrylamide gel electrophoresis.



Used Protein marker

Prestained protein ladder

Source: http://www.fermentas.com

3. Methods

3.1. Nucleic acid methods

3.1.1. Design of *HBD2* and *HD5* genes with optimized codon usage for expression in *Escherichia coli*

The sequences of *human beta-defensin 2 gene* (GenBank accession No AF040153) and *human alpha-defensin 5 gene* (GenBank accession No M97925) were optimized for high expression in *E. coli* according to the codon usage table (http://www.kazusa.or.jp/codon/). Optimized *nHBD2* and *nHD5* genes were produced by Eurofins MWG Operon (Ebersberg, Germany) (*nHBD2*) and Sloning Biotechnology (Munich, Germany) (*nHD5*), respectively. All primers designed for and used in this study are listed in table 5.

3.1.2. Plasmid DNA extractions

3.1.2.1. Rapid method for the isolation of plasmid DNA

3 ml of overnight culture was centrifuged at 12,000 g for 5 min and the pellet of bacterial cells was resuspended in 200 µl of Buffer I and then 200 µl of Buffer II was added and mixed thoroughly by inverting several times. It was incubated at room temperature (RT) for 5 min. After adding 200 µl of ice-cold Buffer III, it was briefly mixed again and incubated on ice for 5 min. Chromosomal DNA and cellular debris are then removed by centrifugation at 12,000 g for 10 min. The clear supernatant was transferred to a new tube. 420 µl of 100 % ethanol was added and incubated on ice for 2 min. After centrifugation at 12,000 g for 10 min, the supernatant was discarded. The pellet was washed with 400 µl 70 % ethanol twice and dried. Finally, the pellet was dissolved in 40 µl of 10 mM Tris-HCl, pH 7.4 and stored at -20 °C.

Buffer compositions :

Buffer I	1 M Tris-HCl, pH 7.5	5 ml
	0.5 M EDTA, pH 8.0	2 ml
	RNase (10 mg/ml)	1 ml
	dH_2O	ad 100 ml

Buffer II	NaOH	0.2 N
	SDS	1 % (w/v)
Buffer III	Sodium acetate, pH 4.8	3 M

3.1.2.2. Plasmid isolation with Plasmid Mini Kit, QIAGEN

Plasmid Mini Kit (QIAGEN) was used for the isolation of plasmid DNA. 3 ml of overnight culture was collected by centrifugation at 12,000 g for 5 min and the pellet was resuspended with 250 µl Buffer P1 and then 250 µl Buffer P2 was added and mixed by inverting. And then, Buffer N3 was added and mixed immediately and thoroughly using inverting. The mixture was then centrifuged at 12,000 g for 10 min and the supernatant was applied to the QIAprep spin column (supplied by the manufacturer) by pipetting. After centrifugation for 1 min, the flow-through was discarded. The column was washed by adding 500 µl Buffer PB (supplied by the manufacturer) and centrifuged for 1 min. After that, it was washed with 750 µl Buffer PE (supplied by the manufacturer). The QIAprep column was placed in a clean and sterile 1.5 ml tube and DNA was eluted by 10 mM Tris-HCl, pH 8.5.

Buffer compositions (supplied by the manufacturer)

P1	Tris/HCl (pH 8.0)	50 mM
	EDTA	10 mM
	RNAse A	100 µg/ml
P2	NaOH	200 mM
	SDS	1 % (w/v)

P3	Potassium acetate (pH 5.5)	3 M

3.1.3. Enzymatic digest of DNA with restriction endonucleases

Approximately, 10 µg plasmid DNA dissolved in dH_2O was mixed with 5 µl of the appropriate 10x NEB buffer and 10 U of restriction enzyme (NEB), so that the final volume of the sample was 50 µl. The plasmid DNA was digested at 37 °C for 2 h. Afterwards, the restriction enzyme was removed by PCR purification kit (QIAGEN).

3.1.4. Horizontal gel electrophoresis

For routine analytical and preparative separation of DNA fragments, horizontal gel electrophoresis under non-denaturing conditions was performed using agarose gels with 1.5 % (w/v) agarose in running buffer (TAE).

To prevent diffusion of the DNA and to have a visible running front, 0.2 volume of loading dye was added to the samples before loading. The electrophoresis was carried out at 150 V and then stained in an ethidium bromide solution (10 mg/ml), washed with water and photographed under a UV-transilluminator.

50x TAE buffer:	Tris		2 M
	acetic acid (99.7 %)		6 % (v/v)
	EDTA (pH 8.0)		50 mM
	dH_2O	ad	1,000 ml

6x loading dye:	bromphenol blue	0.25 % (w/v)
	Xylene cyanol FF	0.25 % (w/v)
	Ficoll (Type 400, Pharmacia)	15 % (w/v)
	glycerol	30 % (v/v)

dH_2O	ad 50 ml

3.1.5. Isolation of DNA fragments from agarose gels

Agarose pieces containing the DNA fragment of interest were cut out of the gel and DNA was purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN). The piece of agarose containing DNA was subsequently melted at 50 °C for 10 min in QG buffer (supplied by the manufacturer). Applying the mixture to a QIAquick spin column was followed and then the column was centrifuged for 1 min. DNA was bound to the column and the rest of the solution was removed by centrifugation. The column was then washed with 750 µl PE buffer (supplied by the manufacturer, supplemented with ethanol) and residual PE buffer was removed with centrifugation for 1 min. Finally, the DNA was eluted from the column with 20 µl dH_2O.

3.1.6. Polymerase chain reaction (PCR)

Nucleotide sequences were amplified by PCR since the DNA sequence of the PCR product can be selectively altered.

The PCR reaction was prepared as following:

5x Phusion HF polymerase buffer	20 µl
10 mM dNTP-Mix	2 µl
0.5 µg/µl Primer 1	2 µl
0.5 µg/µl Primer 2	2 µl
100 ng/µl Template-DNA	1 µl
Phusion HF polymerase	1 µl
dH_2O	ad 100 µl

The reaction samples were boiled at 98 °C for 30 s. The amplification of DNA was performed under the optimal conditions for 25 cycles. Denaturation condition was at 98 °C for 8 s and annealing temperature was determined at Tm+3 °C of the lower Tm primer for 20 s and extension was done at 72 °C for 15 s/1 kb. Final

extension was performed at 72 °C for 5 min to ensure completely synthesis of all DNA fragments.

3.1.7. Dephosphorylation of DNA fragments

In order to prevent religation of vector DNA after enzymatic digestion, the 5' end of DNA fragment should be dephosphorylated. Removal of the 5´-phosphate group was achieved by adding phosphatase from calf intestine (1 unit) and 10x alkaline phosphatase buffer. The samples were incubated at 37 °C for 1 h. Afterwards, purification by PCR purification kit (QIAGEN) was done for inactivation of phosphatase.

3.1.8. Ligation of DNA fragments

Linearized vector and insert DNA after restriction digestion can be ligated either due to the presence of sticky ends or by blunt ends ligation. The enzyme for the ligation process was the T4-DNA ligase (NEB). Best efficiencies were obtained using an insert/vector ratio of 3/1. Reactions were performed at 18 °C for 1 h in a final volume of 20 µl containing 2 µl 10x ligation buffer and 1 µl (1 unit) T4 ligase.

3.1.9. Transformation of bacterial cells

3.1.9.1. Preparation of electrocompetent cells

For construction of recombinant strains, the bacterial cell wall and cell membrane were made permeable (transformation-competent).

For electrocompetent *E. coli*, 100 ml LB medium were incubated containing 1.5 ml of overnight culture at 37 °C and grown to OD_{600} of 0.5. Then the bacterial cells were centrifuged at 2,000 g, 4 °C for 10 min. The pellet was washed 2 times with sterile ice-cold dH_2O. Finally, the washed pellet was resuspended in 150 µl 10 % glycerol (v/v) and aliquots (each 80 µl) were transferred to new 1.5 ml tubes and stored at -80 °C.

3.1.9.2. Transformation of bacterial cells by electroporation

The translocation of recombinant DNA into the corresponding competent cells can be achieved by briefly applying an electrical impulse.

For electroporation, one vial of 80 µl electrocompetent cells was thawed on ice and mixed with 1-2 µl plasmid DNA solution (1-100 ng DNA) and incubated for 15 min on ice. Then the electrocompetent cells were transferred to a cuvette and electroporation was performed in a GenePulser Xcel (Biorad) electroporator with settings of 2.5 kV, 200 Ω and 25 µF in the 1-mm-wide GenePulser electroporation cuvette. After electroporation, 1 ml of SOC medium was added and incubated at 37 °C for 1 h in a shaker. Afterwards, bacteria were plated on a selective agar plate. The plate was incubated at 37 °C for 18 h.

3.1.10. Sequencing with fluorescent labeled nucleotides (AmpliTaq® FS BigDye-Terminator, Perkin Elmer)

The DNA sequencing was based on the chain termination method (Sanger et al. 1977). In order to determine the nucleotide sequences, PCR was carried out with different fluorescent dyes labeled ddNTP and these labeled nucleotides can be detected on the ABI PRISM 310 Genetic Analyzer using the laser (Perkin Elmer).

The sample composition for PCR was:

plasmid DNA	0.5 µg
10 pM primer	1.5 µl
5x buffer (kit component)	2 µl
premix (kit component)	2 µl
dH_2O	ad 10 µl

The subsequent sequencing PCR was performed under the following conditions: First, the plasmid DNA was denatured at 94 °C for 3 min. Denaturation at 94 °C for 30 s, annealing at Tm of the lower Tm primer for 15 s and elongation at 60 °C for 4min were followed by 25 cycles. Subsequently, the reaction sample was precipitated with 90 µl dH_2O, 10 µl 3 M sodium acetate (pH 4.6) and 250 µl 100 %

ethanol. After centrifugation at 13,000 g for 15 min, the supernatant was removed and the pellet was washed with 250 µl 70 % ethanol (v/v). After centrifugation and the removal of ethanol, the pellet was dried. The pellet was resuspended in 25 µl Hidi formamide (ultrapure and deinoized) and the sample was then analyzed in an ABI prism sequencer (Perkin Elmer).

3.2. Protein methods

3.2.1. Protein expression with "PURExpress *In Vitro* Protein Synthesis Kit" (NEB)

PURExpress kit is a novel coupled cell-free transcription/translation system reconstituted from purified components necessary for *E. coli* translation. Overlap extension PCR can be used to generate template DNA for use with "PURExpress *In Vitro* Protein Synthesis Kit". In the first round of PCR, PURERBSF1 and PURER1 were used to add RBS sequences. In the second round of PCR with PURET7F1 and PURER1, flanking primers bind the homologous regions of the first round product and extension occurred to contain the complete regulatory sequences and tags (Fig. 2).

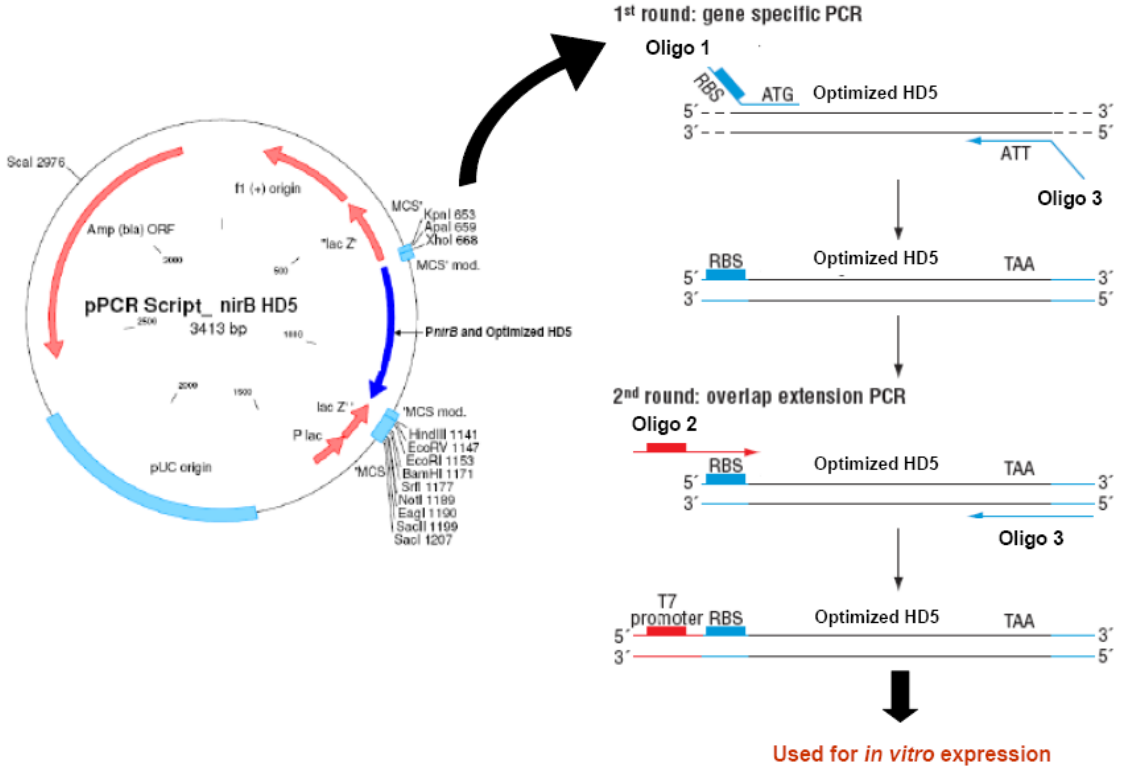


Fig. 2. Overlap extension PCR to generate linear template DNA, *LT_nHD5*.

Firstly, solution A and B which were provided by the manufacturer were thawed on ice.

The reaction sample composition was:

Solution A	12.5 µl
Solution B	5 µl
RNase inhibitor	1 µl
Template DNA	1 µl (100 ng)
dH_2O	ad 25 µl

After adding all components, the sample was mixed gently and incubated at 37 °C for 1 h. The reaction was stopped by placing the tube on ice. Expression of protein was analyzed by Western blot.

3.2.2. Protein expression in recombinant strains

For expression of the target proteins under the control of various promoters such as *nirB*, *lac*, *bla* and *acrB* using pMUT1-Tc, tetracycline (10 µg/ml) and protease inhibitor cocktail at a concentration according to the manufacturer (Roche, Switzerland) were added in LB medium. EcN harbouring plasmid pNIRB5 was grown to an optical density (OD_{600}) of 1.0 and induced without aeration at 37 °C for 24 h for expression of HD5 under the control of *nirB* promoter. Anaerobiosis was achieved by sealing tightly using parafilm and incubation without shaking. For induction of HD5 under the control of *lac* promoter, 1.0 mM IPTG was added to the grown culture at OD_{600} of 1.0 and culture was incubated at 37 °C for 18 h. EcNacrB5 was induced at OD_{600} of 1 with 4 % ethanol (v/v) and 0.5 M NaCl and incubated at 37 °C for 24 h for HD5 expression by the control of *acrB* promoter. EcN containing pBLA5 was grown at 37 °C for 18 h for HD5 production under the control of *bla* promoter.

For induction of protein under the control of the T7 promoter, ten microliters of glycerol culture of each recombinant strain were added to 10 ml of Turbo medium supplemented with ampicillin (20 µg/ml), kanamycin (10 µg/ml) and protease inhibitor cocktail according to the instructions of the manufacturer (Roche, Switzerland). Cultures were grown at 37 °C for 18 h. Subsequently, 8 ml of each grown culture were used to inoculate 800 ml Turbo medium containing ampicillin (20 µg/ml) and kanamycin (10 µg/ml) and an appropriate amount of protease inhibitor cocktail. The cultures were incubated at 37 °C and expression was induced at an OD_{600} of 1.0 with IPTG (final concentration 1.0 mM). The cells harbouring pAR1219 and an expression vector were harvested after incubation at 37 °C or 20 °C at several time points.

3.2.3. Cell lysis by sonication

The induced cells were collected by centrifugation at 6,000 g for 20 min. The cells were lysed in LEW buffer by sonication (15 s bursts, 10 times with a 15 s cooling period between each burst). Lysed extract was separated from cell debris by centrifugation at 10,000 g for 30 min, and then the supernatant was filtrated (0.22 µm, Millipore, USA). The filtrate contained the soluble proteins. To yield the insoluble proteins, the pellet was further dissolved in 8 M urea.

LEW buffer:	NaH_2PO_4	50 mM
	NaCl	300 mM
	pH	7.8

3.2.4. Isolation of soluble fusion protein with an N-terminal His-tag by nickel-affinity chromatography

The respective protein can be enriched by nickel-affinity chromatography due to a His-tag. Protino Ni-TED 2000-packed columns (Macherey-Nagel, Germany) were used for the affinity chromatographic purification of the protein. A column was equilibrated with 8 ml of LEW buffer and then allowed to drain by gravitation. The soluble fraction of cellular proteins was subjected to nickel-affinity chromatography and the His-tagged protein was eluted with 9 ml of elution buffer. Desalination was performed by dialysis with H_2O at 4 °C for 9 h. During this time, water was changed two times and then samples were concentrated with Spectra Absorbent (Spectrum Laboratories, Inc., USA). Bradford protein assay was used for subsequent protein quantification (Bradford 1976) (Roti-Nanoquant, Roth).

Elution buffer:	NaH_2PO_4	50 mM
	NaCl	300 mM
	Imidazole	250 mM
	pH	7.8

3.2.5. SDS tricine western blot analysis

After gel electrophoresis separation of proteins, proteins were transferred to an Immobilon PS-Q membrane. Then the proteins were examined by specific antibodies.

3.2.5.1. Tricine SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis **(**SDS-PAGE**)** is a technique for separating proteins based on their ability to move within an electrical field, which is a function of the length of their polypeptide chains and equivalent to their molecular weight. This is achieved by adding SDS detergent to remove secondary and tertiary protein structures and to maintain the proteins as linear polypeptide chains. The SDS coats the proteins, proportional to their molecular weight and confers a negative electrical charge to all proteins in the sample which is proportional to their molecular weight (Sambrook and Russell 2001). Tricine SDS-PAGE is commonly used to separate proteins in the mass range of 1-100 kDa (Schagger 2006). It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa.

For polyacrylamide gel electrophoresis (PAGE), a mini gel chamber was used for gels with dimensions of 7 x 9 x 0.1 cm (height x width x depth). Separating and stacking gels were prepared with the compositions given below. The prepared gel mixture was poured between the previously cleaned glass plates by ethanol and then dH_2O was overlaid. After polymerization, dH_2O was removed and the stacking gel was poured onto the separating gel. The wells were formed with a comb in the stacking gel.

Total proteins from whole cells were prepared by centrifugation of induced cultures at 13,000 g for 10 min and cells were resuspended in 10 mM Tris-HCl, pH 7.4. Proteins in the cell-free medium were obtained by centrifugation of 2 ml induced culture at 13,000 g for 10 min and sterile filtration (0.22 µm, Millipore, USA). Afterwards, the supernatant was precipitated with 10 % trichloroacetic acid (TCA) (w/v) at 4 °C for 1 h. After centrifugation at 13,000 g and 4 °C for 15 min, the pellet was washed twice by addition of 1 ml 100 % ethanol and once by 1 ml of 70 % ethanol (v/v). The final pellet was dissolved in 10 mM Tris-HCl, pH 7.4, SDS-PAGE

sample buffer was added at a ¼ volume of each sample and then samples were boiled at 95 °C for 5 min.

Electrophoresis was performed at 50 V for running in the stacking gel and at 100 V for the separating gel. “Prestained protein ladder” (Fermentas) was used as the standard of molecular weight.

Electrode buffers:

Anode buffer:	Tris	0.1 M
	HCl	225 mM
	pH	8.9
Cathode buffer:	Tris	0.1 M
	Tricine	0.1 M
	SDS	0.1 % (w/v)
	pH	8.25

Sample buffer:	SDS	12 % (w/v)
	mercaptoethanol	6 % (v/v)
	glycerol	30 % (v/v)
	Coomassie blue G-250	0.05 % (w/v)
	Tris-HCl, pH 7.0	150 mM

12 % separating gel :	3X Gel buffer	4.95 ml
	30 % Acrylamide, 0.8 % Bisacrylamide	4.8 ml
	dH_2O	3.69 ml
	glycerol	1.5 ml
	10 % (w/v) APS	45 µl
	TEMED	6 µl
4% stacking gel:	3X Gel buffer	1.2 ml
	30 % Acrylamide, 0.8 % Bisacrylamide	0.67 ml
	dH_2O	3.09 ml
	10 % (w/v) APS	40 µl

	TEMED	4 µl

3x Gel buffer:	Tris	18.15 g
	SDS	150 mg
	37 % (v/v) HCl	4.31 ml
	dH_2O	ad 50 ml

3.2.5.2. Western blotting

After electrophoresis, proteins were transferred to an Immobilon PS-Q membrane (Millipore, USA) in Western blot buffers. The specific antibody can make visible a particular protein in the membrane. To transfer the protein, the membrane and 12 Whatman papers were cut into pieces of the gel size. The membrane was soaked with methanol for 30 s and incubated in anode buffer II for 5 min. 6 wet papers in anode buffer I were placed on the plate of the blot apparatus and 3 wet papers with anode buffer II followed. The membrane was placed on the papers and the gel on the membrane. 3 layers of paper soaked with cathode buffer covered the gel. Air bubbles were removed before the upper plate of the blot apparatus was placed on top. The transfer was performed at 0.8 mM/cm^2 for 1 h.

Western blot buffers:

Anode buffer I:	Tris	0.3 M
	methanol	20 % (v/v)
	Tween 20	0.1 % (v/v)

Anode buffer II:	Tris	25 mM
	methanol	20 % (v/v)

Cathode buffer:	Tris	25 mM
	methanol	20 % (v/v)
	ε-Amino-n-capronic acid	40 mM

3.2.5.3. Antibody treatment

After transfer of the protein, the membrane was fixed with 0.01 % glutaraldehyde (in TBS) for 20 min and blocked in 5 % (w/v) nonfat milk for 18 h. Subsequently, the blot was incubated with primary antibody solution at RT for 1 h. The ratio of dilution of primary antibody was dependent on the serum and performed in TBS-T with 5 % (w/v) nonfat milk. In order to remove unspecifically bound antibody, the blot was washed 3 times with TBS for 10 min. The blot was incubated with peroxidase coupled secondary antibodies at RT for 1 h (dilution in TBS-T with 5 % (w/v) nonfat milk). Then, three washing steps with TBS-T for 10 min followed and the blot was moistened with the chemiluminescent detection solution for 1 min with gentle shaking. Afterwards, the blots were processed by the luminescence image analyzer (Imagequant LAS 4000).

10x TBS:	Tris-HCl (pH 7.4)	500 mM
	NaCl	1500 mM

TBS-T was prepared with adding of 0.05 % Tween 20 (v/v) to 1x TBS.

Dilution ratios of antibodies:

Anti-HBD2 rabbit polyclonal antiserum	1:2,000
Anti-HD5 rabbit polyclonal antiserum	1:1,000
Anti-ß-galactosidase mouse monoclonal antibody	1:1,000
Anti-Maltose binding protein rabbit polyclonal antiserum	1:5,000
HRP-conjugated polyclonal goat anti-rabbit serum	1:10,000
HRP-conjugated polyclonal rabbit anti-mouse serum	1:5,000

Chemiluminescent detection solution:	Tris, pH 8.5	0.1 M
	coumaric acid	0.2 mM
	Luminol	1.25 mM
	H_2O_2	0.06 % (v/v)

3.2.6. Antimicrobial activity tests

3.2.6.1. Radial diffusion assay

Radial diffusion assay was developed by Robert I. Lehrer to measure antimicrobial susceptibility (Lehrer et al. 1991). For radial diffusion assays, bacteria were grown at 37 °C for 18 h in 5 ml of TSB broth. To obtain mid-logarithmic phase bacteria, 5 µl (*E. coli* K-12 MG1655 and *Salmonella enterica* serovar Typhimurium SL1344) or 50 µl (*Listeria monocytogenes*) of the overnight cultures were used to inoculate 5 ml fresh TSB and incubated for an additional 2.5 h at 37 °C. The bacteria were centrifuged at 900 g, 4 °C for 10 min, washed once with cold 10 mM sodium phosphate buffer, pH 7.4 and resuspended in 2.5 ml of cold 10 mM sodium phosphate buffer. An aliquot containing $4x10^6$ bacterial cells was added to 10 ml of previously autoclaved and warm (42 °C) underlay agar. The agar was poured into a petri dish and allowed to solidify. Thereafter, a sterile glass pipette (5 mm diameter) was used to create wells in the agar. After adding samples to each well, the plates were incubated at 37 °C for 4 h and then were overlaid with 10 ml of sterile overlay agar maintained in liquid state by keeping at 42 °C. After incubation at 37 °C for 18 h, the inhibition zones surrounding the wells were detected.

100 mM Sodium phosphate buffer:	200 mM $NaH_2PO_4 \cdot H_2O$		4.75 ml
	200 mM $Na_2HPO_4 \cdot 2\ H_2O$		20.25 ml
	dH_2O	ad	500 ml
	pH		7.4

10 mM Sodium phosphate buffer:

Dilution of 100 mM Sodium phosphate buffer 1:10 with dH_2O

Underlay gel:	EEO agarose	0.5 g
	100 mM Sodium phosphate butter	5 ml
	TSB medium	0.5 ml

	dH_2O	ad	50 ml
	pH		7.4
Overlay gel:	EEO agarose		0.5 g
	TSB		3 g
	dH_2O	ad	50 ml

3.2.6.2. The killing assay in liquid culture

Bioactivity of secreted mature fusion HBD2 was tested in liquid culture. *E. coli* K-12 MG1655 and *Listeria monocytogenes* EGD were used as indicator strains. 2.5 µg or 5 µg of total proteins from the cell-free supernatant were added to log phase *E. coli* K-12 MG1655 (10^7 cfu/ml) or *Listeria monocytogenes* EGD (10^7 cfu/ml). Each test mixture had a volume of 300 µl and contained secreted proteins and bacteria in 10 mM sodium phosphate buffer, pH 7.4, and 1 % TSB medium. After mixing all components in the samples, a 100 µl aliquot of each sample was withdrawn for analysis of initial values (time point zero). The residual 200 µl of the samples were incubated at 37 °C for 4 h. All samples were serially diluted and aliquots of 100 µl were spread on LB agar plates. The plates were incubated at 37 °C for 18 h and then the number of colonies was counted.

4. Results

4.1. Codon optimization of human beta-defensin 2 and human alpha-defensin 5 gene for expression in *Escherichia coli*

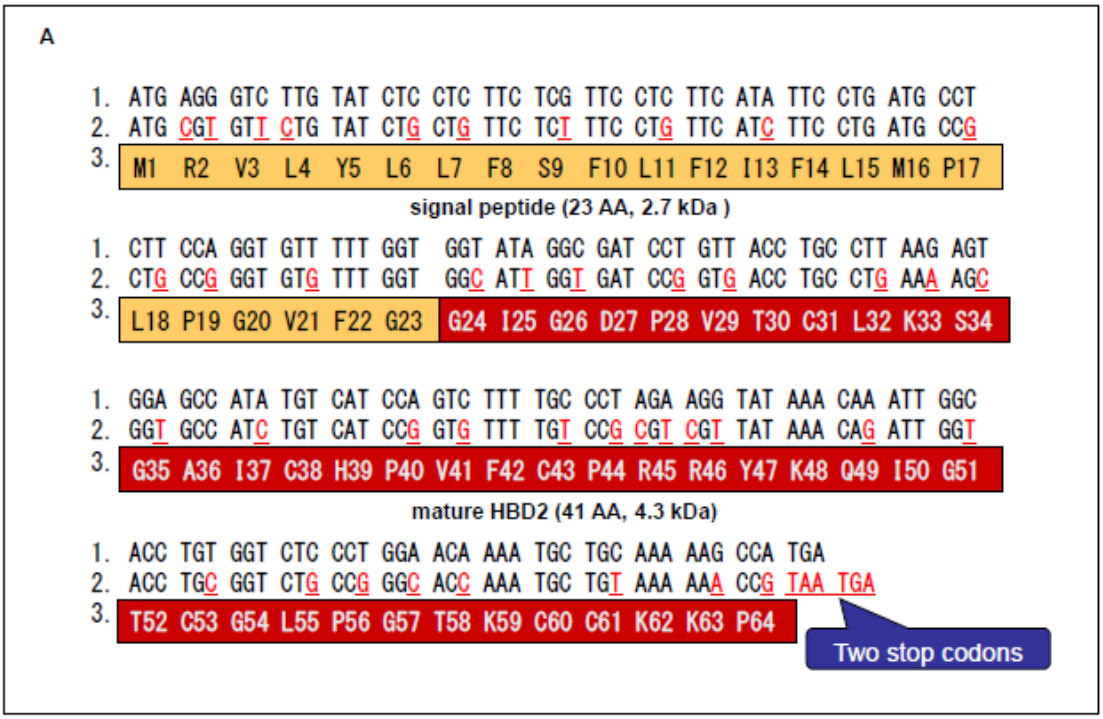
Differences in codon usage between species can affect the quantity and quality of the protein expressed by recombinant techniques. Therefore, codons in the cDNA of human beta-defensin 2 (HBD2) and human alpha-defensin 5 (HD5) were analyzed for rare codons.

HBD2 (GenBank accession No AF040153) consists of 64 amino acids, 27 amino acids (42.2 %) of which are encoded by rarely used codons in *E. coli*. Moreover, 6 codons (coding for R2, I13, I25, I37, R45 and R46) play important roles in adverse effects on heterogeneous protein expression in *E. coli* (Kane 1995). Particularly, R2, R45 and R46 are encoded by the least used codons (AGG and AGA) among the minor codons in *E. coli* (Fig. 3). The presence of single AGG or AGA, and tandem repeats of them were discovered to dramatically reduce the maximum level of protein synthesis and may also cause frameshifts with high frequency (Spanjaard et al. 1990).

HD5 (GenBank accession No M97925) consists of 94 amino acids with 29 amino acids (30.9 %) encoded by low usage codons in *E. coli*. Among these 29 amino acids, 6 amino acids are encoded by codons (coding for R2, R25, R55, R62, R68 and R90) which may cause adverse effects on protein expression in *E. coli* (Fig. 3).

In order to avoid the potential problems of these rare codons on HBD2 and HD5 expression in *E. coli*, corresponding new genes, *nHBD2* (EMBL HE583189) and *nHD5* (EMBL HE583188) (Fig. 3), were designed where the rare codons were replaced by frequently used ones in *E. coli* according to the codon usage table of *E. coli* (http://www.kazusa.or.jp/codon/). To increase the translation termination signal in *E. coli*, the original stop codon TGA was replaced by two stop codons in series, TAATGA, which is the most efficient translational termination sequence in *E. coli* (Hannig and Makrides 1998).

nHBD2 and *nHD5* were synthesized by Eurofins MWG Operon (*nHBD2*) and Sloning Biotechnology (*nHD5*), respectively, according to the sequences designed in this study.



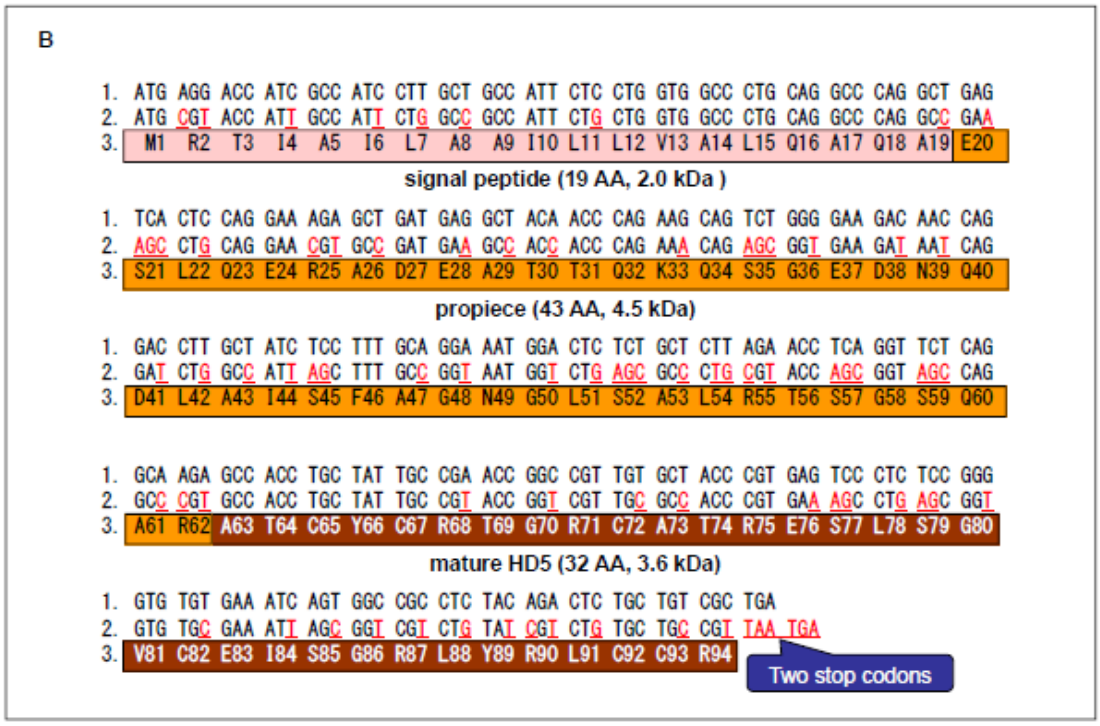


Fig. 3. Comparison of (A) HBD2 and (B) HD5 sequences between original cDNA and modified ones, Lane 1: original sequences of the cDNA, (A) *HBD2*-gene (GenBank accession No AF040153) and (B) *HD5*-gene (GenBank accession No M97925) sequences; Lane 2: optimized sequences of (A) *nHBD2*-gene (EMBL HE583189) and (B) *nHD5*-gene (EMBL HE583188); Lane 3: sequences of corresponding amino acids of (A) HBD2 and (B) HD5.

4.2. Construction of recombinant plasmids for the expression of defensins in *E. coli*

4.2.1. Cloning of *nHD5*-gene in pMUT1-Tc under the control of various promoters

Plasmid pMUT1-Tc mediating tetracycline resistance is a derivate of pMUT1, one of two very stable cryptic plasmids of EcN. pMUT1-Tc was used as a cloning vector for the first attempt to clone and express HD5 protein. For that purpose, *nHD5* gene was cloned under the control of one of several promoters such as *nirB*, *bla*, *lac* and *acrB*, respectively. *nirB* promoter is active under anaerobic condition and *bla* promoter is a constitutive one. Moreover, *lac* promoter is inducible by IPTG and *acrB* promoter is expressed under stress conditions.

Table 6. Sequences of applied promoters

Promoter	Sequences
nirB promoter	CCGTGACTTA AGAAAATTTA TACAAATCAG CAATATACCC ATTAAGGAGT ATATAAAGGT GAATTTGATT TACATCAATA AGCGGGGTTG CTGAATCGTT AAGGTAGGCG GTAATAGAA AAGAAATC (DQ841278)
bla promoter	TTCAAATATG TATCCGCTCA TGAGACAAT (AM403094)

lac promoter	AGCTGTTTCC TGTGTGAAAT TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA GCATAAAGTG TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGCGC (U46017)
acrB promoter	TATCCATCCC GAAGGTGTTC GGTTAGTTTA AGCCACTAAA AAGGGGATGC ATTATGGACG AATACTCACC CAAAAGACAT GATATAGCAC AGCTTAAATT (5501 bp to 5600 bp of pMW211)

(The numbers in parentheses for *nirB*, *bla* and *lac* are Genbank Accession No.)

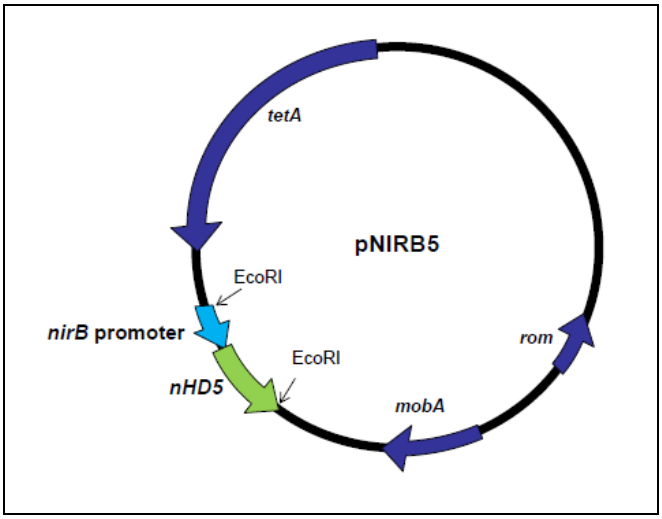


Fig. 4. Physical map of plasmid pNIRB5. pMUT1-Tc harbouring *nHD5* gene under the control of *nirB* promoter.

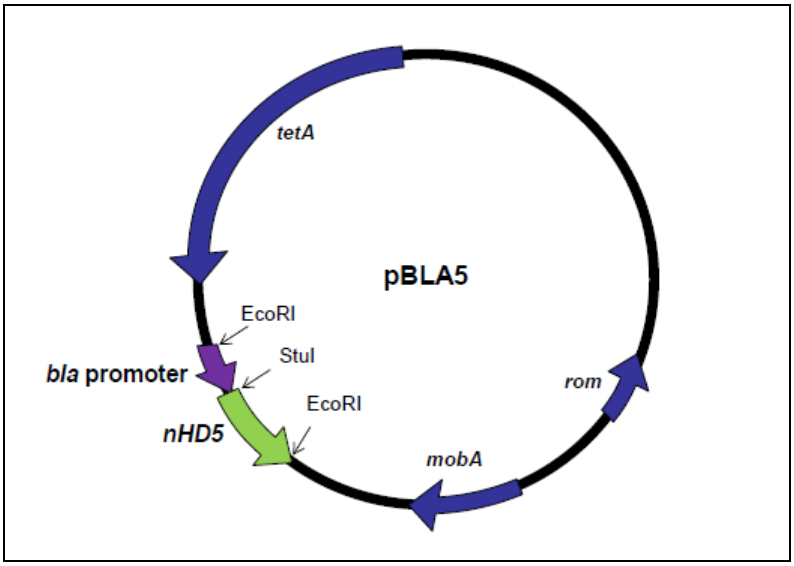


Fig. 5. Physical map of plasmid pBLA5. pMUT1-Tc harbouring *nHD5* gene under the control of *bla* promoter.

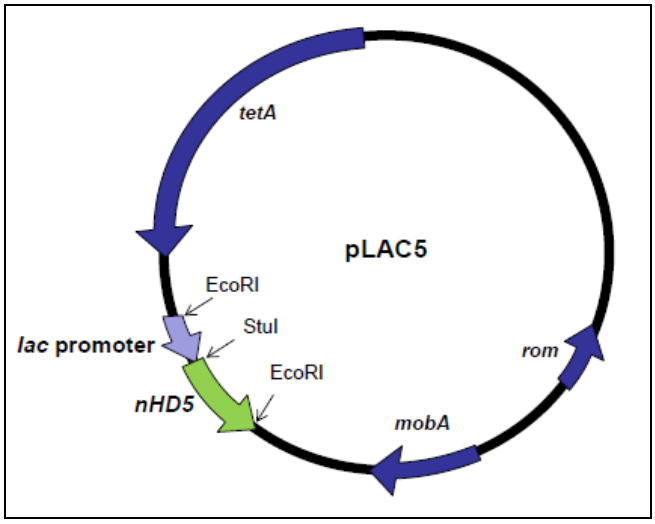


Fig. 6. Physical map of plasmid pLAC5. pMUT1-Tc harbouring *nHD5* gene under the control of *lac* promoter.

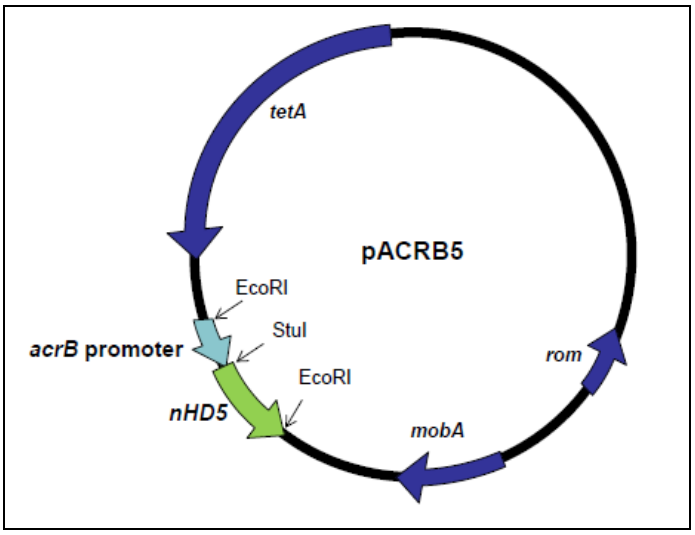


Fig. 7. Physical map of plasmid pACRB5. pMUT1-Tc harbouring *nHD5* gene under the control of *acrB* promoter.

Plasmid DNA of pMUT1-Tc, *nHD5* and promoters were digested with EcoRI and StuI and *nHD5* and promoters were ligated into pMUT1-Tc. This approach resulted in, four different recombinant plasmids each with one of the promoters from the genes *nirB*, *bla*, *lac* and *acrB* (Figs. 4-7). *E. coli* K-12 DH5α was transformed with the resulting plasmid by electroporation. The colonies obtained were proven to contain the favored insert and the boundary between insert and plasmid vector with restriction enzyme analysis and the correct DNA-sequence was determined. EcN was transformed with these recombinant plasmids.

4.2.2. Expression of HD5 in EcN and EcNc after cloning into pMUT1-Tc under the control of various promoters

Screening for expression of HD5 was performed by Western blot with a HD5 antibody and a HD5 antiserum. First of all, the primary antibody was HD5 mouse monoclonal antibody (Hycult biotechnology, 1:100 dilution) and HRP-conjugated polyclonal rabbit anti-mouse serum was followed. However, expression of HD5 was

not detected by Western blot with them. We also tried to detect HD5 expression with other antiserums. HD5 rabbit polyclonal antiserum (provided by Edith Porter, 1:1,000 dilution) was applied and HRP-conjugated polyclonal goat anti-rabbit serum was used as a secondary antiserum. Nevertheless, expression of HD5 could not be detected with any constructs by Western blot analysis using both HD5 mouse monoclonal antibody and HD5 rabbit polyclonal antiserum (data not presented).

4.2.3. Cloning of *nHD5* gene into low-copy number expression vectors for expression by the lactose promoter/operator system

In a second approach the low-copy number expression vectors pCON3-86B, pCON7-71 and pCON7-74 were employed which utilize the lactose promoter/operator system and which permit the stable maintenance and regulated expression of highly toxic gene products (Anthony et al. 2004). *nHD5* gene including the SD box was cloned between SalI and HindIII restriction enzyme sites of pCON3-86B, pCON7-71 and pCON7-74 vectors and the resulting recombinant plasmids pCON86HD5, pCON71HD5 and pCON74HD5 introduced into *E. coli* K-12 BL21 by electroporation. Strain BL21 encodes the T7-DNA-depending RNA polymerase under the control of the *lac* promoter derivate L8-UV5 *lac* (Grossman et al. 1998, Pan and Malcolm 2000). *nHD5* DNA sequence in all three recombinant plasmids was confirmed by restriction enzyme analysis and the preferred sequence was determined.

4.2.4. Expression of HD5 in *E. coli* K-12 BL21 after cloning into low-copy number expression vectors under the control of the lactose promoter/operator system

After induction for HD5 expression via the lactose promoter/operator system by 1 mM IPTG at 37 °C for 18 h of recombinant clones of BL21 harbouring pCON86HD5, pCON71HD5 and pCON74HD5, Western blot analysis was performed using HD5 rabbit polyclonal antiserum (provided by Edith Porter, 1:1000 dilution). However, HD5 expression could not be detected in any of BL21 strains harbouring recombinant plasmids (data not presented).

4.2.5. *In vitro* expression of *nHD5* gene

Since HD5 could not be expressed in any of the recombinant *E. coli* strains, we decided to attempt expression of HD5 in a cell free transcription/translation system using a 'PUREexpress *In Vitro* Protein synthesis Kit (NEB)'. *nHD5* gene which was synthesized commercially (Sloning Biotechnology) was used as the template for generation of *LT_nHD5* DNA by two consecutive PCRs according to the manufacturer (Fig. 8). *LT_nHD5* DNA was employed in protein synthesis and followed by Western blot analysis with HD5 rabbit polyclonal antiserum (provided by Edith Porter, 1:1,000 dilution).

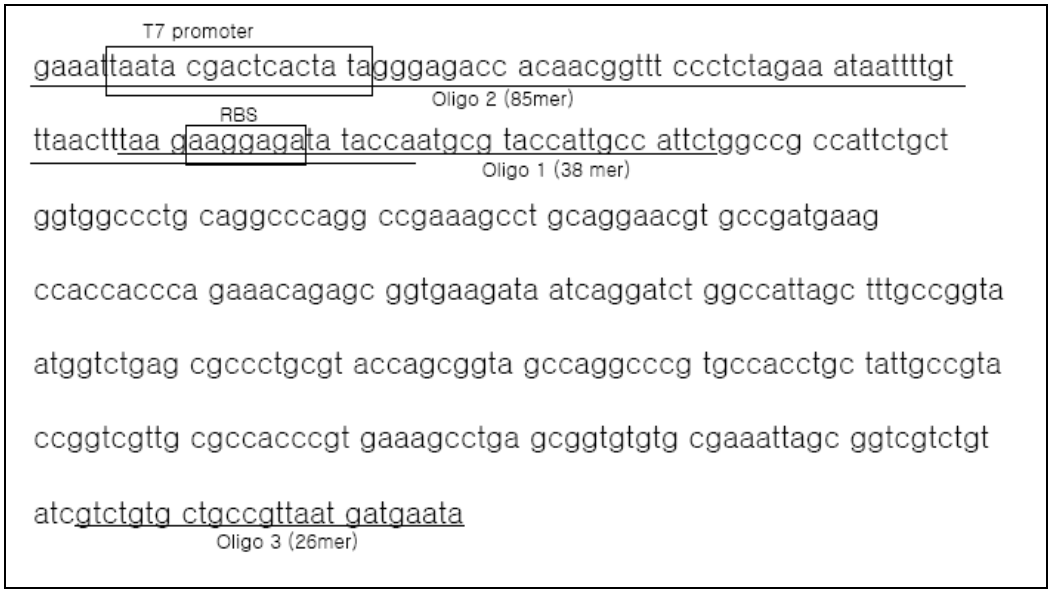


Fig. 8. Sequence of *LT_nHD5* DNA. Generation of *LT_nHD5* DNA for protein synthesis by cell free transcription/translation and three oligos used in the synthesis of *LT_nHD5* (Table 5, Oligos PURERBSF1, PURER1 and PURET7F1 were used).

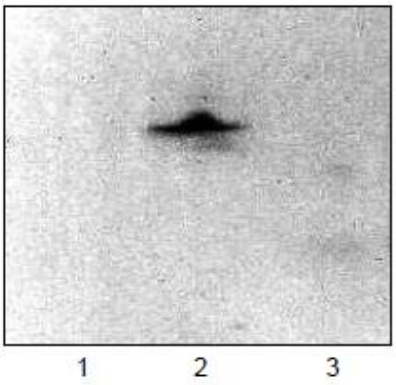


Fig. 9. Western blot analysis with HD5 rabbit polyclonal antiserum of expressed HD5 by cell free transcription/translation after incubation at 37 °C for 1 h. Lane 1: negative control DNA, 100 ng DNA was applied for protein synthesis, supplied by the manufacturer; Lane 2: LT_HD5, 100 ng DNA was applied for protein synthesis, 10.1 kDa; Lane 3: positive control, commercial mature HD5, 0.5 µg, 3.6 kDa.

Western blot analysis showed expression of *nHD5* DNA by "PURExpress *In Vitro* Protein synthesis Kit". This demonstrated *nHD5* gene sequence to encode an expressible gene (Fig. 9). Synthesized HD5 protein (LT_HD5) is the proform of HD5 with the theoretical MW of 10.1 kDa (Fig. 9, Lane 2) and commercial HD5 is the mature form and this theoretical MW is 3.6 kDa (Fig 9, Lane 3).

4.2.6. Expression of *nHD5* gene after cloning into pT7-vectors under control of the T7-RNA polymerase specific phi10 promotor

In a third approach, high copy number vectors of the pT7 series were used as part of another expression vector system into which genes under the control of the T7-RNA-polymerase specific promoter phi10 can be cloned. With vector pT7-3, plasmid pT73HD5 was constructed with *nHD5* gene including the SD box under the control of the phi10 promoter. As a negative control, *nHD5* gene including the SD box was cloned into pT7-4 in antiparallel orientation to the phi10 promoter resulting in pT74HD5. Subsequently *E. coli* K-12 BL21, the *E. coli* strain with the T7 RNA polymerase in the chromosome under the control of the IPTG inducible *lac* promoter, was transformed with both recombinant constructs. The resulting strains, BL21T73HD5 and BL21T74HD5 were induced and subjected to SDS-PAGE and

subsequently Western blot analysis was performed with HD5 rabbit polyclonal antiserum (provided by Edith Porter, 1:1,000 dilution).

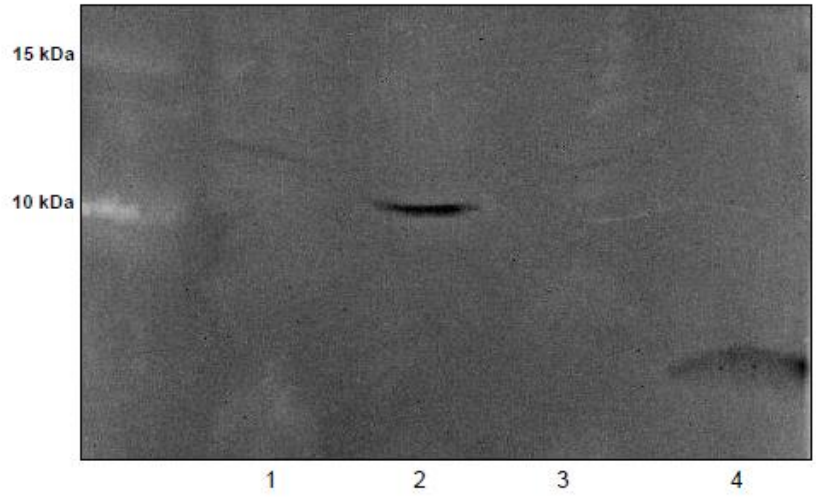


Fig. 10. Western blot analysis with HD5 rabbit polyclonal antiserum of HD5 expressed in BL21 after induction at 37 °C for 4 h (Samples were pellet of whole cell proteins after centrifugation and adjusted to cell density ($1.5x10^8$ cfu cells were loaded for each lane)). Lane 1: BL21; Lane 2: BL21T73HD5, The theoretical MW of expressed HD5 is 10.1 kDa; Lane 3: negative control, BL21T74HD5; Lane 4: positive control, commercial mature HD5, 3.6 kDa, 1 µg.

Western blot analysis demonstrated the expression of the proform of HD5 (theoretical MW: 10.1 kDa) in *E. coli* K-12 BL21 harbouring recombinant pT7-3 with *nHD5* gene including SD box (BL21T73HD5) in contrast to the corresponding strain with the recombinant pT7-4 plasmid (BL21pT74HD5: negative control) (Fig. 10).

4.2.7. Expression of HD5 and HBD2 fusion proteins in EcN and EcNc after cloning into pET-28a(+) vector carrying a His tag and under the control of the T7-RNA polymerase

Plasmid vector pET-28a(+) (Novagen, USA, Fig. 11), which encodes two His tags and allows expression of genes under the control of the T7 promoter was used for the construction of recombinant plasmids coding for fusion proteins consisting of an N-terminal His tag and a defensin. The synthetic full-length *nHBD2*-gene or that

part of *nHBD2*-gene encoding the mature part of HBD2 (nMHBD2: amino acids G24 to P64, Fig. 3 A) was ligated with the cleaved pET-28a(+) to construct the expression plasmids pEAS101 and pEAS102, respectively (Figs. 12, 13). Plasmid pEAS101 harbors *nHBD2*- and pEAS102 encodes *nMHBD2*-gene. Both genes were under the control of the T7 promoter and each of the resulting defensins (HisHBD2, HisMHBD2) contained an N-terminal His-tag. Plasmids pEAS103 and pEAS104 were constructed in order to express both the proform of HD5 and the mature part of HD5 (*nMHD5*: amino acids A63 to R94, Fig. 3 B), respectively. Again, the resulting defensin proteins contained an N-terminal His-tag. The gene product encoded by pEAS103 was HisHD5, and the one encoded by pEAS104 was HisMHD5 (Figs. 14, 15).

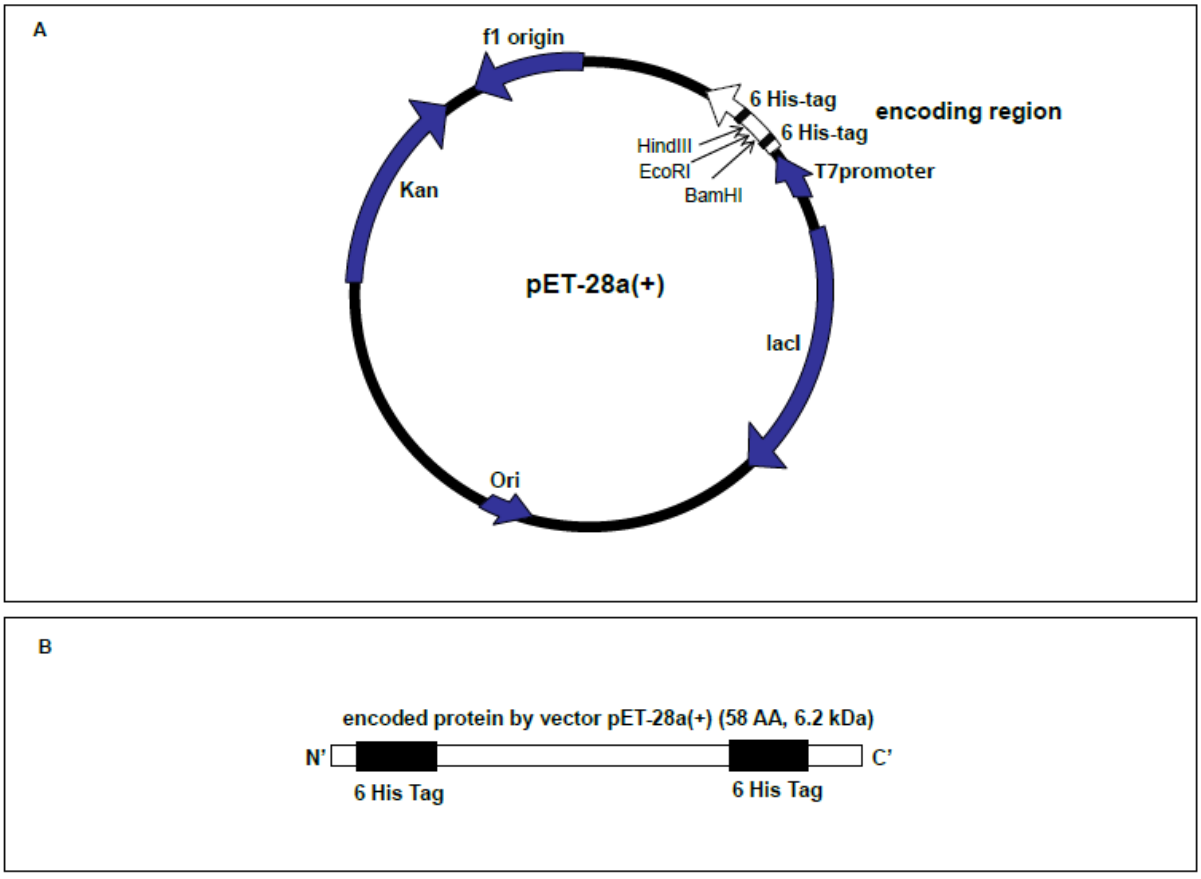


Fig. 11. Expression vector pET-28a(+). (A) Physical map of vector pET-28a(+) harboring the gene (open arrow in A) encoding the protein depicted in. (B) Schematic composition of the protein encoded by vector pET-28a(+).

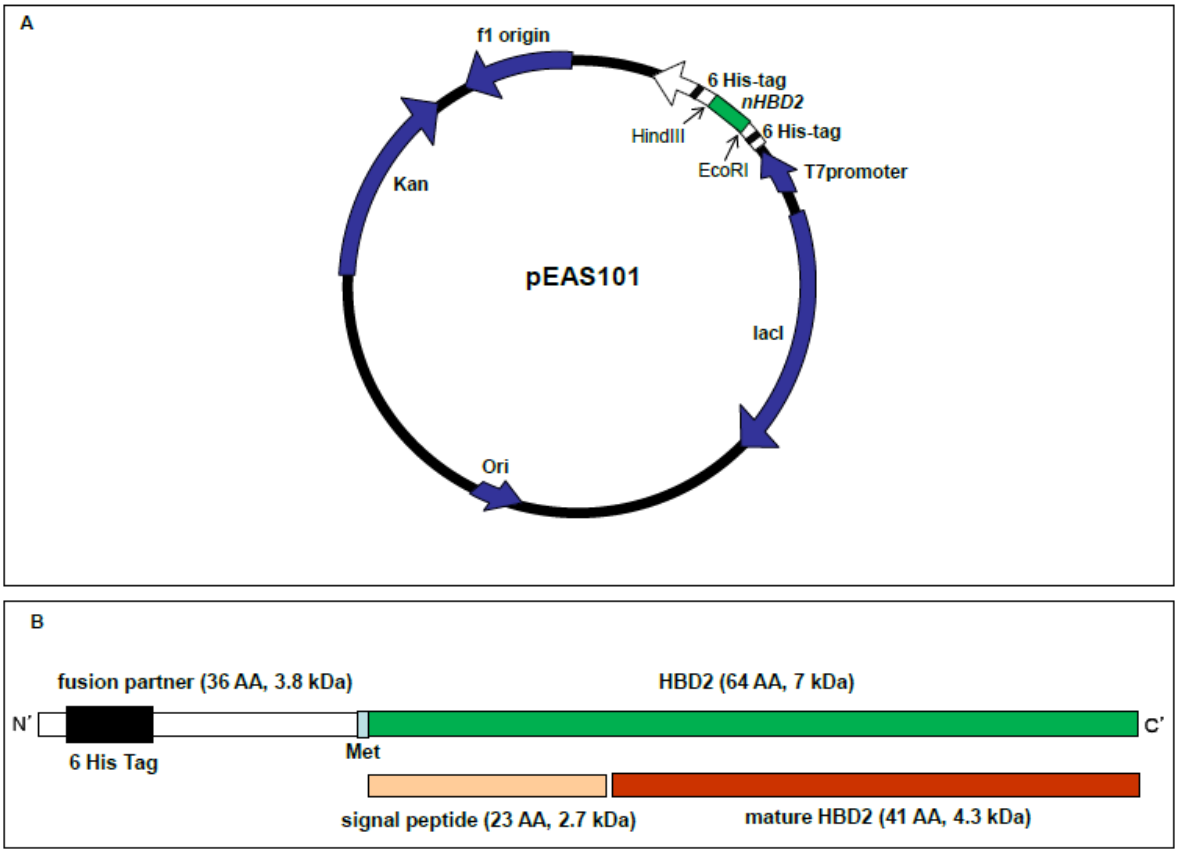


Fig. 12. Expression plasmid pEAS101. (A) Physical map of the recombinant plasmid pEAS101 encoding the fusion gene composed of the fusion partner including a His-tag and *nHBD2*-gene. (B) Schematic composition of the fusion protein HisHBD2.

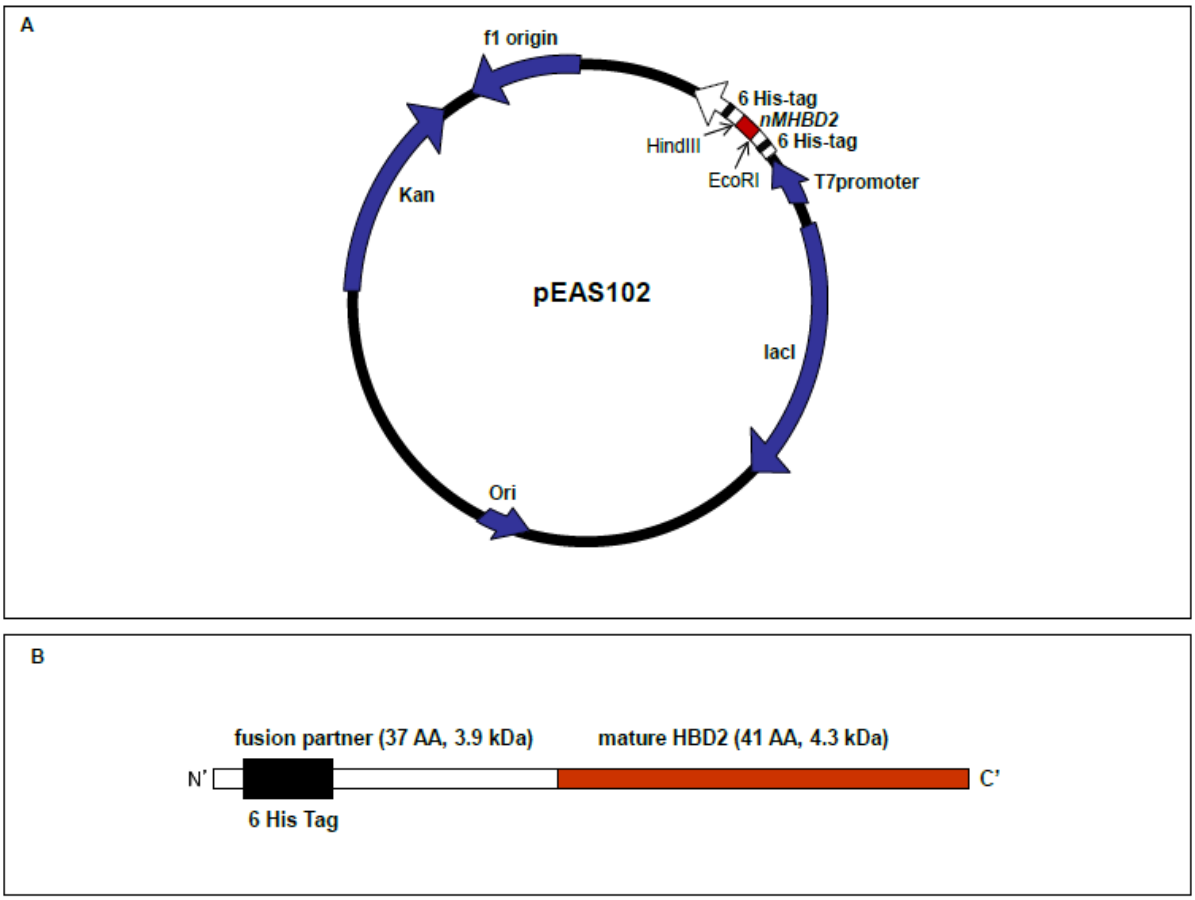


Fig. 13. Expression plasmid pEAS102. (A) Physical map of the recombinant plasmid pEAS102 harboring the fusion gene composed of the fusion partner including a His-tag and that part of *nHBD2*-gene encoding the mature form of HBD2. (B) Schematic composition of the fusion protein HisMHBD2.

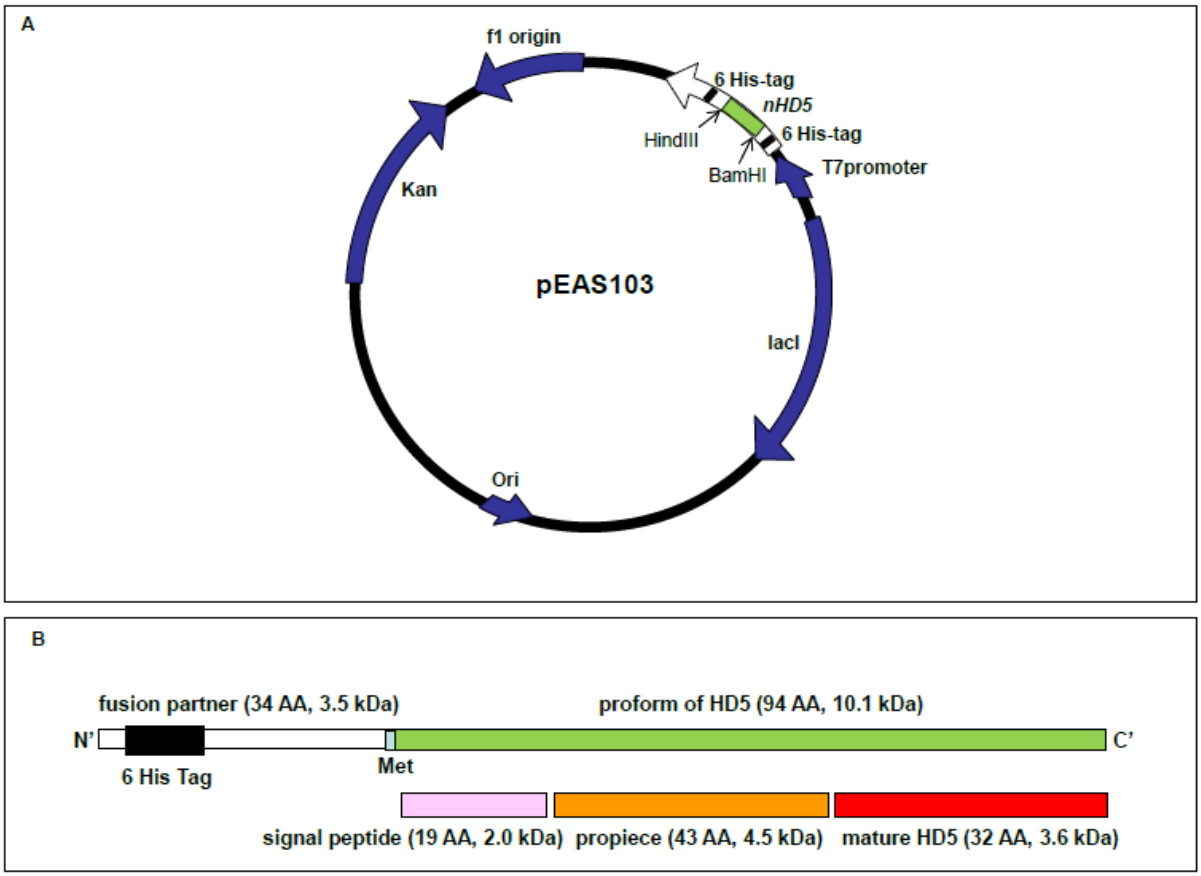


Fig. 14. Expression plasmid pEAS103. (A) Physical map of pEAS103 containing the fusion gene composed of the fusion partner with a His-tag and *nHD5*-gene. (B) Physical map of the composition of the fusion protein HisHD5.

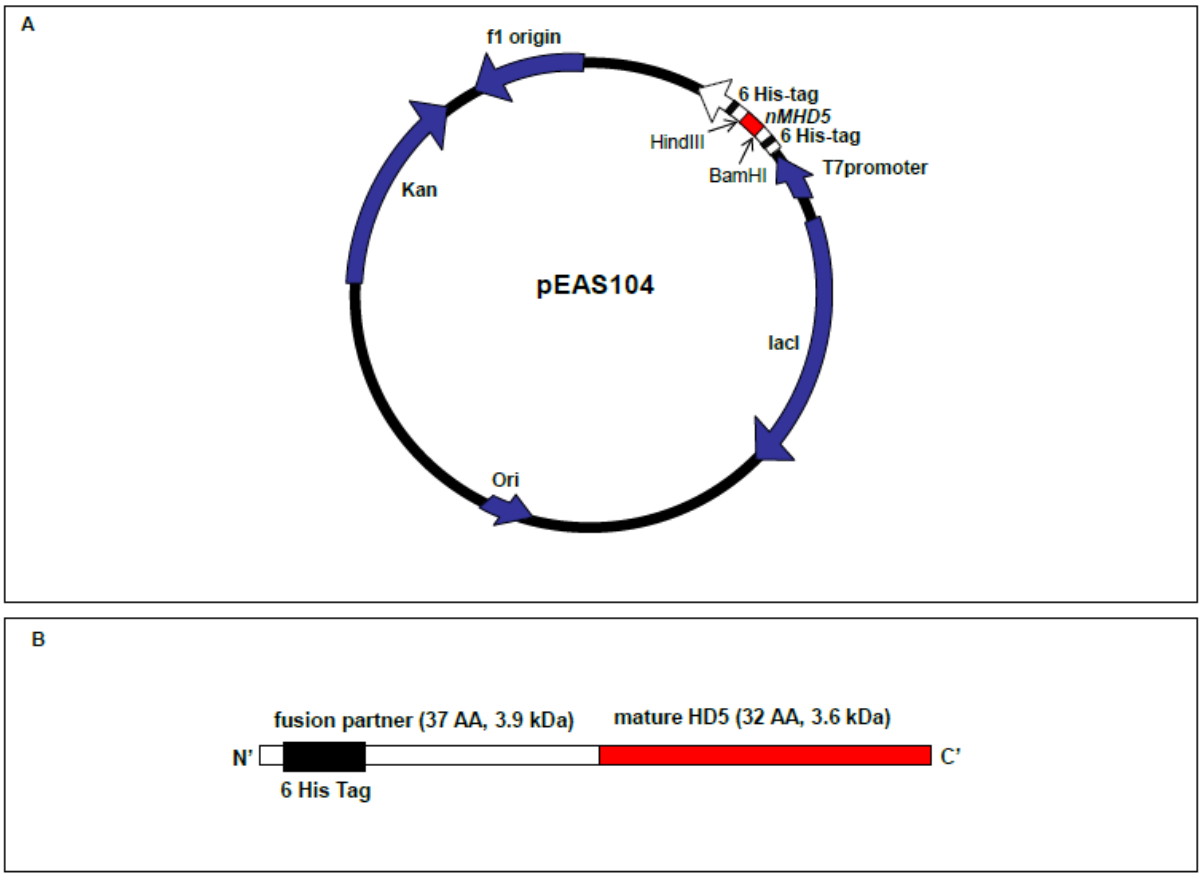


Fig. 15. Expression plasmid pEAS104. (A) Physical map of the recombinant plasmid pEAS104 encoding the fusion gene composed of the fusion partner encoding a His-tag and that part of *nHD5*-gene encoding mature HD5. (B) Fusion protein HisMHD5 and the parts it is composed of.

4.3. Expression of HBD2 and HD5 fusion proteins in *Escherichia coli*

Western blot analysis of whole cellular protein of the induced recombinant strains EcN100, EcN101 and EcN102 was performed with anti-HBD2 rabbit polyclonal antiserum for detection of HisHBD2 and HisMHBD2 expression. After cell lysis, also soluble and insoluble protein fractions were screened by Western blot analysis with HBD2 rabbit polyclonal antiserum. The negative control, EcN100, was always treated like EcN101 and EcN102 with respect to growth, induction, sample preparation, SDS-Page and Western blot.

Fig. 16. (A) Western blot analysis with anti-HBD2 rabbit polyclonal antiserum of whole cell proteins after induction of expression at 20 °C for 6 h (Samples were pellet of whole cell proteins after centrifugation and adjusted to cell density ($1.5x10^8$ cfu cells were loaded for each lane)). Lane 1: EcN100, Lane 2: EcN101. (B) Western blot analysis with anti-HBD2 rabbit polyclonal antiserum of soluble and insoluble forms of HisHBD2 expressed in EcN101 after induction at 20 °C for 6 h (Samples were adjusted to the number of bacterial cells ($1.5x10^8$ cfu)). Lane 1: supernatant of whole cell proteins after cell lysis and centrifugation and filtration (i.e. soluble protein fraction); Lane 2: pellet of whole cell proteins after cell lysis and centrifugation (i.e. insoluble protein fraction).

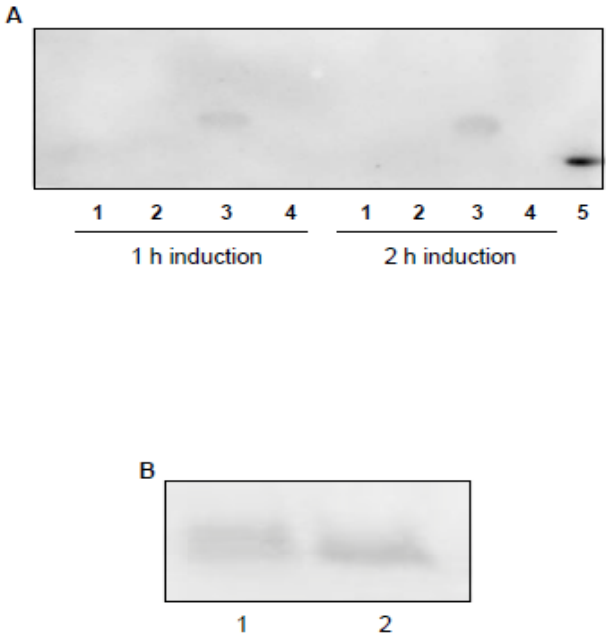


Fig. 17. (A) Western blot analysis with anti-HBD2 rabbit polyclonal antiserum of whole cell proteins after induction of expression at 37 °C (Samples were adjusted to the number of bacterial cells ($1.5x10^8$ cfu)). Lane 1: EcN100, pellet of whole cell proteins after centrifugation; Lane 2: EcN100, cell-free culture medium after centrifugation and filtration; Lane 3: EcN102, pellet of whole cell proteins after centrifugation; Lane 4: EcN102, cell-free culture medium after centrifugation and filtration; Lane 5: positive control (commercial HBD2, 1 µg). (B) Western blot analysis with anti-HBD2 rabbit polyclonal

antiserum of soluble and insoluble forms of HisMHBD2 expressed in EcN102 after induction at 37 °C for 2 h (Samples were adjusted to the number of bacterial cells ($1.5x10^8$ cfu)). Lane 1: supernatant of whole cell proteins after cell lysis and centrifugation and filtration (i.e. soluble protein fraction); Lane 2: pellet of whole cell proteins after cell lysis and centrifugation (i.e. insoluble protein fraction).

HisHBD2 expression was assayed at several time points after induction at 37°C, because in the clinical setting, after oral administration of the defensin-producing EcN strain, defensin expression should take place in the human gut at the physiological body temperature. However, HisHBD2 was not detected after induction at 37 °C at any time point (data not shown). HisHBD2 was observed after induction at 20 °C for 6 h and present in the insoluble pellet of whole cell proteins (Fig. 16). In contrast, after induction of HisMHBD2 expression at 37 °C for 2 h, HisMHBD2 was detected in the supernatant (soluble protein fraction) as well as in the pellet (insoluble protein fraction) after cell lysis (Fig. 17). No defensin protein was detected in corresponding samples of the negative control (EcN100).

Expression of HisHD5 and HisMHD5 in EcN103 and EcN104 was screened by Western blot with HD5-specific rabbit polyclonal antiserum of whole cell protein and soluble as well as insoluble protein fraction.

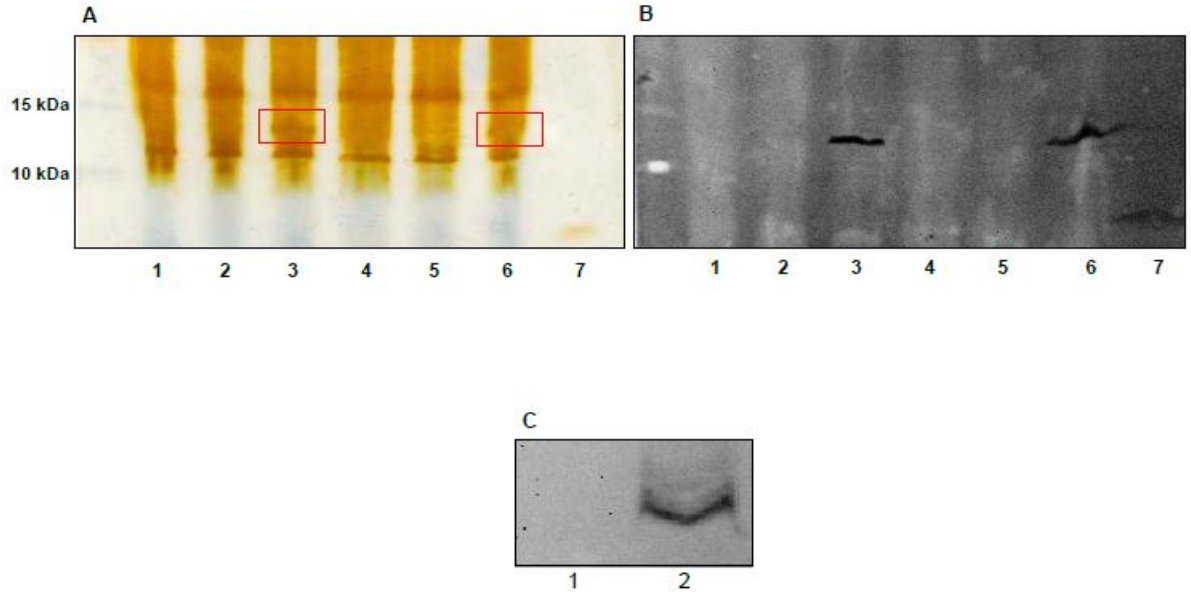


Fig. 18. (A) Silver-stained gel after SDS-PAGE (the boxes indicate expressed HisHD5) and (B) Western blot analysis with anti-HD5 rabbit polyclonal antiserum of whole cell proteins after induction of expression at 37 °C for 4 h (Samples were pellet of whole cell proteins after centrifugation, and identical optical densities (corresponding to$1.5x10^8$ cfu) were loaded for each lane). Lane 1: EcN1219, Lane 2: EcN100, Lane 3: EcN103, Lane 4: EcNc1219, Lane 5: EcNc100, Lane 6: EcNc103, Lane 7: positive control (commercial HD5, 1 µg). (C) Western blot analysis with anti-HD5 rabbit polyclonal antiserum of soluble and insoluble forms of HisHD5 expressed in EcN103 after induction at 37 °C for 4 h (Samples were adjusted to the number of bacterial cells ($1.5x10^8$ cfu)). Lane 1: supernatant of whole cell proteins after cell lysis and centrifugation and filtration (i.e. the soluble protein fraction); Lane 2: pellet of whole cell proteins after cell lysis and centrifugation (i.e. the insoluble protein fraction).

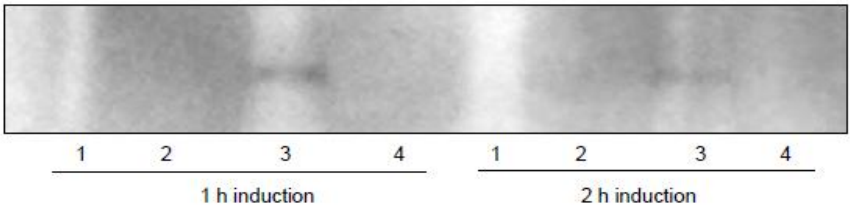


Fig. 19. Western blot analysis with anti-HD5 rabbit polyclonal antiserum of whole cell proteins after induction at 37 °C (Samples were adjusted to the number of bacterial cells ($1.5x10^8$ cfu)). Lane 1: EcN100, pellet of whole cell proteins after centrifugation; Lane 2: EcN100, cell-free culture medium after centrifugation and filtration; Lane 3: EcN104, pellet of whole cell proteins after centrifugation; Lane 4: EcN104, cell-free culture medium after centrifugation and filtration.

HisHD5 was detected after induction at 37 °C for 4 h, however, it was present in the insoluble fraction of total cellular proteins (Fig. 18). HisMHD5 was detected as well, but only a very low amount of HisMHD5 was produced after induction at 37 °C for 1 h and 2 h (Fig. 19). HisMHD5 was not detectable at all after induction at 37 °C for more than 2 h in the presence of the protease inhibitor cocktail (data not shown).

4.4. Isolation by nickel-affinity chromatography and antimicrobial activity of HisMHBD2

HisMHBD2 fusion protein expressed in EcN103 was eluted from Ni-column and subsequently dialysis and protein quantification were performed as described in Materials and Methods. The resulting material used for antimicrobial activity tests. Ni-

column eluates from EcN100 samples (negative control) were treated like eluates from HisMHBD2 samples. Antimicrobial activity of eluted proteins was evaluated by radial diffusion assays employing *E. coli* K-12 MG1655, *Salmonella enterica* serovar Typhimurium SL1344 *and Listeria monocytogenes* EGD as indicator strains.

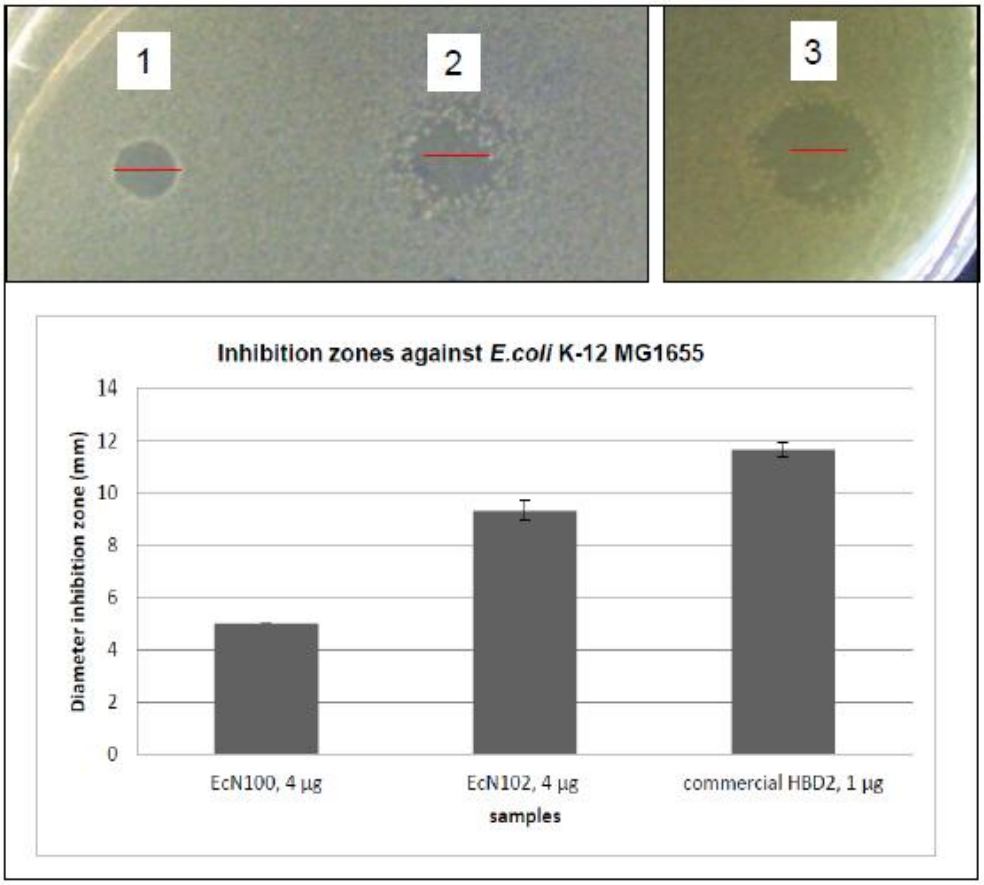


Fig. 20. The antimicrobial activity of HisMHBD2 against *E. coli* K-12 MG1655. 1: negative control (EcN100), 4 µg of fraction 0.03 eluted from Ni-column; 2: eluted proteins including HisMHBD2 (EcN102), 4 µg of fraction 0.03 eluted from Ni-column; 3: positive control, commercial HBD2, 1 µg; The lines indicate the 5 mm diameters of wells, Experiments were carried out in triplicates and the mean values and standard deviations are presented.

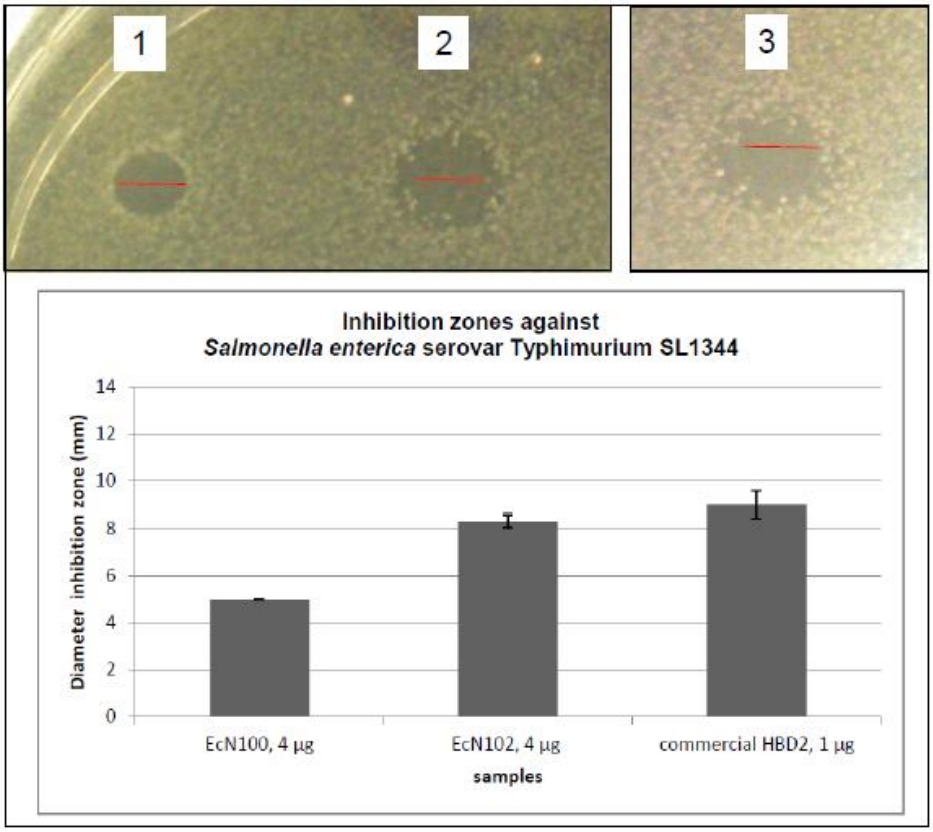


Fig. 21. The antimicrobial activity of HisMHBD2 against *Salmonella enterica* serovar Typhimurium SL1344. 1: negative control (EcN100), 4 µg of fraction 0.03 eluted from Ni-column; 2: eluted proteins including HisMHBD2 (EcN102), 4 µg of fraction 0.03 eluted from Ni-column; 3: positive control, commercial HBD2, 1 µg; The lines indicate the 5 mm diameters of wells, Experiments were carried out in triplicates and the mean values and standard deviations are presented.

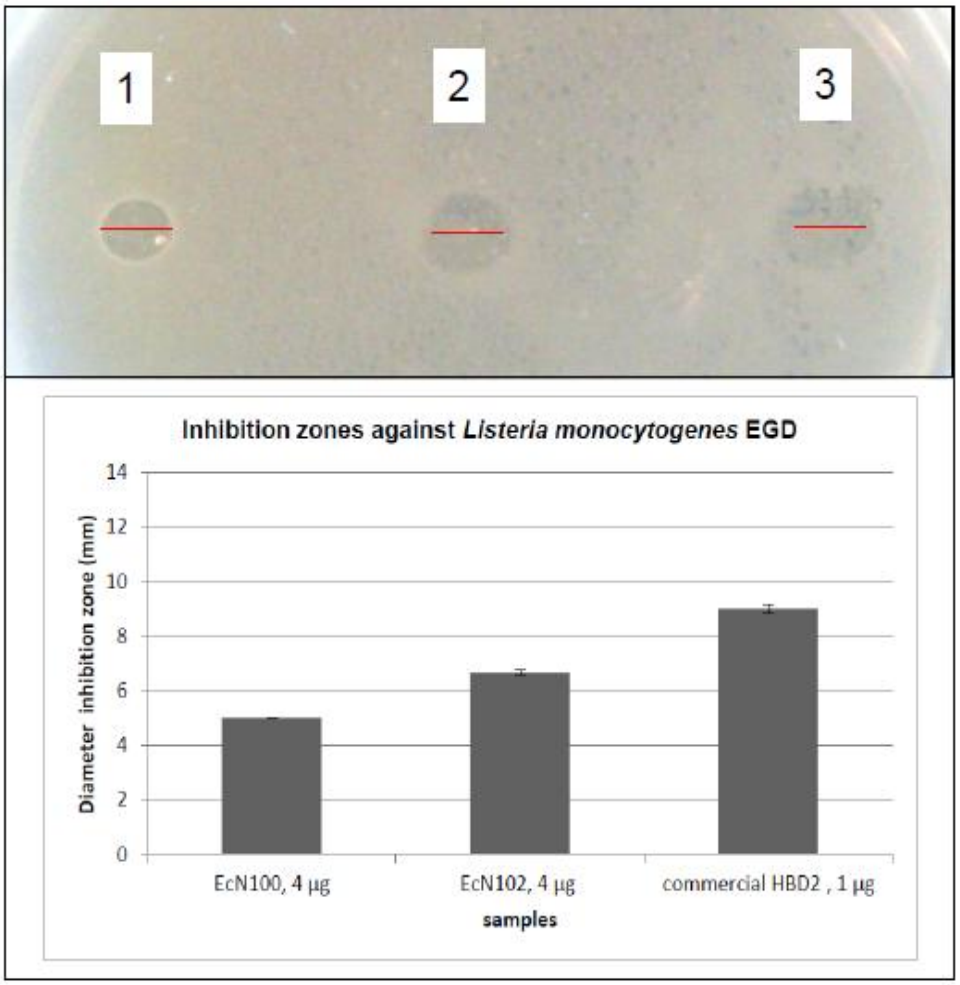


Fig. 22. The antimicrobial activity of HisMHBD2 against *Listeria monocytogenes* EGD. 1: negative control (EcN100), 4 µg of fraction 0.03 eluted from Ni-column; 2: eluted proteins including HisMHBD2 (EcN102), 4 µg of fraction 0.03 eluted from Ni-column; 3: positive control, commercial HBD2, 1 µg; The lines indicate the 5 mm diameters of wells, Experiments were carried out in triplicates and the mean values and standard deviations are presented.

Inhibition zones could be detected with eluted proteins from strain EcN102 encoding HisMHBD2 and HBD2 (the positive control) while the negative control (eluted protein from EcN100) did not show any antimicrobial effect (Figs. 20-22).

Insoluble HisHBD2 from EcN101 and HisHD5 encoded by EcN103 were examined for bioactivity after the refolding procedure by “Protein Refolding kit” (Novagen, USA), however, these protein fractions did not show any activity. HisMHD5 from EcN104 was expressed at a very low yield in the presence of the

protease inhibitor cocktail. Therefore it was not possible to obtain enough material to get a solution of comparable concentration for activity tests.

4.5. Secretion of a MHBD2 fusion protein by recombinant *E. coli* strains

E. coli protein YebF (10.8 kDa) is a soluble endogenous protein secreted into the medium and it is used as a carrier for transgenic proteins (Zhang et al. 2006). It has been shown that passenger proteins linked to the carboxyl end of YebF are efficiently secreted by the respective recombinant *E. coli* K-12 strain. Therefore, we decided to design a fusion protein consisting of the mature part of HBD2 fused to the C-terminus of YebF in order to achieve secretion of the resulting fusion protein YebFMHBD2 by the respective recombinant *E. coli* strains (Figs. 23, 25). EcN105 harbouring plasmids pAR1219 and pEAS105 encoding only *yebF* (NCBI (B1847)) was used as the negative control and treated as the recombinant strain encoding a defensin gene (Fig. 24).

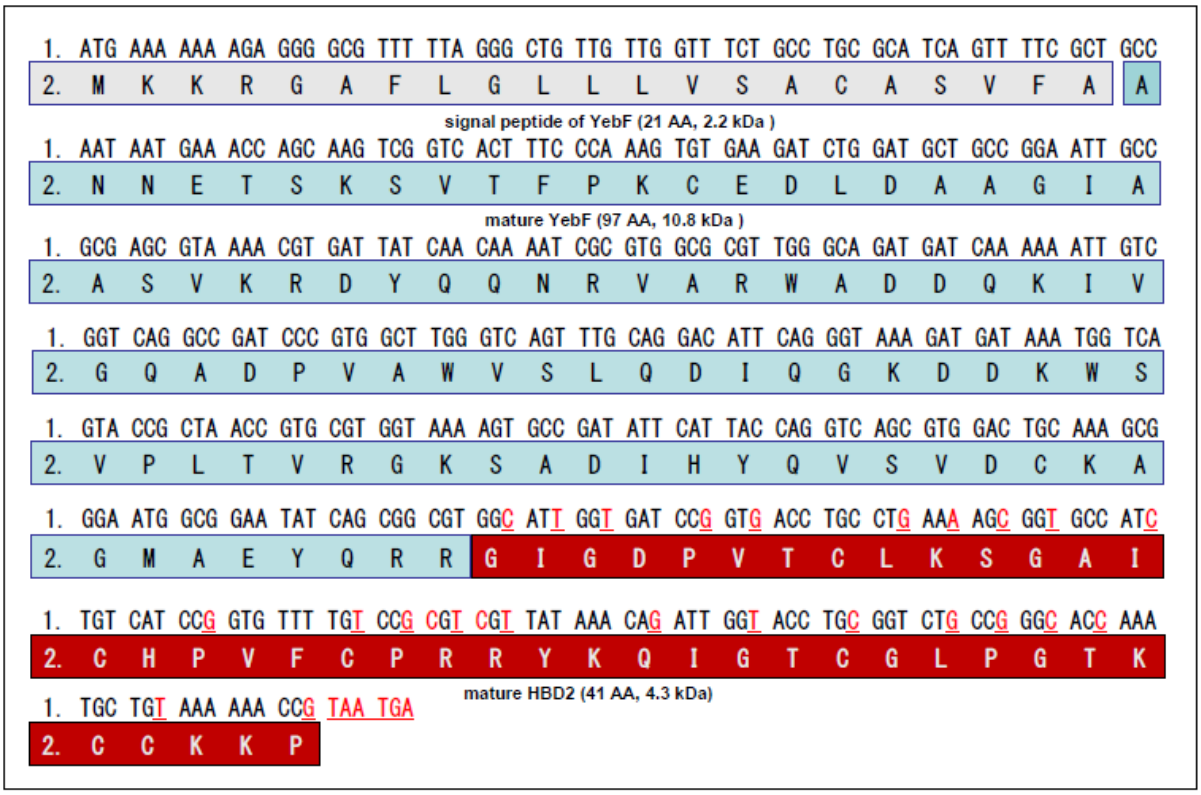


Fig. 23. DNA and amino acid sequences of the constructed fusion gene and the corresponding fusion protein YebFMHBD2; Lane 1: cDNA sequence of *yebFMHBD2*-gene; Lane 2: sequence of corresponding amino acids of YebFMHBD2.

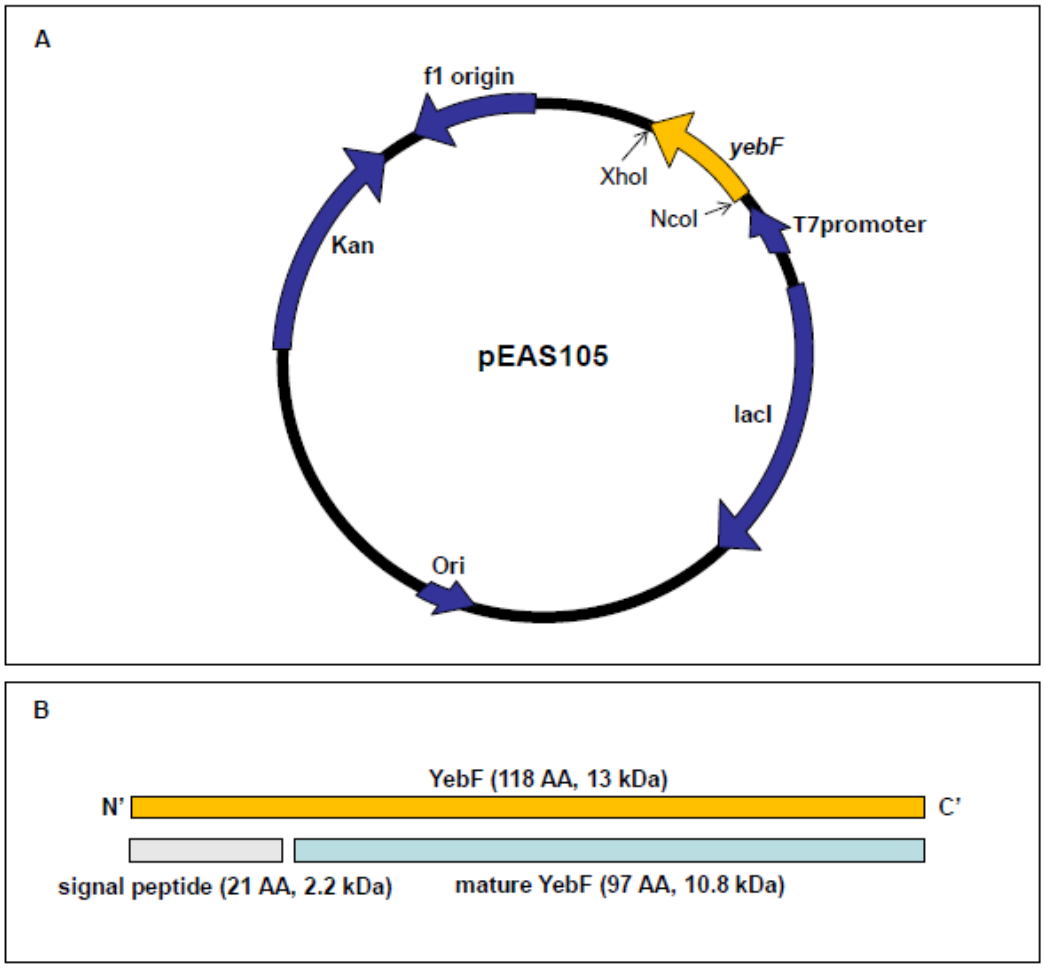


Fig. 24. Expression plasmid pEAS105. (A) Physical map of plasmid pEAS105 harboring *yebF* gene. (B) Schematic presentation of the corresponding protein YebF.

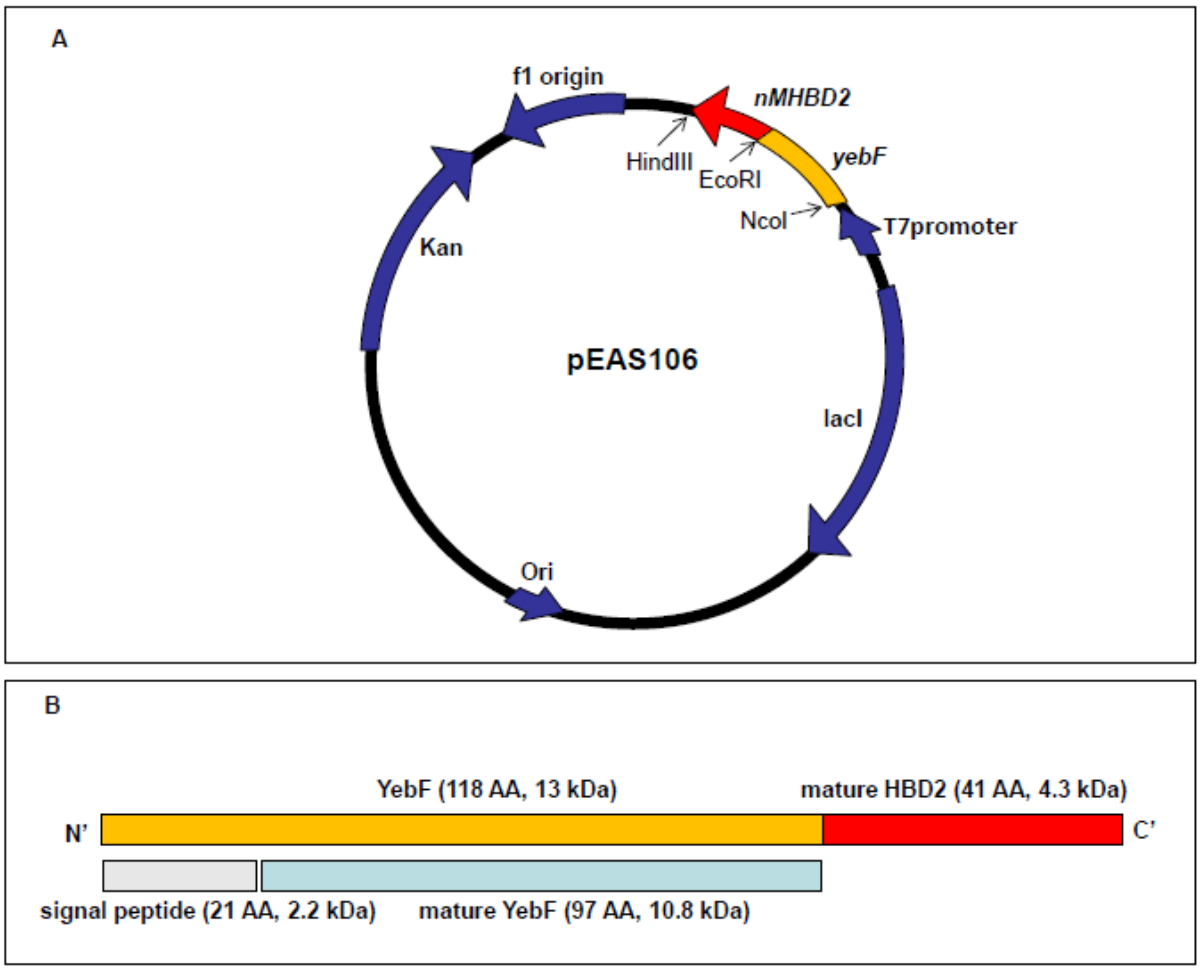


Fig. 25. Expression plasmid pEAS106. (A) Physical map of the recombinant plasmid pEAS106 encoding the fusion gene composed of the fusion partner, *yebF* and *nMHBD2*-gene. (B) Schematic composition of the fusion protein YebFMHBD2.

Secretion of YebFMHBD2 was tested by analysis of whole cellular protein and cell-free supernatant samples of induced EcN105 and EcN106 by Western blot. Since the presence of the fusion protein in the culture medium could be a result of the achieved secretion via YebF, cell lysis, leakage through the outer membrane, a natural cell secretion process, or some combination thereof, we analyzed cells and growth medium by Western blot analysis, not only with anti-HBD2 rabbit polyclonal antiserum, but also with a monoclonal antibody specific for the cytoplasmic ß-galactosidase and with a rabbit polyclonal antiserum specific for the periplasmic maltose-binding protein (MBP) (Fig. 26).

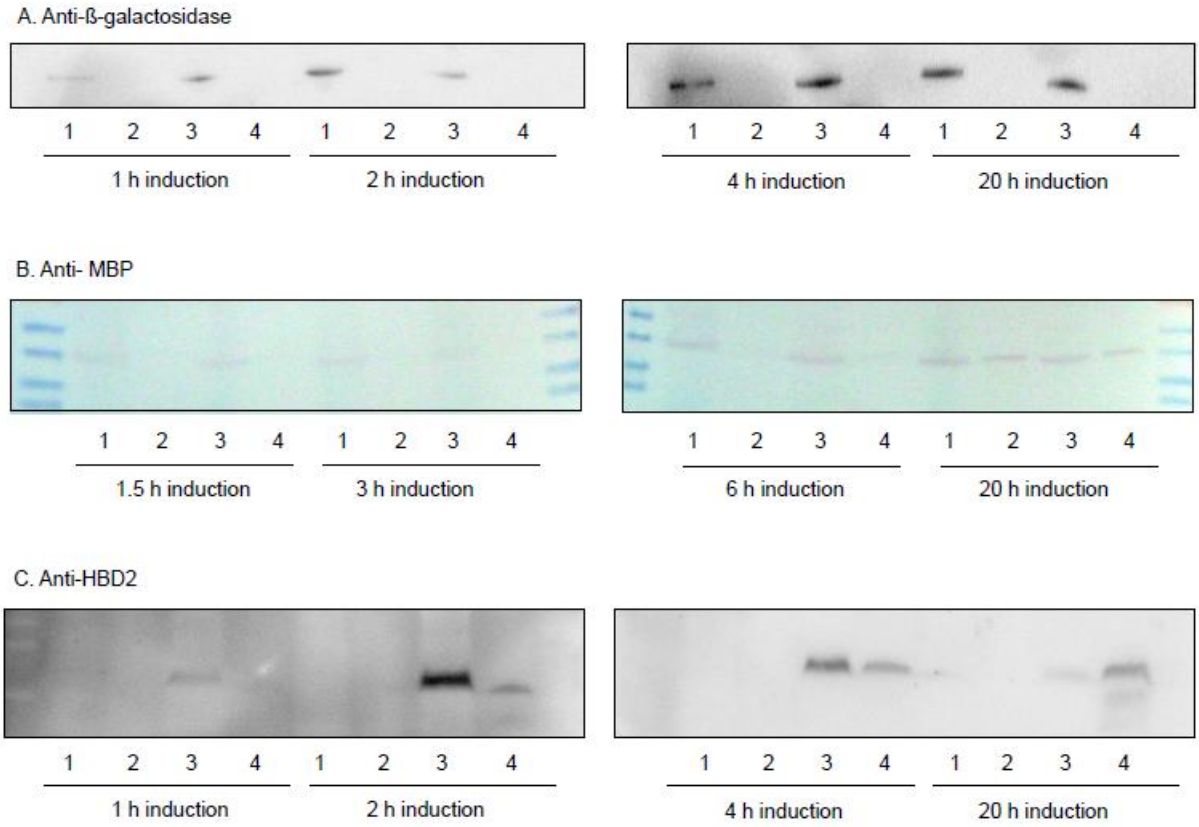


Fig. 26. Analysis of intracellular and secreted polypeptides from recombinant strains EcN105 and EcN106 (Samples were adjusted to the number of bacterial cells ($1.1x10^8$ cfu)). Lane 1: EcN105, cellular proteins (pellet after centrifugation); Lane 2: EcN105, proteins in the cell-free supernatant (supernatant after centrifugation and filtration); Lane 3: EcN106, cellular proteins (pellet after centrifugation); Lane 4: EcN106, proteins in the cell-free supernatant (supernatant after centrifugation and filtration).

YebFMHBD2 was found in the cell-free supernatant after centrifugation and sterile filtration of induced EcN106 cultures, in contrast to EcN105 cultures. Fig. 26 shows the accumulation of YebFMHBD2 in the medium during induction. In the cell-free supernatant, YebFMHBD2 was clearly detected at 2 h and 4 h after induction. After induction for 20 h, most of YebFMHBD2 was present in the medium. Two protein bands appeared at this time point, the reason might be protein degradation. In samples representing the bacterial cells, ß-galactosidase, MBP and HBD2 were readily detected. ß-galactosidase, however, was not observed in the medium at all at any time point analyzed. MBP was not detected in the medium at 1.5 h after

induction. A trace amount of MBP was detected in the medium at 3 h and 6 h after induction, which indicates the beginning of some inevitable cell lysis.

Moreover, the location of synthesized YebFMHBD2 from EcN106 was compared with the location of HisMHBD2 from EcN102 by Western blot analysis (Fig. 27).

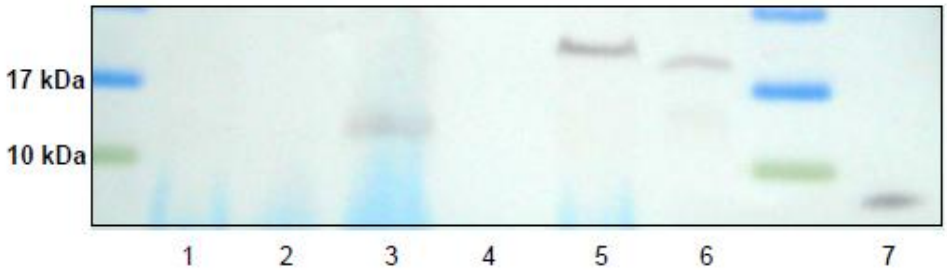


Fig. 27. Comparison of intracellular and secreted polypeptides from recombinant strains of EcN102 encoding HisMHBD2 and EcN106 encoding YebFMHBD2 at 2 h after induction by Western blot (Samples were adjusted to the number of bacterial cells ($1.1x10^8$ cfu)). Lane 1: EcN100, cellular proteins (pellet after centrifugation); Lane 2: EcN100, proteins in the cell-free supernatant (supernatant after centrifugation and filtration); Lane 3: EcN102, cellular proteins (pellet after centrifugation); Lane 4: EcN102, proteins in the cell-free supernatant (supernatant after centrifugation and filtration); Lane 5: EcN106, cellular proteins (pellet after centrifugation); Lane 6: EcN106, proteins in the cell-free supernatant (supernatant after centrifugation and filtration); Lane 7: positive control, commercial HBD2, 1 µg, The theoretical molecular masses are: for HisMHBD2 8.2 kDa, for YebFMHBD2 with signal peptide 17.3 kDa, secreted YebFMHBD2 without signal peptide 15.1 kDa and for mature HBD2 4.3 kDa.

YebFMHBD2 was detected in bacterial cells and in the culture medium, whereas HisMHBD2 was present only in the fraction representing total cellular proteins and not in the cell-free supernatant (i.e. culture medium). The fusion defensins were observed to migrate slower than expected from their theoretical molecular mass (HisMHBD2: 8.2 kDa, YebFMHBD2 with signal peptide: 17.3 kDa, secreted YebFMHBD2 without signal peptide: 15.1 kDa) in Western blot analysis (Fig. 27). Small peptides often fail to follow the standard relationship between mass and mobility in SDS-PAGE (Faurschou et al. 2005, Huang and Matthews 1990, Weber et al. 1972). Moreover, secreted YebFMHBD2 from the spent culture medium (Fig. 27.

Lane 6) was detected as a smaller protein than YebFMHBD2 from the cellular protein pool (Fig. 27. Lane 5), because YebF is cleaved after the 21-amino acid *sec*-leader during secretion process (Zhang et al. 2006).

These results demonstrate YebFMHBD2 to be secreted into the medium and not just being released by cell lysis.

4.6. Antimicrobial activity of the secreted fusion protein YebFMHBD2

Firstly, YebFMHBD2 encoded by EcN106 was tested for antimicrobial activity by radial diffusion assays. However, inhibition zones were observed with the negative control, secreted proteins from EcN105. The reason could be that EcN produces and secretes the two microcins M and H47 (Patzer et al. 2003).

Therefore, to test for antimicrobial activity of the secreted YebFMHBD2 protein supernatants of the microcin-negative isogenic SK22D strain harboring pAR1219 and pEAS106 were employed. Cell-free supernatants of induced cultures from SK22D105 and SK22D106 were obtained by centrifugation and sterile-filtration. Before antimicrobial tests by radial diffusion assays and killing assays in liquid medium were performed, the respective cell-free supernatant was dialyzed and concentrated.

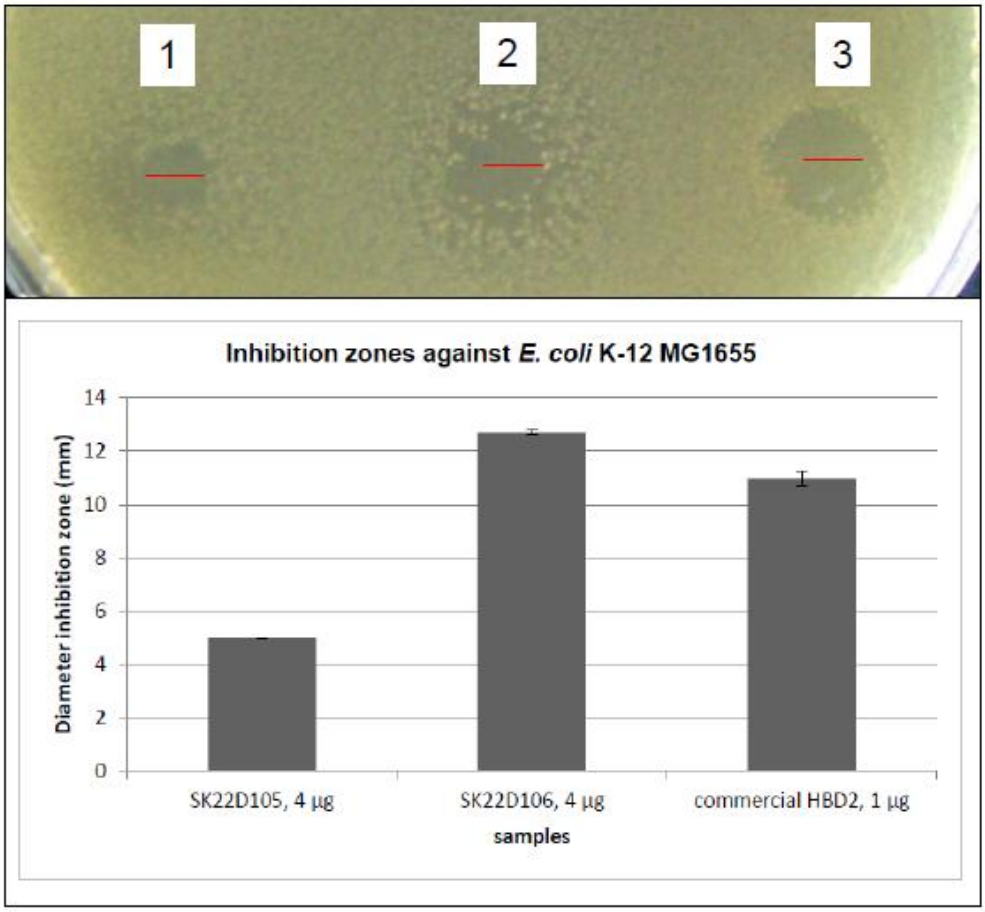


Fig. 28. The antimicrobial activity of YebFMHBD2 by radial diffusion tests with *E. coli* K-12 MG1655. 1: negative control, proteins in the cell free supernatant after centrifugation and filtration from SK22D105, 4 µg; 2: proteins in the cell free supernatant including YebFMHBD2 after centrifugation and filtration from SK22D106, 4 µg; 3: positive control, commercial HBD2, 1 µg; The lines indicate the 5 mm diameters of wells, Experiments were carried out in triplicates and the mean values and standard deviations are presented.

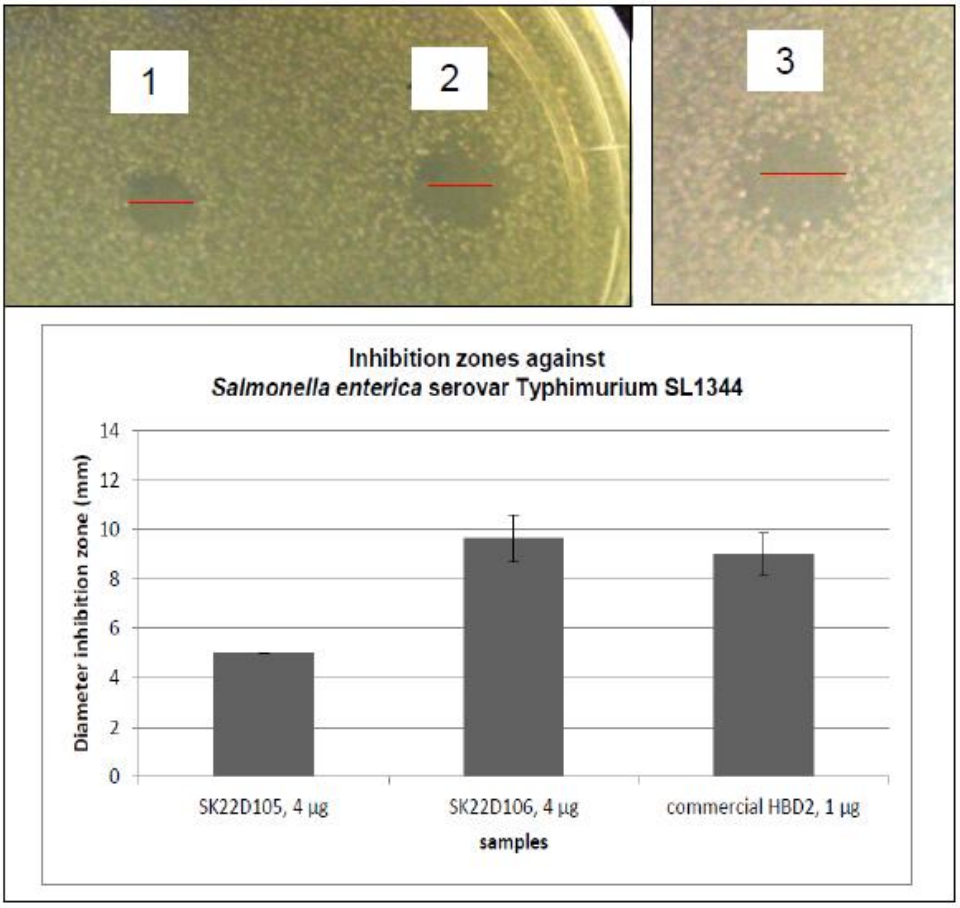


Fig. 29. The antimicrobial activity of YebFMHBD2 by radial diffusion tests with *Salmonella enterica* serovar Typhimurium SL1344, 1: negative control, proteins in the cell free supernatant after centrifugation and filtration from SK22D105, 4 µg; 2: proteins in the cell free supernatant including YebFMHBD2 after centrifugation and filtration from SK22D106, 4 µg; 3: positive control, commercial HBD2, 1 µg; The lines indicate the 5 mm diameters of wells, Experiments were carried out in triplicates and the mean values and standard deviations are presented.

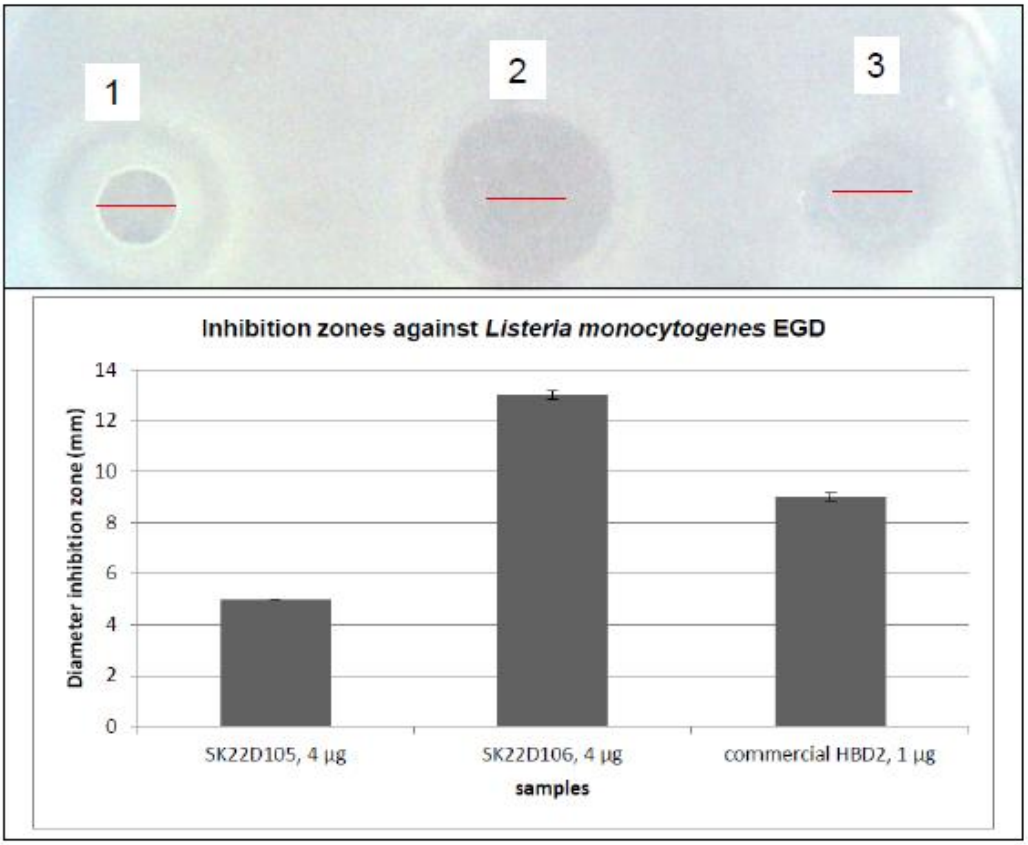


Fig. 30. The antimicrobial activity of YebFMHBD2 by radial diffusion tests with *Listeria monocytogenes* EGD. 1: negative control, proteins in the cell free supernatant after centrifugation and filtration from SK22D105, 4 µg; 2: proteins in the cell free supernatant including YebFMHBD2 after centrifugation and filtration from SK22D106, 4 µg; 3: positive control, commercial HBD2, 1 µg; The lines indicate the 5 mm diameters of wells, Experiments were carried out in triplicates and the mean values and standard deviations are presented.

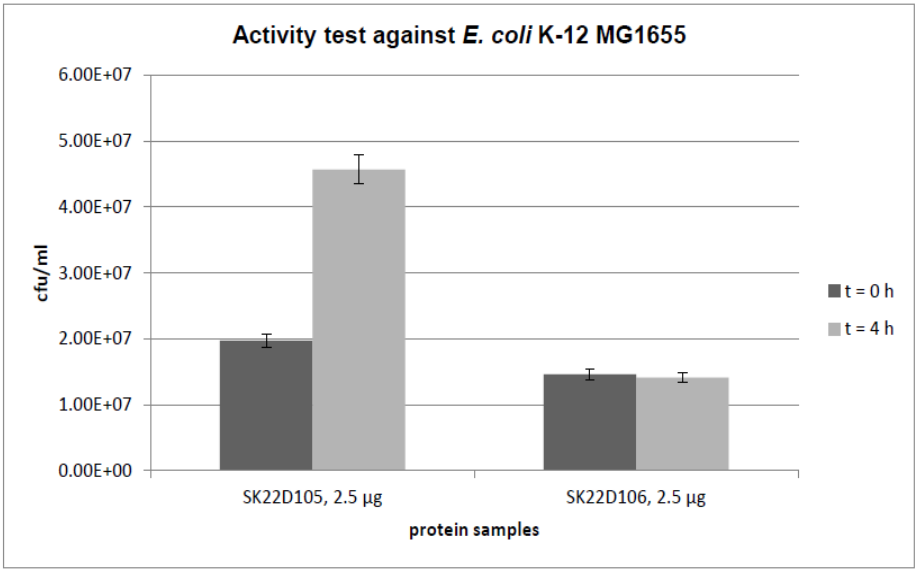


Fig. 31. Antimicrobial activity of secreted proteins from SK22D105 (negative control) and secreted proteins including YebFMHBD2 from SK22D106 against *E. coli* K-12 MG1655 as determined by killing assays in liquid medium. Experiments were carried out in triplicates and the mean values and standard deviations are presented.

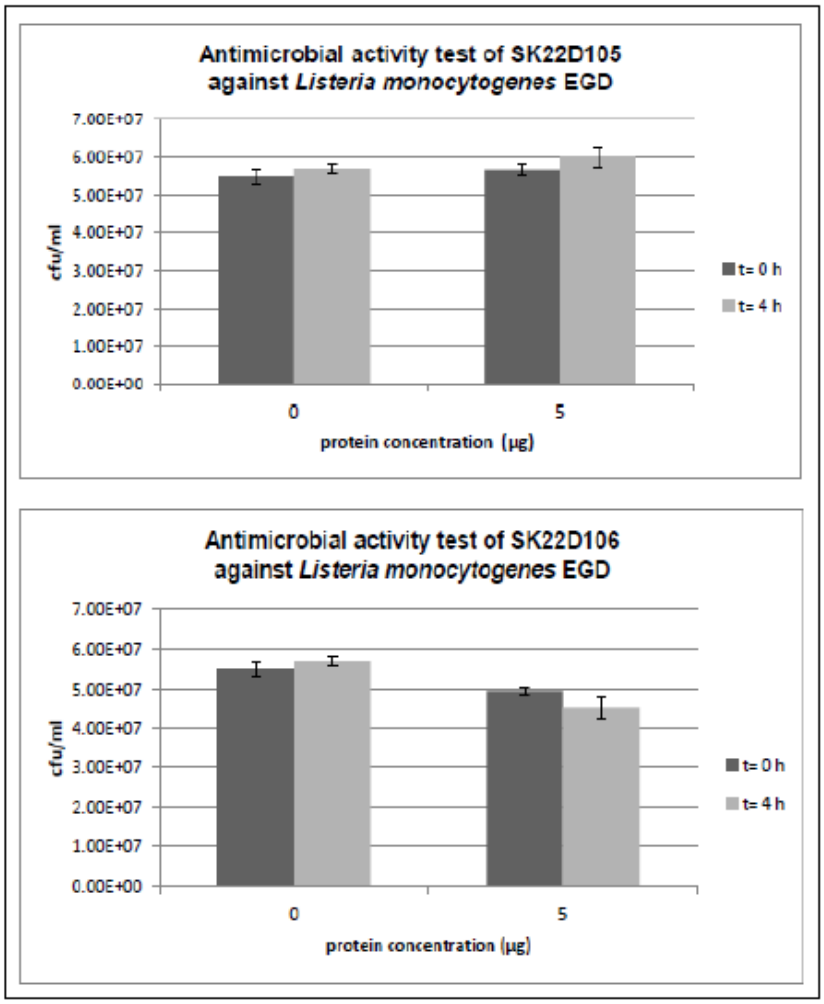


Fig. 32. Antimicrobial activity of secreted proteins from SK22D105 (negative control) and secreted proteins including YebFMHBD2 from SK22D106 against *Listeria monocytogenes* EGD as determined by killing assays in liquid medium. Experiments were carried out in triplicates and the mean values and standard deviations are presented.

Only supernatant from SK22D106 showed killing of *E. coli* K-12 MG1655, *Salmonella enterica* serovar Typhimurium SL1344 and *Listeria monocytogenes* EGD in radial diffusion assays (Figs. 28-30). The same supernatant caused growth inhibition of *E. coli* K-12 MG1655 and *Listeria monocytogenes* EGD in liquid culture (Figs. 31, 32). Since the killing assay in liquid culture was performed in the presence of only 1 % TSB medium, *E. coli* K-12 MG1655 growth was also reduced in the presence of the negative control (secreted proteins from SK22D105) after 4 h incubation.

5. Discussion

This study was undertaken to do the first step in the development of a microbial delivery vector for defensins using a well-known probiotic *E. coli* strain, *E. coli* Nissle 1917 (EcN) (Sonnenborn and Schulze 2009). We have chosen EcN because this probiotic has an excellent biosafety profile and is used in medical practice since 1917. Moreover, it is marketed as a licensed drug in 8 European countries as well as in Iran, Lebanon, Peru and South Korea.

5.1. Construction and expression of cationic antimicrobial peptides, i.e. defensins in *E. coli*

To achieve expression of small cationic peptides with antibacterial activity in bacteria, two major barriers must be overcome. These include the potential ability of the cationic peptides to kill the producing strain and the susceptibility of the cationic peptides to proteolytic degradation. Piers and coworkers found that they could overcome such barriers by using a fusion protein expression system (Piers et al. 1993).

Efficient expression of defensin genes in *E. coli* is not achieved with the original DNA sequence, because of different codon usage in humans and in *E. coli*. Therefore, the defensin genes to be cloned were optimized for expression in *E. coli*. It was shown that codon optimization can significantly improve the expression level. About nine-fold improvement of cellular soluble HBD2 expression was observed by optimizing the HBD2 gene with *E. coli*-preferred codons, as reported by Peng and co-workers (Peng et al. 2004). Therefore, two new sequences encoding human beta-defensin 2 (*nHBD2*) and human alpha-defensin 5 (*nHD5*) were synthesized with the codon-preference of *E. coli*.

In this study, the first defensin to be cloned and produced in EcN was human alpha-defensin 5 (HD5), which together with human alpha-defensin 6 is specifically reduced in small intestinal Crohn's disease (Wehkamp and Stange 2010). We have

chosen HD5 because it is encoded as an inactive proform and production of the inactive proform of HD5 should not affect viability of EcN. The other defensin selected for production by EcN was HBD2. This defensin is induced in Caco-2 cells by EcN via its flagellin and its induction in Crohn's colitis is typically impaired (Gersemann et al. 2008, Schlee et al. 2007).

In a first approach, we tried to express human α-defensin 5 (HD5) after cloning the optimized gene in pMUT1-Tc, which is a derivate of the very stable cryptic plasmid pMUT1 of EcN. The recombinant plasmid constructs designed harboured the HD5 gene under the control of one of several promoters such as *nirB*, *bla, lac* and *acrB. nirB* promoter is active under anaerobic condition and *bla* promoter is a constitutive one. And *lac* promoter is inducible by IPTG and *acrB* promoter is expressed under stress conditions. Since pMUT1 is the very stable cryptic plasmid of WT EcN and several promoters employed in this study in order to control of expression of HD5 into pMUT1-Tc might be suitable for production of defensins in the human gut condition, we decided to attempt to construct recombinant plasmids with this plasmid and these promoters. Moreover, the low-copy number expression vectors pCON3-86B, pCON7-71 and pCON7-74 were applied which utilize the lactose promoter/operator system and allow the stable maintenance and regulated expression of highly toxic gene products (Anthony et al. 2004). HD5 expression was induced by adding 1.0 mM IPTG to the grown culture at OD_{600} of 1.0 and culture was incubated at 37 °C for 18 h. However, expression of the target defensin was not detected with these constructs by Western blot. The promoters applied might not be proper to produce HD5. It was reported that the many different types of promoters can affect the level of gene expression in *E. coli*. The suitability of promoters for high level gene expression is governed by several criteria. First, the promoter must be strong, capable of protein production in excess of 10-30 % of the total cellular protein. Second, the promoter should exhibit a minimal level of basal transcription; a highly repressible promoter is particularly important for cases in which the protein of interest is toxic or detrimental to the growth of the host cell. Third, promoters should be capable of induction gene (Hannig and Makrides 1998). Besides, the strength of different promoters is determined by the relative frequency of transcription initiation. This is mainly affected by the affinity of the promoter sequence for RNA polymerase

(Tegel et al. 2011). Since several promoters which we tried do not meet all criteria for high level gene expression in *E. coli*, a yield of expression of the target protein might not be enough to be detected via Western blot analysis.

Many promoter systems of *E. coli* are described as tools for protein expression, but only a few of them are commonly used (Table 7). The expression system based on T7 RNA polymerase promoter was employed in this study which allowed expression of HD5 is the most powerful according to Terpe (Terpe 2006).

The preferential codon optimized HD5 (*nHD5*) gene was checked for expression in a cell free transcription/translation system under the control of the T7 RNA polymerase promoter using "PURExpress *In Vitro* Protein synthesis Kit (NEB)". The result showed that the gene sequence encodes an expressible gene.

Since we confirmed that *nHD5* gene was able to be expressed under the control of the T7-RNA-polymerase, in a second approach high copy number vectors of the pT7 series were used as another expression vector system into which genes under the control of the T7-RNA-polymerase can be cloned. This approach was successful and expression of the proform of HD5 in *E. coli* K-12 BL21 harbouring recombinant pT7-3 with the *nHD5* gene by Western blot could be demonstrated.

This expression system is one of the most widely used ones in *E. coli* (Studier and Moffatt 1986). The T7-RNA-polymerase is very selective and efficient, resulting both in a high frequency of transcription initiation and effective elongation. These features result in a RNA elongation that is approximately five-fold faster than for *E. coli* RNA polymerase; hence, the T7 promoter is a much stronger promoter than *E. coli* promoters (Golomb and Chamberlin 1974). The T7-RNA-polymerase that is able to bind and start transcription from the phi10 promoter from bacteriophage T7. This promoter is present in the plasmid vectors pT7-3 and pT7-4 employed in this study.

The combination of the IPTG-inducible T7-polymerase gene and the gene of interest cloned in pT7-3 or pT7-4 under the control of the phi10 promoter results in high level protein expression. However, as reported by Tegel et al. the fraction of soluble protein is rather small in contrast to the expression by the very weak

promoter *lac*UV5, which results in very low protein amounts but with the largest fraction of soluble protein (Tegel et al. 2011).

According to our study, defensin genes may be necessary to be under the control of the promoter resulting in high protein yields.

Table 7. *E. coli* promoter systems that are in use for heterologous protein production and their characteristics (modified from Terpe 2006).

Expression system based on	**Induction (range of inducer)**	**Level of expression**	**Key features**	**Original reference**
lac promoter	Addition of IPTG 0.2 mM (0.05~2.0 mM)	Low level up to middle	Weak, regulated suitable for gene products at very low intracellular level Comparatively expensive induction	(Gronenborn 1976)
trc and *tac* promoter	Addition of IPTG 0.2 mM (0.05~2.0 mM)	Moderately high	High-level, but lower than T7 system regulated expression Comparatively expensive induction High basal level	(Brosius et al. 1985)
T7 RNA polymerase	Addition of IPTG 0.2 mM (0.05~2.0 mM)	Very high	Utilizes T7 RNA polymerase High-level inducible over expression T7*lac* system for tight control of induction needed for toxic clones Relatively expensive induction Basal level depends on used strain (pLys)	(Studier and Moffatt 1986)
Phage promoter P_L	Shifting the temperature from 30 to 42 °C (45 °C)	Moderately high	Temperature-sensitive host required Less likelihood of “leaky” uninduced expression Basal level, high basal level by temperatures below 30°C No inducer	(Elvin et al. 1990)

tetA promoter/operator	Anhydrotetracycline 200 µg/l	Variable from middle to high level	Tight regulation Independent on metabolic state Independent on *E. coli* strain Relative in expensive inducer Low basal level	(Skerra 1994)
araBAD promoter (P_{BAD})	Addition of L-arabinose 0.2 % (0.001-1.0 %)	Variable from low to high level	Can fine-tune expression levels in a dose-dependent manner Tight regulation possible Low basal level Inexpensive inducer	(Guzman et al. 1995)
rhaP$_{BAD}$ promoter	L-rhamnose 0.2 %	Variable from low to high level	Tight regulation Low basal activity Relative expensive inducer	(Haldimann et al. 1998)

Therefore, we made use of the low copy number vector, pET-28a(+) expression vector, for cloning defensin genes. The defensin gene was ligated in this vector in a way that gives rise to a fusion gene encoding a protein consisting of an N-terminal peptide harboring a His-tag and the defensin. In addition, the cloning of the respective defensin gene was performed in a way that allowed the expression of the fusion gene under the control of the phi10 T7-polymerase-specific promoter. The T7 polymerase gene, the expression of which is IPTG-inducible, was provided by plasmid pAR1219. Both plasmids, pAR1219 and the recombinant pET-28a(+), were introduced into WT EcN and the EcN strain EcNc cured of both of its cryptic plasmids pMUT1 and pMUT2. To reduce the risk of killing the producer strain of the His-tagged defensin induction of expression was performed in Turbo medium containing 86 mM NaCl. As reported by Xu and colleagues (Xu et al. 2006), this concentration of NaCl led to the survival of 80 % of an *E. coli* D31 culture producing HBD2, in contrast to only 40 % survival in a culture without NaCl. In our hands, these measures helped the recombinant EcN strains to grow even during expression of the recombinant defensin proteins.

Another potential problem, defensin degradation, was avoided by adding a protease inhibitor cocktail during incubation. A further approach to impede protein degradation in the absence of a protease inhibitor cocktail is the deletion of genes encoding proteases such as OmpT and Lon in EcN to construct a suitable strain for protein expression comparable to *E. coli* K-12 strains BL21 (DE3) and KRX (Derbise et al. 2003). This will be part of the ongoing work to optimize defensin production by EcN.

Western blots demonstrated successful expression of HBD2 and HD5 as fusion proteins with a His-tag at the N terminus in probiotic EcN under the control of the T7 promoter. The recombinant EcN strains harbored the recombinant plasmid pET-28a(+) and the T7-polymerase encoding plasmid pAR1219. The full-length HBD2 fusion protein (HisHBD2) was mainly present in the insoluble protein fraction, but the fusion protein containing the mature part of HBD2 (HisMHBD2) was detected in the soluble and insoluble protein fraction. This is in accordance with an earlier study showing mature HBD2 without signal peptide to have a higher solubility than full-length HBD2 (Xu et al. 2006).

This is the consequence of the presence of the signal peptide being rich in hydrophobic amino acids (78.3 %) in comparison with the mature part of HBD2 (34.1 %). The high hydrophobicity of the signal peptide resulted in the lower solubility of HisHBD2 protein, because the exposure of the hydrophobic part might induce the aggregation. The signal peptide might also reduce the folding rate or accuracy of disulfide bonds in that part of the molecule representing mature HBD2.

The proform of HD5 fusion protein (HisHD5) consisting besides the part of the fusion partner with the His tag of the HD5 signal sequence, of the propiece and of the piece representing the mature HD5, was found only in the insoluble protein fraction without showing bioactivity. Only very low amount of the fusion protein with the mature HD5 (HisMHD5) could be detected in EcN. Since the signal peptide of HD5 also contains a high percentage of hydrophobic amino acids (78.9 %), this might be the cause for the insolubility of HisHD5. Production of the recombinant mature forms of α-defensins is considered challenging, since the presence of an anionic pre-piece is believed to be important for the inhibition of cytotoxicity of the cationic peptides (Valore et al. 1996). It was reported that the expression of several human alpha-defensins (hNP-1, hNP-2, hNP-3, hD-5 and hD-6) in bacteria as mature fusion defensins containing a portion of the tryptophan operon of *E. coli* including a His tag (111 AA) did not result in soluble proteins, since they were mainly found in inclusion bodies (Pazgier and Lubkowski 2006).

Moreover, α-defensins could be produced only with low yields by engineered bacterial systems, and α-defensins were sensitive to rapid degradation by bacterial proteases (Piers et al. 1993, Valore and Ganz 1997). Up to now, successful expression of human alpha-defensins in soluble and active form in *E. coli* has not been reported.

5.2. Antimicrobial activity of expressed fusion mature HBD2 (HisMHBD2)

In this study, mature fusion HBD2 (HisMHBD2) was produced in a soluble form. It is well-known that soluble recombinant proteins are often correctly folded and are usually bioactive (Sorensen H. P. and Mortensen 2005). Therefore, the heterologous expression in bacteria resulting in a soluble protein is very attractive. In

fact, the soluble HisMHBD2 obtained in this study showed antimicrobial activity in radial diffusion assays against *E. coli* K-12 MG1655, *Salmonella enterica* serovar Typhimurium SL1344 and *Listeria monocytogenes* EGD. According to the antimicrobial mechanism of action of defensins, cationic parts of the peptides are capable of interacting with negatively charged structures of the bacterial membrane and then lead to its permeabilization (Ganz 1999, Peschel 2002). HisMHBD2 is cationic containing the fusion partner (pI 8.0), therefore, even the fusion protein HisMHBD2 seems to be able to interact with the cell membranes of microbes. However, as other modes of defensin action are discussed, we cannot exclude other mechanisms of antimicrobial activity exhibited by HisMHBD2 (Yount et al. 2009).

5.3. Secretion of a MHBD2 fusion protein by recombinant *E. coli* strains

Although leakage or lysis of EcN bacteria in the human gut might occur and could be sufficient to release HBD2 in significant amounts we constructed a recombinant EcN stain encoding a fusion protein consisting of YebF and the mature part of HBD2, in order to achieve secretion of YebFMHBD2 product. The carrier protein YebF was used as the fusion partner since “passenger” proteins linked to the C-terminus of YebF were reported to be efficiently secreted from *E. coli* K-12 strains (Zhang et al. 2006). As expected, secretion of YebFMHBD2 from recombinant EcN strains could be demonstrated and we could show that the appearance of this fusion protein in the cell-free supernatant was not due to cell lysis at early time points. Occurrence of cell leakage/lysis was checked by Western blots specific for the cytoplasmic ß-galactosidase and the periplasmatic maltose-binding protein in samples from bacterial cell extracts and samples from the cell-free culture supernatants. The results obtained with YebF resembled closely those reported in the publication by Zhang and colleagues (Zhang et al. 2006). They compared the release of YebF and maltose-binding protein (MBP).

In their study, YebF was present in the culture medium at 1.5 h and 3 h after induction, whereas MBP was not present in the medium at 1.5 h and was hardly detected in the medium at 3 h after induction. After induction for 6 h, MBP was primarily localized in the cellular fraction, although some leakage into the culture

medium was observed. In contrast, YebF was almost entirely present in the culture medium at 6 h after induction. The authors believed that these data support YebF to be secreted rather than leaking across the outer membrane. We achieved comparable results for the location of MBP. Moreover, we observed that YebFMHBD2 was present in the culture medium at 2 h and 4 h after induction, and its main amount was present in the medium at 20 h after induction. In addition, the location of cytoplasmic ß-galactosidase protein was analyzed in our study, and this protein was only detected in the cell fraction but not in the medium. Moreover, in comparison to the location of YebFMHBD2, HisMHBD2 was only present in the cells at 2 h after induction, whereas YebFMHBD2 was present at this time point in the cells and in the culture medium as well. These results demonstrated that fusion of MHBD2 to the C-terminus of YebF led to efficient secretion of the fusion protein from the producing bacterial cells.

5.4. Antimicrobial activity of secreted YebF-mature HBD2 fusion protein (YebFMHBD2)

Interestingly, cell-free supernatant samples from bacterial cultures expressing YebFMHBD2 had antimicrobial activity. Such samples in contrast to control samples of cultures expressing only YebF inhibited growth of the Gram-negative strains *E. coli* K-12 MG1655 and *Salmonella enterica* serovar Typhimurium SL1344 as well as of the Gram-positive *Listeria monocytogenes* strain EGD. Since secreted YebFMHBD2 without signal peptide is cationic including the fusion partner (theoretical pI 8.93), thus, the fusion protein YebFMHBD2 is assumed to interact against negatively charged structures of the bacterial membrane. Obviously, at least part of the YebFMHBD2 protein becomes folded after secretion in a way that allows the MHBD2-part to execute its antimicrobial activity. It can be speculated that MHBD2 is released from YebFMHBD2 in the human gut by the activity of trypsin as was shown for the proteolytic activation of the inactive proHD5. Trypsin cleaves after arginine and lysine residues and two arginine residues are the last two amino acid residues of the YebF-part in YebFMHBD2.

Zhang and colleagues also found that by using YebF, e. g. as YebF-hIL2 (human interleukin-2, 15 kDa and very hydrophobic), YebF-α-amylase (α-amylase,

48 kDa and hydrophilic) and YebF-alkaline phosphatase (94 kDa), YebF could carry these fusion proteins in their active states out of the producing bacterial cell and into the medium (Zhang et al. 2006).

They compared the simultaneous release of YebF-amylase and alkaline phosphatase, a well-defined periplasmic enzyme, from cells hosting pYebF-AmyH_6. As alkaline phosphatase is induced by phosphatate starvation, it is poorly expressed in *E. coli* grown in rich medium containing inorganic phosphate salts. This study showed that 42.6 % of YebF-α-amylase was secreted to the medium compared to only 5.6 % of alkaline phosphatase (Zhang et al. 2006). Moreover, activity assays for the YebF-hIL-2 fusion protein based on the growth of cytotoxic T-lymphocyte line (CTLL)-2 *in vitro* showed the secreted protein in the medium to be active (43,800 units of hIL-2/ml) (Zhang et al. 2006).

These data are in agreement with our results that YebF has the ability to carry proteins of varying size and hydrophobicity/hydrophilicity from the cytoplasm of *E. coli* cells into the medium where the fusion protein shows the activity of the passenger protein.

5.5. Concluding remarks

This study showed the successful construction of recombinant strains of the probiotic *E. coli* Nissle 1917 which not only were able to produce HD5 and HBD2 defensin proteins but also produced antimicrobially active mature HBD2. Furthermore, a microcin-negative EcN mutant harboring plasmid pEAS106 secreted a fusion protein consisting of YebF and the mature part of HBD2 into the culture medium, and the secreted fusion protein even showed growth inhibition of *E. coli*, *Salmonella* and *Listeria monocytogenes*.

In contrast to earlier reports about defensin production in *E. coli* K-12 strains (Huang et al., 2006; Pazgier and Lubkowski, 2006; Xu et al., 2006), this study reports for the first time the production of active HBD2 defensin in a probiotic *E. coli* strain (Nissle 1917), which is licensed as a drug for the treatment of patients suffering from e.g. diarrhea or ulcerative colitis. This probiotic strain is a good transient colonizer of

the human gut as opposed to *E. coli* K-12 strains which are not able to survive in the human digestive tract. Furthermore, to our knowledge, this is also the first study showing not just production but also secretion of mature HBD2 by an *E. coli* strain which showed activity against pathogenic *Salmonella* and *Listeria* strains.

These constructs and the results achieved with them laid the foundation for the development of EcN as a delivery vehicle for defensins to patients suffering from a deficiency of such antimicrobial peptides. In Crohn´s disease, for example, the induction of defensin molecules, such as HBD2, is impaired, in part due to a genetic defect resulting in reduced amounts of HBD2 in the gut (Wehkamp and Stange 2006). Therefore, targeted delivery of HBD2 by a recombinant EcN-strain producing this defensin locally in the gut after oral administration could have a therapeutic effect in Crohn's disease patients.

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Supplementary results

Table S1. Diameters of radial diffusion assays for antimicrobial activity tests of HisMHBD2 against *E. coli* K-12 MG1655.

Protein samples	Diameters (mm)		
Negative control, EcN100 [a]	5	5	5
EcN102 [b]	8	8	8
commercial HBD2 [c]	13	11	11

[a] negative control (EcN100), 4 µg of fraction 0.03 eluted from Ni-column

[b] eluted proteins including HisMHBD2 (EcN102), 4 µg of fraction 0.03 eluted from Ni-column

[c] positive control, commercial HBD2, 1 µg

Table S2. Diameters of radial diffusion assays for antimicrobial activity tests of HisMHBD2 against *Salmonella enterica* serovar Typhimurium SL1344.

Protein samples	Diameters (mm)		
Negative control, EcN100 [a]	5	5	5
EcN102 [b]	8	7	10
commercial HBD2 [c]	13	7	7

[a] negative control (EcN100), 4 µg of fraction 0.03 eluted from Ni-column

[b] eluted proteins including HisMHBD2 (EcN102), 4 µg of fraction 0.03 eluted from Ni-column

[c] positive control, commercial HBD2, 1 µg

Table S3. Diameters of radial diffusion assays for antimicrobial activity tests of HisMHBD2 against *Listeria monocytogenes* EGD.

Protein samples	Diameters (mm)		
Negative control, EcN100 [a]	5	5	5
EcN102 [b]	6	7	7
commercial HBD2 [c]	10	9	8

[a] negative control (EcN100), 4 µg of fraction 0.03 eluted from Ni-column

[b] eluted proteins including HisMHBD2 (EcN102), 4 µg of fraction 0.03 eluted from Ni-column

[c] positive control, commercial HBD2, 1 µg

Table S4. Diameters of radial diffusion assays for antimicrobial activity tests of YebFMHBD2 against *E. coli* K-12 MG1655.

Protein samples	Diameters (mm)		
Negative control, SK22D105 [d]	5	5	5
SK22D106 [e]	12	13	13
commercial HBD2 [f]	13	10	10

[d] negative control, proteins in the cell free supernatant after centrifugation and filtration from SK22D105, 4 µg

[e] proteins in the cell free supernatant including YebFMHBD2 after centrifugation and filtration from SK22D106, 4 µg

[f] positive control, commercial HBD2, 1 µg

Table S5. Diameters of radial diffusion assays for antimicrobial activity tests of YebFMHBD2 against *Salmonella enterica* serovar Typhimurium SL1344.

Protein samples	Diameters (mm)		
Negative control, SK22D105 [d]	5	5	5
SK22D106 [e]	13	8	8
commercial HBD2 [f]	12	8	7

[d] negative control, proteins in the cell free supernatant after centrifugation and filtration from SK22D105, 4 µg

[e] proteins in the cell free supernatant including YebFMHBD2 after centrifugation and filtration from SK22D106, 4 µg

[f] positive control, commercial HBD2, 1 µg

Table S6. Diameters of radial diffusion assays for antimicrobial activity tests of YebFMHBD2 against *Listeria monocytogenes* EGD.

Protein samples	Diameters (mm)		
Negative control, SK22D105 [d]	5	5	5
SK22D106 [e]	13	14	12
commercial HBD2 [f]	10	9	8

[d] negative control, proteins in the cell free supernatant after centrifugation and filtration from SK22D105, 4 µg

[e] proteins in the cell free supernatant including YebFMHBD2 after centrifugation and filtration from SK22D106, 4 µg

[f] positive control, commercial HBD2, 1 µg

Table S7. Bacterial cfu/ml of samples after incutabion at 37 °C for 4 h for antimicrobial test in liquid culture against *E. coli* K-12 MG1655.

Protein samples (2.5 µl)	Bacterial numbers (cfu/ml)					
SK22D105 before incubation [g]	$6.20x10^6$	$6.10x10^6$	$1.96x10^7$	$2.36x10^7$	$3.14x10^7$	$3.12x10^7$
SK22D105 after incubation [g]	$1.38x10^7$	$1.76x10^7$	$7.40x10^7$	$7.30x10^7$	$4.70x10^7$	$4.90x10^7$
SK22D106 before incubation [h]	$7.90x10^6$	$4.70x10^6$	$1.14x10^7$	$1.14x10^7$	$2.83x10^7$	$2.38x10^7$
SK22D106 after incubation [h]	$3.60x10^6$	$3.70x10^6$	$1.55x10^7$	$1.65x10^7$	$2.10x10^7$	$2.40x10^7$

[g] SK22D105 contained secreted proteins from SK22D105 and bacteria in 10 mM sodium phosphate buffer, pH 7.4, and 1 % TSB medium.

[h] SK22D106 contained secreted proteins from SK22D106 and bacteria in 10 mM sodium phosphate buffer, pH 7.4, and 1 % TSB medium.

Table S8. Bacterial cfu/ml of samples after incutabion at 37 °C for 4 h for antimicrobial test in liquid culture against *Listeria monocytogenes* EGD.

Protein samples	Bacterial numbers (cfu/ml)					
Negative control before incubation [i]	$8.27x10^7$	$7.19x10^7$	$2.19x10^7$	$5.80x10^7$	$5.20x10^7$	$4.41x10^7$
Negative control after incubation [i]	$7.50x10^7$	$7.10x10^7$	$5.00x10^7$	$4.00x10^7$	$5.04x10^7$	$5.55x10^7$
SK22D105 5µl before incubation [g]	$8.14x10^7$	$6.86x10^7$	$5.52x10^7$	$2.83x10^7$	$5.43x10^7$	$5.30x10^7$

SK22D105 5µl after incubation [g]	$7.00x10^7$	$1.17x10^8$	$4.70x10^7$	$1.90x10^7$	$5.59x10^7$	$5.19x10^7$
SK22D106 5µl before incubation [h]	$5.66x10^7$	$6.54x10^7$	$4.26x10^7$	$4.45x10^7$	$3.59x10^7$	$5.17x10^7$
SK22D106 5µl after incubation [h]	$7.40x10^7$	$8.30x10^7$	$3.30x10^6$	$1.66x10^7$	$4.70x10^7$	$4.80x10^7$

[i] Negative control contained bacteria in 10 mM sodium phosphate buffer, pH 7.4, and 1 % TSB medium without any proteins.

[g] SK22D105 contained secreted proteins from SK22D105 and bacteria in 10 mM sodium phosphate buffer, pH 7.4, and 1 % TSB medium.

[h] SK22D106 contained secreted proteins from SK22D106 and bacteria in 10 mM sodium phosphate buffer, pH 7.4, and 1 % TSB medium.

Abbreviations

α	alpha
A	alanine
Ap^R	ampicillin resistance
β	beta
bp	base pairs
C	cysteine
cDNA	complementary DNA
CFU	colony forming unit
D	aspartic acid
Da	Dalton
dH_2O	destilled H_2O
Δ	Delta, difference operator
DNA	desoxyribonucleic acid
dNTP	2'-desoxyribonuclesid-5'-triphosphate
ε	epsilon
E	glutamic acid
EcN	*Escherichia coli* strain Nissle 1917
EcNc	EcN cured from both cryptic plasmids
e.g.	exempi gratia
EDTA	ethylendiamintetraacetate
et al.	et altera
EtOH	ethanol
g	gram
G	glycine
H	histidine

HBD2	human beta defensin 2
HD5	human alpha defensin 5
hrs	hours
I	isoleucine
IPTG	Isopropyl-β-D-thogalactopyranosid
K	lysine
kb	kilo bases
Km	kanamycin
µ	micro
m	milli
M	methionine
M	molar
MBP, MalE	Maltose Binding Protein
min	minute
N	asparagine
OD_{600}	optical density at 600nm
P	proline
PCR	polymerase chain reaction
Q	glutamine
R	arginine
RNA	ribonuclease
S	serine
SDS-PAGE	sodium dedecyl sulfate-polyacrylamide gel electrophoresis
T	threonine
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline

TCA	trichloroacetic acid
Tc^R	tetracycline resistance
TEMED	N,N,N', N'-tatramethyldiamin
Tris	Trishydroxylmethylaminomethan
TLR	Toll-like receptor
V	valine
V	Volt
Y	tyrosine

Physical maps of expression plasmids

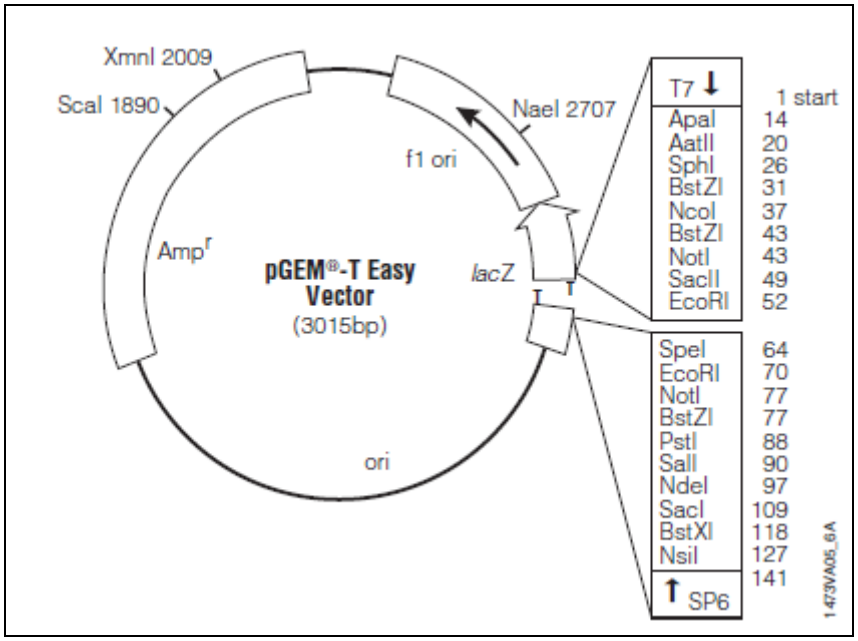

Fig. S1. Physical map of pGEM-T Easy vector (3015 bp) which is the convenient system for the cloning of PCR products with single 3'-T overhangs (Promega, USA).

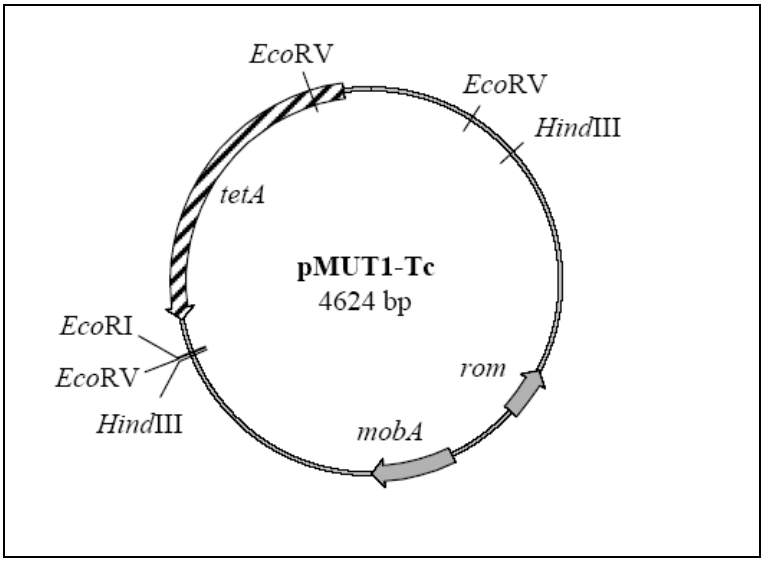

Fig. S2. Schematic representation of expression vector, pMUT1-Tc (4624 bp) mediating tetracycline resistance is a derivate of pMUT1, one of two very stable cryptic plasmids of EcN.

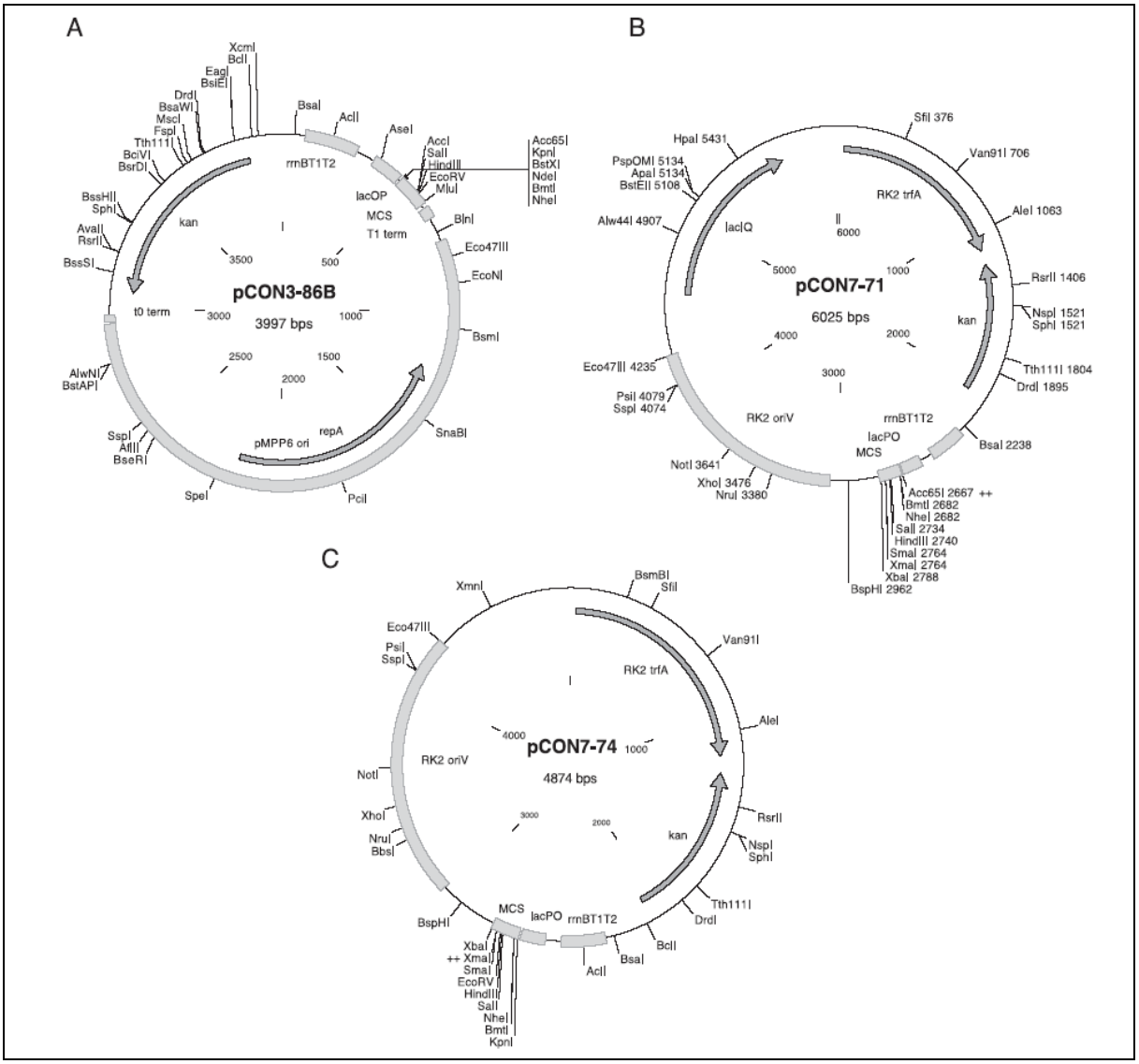

Fig. S3. Physical maps of the expression vectors (A) pCON3-86B (3997 bp), (B) pCON7-71(6025 bp) and (C) pCON7-74 (4874 bp) indicating unique restriction sites and showing relevant features (Anthony et al. 2004).

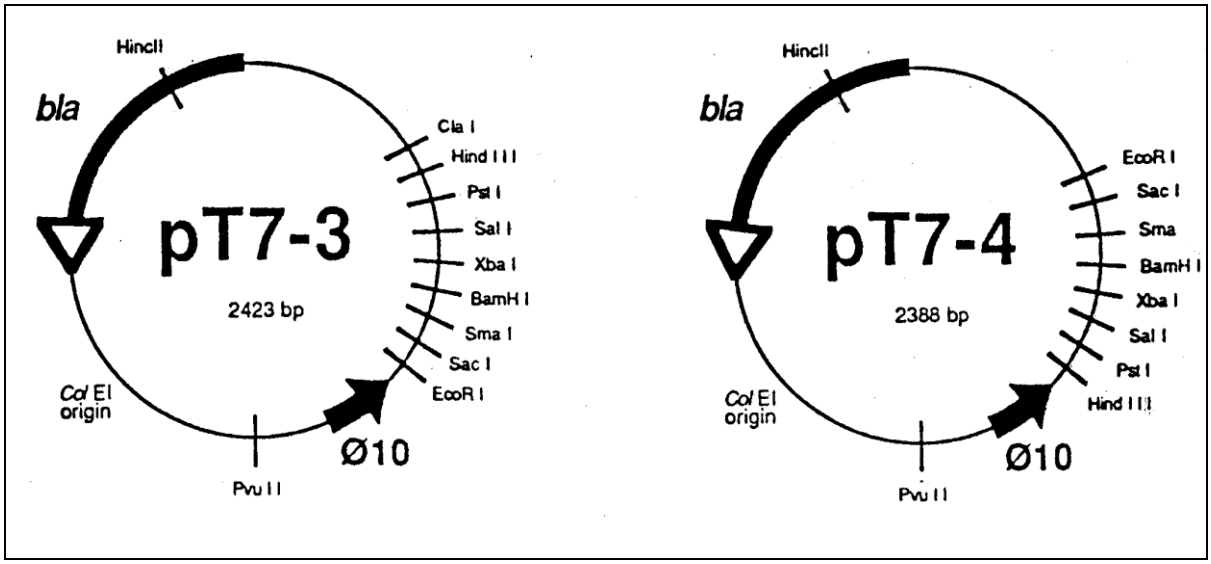

Fig. S4. Plasmid maps of expression vectors pT7-3 (2423 bp) and pT7-4 (2388 bp) which are expression vector system into which genes under the control of the T7-RNA-polymerase specific promoter phi10 can be cloned (Tabor and Richardson 1985).

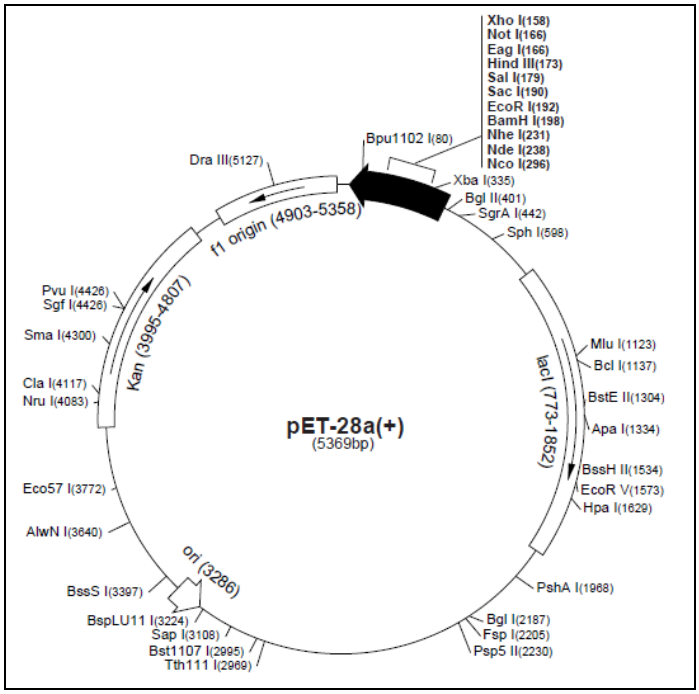

Fig. S5. Plasmid map of expression vector, pET-28a(+) (5369 bp) which encodes two His tags and allows expression of genes under the control of the T7 promoter (Novagen, USA).

Sequences

nHBD2-gene (EMBL HE583189)

atgcgtgttctgtatctgctgttctctttcctgttcatcttcctgatgccgctgccgggtgtgtttggtg

gcattggtgatccggtgacctgcctgaaaagcggtgccatctgtcatccggtgttttgtccgcgtcgtta

taaacagattggtacctgcggtctgccgggcaccaaatgctgtaaaaaaccgtaatga

nHD5-gene (EMBL HE583188)

atgcgtaccattgccattctggccgccattctgctggtggccctgcaggcccaggccgaaagcctgcagg

aacgtgccgatgaagccaccacccagaaacagagcggtgaagataatcaggatctggccattagctttgc

cggtaatggtctgagcgccctgcgtaccagcggtagccaggcccgtgccacctgctattgccgtaccggt

cgttgcgccacccgtgaaagcctgagcggtgtgtgcgaaattagcggtcgtctgtatcgtctgtgctgcc

gttaatga

Publication

Ean-jeong Seo, Stephanie Weibel, Jan Wehkamp, Tobias Oelschlaeger, Construction of recombinant *E. coli* Nissle 1917 (EcN) strains for the expression and secretion of defensins (submitted to International Journal of Medical Microbiology)

Presentations

Ean-jeong Seo, Jan Wehkamp and Tobias Oelschlaeger, Construction of recombinant *E. coli* Nissle 1917 (EcN) strains expressing/secreting defensins, The symposium of Bile acids, Defensins and Disease, 2011.

Ean-jeong Seo, Jan Wehkamp and Tobias Oelschlaeger, Construction of recombinant *E. coli* Nissle 1917 (EcN) strains expressing defensins: a novel therapy for treatment of inflammatory bowel disease?, The 5th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Wuerzburg, 2010.

Ean-jeong Seo, Jan Wehkamp and Tobias Oelschlaeger, Construction of recombinant *E. coli* Nissle 1917 (EcN) strains expressing defensins: a novel therapy for treatment of inflammatory bowel disease?, The 3rd Seeon conference, Microbiota, Probiota and Host, 2010.

Ean-jeong Seo, Jan Wehkamp, Ulrich Dobrindt and Tobias Oelschlaeger, Construction of recombinant *E. coli* Nissle 1917 (EcN) strains expressing defensins: a novel therapy for treatment of inflammatory bowel disease?, The 4th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Wuerzburg, 2009.

Affidavit

I hereby confirm that my thesis entitled Construction of recombinant *E. coli* Nissle 1917 (EcN) strains for the expression and secretion of defensins is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 23.02.2012, Ean-jeong Seo

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation Konstruktion von rekombinanten E. coli Nissle 1917 (EcN) Stämmen, die Defensine exprimieren bzw. sekretieren eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 23.02.2012, Ean-jeong Seo