CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF DENTISTRY

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GENERATION OF RECOMBINANT HUMAN β-DEFENSIN-3 AND ITS MICROBICIDAL ACTIVITY AGAINST ORAL BACTERIA

by

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Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Dentistry

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CASE WESTERN RESERVE UNIVERSITY

January 17, 2003

GENERATION OF RECOMBINANT HUMAN BETA-DEFENSIN-3 AND ITS MICROBICIDAL ACTIVITY AGAINST ORAL BACTERIA

Abstract

by

Laurence Anthony Golden

Mucosal epithelium provides a first line of host defense against bacteria. Oral epithelium is a source of human β-defensin 3 (hBD-3), a member of a family of inducible small cationic peptides, having a broad range of microbicidal activity. The objective of this study was to generate recombinant hBD-3 (rhBD-3) and test its antimicrobial activity against *Actinobacillus actinomycetemcomitans* (*A.a.*), *Fusobacterium nucleatum* (*F.n.*), *Streptococcus sanguis* (*S.s.*), and *Porphyromonas gingivalis* (*P.g.*). The methods involved the generation of rhBD-3 using hBD-3 cDNA. This was produced by RT-PCR and cloned into pET-30c (Novagen). The resulting plasmid was transformed into *E. coli* [BL21trx(DE3), Novagen], where expression of rhBD-3 fusion protein was regulated by IPTG. rhBD-3 (with His-tag) was isolated and concentrated by nickel column chromatography, followed by reverse phase high performance liquid chromatography (RP-HPLC). rhBD-3 was released from the fusion protein by enterokinase and purified by RP-HPLC. The identity of rhBD-3 was confirmed by MALDI-MS,

and nickel affinity blot, and demonstrated comparable microbicidal activity against the standard *E. coli ML35p*. *A.a., F.n., S.s.*, and *P.g.* were separately incubated in 10mM phosphate buffer containing 1% tryptic soy broth and various concentrations (0, 5, 10, 20, or 40 μg/ml) of rhBD-3 at 37°C, anaerobically for 3 hrs, followed by serial dilution and plating. There was a dose dependent inhibition of microbicidal activity against *A.a, S.s.,* and *P.g.* *F.n.* resisted rHBD-3 killing. This is consistent with previous observations that *F.n.* was resistant to rhBD-1 and –2; and that *F.n.* induced hBD-2 in oral epithelial cells. The data strongly suggests an adaptive advantage of this organism and possible involvement in promoting homeostasis in the oral cavity.

DEDICATION

To Our Lord, who makes all things possible,

To Our Lady, through whom my father, Laurence, and my mother, Mary Lynn, my Grandmother, Mary, my Aunt Jean, my friends, and I asked Our Lord that I may have this opportunity.

To St. Anthony of Padua, through whose intercession my mother asked Our Lord for a child, after having been told by physicians, for twelve years, this was an impossibility.

To St. Josemaria Escrivá de Balaguer, through whose intercession I have asked for help.

To my parents who have unselfishly struggled and sacrificed all my life to give me every opportunity.

To my Grandparents, Mary and Ernest who also unselfishly supported all of my endeavors.

To my wife Fani, who has given up all that she knows and has changed everything familiar in her life to begin with me. I pray that I always make you happy and that we struggle to live God's will.

ACKNOWLEDGMENTS

To Dr. Aaron Weinberg, Thesis Advisor, thank you for the opportunity to work in your laboratory, for all of your effort in the writing of this document and in preparing me to defend this thesis. I appreciate that you are about excellence and that you held me to such standards. Your enthusiasm about the science made me enthusiastic. I am proud to have worked with you.

To Dr. Zhimin Feng, Co-advisor, without whose friendship and help this work would not be possible. Your patience and kindness knows no limit.

To Dr. Thomas McCormick, for your help, care, and patience.

To Dr. Mark Hans, Graduate Program Director and Chairman of the Department of Orthodontics, thank you for this opportunity, I have longed for it all of my adult life. It seemed important to me to "maintain the ranks" as a resident," and not be too casual with the boss, but I like and admire you far more than I allowed myself to let on. You work especially hard to make Case Western's Orthodontic program excellent. I wish I could continue to learn and practice orthodontics with you. You were a friend to me, and I am grateful to you.

To Dr. J. Martin Palomo, for your support and friendship. I admire and appreciate that fact that when asked for help, you always made the effort to give it.

To Dr. Manish Valiathan, for your teaching and support. I appreciate your friendship and admire and envy your precocious knowledge of orthodontics.

To Dr. Arthur Phelps, Clinical Director, its difficult to thank you or to describe the impression that you have imparted to me, in the same way it is difficult for a son to articulate appreciation to his parents. I think it's fair to say that when my colleagues and I have practiced for as many years as you have, we hope to be respected the way that you are. However, to be respected and liked on the scale that you are, is probably beyond our reach.

To the orthodontic faculty: thank you for giving of your time, knowledge, and wisdom. I look forward to thanking you by individual correspondence.

To my colleagues and friends from the class of 2001: Jay Galati, Jed Gass, Don Hunt, my "big brother", and Sally Park.

To my colleagues and friends from the class of 2002: Yumi Abei, Jerry Hawk, Rachel Hamilton, and my brother Alexandros Moullas. Thank you for all of your help Alex, I am certain that I could not have gained a better brother.

To my classmates of 2003: Gordon Groisser, Mandana Mozayeni-Azar, and Nawaf Masri. We have become such close friends. I will miss you.

To my colleagues and friends from the class of 2004: Sean Bates, Lance Bruntz, David Lenhart, and Chris Spoonhower. It has been a pleasure to know you. I hope that our friendship continues to grow.

To Laverne Vogel, it has been my great pleasure to work with you.

To Dr. Paula Stern, who gave me my first opportunity to perform research; and then spent three years writing innumerable letters of recommendation, thank you for your friendship and support.

To my faculty at Northridge Preparatory School who gave me tools to study and the confidence to be successful.

I feel that I have gained so many friends these past two and a half years, who for the sake of time I should not attempt to thank here. These two and one-half years in the Case Western Reserve University Orthodontic Department have been the happiest of my life; not only because I married my best friend, the person I met in my first week in Cleveland and spent almost every single day studying with, in the University Hospital cafeteria; not only because my parents and grandparents had the opportunity to see me accomplish my dream; not only because working with young people and having the opportunity to boost their self esteem is so noble and enjoyable; but because my interaction with the faculty, staff, and residents in orthodontic program has allowed me to make friendships that are extremely special to me. I will miss my interaction with them very much.

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INTRODUCTION

More than 10^{14} bacteria live on and surround the 10^{13} human cells that make up the human body. Intuitively, we might think that this would be a deadly situation, but that is the paradigm. Four hundred different species of oral bacteria are present in the oral cavity. The oral mucosal barrier is compromised by dental eruption and exfoliation, traumatic abrasion, and occasionally by an untoward orthodontic wire. How is it that we protect ourselves from this microbial challenge? Human life is believed to have evolved over time; is it possible that some of our microbial guests have evolved with us? Could some of these guests have developed beneficial relationships with us rather than pathogenic ones?

The interaction between bacteria and epithelial cells is now understood to be a relationship where the host's epithelial cells actively respond to the host's microbial guests. The epithelial response is particularly important in the oral cavity where exposure to pathogenic microbes is ever present. Humans have many mechanisms that are responsible for keeping the balance between health and disease. These include the natural defenses of saliva and the host innate immune response, as well as the ability to generate antibodies via acquired immune responses. Antimicrobial peptides protect mucosa and dry epithelial surfaces of all multicellular organisms (Zasloff

2002). Antimicrobial peptides are part of the innate immune system; they have broad-spectrum antibacterial and antifungal activity and they may link innate immunity with the acquired immune system via activation of cytokine and inflammatory responses in oral epithelia. Antimicrobial peptides target the microbial membranes and kill rapidly.

Beta defensins are a cationic family of antimicrobial peptides. *Fusobacterium nucleatum* (*F.n.*) is a bacterium that is ubiquitous in the oral cavity. *F.n.* induces human β-defensin-2 expression in primary normal human epithelial cells and thereby, confers protection to these cells against the pathogen *Porphyromonas gingivalis* (*P.g.*), which is known to destroy periodontal tissue. *P.g.* does not induce expression of human β-defensin-2 and is sensitive to human β-defensin 1 and 2 while *F. n.* resistant. *F.n.* may have developed a novel strategy of using the host's β-defensins to kill other bacteria. This strategy may be an expression of adaptive co-evolution between this organism and the human host, and that contributes to homeostasis and health in body sites constantly challenged with bacteria (Weinberg et al. 2003).

Human beta defensin-3 (hBD-3) was described by Harder et al. (2001) and is found to be expressed in the oral epithelium. Is *F.n.* resistant to hBD-3 as it is to hBD-1 and 2? Is *P.g.* susceptible to hBD-3 as it is to hBD-1 and 2. If *F.n.* is found to be resistant to yet another beta defensin that kills *P.g.* then

the case for a beneficial, commensal relationship between *F.n.* and its human host is strengthened.

LITERATURE REVIEW

Antimicrobial Peptides

Epithelial tissues provide the first line of defense between an organism and the environment. Disruption of this barrier leads to bacterial invasion and subsequent inflammation. Human oral epithelium is constantly exposed to a variety of microbial challenges that can lead to bacterially induced periodontal diseases, and to various mucosal infections. Epithelium is not only a physical barrier to noxious intruders; it also generates potent antimicrobial peptides (APs). Antimicrobial peptides are defined as proteins with less than one-hundred amino acids and molecular weights from approximately 3,500-6,500 Da (Dale 2002). These agents are a key component of innate immunity. Unlike the acquired immune system, which features memory and high specificity, APs can act rapidly against multiple bacterial species, with less specificity. Innate responses occur within minutes after infection, much earlier than the acquired immune system (Weinberg et al. 1998).

APs are present throughout the animal kingdom. This ubiquitous presence suggests the importance of these molecules in host defense (Zasloff 1992). These agents exhibit a broad spectrum of microbicidal activity. It is hypothesized that epithelial-cell-derived APs act to keep the natural flora of microorganisms in a steady state in areas such as the skin,

the airway, the endocervix, the gastrointestinal tract, and the mouth. Antimicrobial peptides include the α and β defensins and the cathelicidin family member, LL-37 (Hancock and Scott 2000). The antimicrobial protein calprotectin is also expressed in the cytoplasm of oral epithelia and neutrophils (Eversole et al. 1993).

The small cationic APs produced by oral epithelial cells are postulated to play an important role in determining the outcome of the host-pathogen interaction at the oral mucosal barrier (Weinberg et al. 1998). These peptides may work in concert with other large APs, such as leukocyte-granule-associated bactericidal/permeability-increasing protein (BPI) (Elsbach 1994), anionic antimicrobial peptides such as leukocyte-associated and keratinocyte-associated calprotectin (Eversole et al. 1993), and salivary APs, such as the histidine rich histatins (Oppenheim et al. 1988).

Eukaryotic small cationic APs have been divided into four distinct groups, based on peptide structure, amino acid composition, and number of disulfide bonds (Weinberg et al. 1998). Over seventy cationic AP structures have been characterized in insects, amphibians, birds, and various mammals; thus it is clear that these agents are widespread in nature, and represent an evolutionarily ancient mechanism of host defense. Moreover, unlike conventional antibiotics, acquisition of resistance by a sensitive organism against APs is surprisingly rare and difficult to generate (Ge et al. 1999). Although the peptides differ greatly in primary structures, they are nearly all

cationic by virtue of their numerous arginine and lysine residues. Their strong cationic charge is important for binding to microbial surfaces, where they displace native divalent cations on cell-surface lipopolysaccharides (LPS) and disrupt the normal barrier property of the Gram-negative outer membrane, eventually killing the organism (Hancock et al. 1997). Other anionic membrane associated targets include lipoteichoic acid in gram-positive organisms and lipopeptide and porin proteins present in both gram-negative and gram-positive bacteria.

Defensins

Among the groups of antimicrobial peptides studied are the mammalian defensins. Defensins are cationic, cysteine-rich peptides that display broad-spectrum antimicrobial activity. They are found in the granules of inflammatory cells, where they play a role in nonoxidative microbial killing (Lehrer et al. 1993) and in mucosal epithelium (β-defensins), where they are believed to play an important role in maintaining homeostasis between the host and resident organisms (Weinberg et al. 1998). Their structure is characterized by a conserved cysteine motif that forms three disulfide linkages, imposing a characteristic β-sheet structure (Hill et al. 1991; White et al. 1995). Associated with this structure is an amphiphilic charge distribution that enables the defensins to interact with and disrupt target cell membranes (Lehrer et al. 1989). They are believed to carry out this function by forming

channels in the microbial target membrane, leading to cell lysis and eventual death (Kagan et al. 1990).

Several families of natural antibiotic peptides or proteins are expressed in epithelia, as well as in neutrophils. These peptides have broad-spectrum activity against gram-positive and gram negative bacteria, yeasts and some viruses (Hancock 1997). They complement the antimicrobial factors of saliva, such as the histatins, lysozyme, and salivary immunoglobulins. Both constitutive and inducible antimicrobial peptides are expressed in gingival epithelium, suggesting that they have a normal surveillance function as well as a specific role in innate host defense in response to infection. A person's individual variation in expression of the innate defenses may contribute to disease susceptibility (Dale and Krisanaprakornkit 2001).

The small (3-5 kDa) cationic defensins represent an important peptide family among antimicrobial peptides. Two subfamilies, the α-defensins and β-defensins, are distinguished on the basis of the location of their six-cysteine residues. Most recently discovered is the cyclic θ defensin from macaque leukocytes (Tang et al. 1999). No human equivalent of the cyclic θ defensins has been found. Human α-defensin (hD-1, hD-2, hD-3, hD-4) are produced and stored in azurophilic granules of polymorphonuclear cells (PMNs) and monocytes, and hD-5 and hD-6, are produced by Paneth cells found in the lower segments of the crypts of Liberkühn in the human ileum (Jones and Bevins 1993).

β-defensins

The first β-defensin was isolated from bovine tracheal mucosa (Diamond et al. 1991). Subsequently, thirteen novel β-defensins were purified from bovine neutrophils (Selsted et al. 1993), and the three-dimensional structure, including the disulfide array of one of these β-defensins, has been determined (Tang et al. 1993). Human equivalents of bovine neutrophil β-defensins have not been found. β-defensins are expressed in all human epithelial tissues (Huttner et al. 1999).

human β-defensin-1 (hBD-1)

The first human β-defensin, human β-defensin-1 (hBD-1) was isolated from plasma hemofiltrates, from patients with end stage renal disease (Bensch et al. 1995). hBD-1 was later found in urine, and was shown to be a potent Gram-negative bacteria-killing antibiotic (Valore et al. 1998). An important characteristic of this AP is that it is constitutively expressed in various epithelia, including the oral mucosa (Valore et al. 1998; O'Neil et al. 1999; Goldman et al. 1997; Fulton et al. 1997; Krisanaprakornkit et al. 1998).

human β-defensin-2 (hBD-2)

The second human β-defensin, hBD-2, was discovered in extracts from psoriatic lesions (Harder et al. 1997; Schröder and Harder 1999), and later found in human tracheal epithelium (Liu et al. 1998). In contrast to hBD-1, hBD-2 is inducible upon treatment with tumor necrosis factor α (TNF-α)

(Harder et al. 1997; Harder et al. 2000), interleukin-1β (IL-1β) (Harder et al. 2000; Singh et al. 1998), and contact with mucoid forms of gram-negative bacteria, *Pseudomonas aeruginosa* (Harder et al. 2000) and the oral commensal *Fusobacterium nucleatum* (*F.n.*) (Weinberg et al. 1998; Krisanaprakornkit et al. 2000). Both human β-defensins show microbicidal activity predominantly against Gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*.

However, they demonstrate only low microbicidal activity against Gram-positive bacteria such as *Staphylococcus aureus* (Schröder and Harder 1999; Harder et al. 1997; Zucht et al. 1998), a bacterium that causes infections ranging from skin abscesses to life-threatening conditions such as endocarditis and toxic shock (Turnridge and Grayson 1993).

Since hBD-2 is apparently not effective against *S. aureus*, yet psoriatic lesions are remarkably free of this skin inhabiting organism, the scientific community hypothesized that other APs must also be expressed in these lesions, that do effectively kill *S. aureus*.

human β-defensin-3 (hBD-3)

A search began for another AP that was active in these psoriatic lesions. Harder et al. (2001) found that AP. Harder described the presence of hBD-3, the third member of the β defensin family, which is induced in psoriatic lesions and exquisitely shows microbicidal activity against *S. aureus*. Moreover, while hBD-2 was found to be salt sensitive; i.e. increasing salt

concentrations inhibit its antimicrobial activity, hBD-3 is salt insensitive, suggesting that it works well on skin, which may be subjected to the salty conditions of sweat (Harder et al. 2001). hBD-3 has broad-spectrum antibiotic activity that kills, at low micromolar concentrations, multiresistant *S. aureus*, *P. aeruginosa*, *S. pyogenes*, vancomycin-resistant *E. faecium*, and the yeast *C. albicans* (Harder et al. 2001).

hBD-1, 2, & 3 have been shown to be expressed in human oral epithelial cells by Weinberg and his colleagues (Weinberg et al. 1998; Krisanaprakornkit et al. 1998; 2000; hBD-3 recently shown at the mRNA level). Skin and tonsils have been found to be major sources of hBD-3 (Harder et al. 2001). Tumor necrosis factor-α (TNF-α) and a clinical isolate of *Pseudomonas aeruginosa* were found to induce hBD-3 mRNA expression. Because of its broad spectrum microbicidal activity,
hBD-3 may very well be important in the innate epithelial defense against various microorganisms, inhabiting the skin and particularly in maintaining steady state conditions in the oral cavity.

Localized Expression of Antimicrobial Peptides in Gingiva

There is a pattern of β-defensin expression within the gingiva that is consistent with function as a microbial barrier in the stratified epithelial tissues (Dale et al. 2001). The expression of hBD-1 and hBD-2 are greatest in the upper portion of the tissue, and particularly strong at the gingival margin where tissue may be in almost continuous contact with supragingival plaque.

The β-defensins hBD-1 and hBD-2 are expressed in areas of differentiation and inflammation, but are expressed poorly or not at all in the junctional epithelium (Schroeder et al. 1997). α-defensins and LL-37 are present in high amounts in neutrophils that migrate through the junctional epithelium to the gingival sulcus (Dale et al. 2001).

Periodontitis

Periodontitis is a complex interplay of bacterial infection and host response, which may be modified by behavioral factors. The present model of periodontitis holds that only 5-20% of any population suffers from severe generalized periodontitis, and that only moderate disease affects a majority of adults. One major risk factor is smoking; which is associated with periodontal disease independent of oral hygiene, age, or any other risk factor (Fenesy 1998).

Periodontitis may also be associated with systemic conditions such as metabolic disorders (diabetes mellitus, female hormonal alterations), drug-induced disorders, hematologic disorders, and alteration in neutrophil chemotaxis.

In human periodontitis, there is no simple direct pathogen-disease link. Three pathogens have a strong association with periodontal disease: *Actinobacillus actinomycetemcomitans* (*A.a.*), spirochetes (of acute necrotizing gingivitis) and *Porphyromonas gingivalis*. These pathogens may be the cause of the continued loss of periodontal attachment, or destruction of

the periodontal ligament and loss of supporting bone. This loss of attachment is seen in adult periodontitis and the three types of early-onset periodontitis: prepubertal periodontitis, localized, and generalized juvenile periodontitis (Fenesy et al. 1998).

Orthodontics also plays a role in periodontitis. Metallic orthodontic brackets create a favorable environment for *A.a.* and *F.n.* (Anhoury et al. 2002).

The bacterial etiology of periodontal disease is complex, with a variety of organisms responsible in the initiation and progression of disease. Many, if not all, of these organisms, may be present in periodontally healthy individuals. Our model holds that the disease develops from a shift in the balance between bacterial and host factors.

Periodontal destruction results from the hosts' response to bacterial invasion, that is, attachment and bone loss are due to episodes of acute inflammation (Page et al. 1986). In general, severe forms of periodontitis are associated with a number of gram-negative anaerobic bacteria. Of this group, most evidence points to a pathogenic role for *Porphyromonas gingivalis* (Haffajee and Socransky 1994).

Oral gram-negative bacteria, including: *Actinobacillus actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.*), *Prevotella intermedius* (*P.i.*) have been associated with destructive periodontal infection. Oral gram-negative bacteria are found to be sensitive to the cidal mechanism

of human neutrophil defensins (Zhong et al. 1998). *A.a.*, *P.g.*, *P.i.*, *Bacteroides forsythus* and *Treponema* are associated with site- specific periodontal inflammation (Hultin et al. 2002).

Aggressive periodontitis comprises a group of rapidly progressive forms of periodontitis. Besides bacteria, a high level of subject susceptibility must be involved in the expression of disease. *A.a.* and *P.g.* are detectable in patients with aggressive periodontitis (Trevilatto et al. 2002). *A.a.JP2* is associated with localized aggressive periodontal disease in juveniles (Fine et al. (2002). Prepubertal periodontitis, also known as generalized aggressive periodontitis, exhibits generalized gingival inflammation, extensive alveolar bone loss, and tooth mobility. *A.a.* is suspected to play a key role in the etiology of prepubertal periodontitis (Bodur et al. 2001 and Anhoury et al. 2002).

Actinobacillus actinomycetemcomitans

A.a. is a facultative gram-negative small, non-motile, short, straight or curved rod and was one of the first organisms to be associated with localized early onset periodontitis (Slots 1976). *A.a.* has been serotyped into categories (a, b, c, d, e, f), with serotype b being the most virulent of the species. Serotype b, aka *A.a.JP2*, expresses an important virulence factor referred to as leukotoxin since it kills polymorphonuclear leukocytes (Kaplan et al. 2002). *A.a.JP2* has been associated with localized early-onset periodontitis by increased prevalence and proportions in subgingival plaque in

active sites of patients with localized early-onset periodontitis and low numbers in healthy sites (Darby et al. 2001).

Porphyromonas gingivalis (P.g.)

P.g. is a gram-negative anaerobic nonmotile short rod that forms black-pigmented colonies with zones of hemolysis on blood agar plates. *P.g.* has been implicated primarily in adult periodontitis, but has also been associated with localized early-onset periodontitis, though not as strongly as *A.a.* (Darby and Curtis 2001). Along with the genus *Prevotella* comprises the second most common cause of human infection by anaerobic gram-negative bacilli (Ryan 1994). *P.g.* is rarely detected in healthy sites (Socransky and Haffajee 1992). *P.g.* can outmanuver innate immune defenses (Madianos et al. 1997) and invade normal human oral epithelial cells (NHOECs) (Lamont et al. 1995) and periodontal tissues (Rudney et al. 2001).

Fusobacterium nucleatum (F.n.)

F.n. is an ubiquitous gram-negative fusiform anaerobe, found in healthy sites. In diseased sites it can increase by 1000 fold. *F.n.* is an oral commensal associated with dental plaque formation (Kolenbrander and London 1993). *F.n.* is ubiquitous in health and diseased oral sites (Lee et al. 1999). In patients with no known systemic disease or immune dysfunction, necrotizing ulcerative periodontitis demonstrates rapid loss of periodontal attachment and alveolar bone. *F.n.* and *P.g.* are associated with this disease (Krisanaprakornkit et al. 2000).

Streptococcus sanguis

S.s. is a gram-positive, anaerobic, cocci. *S.s.* is one of the early tooth colonizers; apparently important in generating early plaque that permits exogenous bacterial colonization. Studies have shown that this organism adheres to *F.n.* a later plaque forming bacterium, as well as to *P.g.* (Yao et al. 1996).

Localized Expression of Antimicrobial Peptides in Gingiva

Expression of β-defensin within the gingiva shows a pattern of expression consistent with function as a microbial barrier in the stratified epithelial tissues (Dale et al. 2001). The expression of hBD-1 and hBD-2 are greatest in the upper portion of the tissue, and particularly strong at the gingival margin where tissue can be expected to be in almost continuous contact with supragingival plaque. The β-defensins hBD-1 and hBD-2 are expressed in areas of differentiation and inflammation, but are expressed poorly or not at all in the junctional epithelium (Schroeder et al. 1997). α-defensins and LL-37 are present in high amounts in neutrophils that migrate through the junctional epithelium to the gingival sulcus (Dale et al. 2001).

Virulence Factors of Periodontal Pathogens

Tissue plasminogen activator (t-PA) and plasminogen activator inhibitor 2 are important proteolysis factors present in inflamed human periodontal tissues. The effect of lipopolysaccharide (LPS) from *A.a*, *P.g.* and *F.n.* has been shown to increase t-PA synthesis. LPS from *A.a.*, *F.n.*, but not

P.g. also increased PAI-2 level. This indicates that LPS derived from periodontal pathogens may cause unbalanced regulation of plasminogen activator and plasminogen activator inhibitor 2 by human gingival fibroblasts and this may contribute to the destruction of periodontal connective tissue through dysregulated pericellular proteolysis (Xiao et al. 2001).

Virulence Factors of *F.n.*

Hemagglutation activities, adherence factors, for *F.n.*, are suspected to indicate a higher pathogenicity of certain strains of *F.n.*, which is closely related to their implication in the development of periodontal disease. (Roques et al. 2000).

Virulence Factors of *A.a.*

A.a. possesses a number of virulence factors including: its capsule, fimbriae as attachment factors, endotoxin, immunosuppressive factor, leukotoxin, and hemolysins. Serotype b, also called JP2 is the predominant type found in LJP. (Serotypes a-f have been identified) Serotype b LPS brings about high IgM and IgA and type 2 cytokines responses (Kato et al. 2000). *A.a.* synthesizes a leukotoxin that destroys human polymorphonucleocytes (PMNs), and this toxin is thought to be responsible for the virulence of *A.a.* Histatins, components of the nonimmune oral defense system neutralized the toxic effect of the leukotoxin preparation in a concentration dependent manner (Kesavalu et al. 2002). Komatsuzawa et al. 2002, found a major sarcosyl-insoluble outer membrane protein of *A.a.*, Omp

A can be associated with periodontal disease. It has homologies similar with some bacterial virulence factors responsible for adhesion, invasion, serum resistance, or protein antigenicity.

A.a has been shown to be internalized by transformed cells such as the KB cell line. Initial adhesion to the epithelial cell transferring receptor appears to be the primary stimulus for invasion, although binding to integrins may constitute a second entryway (Meyer et al. 1996). Invasion of most strains requires remodeling of actin microfilaments that translocate from the periphery of the cell to focus surrounding the bacteria (Fives-Taylor et al. 1999). Other strains are taken up through actin-independent receptor-mediated endocytosis (Brissette and Fives-Taylor 1999). Internal bacteria are initially confined within a host-derived membrane vacuole, but this membrane is soon broken down and the bacteria reside in the cytoplasm (Sreenivasan et al. 1993). Not content with this location, *A.a.* can move to adjacent cells by inducing the formation of surface membrane protrusions through which the organism can migrate and enter the temporarily adjoined neighboring cell. The formation of these protrusions is a consequence of bacterial interaction with the plus ends of microtubules and movement through them may be driven by bacterial cell division (Fives-Taylor et al. 1999).

Virulence Factors of *P.g.*

P.g. clonal types that participate in periodontal infections express serologically distinct surface antigens, K antigens and LPS, which are

virulence factors (Sims et al. 2001). These black-pigmented bacteria produce cytotoxic metabolic end products and cell membrane proteases (Saito et al. 2001). Mitogen–activated protein (MAP) kinase pathways are used by the host for signal transduction, but may also be used by *P.g.* to invade human gingival epithelial cells (GECs) (Wantanabe et al. 2001). Extracellular cysteine proteinases, referred to as gingipains, are also considered important virulence factors for *P.g.* (Imamura et al. 2001).

Various forms of periodontal diseases are closely associated with specific subgingival bacteria. Adherence of bacteria to host tissues is a prerequisite for colonization (Abiko et al. 2000). The arginine gingipains RgpA and RgpB of *P.g.* are virulence factors of *P.g.* Immunization with RgpA stimulates the production of hemagglutinin domain-specific antibodies, which contribute to the prevention of *P.g.*-mediated periodontal disease. Following colonization of the gingival crevice, *P.g.* initiates inflammation of the gingival, destruction of periodontal tissue, loss of alveolar bone, and exfoliation of teeth. *P.g.* posseses a wide variety of virulence factors, including fimbriae, lectin-like adhesions, capsular polysaccharide, lipopolysaccharide, hemagglutinins, and hemolysins. Gingipains R (RgpA and RgpB) cleave proteins after arginine residues, while gingipains are able to degrade both collagen and fibronectin, inactivate protease inhibitors, degrade immunoglobins, and facilitate iron acquisition. They destroy host coagulation cascade proteins, degrade complement, and digest various cytokines.

Immunization of animals with relevant *P.g.* antigens including fimbriae and porphypain 2 (gingipain K) provide protection against subsequent *P.g.* challenge in some animal models (Gibson and Genco 2001).

P.g. binds through adhesions such as fimbriae to surface receptors on gingival cells. Microtubules and microfilaments facilitate invagination of the membrane that results in the engulfment of bacterial cells. *P.g.* rapidly relocates to the perinuclear area where they replicate. Calcium ions are released from intracellular stores and other signaling molecules such as the MAP-kinase family can be phosphorylated/dephosphorylated or degraded. *P.g.* can also selectively target components of the mitogen-activated protein (MAP) kinase pathways that are central to many host signaling response. *P.g.* directs its own entry into human gingival epithelial cells by modulating important gingival epithelial cell signaling pathways (Lamont and Yilmaz 2002).

Potential Relevance of Invasion

Three features characterize every epithelial tissue. Epithelial cells are closely opposed and attached via specialized cell-cell junctions. Epithelial cells produce basal lamina on which cells become organized as a tissue, and which separates the epithelium from the adjacent connective tissue. Epithelial cells are also characterized by the presence of a cytoskeleton composed of keratin intermediate filaments in addition to the typical actin filaments and microtubules. The integrity of the epithelial barrier is

specifically disrupted by various microbial pathogens that attack cell-cell junctions and thereby dissociates cells from each other. Another mechanism of bacterial invasion is by signaling rearrangement of the actin cytoskeleton, resulting in bacterial uptake. This seems to be the mechanism used by *P.g.* (Belton et al. 1999; Lamont and Yilmaz 2002).

Inter and intracellular invasion may play a role in the progression of disease. The highly proteolytic *P.g.* and the leukotoxic *A.a.* are both able to invade gingival epithelium. Invasion allows closer proximity to host targets for destructive bacterial products such as proteases, leukotoxin, and LPS to lay waste to the structural integrity of the periodontal tissues and also provide a sheltered nutritious environment. Human gingiva produces a chemokine, interleukin-8, which is transcriptionally down regulated by *P.g.* even in the presence of otherwise stimulatory organisms such as *F.n.* (Darveau et al.1998). In clinically healthy periodontium, IL-8 forms a gradient of expression that is highest at the surface and decreases deeper in the gingiva (Tonetti et al. 1994). This gradient directs neutrophils to sites of bacterial accumulation helping to protect against both bacteria and PMN damage. Inhibition of IL-8 by *P.g.* could have a debilitating effect on innate host defense.

Bacterial antigens are released from supra and sub gingival biofilm on the tooth surface as well as from bacteria attached to mucosal surfaces. Epithelial cells respond to bacteria by secreting interleukin-8 and other

chemokines to alert various cell types and to attract neutrophils. They also produce natural antimicrobial peptides in response to bacterial plaque. These antimicrobial peptides are part of the innate immune system; a complex set of responses that keep microbial invaders in check and maintains the microbial ecology of the healthy periodontal pocket (Weinberg et al. 1998). The epithelium responds to infection and signals further host responses and integrates innate and acquired immune responses. The acquired immune system is linked to cytokines, Langerhans cells, and dendritic cells of bone marrow origin by the epithelium in which these cells are located within. This understanding of epithelial innate defense responses, the interaction of epithelium and the immune system, and the active nature of the interaction of epithelial cells with bacteria is a new model of the host's response to infection. The relevance of this is the role of the epithelium in periodontal health and disease (Dale 2002).

Smart Bugs and Commensalism

A.a, *P.g*, *F.n.*, and *S.s.* are all oral bacteria, which have varying degrees of association with periodontal disease. Each of these bacteria may seek to modulate the human oral immune response. It is our interest to determine if human beta defensin-3 is antimicrobially effective against these oral bacteria.

Among the 10^{14} bacteria that have evolved to reside on our mucosal surfaces (Henderson et al. 1998) some have mutualistic relationships with the

human host (Hooper et al. 2001). Recall that hBD-1 is constitutively expressed and that hBD-2 is inducible by *F.n.* (Krisanaprakornkit et al. 2000). Interestingly, however, challenge of NHOECs by *P.g.* (the bacterium which is most strongly associated with periodontal disease) results in no hBD-2 mRNA induction. It is very advantageous for a successful pathogen, like *P.g.*, to not trigger the production of the potent gram-negative antimicrobial hBD-2 (Weinberg et al. 1998; Krisanaprakornkit et al. 1998; Krisanaprakornkit et al. 2000).

hBD-2 is expressed only in the presence of infection or inflammation in most tissues (including skin, trachea, gut epithelium), but interestingly it is expressed in normal uninflamed gingival tissue. The high level of hBD-2 expression may be a result of exposure of the tissue to commensal, nonpathogenic bacteria (Krisanaprakornkit et al. 2000). For example, normal oral gingival epithelial cells do not express hBD-2 in cell culture unless stimulated. Bacterial products, proinflammatory cytokines and phorbol ester, a general cell activator, are all effective stimulants. Cell wall preparations of the commensal organism, *F.n.*, are very effective in up regulating hBD-2 mRNA expression. This upregulation is not coupled with general signaling of other aspects of the innate immune system, such as IL-8 expression, a neutrophil chemoattractant (Krisanaprakornkit et al. 2000). Furthermore, it does not utilize the NF-κB intracellular signaling cascade typically used by proinflammatory stimuli, but instead utilizes a mitogen activated protein (MAP)

kinase signaling cascade that is associated with cytokine and stress responses. This may be interpreted as evidence that *F.n.* is a commensal organism, and that *F.n.* is aiding the epithelial tissue in preparation for possible exposure to pathogens, that is, the balance of health and disease is aided by the presence of the commensal organism, and that this is accomplished without fully unleashing other innate immune responses (Dale and Krisanaprakornkit 2001).

Weinberg generated stable lines of transfected KB cells (an oral cell line that originated from a human oral squamous cell carcinoma, which does not express hBD-1 or hBD-2. These cells were transfected so that they would express hBD-1 and hBD-2. These cells were challenged with *P.g.* (ATCC 33277; type strain). and invasion by *P.g.* was shown to be inhibited 44% by hBD-1 and 66% by hBD-2, respectively, when compared to control KB cells. Weinberg's lab then challenged induced NHOECs and compared them to uninduced quiescent NHOECs to evaluate protection against *P.g.* invasion. These cells were challenged with *F.n.* cell wall. The prestimulated cells were more than 50% protected when compared to unstimulated cells (Krisanaprakornkit et al.1998). There was no evidence that *F.n.* induced any other β-defensin in NHOECs, suggesting that the protection conferred to the NHOECs from *F.n.* was due solely to hBD-2 induction. LL-37, the only other cationic antimicrobial peptide known to be expressed in mucosal epithelia, is not know to be induced by *F.n.* (Weinberg et al. 1998) and has been localized

only to PMNs in human gingival (Dale and Krisanaprakornkit 2001). In order to determine that protection from *P.g.* invasion was not the result of the two species competing for epithelial cell binding sites, invasions assays were conducted one and two hours after challenge with *F.n.*; a time that is insufficient for hBD-2 induction by *F.n.* (Krisanaprakornkit et al. 2000). In both cases, *P.g.* invaded NHOECs at levels comparable to untreated cells, suggesting that the presence of *F.n.* alone was not inhibiting *P.g.* invasion. Because it appeared that *F.n.* suppressed *P.g.* invasion of oral epithelial cells through induction of hBD-2, Weinberg et al investigated if these organisms differed in their sensitivities to β-defensins. They generated recombinant hBD-1 and hBD-2 using a baculovirus expression system (Valore et al. 1998) with S*f*21 insect cells. *P.g.* was shown to be killed by rhBD-1 and –2 at low micromolar concentrations, over 100 μg/mL hBD-1 = 2.55 μM; 10 μg/mL hBD-2 = 2.31 μM), *F.n.* was not. Moreover, even at concentrations, over 100 μg/mL, neither rhBD-1 nor hBD-2 killed *F.n.* It appears as though *F.n.* has an adaptive advantage an organism gains by producing an antimicrobial agent to which it is resistant, while competitive bacteria are susceptible (Weinberg et al. 2003).

MATERIALS and METHODS

The objective of this project was to generate recombinant hBD-3 and to evaluate its antimicrobial activity against selected bacteria of the human oral flora. hBD-3-His tag fusion protein was generated by reverse transcription (RT) polymerase chain reaction (PCR) and cloned into the pET-30c (Novagen®, Madison, WI) plasmid that was kindly provided to Dr. Aaron Weinberg's laboratory at Case Western Reserve University by Drs. Jürgen Harder and Jens Schröder, Kiel University, Germany.

Transformation

Transformation is the direct uptake and recombination of naked DNA fragments through a cell wall by competent bacteria. The natural occurrence of this process is uncommon. Competent (chemically or electrically treated to increase the uptake of DNA) E. coli bacteria BL21(DE3) (Novagen®) were incubated with the pET-hBD-3 plasmid, from Dr. Jürgen Harder. The plasmid was introduced into E. coli BL21(DE3). The plasmid was engineered to carry the code for production of hBD-3-His tag fusion protein.

pET-30c (Novagen) Plasmid

The plasmid/vector, named pET-30c, is from the Novagen® company. The plasmid is used for cloning and expressing recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong

bacteriophage T7 transcription and translation signals induced by providing a source of T7 RNA polymerase in the host cell. The pET vectors were originally constructed by Studier and colleagues (1986). Translation vectors (used for the expression of target genes derived from eukaryotic sources) contain the highly efficient ribosome-binding site from the phage T7 major capsid protein. Vectors with the suffix "c" express from the ATC triplet of the BamH I recognition sequence.

Various pET vectors contain different sequences adjacent to the cloning sites that encode a number of peptide "tags", which perform various functions when fused with the target protein. Some of the fusion tags facilitate detection and purification of the target protein, whereas others increase the probability of biological activity by affecting the solubility in the cytoplasm or export to the periplasm. The selective markers Amp (ampicillin resistance) and Kan (kanamycin resistance) are available with the pET vectors.

A powerful feature of the pET system is the ability to clone target genes under conditions of extremely low transcriptional activity, that is, in the absence of a source of T7 RNA polymerase. Background expression is minimal in the absence of T7 RNA polymerase because the host RNA polymerases do not initiate from T7 promoters, and cloning sites in pET plasmids are in regions weakly transcribed by read-through activity of bacterial RNA polymerase.

T7 RNA polymerase is so selective and active that when fully induced, almost all of the cell's resources are converted to target gene expression. The desired product can comprise more than 50% of the total cell protein a few hours after induction. Lowering the concentration of the inducer can attenuate expression levels. In our case, isopropyl β-D-thiogalactopyranoside (IPTG) is the inducer. Decreasing the expression level may increase the soluble yield of target protein. pET maintains target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expressing host, target protein expression may be initiated by transferring the target gene under *lacUV5* control. Expression of the target protein (in this case, HBD-3 fusion protein) is induced by addition of IPTG to the bacterial culture (Novagen® pET System Manual 5/00). Figure 1 shows the 5423 base pair pET-30c plasmid. It illustrates circular DNA and the host and vector elements available for control of T7 RNA polymerase levels and the subsequent transcription of a target gene in a pET vector.

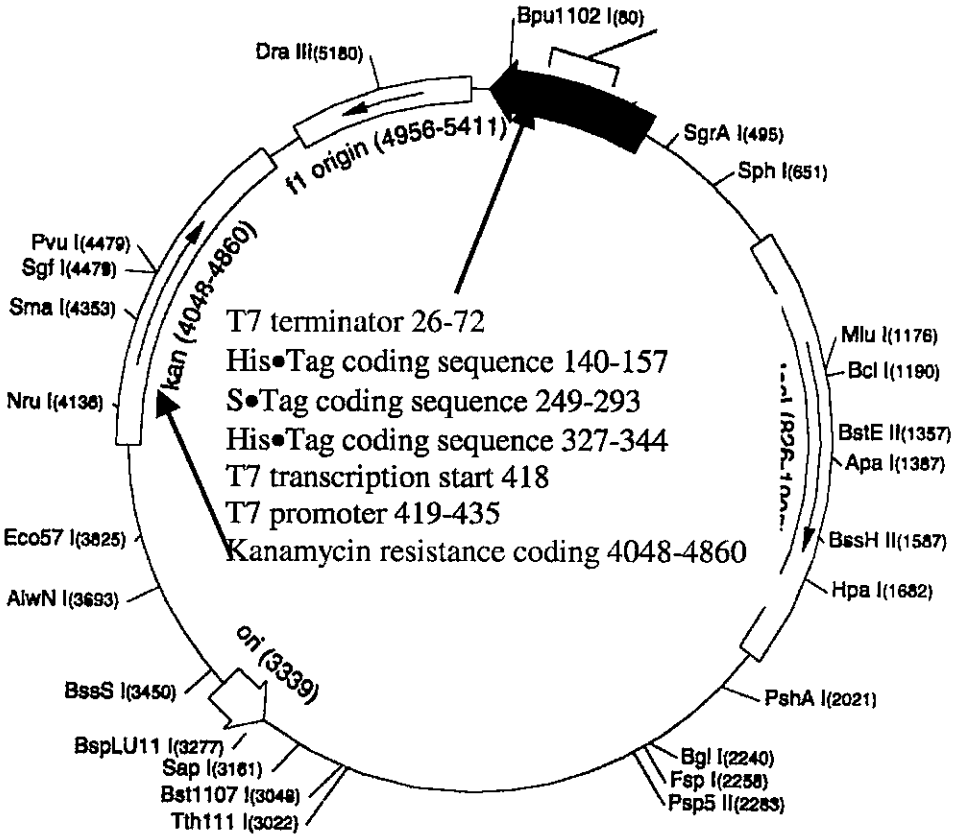
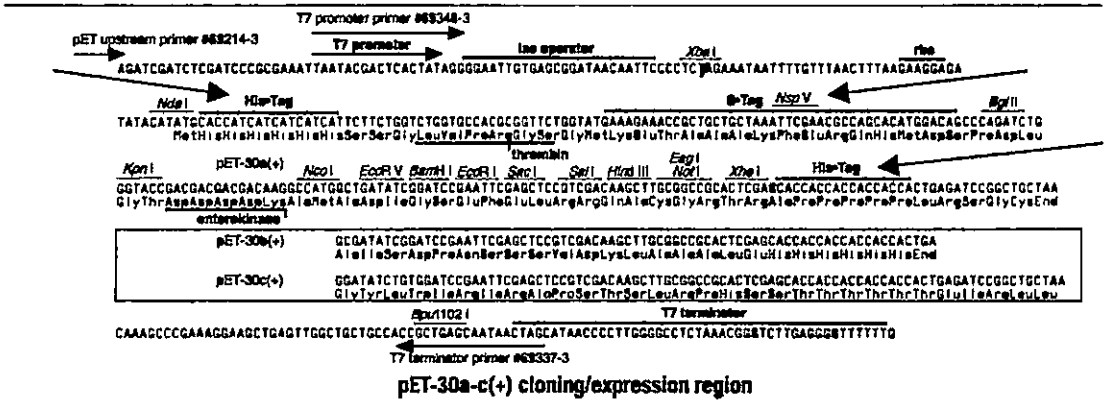


Figure 1. Gene map of pET-30c plasmid. The base pair coding sequence is number 1-5423 and increases in a clockwise fashion. Arrows point to the T7 terminator sequence and to the kanamycin resistance sequence on the circular DNA. (pET system manual, Novagen® 5/2000).

The most widely used host is BL21, which has the advantage of being deficient in both *lon* and *ompT* proteases (increase the stability of target protein). The pET-30c plasmid carries an N-terminal His•Tag® and S•Tag™/enterokinase configuration plus an optional C-terminal His•Tag® sequence to allow for identification and separation of the hBD-3 fusion protein

(pET system manual, Novagen® 5/2000). These areas are marked by arrows in Figure 2.



pET-30a-c(+) cloning/expression region

Figure 2. The pET30-c plasmid nucleic acid sequence. Areas that code for His•Tag and S•Tags that facilitate identification and isolation of the rhBD-3 protein are indicated by arrows. (pET system manual, Novagen® 5/2000).

The pET plasmid uses the T7 promoter to direct the expression of target genes. Since *E. coli* RNA polymerase does not recognize the T7 promoter, there is virtually no transcription of the target gene in the absence of a source of tRNA polymerase and the cloning step is thus effectively uncoupled from the expression step (iNovations Volume 11, Number 1).

After plasmids are established in a nonexpression host, they are most often transformed into a host bearing the T7 RNA polymerase gene (λDE3 lysogen [a bacterium that carries a repressed prophage as part of its genome]) for expression of target proteins (iNovations Volume 11, Number 1).

In λDE3 lysogens the T7 RNA polymerase gene is under the control of the *lacUV5* promoter, which allows some degree of transcription in the uninduced state and, in the absence of further controls, is quite suitable for expression of many genes whose products have innocuous effects on host cell growth. The λDE3 lysogen is induced with IPTG. As seen in Figure 2, the T7 lac promoter sequence contains a 25bp *lac* operator sequence immediately downstream from the 17bp promoter region. Binding of the *lac* repressor at this site effectively reduces transcription by T7 RNA polymerase, thus providing a second *lacI*-based mechanism (*besides the lacUV5)* to suppress basal expression in λDE3 lysogen. Figure 2 shows the nucleic acid sequence of the plasmid. The pET-30c plasmid has 5423 base pairs (Novagen®, Competent Cells Manual, 6/01).

BL21(DE3) strain of *E. coli*

The BL21(DE3) strain of *E. coli* was purchased from Novagen®. *E. coli* BL21(DE3) are called competent cells because they enable convenient, efficient construction of plasmid recombinant proteins. The BL21(DE3) strain of *E. coli* contains a lysogen of λDE3. This lysogen carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter. This strain is suitable for the production of protein from target genes cloned in pET vectors. The designation DE3 means that the strain is lysogenic for a λ prophage that contains an isopropyl β-D-thiogalactopyranoside (IPTG) inducible T7 RNA polymerase. λDE3 lysogens

are designed for protein expression from Novagen®'s pET plasmids. BL21 is the most widely used host background for protein expression and has the advantage of being deficient in both lon and ampT proteases. Deficiencies in *lon and ompT* proteases help to reduce proteolytic degradation (Novagen®, Competent Cells Manual, 6/01). This strain of *E.coli* bacteria is lysogenic for a prophage that contains an IPTG inducible T7 RNA polymerase. So when this strain is transformed and is in the presence of IPTG, T7 RNA polymerase initiates the production of protein, including recombinant hBD-3 fusion protein, as shown in Figure 3.

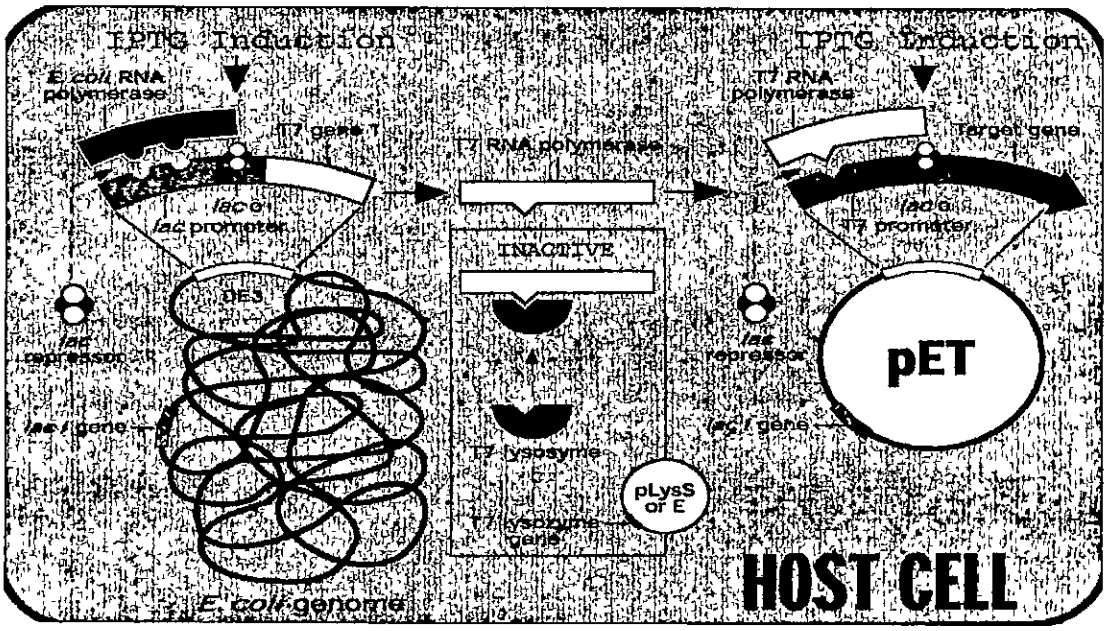


Figure 3. IPTG induction of rhBD-3 expression. IPTG is shown to induce the binding of *E. coli* RNA polymerase to the *lac* operon and *lac* promoter in the *E.coli* genome which ultimately induces translation of the T7 RNA polymerase and expression of the rhBD-3. (Novagen®, Competent Cells Manual, 6/01).

Recombinant hBD-3-His Tag Fusion Protein

This protein is called a recombinant because it refers to the generation of a foreign protein in a life system that normally does not produce that protein. It is called a fusion protein because it contains the HBD-3 antimicrobial peptide linked to bioengineered tags, which facilitate the identification and isolation of the fusion protein. The cDNA coding sequences for the 67-amino acid precursor peptide and the 45-amino acid, mature peptide are shown below in Figure 4.

HBD-3 Mature peptide (45 aa)

```
gga atc ata aac aca tta cag aaa tat tat tgc aga gtc aga
 G   I   I   N   T   L   Q   K   Y   Y   C   R   V   R
ggc ggc cgg tgt gct gtg ctc agc tgc ctt cca aag gag gaa cag atc ggc aag
 G   G   R   C   A   V   L   S   C   L   P   K   E   E   Q   I   G   K
tgc tcg acg cgt ggc cga aaa tgc tgc cga aga aag aaa taa
 C   S   T   R   G   R   K   C   C   R   R   K   K
```

Figure 4. Nucleic and amino acid sequence of hBD-3. (Harder et al. 2001).

Overview of Methods

The cDNA that contains the natural form of hBD-3 was cloned into the expression vector pET-30c (Novagen®), which contains an NH2-terminal His-Tag sequence, by Dr. Jürgen Harder (Harder et al. 2001). The resultant plasmid was transformed into *E. coli* BL21(DE3) (Novagen®) which was then grown to an optimal density of 0.6 (representing mid-log phase) so that the expression of the recombinant hBD-3 (rhBD-3) fusion protein was regulated

by the concentration of Isopropyl β-D-thiogalactopyranoside (IPTG) in the growth medium. Expression was carried out for 4 hours, and bacteria were harvested by centrifugation and lysed by sonication. The generated recombinant hBD-3 (rhBD-3) fusion protein was purified from other non-specific proteins by passing through a nickel affinity column (Novagen®) and eluting the recombinant hBD-3 (rhBD-3) fusion protein. Column bound material was digested with enterokinase (Invitrogen) and the released 45 amino acid containing hBD-3 was initially screened for microbicidal activity using radial diffusion assays (see Materials and Methods section entitled Radial Diffusion Assays).

Once the fusion protein was identified via Nickel Affinity Blot and isolated, the HBD-3 antimicrobial peptide could be purified with high performance liquid chromatography. Verification that the rhBD-3 was identical to native hBD-3 (Harder et al. 2001) was conducted by reforming N-terminal sequencing and matrix assisted laser disruption ionization time of flight-mass spectrometry, (MALDI-TOF-MS), (to be referred to as MS). To initially test the activity of the rhBD-3, radial diffusion assays (RDAs) were conducted against *E.coli* ML35p, in low μM concentrations. RDAs indicated that our protein possessed microbicidal activity. The antimicrobial capacity of hBD-3 was then further tested against *Actinobacillus actinomycetemcomitans, Streptococcus sanguis, Porphyromonas gingivalis, and Fusobacterium*

nucleatum) by conducting microbial killing assays, as described in the section entitled Microbial Killing Assays.

Transformation, Antibiotic Selection, and Storage of Strains

Preparation of Media

A water bath was set at 56° C. Luria-Bertani (LB) broth (Fischer, Scientific International Inc., Hampton, New Hampshire) (12.5 g) was weighed out, using a standard laboratory scale, into a 1 L flask. Five hundred milliliters of deionized water (dH_20) was added and the contents were solubilized, after adjusting the pH (7.5). The media was autoclaved and stored at room temperature.

Preparation of Kanamycin

Kanamycin (0.9 g of stock powder) was weighed out into a 50 mL Falcon tube, and 30 mL of autoclaved, dH_20 were added (final concentration 30 mg/mL). The kanamycin was solubilized in the dH_20 by inverting the 50 mL tube. This 30 mg/mL kanamycin solution was filtered through a 0.2 μm filter, that was attached to a 10 mL syringe, and aliquoted into six-15 mL Falcon tubes.

Preparation of LB-agar Plates for *E. coli*

Agar (7.5 g) was weighed out and added to the LB broth (described above), followed by autoclaving. In the hood, 200 mL of the (12.5 g LB broth, 7.5 g agar, 500 mL dH_20) broth/agar solution were aliquoted to an empty autoclaved flask. These flasks were placed into a 56° C water bath, for 30

minutes. Twelve plates were labeled "30 μg/mL kanamycin", and 8 plates "100 μg/mL ampicillin." In the hood, 300 μL of the 30 mg/mL kanamycin stock solution were added to the 300 mL agar/broth solution. This solution was then mixed and 23 mL per plate was aliquoted to twelve, 100 mm plates. The plates were left half-uncovered until the gel cooled, so that condensation did not collect on the plate cover. In the hood, 400 μL of the 50 mg/mL ampicillin stock solution was added to the 200 mL agar/broth solution and mixed. Twenty-three milliliters of the solution were aliquoted into each plate of eight, 100 mm plates. The plates were stored, with the gel side up at 4°C (cold room).

Preparation of pET-30c Plasmid Containing hBD3 cDNA (pET-hBD3)

We started with a stock solution of 20 ng pET-hBD3 plasmid/μL in dH_20. One microliter of the plasmid solution was added to 9 μL dH_20, which yielded 2 ng/mL pET-hBD3 plasmid.

Preparation of BL21(DE3) competent cells (Novagen®)

The water bath was set to 42° C. Ten sterilized 1.5 mL tubes were labeled and pre-cooled on ice. A tube containing 200 μL of BL21(DE3) competent cells (stored in 1 mL microcentrifuge tubes with caps) was removed from -70° C storage and promptly put on ice and allowed to thaw. The tube was "flit" three times to mix. No vortexing of the tube containing the competent cells was performed. Twenty microliters of competent cells were removed from the middle of the cell solution. The stock was immediately

replaced on ice. The 20 µL aliquot was placed immediately at the bottom of one of the pre-cooled 1.5 mL tubes. The aliquot was pipetted up & down once, to mix. This step was repeated until all the cells were aliquoted. Three tubes were placed on ice, and all left over tubes were returned to -70° C storage.

Transformation

One microliter of the following was added to BL21(DE3) competent cells, respectively, (a) 20 ng/µL "test plasmid", generated for Novagen®'s plasmid standard assay kit, (b) 20 ng/µL pET-hBD-3 (stock solution), (c) 2 ng/µL pET-hBD-3 (prepared dilution). The three-cell/plasmid solutions were mixed and returned to ice. The three tubes containing the cell/plasmid solutions were placed in a rack. The rack was placed in a 42° C water bath for exactly 30 seconds. The rack was returned to ice for 2 minutes. Eighty microliters of room temperature SOC™ medium, from Novagen®, was added to each tube and then kept on ice. The pET-hBD-3 transformed cells were shaken, at 37° C, 250 rpm, for 60 minutes. Three agar plates containing ampicillin & six plates containing kanamycin were warmed to 37° C, for 20 mins. One hundred microliter volumes of a 2:1, 4:1, and 9:1 dilution of transformed cells in SOC medium were plated on LB plates, respectively. A glass rod was used to spread the cells evenly on the whole plate. The plates were left on the bench top for about 15 minutes.

Antibiotic Selection

The plates were cultured overnight, with the gel side up, at 37° C. After 24 hours, transformed colonies were identified, counted and recorded.

Storage of Strains

A single transformed colony was inoculated to 50 mL of LB broth containing 30 μg/mL kanamycin, in a 250 mL flask. This culture was incubated with vigorous shaking, at 37° C, until the OD_{600} reached 0.6-0.8. Aliquots of 0.9 mL were removed and transferred to 2 mL cryovials. Aliquots of 0.1 mL of 80% glycerol were added to each cryovial. These were mixed well and stored at -70° C.

Interim Summary

Nine agar plates were streaked with BL21(DE)3, competent *E.coli* cells and the pET-hBD3 plasmid which confers kanamycin resistance and encodes for the transcription of HBD-3. Twenty-five milliliters of transformed BL21(DE)3, competent *E.coli* cells were frozen at -70° C and stored for future use.

Mini-preparation of pET-hBD3 plasmid

Preparation of Cell Culture

One liter of LB broth was prepared, adjusted to a pH of 7.5 with 1N NaOH, autoclaved, and cooled to -56° C before adding kanamycin (30 μg/ml final). A sterile inoculation loop was used to transfer a well-isolated colony from kanamycin selected, pET-hBD-3 transformed *E.coli* BL21(DE3) plates

into 3 mL of LB broth supplemented with 30 μg/ml kanamycin, in a 10 mL Falcon culture tube. This culture was shaken overnight at 240 rpm, 37°C.

Cell Collection

The morning after overnight culture, 50 μL per prep of Spin Prep Elution Buffer (Novagen®) was warmed to 50ºC (300 μL total). An aliquot (1.5 mL) of overnight culture was transferred to a tube and centrifuged at 10,000 rpm for 1 minute. After decanting the supernatant, the resulting pellet was suspended in a second 1.5 mL aliquot of cell culture, centrifuged, and the supernatant was discarded.

Cell Lysis

The cell pellet was gently vortexed to resuspend the cells in 100 μL, of ice-cold, SpinPrep Resuspension Buffer® (Novagen®) (50 mM glucose, 25 mM Tris-HCl, pH = 8.0, 10 mM EDTA, and RNase). Complete resuspension of the pellet was critical to obtain the best yield. Lysis Buffer (200 μL) was added to the cell suspension and mixed gently by inverting the tube six times. The tubes were not vortexed, since this could cause shearing of genomic DNA. The tubes were incubated for 5 minutes at room temperature. The tubes were carefully opened, 400 μL of Spin Prep Neutralization Buffer (Novagen®) were added, and the contents were mixed gently by inverting each tube. Again tubes were not vortexed. The tubes were centrifuged, 12,000 rpm in a microcentrifuge, 10 minutes.

Absorption, Washing, and Elution

A SpinPrep Filter (Novagen®) was placed in a 2 mL receiver tube. Most of the supernatant (650 µL) was transferred to the Spin Prep Filter/ receiver tube from the Spin Prep Kit (Novagen®) followed by centrifugation. The white pellet of debris was not disturbed while the supernatant was removed. Spin Prep Wash Buffer (Novagen®) (650 µL) was added to each filter and centrifuged for 30 seconds. The supernatant was removed. The Spin Prep Filter/ receiver tube (Novagen®) was centrifuged for another 2 minutes to remove residual SpinPrep Wash Buffer (Novagen®). The SpinPrep Filter was transferred to the 1.5 mL eluate receiver tube (Novagen®). Fifty microliters of SpinPrep Elute Buffer (Novagen®) (pre-warmed to 50° C) was pipetted onto the SpinPrep Filter membrane. The cap of the receiver tube was closed and incubated for 3 minutes at 50° C. The eluate receiver tube/ SpinPrep Filter membrane (Novagen®) was immediately centrifuged for 1 minute to collect eluted plasmid DNA. For maximum DNA yield, a second elution was performed and repeated 5 times. The SpinPrep Filter membrane (Novagen®) was discarded after use.

Interim Summary

A well-isolated colony of pET-hBD3 transformed *E.coli* BL21(DE3) was transferred from kanamycin-selected agar plates into 3 ml of LB broth supplemented with 30 µg/ml kanamycin and cultured overnight. The bacteria

grew and were collected by centrifugation. The bacterial cells were lysed using Lysis Buffer (Novagen®), emptying their protein contents, including intact pET-hBD-3 plasmid.

DNA Quantification by UV Spectroscopy

DNA yield can be determined spectrophotometrically by measuring the absorbance of a dilution of the purified plasmid at 260 nm. The normal calculation for dsDNA is 1 A_{260} = 50 µg/ml, for a 1 cm path cuvette. For optimal accuracy, the target absorbance for UV spectrophotometer readings should be between 0.1-0.5. Therefore, the sample plasmid DNA was diluted between 1:40 to 1:200 in deionized water prior to measuring UV absorbance. The recorded A_{260} was multiplied by the dilution and the extinction coefficient (50 µg/ml/ 1.0 A_{260}) to obtain the concentration of plasmid DNA in µg/ml.

Small Scale Expression

Induction of Overnight Culture with IPTG

Six, well isolated, transformed *E. coli* BL21(DE3) colonies were taken from a kanamycin plate and inoculated to six tubes, respectively, each containing five milliliters of LB broth (30 µg/mL kanamycin). These six tubes were cultured overnight, at 37°C, mixing at 160 rpm. Following overnight culture, 2 mL from each of the six tubes were added to six flasks containing 50 mL LB broth (30 µg/mL kanamycin). Turbidity assessments were conducted using a Genesys™ Spectronic Instruments® spectrophotometer (Spectronic Instruments®, Rochester, New York). The OD_{600} was measured

at this point (time (t) = 0) was about 0.1 against an LB broth blank. The six, 52 mL cultures were incubated at 37° C, with shaking at 250 rpm, for 90 minutes. The OD_{600} was monitored during incubation by removing aliquots aseptically. The optical density was measured after the first 70 minutes, and was about 0.4. The cultures were shaken at the desired temperature until the OD_{600} was approximately 0.6-1.0 (90 minutes) in LB broth with 30 μg/mL kanamycin, at 37° C. IPTG (100mM; 500 μL) was added to each flask to yield 1mM IPTG. These cultures were incubated with mixing at 250 rpm, 37°C, for 4 hours. The flasks were placed on ice for 5 minutes. The cells were then harvested by centrifugation at 880 x g for 10 minutes at 4° C, and the supernatant was aspirated off and discarded. The cells were resuspended in 10 mL cold 20 mM Tris-HCl, pH 8.0, and centrifuged as above (this stopped the growth). A 1 mL pipetter was used to aspirate up and down, followed by vortexing to resuspend the pellet. This cell suspension was centrifuged again for 10 minutes at 880 x g. The supernatant was transferred to a fresh tube. The pellet was resuspended in 1 mL cold 20 mM Tris-HCl, pH 8.0. The cell suspension was transferred to a marked microcentrifuge tube and placed on ice. The pellet and supernatant were pulsed 3 times (Sonic Dismembrator 60®, Fisher, North Fayette, Pennsylvania) at setting 9, with 40 seconds intervals. This sonciation lysed the bacterial cell walls and allowed the cellular contents to be collected for initial isolation of recombinant hBD-3.

S-Tag Assay

The S•Tag System is a protein tagging and detection system based on the interaction of the 15aa S•Tag peptide (which is present in the rhBD3 fusion protein) with ribonuclease S-protein. This specific binding is of high affinity ($K_d = 10^{-9}M$). Whereas neither the S•Tag sequence nor the S-protein alone has enzymatic activity, the two components together form active ribonuclease. Therefore, the assay of ribonuclease activity following the addition of purified S-protein provides a direct measurement of the concentration of S•Tag fusion protein. In practice, a sample is added to a buffer containing purified S-protein and the ribonuclease substrate poly(C). After a brief 5-minute incubation, the reaction is stopped with trichloroacetic acid. The resulting precipitate is removed by centrifugation. Activity is measured by reading the absorbance of the supernatant at 280nm, which increases as the poly(C) is broken down into acid-soluble nucleotides by the enzyme. By comparing the results with a known S-peptide standard included in the kit, the molar concentration of the target protein in the sample can be determined. As little as 20 fmol of target protein can be detected in a 5-minute incubation with the S•Tag Rapid Assay. An optimized protocol allows fully developed blots to be produced in 45 minutes with detection of nanogram amounts of target proteins. The kit also includes a positive control. The molecular weight markers are a novel set of seven recombinant proteins with defined sizes at convenient intervals covering 15-150 kDa. Since each

protein carries the S•Tag peptide, the markers serve as highly accurate size standards on Western Blots. The sizes of the markers are 15,000, 25,000, 35,000, 50,000, 75,000, 100,000 and 150,000 Da. In general, samples need to be diluted from 1:10 to 1:500 in water to be in the linear range of the assay. If the target protein is highly expressed, greater dilution may be necessary. For each set of samples, a blank without added target protein and the S•Tag Standard included in the kit are run in parallel.

The following components were assembled in a set of sterile 1.5 mL microcentrifuge tubes as shown in Table 1. The tubes were incubated at 37° C, for exactly 5 minutes. The reactions were stopped by adding 100 µL ice-cold 25% TCA, vortexing and placing on ice for 5 minutes. The tubes were centrifuged at 880 x g for 10 minutes. The absorbance of the supernatant was read at 280 nm. The spectrophotometer was zeroed with tube #1.

TABLE 1.--Reagents for S•Tag Standard Assay

Component	Tube 1 (Blank)	Tube 2 (Standard)	Tube 3 (Experimental)
Sterile water	348 µL	346 µL	348 µL
S•Tag Standard	-	2 µL	-
Sample extract	-	-	2 µL
Blank extract	2 µL	2 µL	-
10X S•Tag Assay Buffer	40 µL	40 µL	40 µL
S•Tag Grade S-protein	10 µL	10 µL	10 µL

After completion of the S•Tag assay, the protein produced from small scale expression was run through a nickel affinity column, as will be described in the Materials and Methods section entitled hBD-3 Fusion Protein Isolation/Nickel Affinity Chromatography. This step isolated the target protein from the nonspecific cell protein. The isolated target protein was then acidified, as will be described in the Methods section entitled Acidification after Ni-NTA Resin Column. This target protein was further isolated using an analytical reverse phase (RP-C_{18}) column (Waters, Milford, Massachusetts) HPLC according to Harder et al., 2001, as will be described in the Methods section entitled HPLC Isolation of HBD-3 Peptide. The purified protein peaks were pooled after HPLC and a Bradford protein assay was performed to quantify the protein.

Bradford Protein Assay

This method quantitates the binding of Coomassie brilliant blue to an unknown protein and compares this binding to that of different amounts of a standard protein, bovine serum albumin. Duplicate aliquots of 0.5 mg/mL bovine serum albumin (BSA) (5,10,15, and 20 µL) were placed into microcentrifugation tubes. The volume was brought in each tube to 100 µL with 0.15 M NaCl. Two blank tubes were prepared with only 100 µL of 0.15 M NaCl. One milliliter of Coomassie brilliant blue solution (100 mg Coomassie brilliant blue G-250, 50 mL of 95% ethanol, 100 mL of 85% phosphoric acid, 750 mL dH_2O, filtered through Whatman No. 1 filter paper) was added to

each tube and vortexed. The optical density was measured with a spectrophotometer using a 1-cm pathlength microcuvette, at a wavelength of 595 nm. A standard curve was generated by plotting absorbance versus protein concentration. The absorbance of the unknown concentration of target fusion protein was measured and the protein concentration was determined from the standard curve. The quantitiated fusion protein yielded from the small scale expression was treated with enterokinase to cleave off the tags, hopefully leaving a peptide with microbicidal activity.

Enterokinase Enzyme Treatment

Recombinant enterokinase (rEK) from Novagen® is a highly purified preparation of the catalytic subunit of bovine enterokinase, which recognizes the identical cleavage site AspAspAspAspLys↓ as the native enzyme and has similar enzymatic activity. rEK exhibits superior rates of cleavage of fusion proteins containing the recognition sequence when compared to native enzyme. Novagen®'s rEK is purified to near homogeneity and exhibits no secondary cleavage arising from contaminating proteases. When analyzed by SDS-PAGE on a 4-20% gradient gel under reducing conditions, rEK migrates as a single band with apparent molecular weight of 26 kDa. One unit of rEK cleaves 50 μg fusion protein in 16 h at 20° C in a buffer containing 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$. However, because each target protein presents the cleavage site somewhat differently, the efficiency can be variable with the target protein. Excess rEK may result in unwanted

proteolysis at secondary sites. Incubation temperatures ranging from 4°C to 37°C can be used, although 20°C is recommended as the starting point for most proteins.

The highest reaction rates and best cleavage efficiencies are demonstrated when the target protein is maintained at a concentration of 2.5 μM or above. Based on this target concentration, 10 μg of a target protein with a molecular weight of 50 kDa should be cleaved in a total volume of 80 μL or less. If the target protein is too dilute, pilot digestions of the control protein can be performed to determine the amount of rEK necessary to achieve cleavage, or the target protein can be concentrated (Recombinant Enterokinase Kits, Novagen®).

In a 5 mL reaction system, 10X rEK cleavage buffer (500 μL) was added to Ni-affinity column purified hBD-3 fusion protein (2.5 mg) and 50U of rEK. Distilled water was added to this solution to make the volume 5 mL. This solution was incubated at room temperature overnight (16 hours). Prior to performing large-scale production and purification of rhBD-3, the microbicidal activity of the cleaved target protein was confirmed by radial diffusion assay.

Activity Screening (Radial Diffusion Antimicrobial Plate Assay)

The radial diffusion assays were performed as described by Lehrer et al (1989) with modifications by Steinberg and Lehrer (1997). *E. coli* strain ML-35p, a standard for antimicrobial activity tests, was used in this assay, as

described in Hanahan (1983). A single colony of *E. coli* strain ML-35p was picked with a polystyrene loop, transferred to a 125 mL capacity bottle that contained 50 mL of trypticase soy broth (TSB) (Difco, Detroit, MI) (with 100 μg/mL of ampicillin), and incubated at 37° C in a shaking water bath for 24 hours. A 50 μL aliquot of the resulting culture was transferred to 50 mL of fresh TSB (with 100 μg/mL of ampicillin), and incubated for 2.5 hours at 37° C in a shaking water bath (to mid-log phase). The bacterial pellet was washed once with 10 mL of cold sterile 10 mM sodium phosphate buffer, pH 7.4, the cells were centrifuged to pellet, and resuspended in 5 mL of the same cold buffer.

Underlay gels were prepared by mixing 50 mL of 100 mM sodium phosphate buffer with 5 mL full strength trypticase soy broth in a 1000 mL beaker. Eeo agarose (5 g) (Sigma) was added and the volume and brought to 500 mL with dH_2O. The final pH was adjusted to 7.4 with 1N NaOH. The suspension was placed on a hot plate, and stirred it under heat until the agarose dissolved. Aliquots of 50 mL were dispensed into the 125 mL bottles, autoclaved at 121° C for 20 minutes and stored the sterilized media at room temperature. The solidified medium was fluidized, aliquots of 10 mL were placed into a water bath maintained at 42° C. The underlay gel contained 10 mM sodium phosphate buffer, 0.3 mg/mL of TSB powder, and 1% (w/v) of agarose.

Turbidity assay determined $5x10^6$ colony forming units (CFU). Using the formula (where: OD_{620} = 1.820) CFU / mL= OD_{620} x $2.5x10^8$ = $4.55x10^8$, a 1:1000 inoculation was performed to fresh TSB additionally containing 1% agarose. $5x10^6$ colonies were plated. The agar /cells were poured into 150 mm plates and cooled. Preformed wells were made in the agar. Five µL of the RP-HPLC purified peptide, (reconstituted in 0.01% acetic acid was pippetted into the wells and allowed to incubate at 37° C, for 3 hours. An overlay agar was poured into the plates. This agar contained 60 g (twice the customary amount) of trypticase soy broth (TSB, Difco) and 10 g of agarose (Sigma) per liter of deionized water (the use of a low electroendosmosis (EEO)-type agarose (Sigma) was critical to limit electrostatic interactions between the positively charged antimicrobial peptide and the polyanionic, sulfated moieties of the agaropectin component of the standard agar (Steinberg and Lehrer, 1997). Acetic acid (0.01%) was used as a negative control and a 5% acetic acid as positive control. Following overnight incubation at 37° C, zones of inhibition was detected and recorded.

An overlay gel was prepared with 60 g of TSB (twice the regular amount) (Difco) and 10 g of agarose (Sigma) added to 1 L of deionized water. The suspension was placed in a beaker and stirred on a hot plate until the agarose dissolved. It was dispensed in 50 mL aliquots into 125 mL bottles that were autoclaved for 20 minutes at 121° C. The sterilized overlay agar was stored at room temperature. Prior to use, it was heated in a microwave

oven to redissolve the agarose, and then placed in a 42° C water bath to keep it molten, and transferred in 10 mL aliquots to 15 mL centrifuge tubes shortly before they were used to pour the overlay gels. Sodium phosphate buffer, (10 mM) pH 7.4 was prepared by diluting 100 mM sodium phosphate buffer, sterilized and maintained at room temperature. It was chilled in an ice bucket before using to wash bacteria.

The target fusion protein was introduced into small (3 mm diameter) sample wells, from which it diffused radially into the gel. Because the limited nutrient content of this underlay gel only allows for 6-8 bacterial doublings, a nutrient-rich top overlay gel was poured after 3 hours of incubation, so that additional colony development would occur. Using target cells entrapped in the gel also prevented microbial aggregation, an event that confounds standard colony counting assays (Steinberg and Lehrer, 1997).

Large Scale rhBD-3 Expression

Cell Preparation

A single, well isolated, transformed *E. coli* BL21(DE3) colony was taken from a kanamycin plate and inoculated to a flask containing 50 mL LB broth (30 µg/mL kanamycin). This flask was cultured overnight, at 37° C, shaking at 160 rpm. Following overnight culture, 30 mL of overnight culture was used to inoculate one liter of LB broth. Turbidity assessments were conducted using a Genesys™ Spectronic Instruments® spectrophotometer. Sterile LB broth was used as the blanking cuvette. The OD_{600} was measured

at this point (time (t) = 0) and the OD was about 0.1 against an LB broth blank.

IPTG Induced HBD-3 Fusion Protein Expression

The fresh inoculation was incubated at 37° C, 240 rpm, for 70 minutes. The OD_{600} was measured after 70 minutes; OD_{600} was about 0.4. The OD_{600} was checked every ten minutes, until the OD_{600} reached 0.6-1 (90 minutes). IPTG was added (10 mL of 100 mM IPTG) to the 1 L culture to yield a concentration of 1mM IPTG. This culture was incubated with mixing at 250 rpm, 37° C, for 4 hours. The flask was placed on ice for 5 minutes.

Cell Lysis

The culture was then loaded into four 250 mL tubes with caps and centrifuged at 8000 rpm, for 10 minutes, at 4° C. The supernatant was aspirated and the cells were washed with ice-cold 20 mM Tris-HCl pH 8.0 and centrifuged again at 8000 rpm, for 10 minutes, at 4° C. Thirty milliliters of cold 1X Ni-NTA binding buffer, pH 8.0, was used to completely resuspend and pool all of the cells together. Lysozyme (400 µg/mL) was added and the cell suspension was placed in a 37° C water bath for 15 minutes and cooled on ice for 20 minutes. The cell suspension was sonicated on ice, to completely break all of the cell membranes. The lysate was pooled and centrifuged at 10,000 rpm for 20 minutes, at 4° C. The cellular debris was pelleted. The pellet and lysate were placed in 30 mL tubes with caps. The supernatant was saved for further processing and stored at -70° C. The pellet was suspended

within 1% sodium dodecyl sulfate (SDS), saved for further processing and stored at -70° C.

hBD-3 Fusion Protein Isolation/ Nickel Affinity Chromatography

Before purifying proteins under native (non-denaturing) conditions, it is important to check that the protein is soluble. However, even if most of the protein is insoluble, it is often possible to purify traces of soluble material on Ni-NTA His•Bind Resin. In the absence of strong denaturing agents such as urea, unstable proteins in cell lysates may be subject to degradation. It is best to work quickly and to keep the lysate at 0-4° C at all times.

The 1X Ni-NTA Bind Buffer contains 10 mM imidazole to minimize binding of untagged, contaminating proteins and increase purity with fewer wash steps. The reagents for this procedure are listed in Table 2.

Table 2.--Reagents for Purification w/ Ni-NTA His•Bind Resins

Ni-NTA His•Bind matrix® Novagen: 6 mL
1X Ni-NTA Bind Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole*
1X Ni-NTA Wash Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole*
1X Ni-NTA Elute Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole*

*These buffers all have a pH 8.0

Equilibration

Three milliliters of the 50% Ni-NTA His•Bind slurry were added to 12 mL of 1X Ni-NTA Bind Buffer and gently mixed. The resin was allowed to settle by gravity (on ice), and 12 mL of the supernatant were removed with a

pipette. The OD_{280} of the lysate was detected before introducing the lysate into the resin column.

Absorbtion

Cell lysate (12 mL) produced from sonication after large-scale expression was added to the Ni-NTA His•Bind slurry and mixed gently by shaking (250 rpm on a rotary shaker) at 4° C for 120 minutes.

Column Packing

The lysate-Ni-NTA His•Bind mixture was loaded into a column with the bottom outlet capped. All the resin was loaded into the column, by pouring it slowing along a glass stirrer.

Washing

The cell lysate and pellet was passed through the column and collected. This first pass, called the flow through, was collected and saved for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Thirty milliliters of wash buffer was prepared by diluting 5X Bind buffer and 1M imidazole with deionized water. The column was washed with 4x6 mL 1X NiNTA wash buffer, and the wash fractions were collected. The OD_{280} was detected and the wash fractions were saved for SDS-PAGE analysis. The optical density is expected to decrease with each wash and increase when the target protein is eluted. Optical density of the flow through should be high as the bulk of nonspecific protein is washed through the column. Following each consecutive wash, the optical density should

decrease as the remaining non-specific protein is washed through the column.

Eluting

Elution buffer (10 mL) were prepared by diluting 5X Bind buffer and 1M imidazole with deionzed water. The protein was eluted with 4 x 1.5 mL 1X Ni-NTA elution buffer. The four elutions were collected in four tubes. The OD_{280} was detected. The optical density should increase with the first few elutions as the target protein with nickel affinity is eluted. The target protein is eluted by increasing the concentration of imidazole. The imidazole competitively binds the nickel in the column freeing up the histidine tags on the target protein to be eluted through the column. Twenty microliters of each elution was aliquoted for SDS-PAGE. All of the flow-through, wash-through, and elutions were stored at -70° C.

Acidification After Ni-NTA Resin Column

Fusion protein acidification was done immediately following elution in order to avoid precipitation. Thirty-five microliters of trifluoroacetic acid (TFA) was added to every 1.5 mL of eluant and tested with pH paper to have a pH 2.0. Following acidification, the eluted fusion protein was placed in the refrigerator.

SDS-PAGE

Preparation of Separating Gel

A water bath was brought to 37°C, and 4X Tris-HCL/SDS, pH 8.8, (stored at 4°C) and 60% acrylamide bis gel, (stored at 4°C) were warmed. Ammonium

persulfate (A.P.) (20 mg) was added to 200 μL dH_2O to yield 10% A.P. The amount necessary for one gel, as shown in Table 3, was prepared in a 10mL Falcon tube.

TABLE 3.--Separating Gel Reagents

Reagents	Volume (mL)
60% AcrylimideBis (1.6%)	1.500
4X Tris-HCL/SDS, pH 8.8	1.875
dH_2O	4.125
TEMED(Electrophoresis Grade)	0.005
10% Ammonium Persulfate	0.025

A 1 mL pipetter was used to place the separating gel solution in between the 2 glass sheets. To separate the gel solution from air and get a smooth surface, dH_2O was placed on top of the separating gel without disturbing the gel.

Preparation of Stacking Gel

The amount necessary for one gel, as shown in Table 4 was prepared in a 10 mL Falcon tube. The tube was placed in a degassing chamber and the reagents were degassed for 10 minutes.

Table 4.--Stacking Gel Reagents

To Be Degassed	Reagents	Volume (mL)
	60% AcrylimideBis (1.6%)	0.175
	4X Tris-HCL/SDS, pH 6.8	0.625
Not to be degassed	TEMED (Electrophoresis Grade)	0.0025
	10% Ammonium Persulfate	0.012

The reagents listed as "Not to be Degassed" in Table 4, were added to the degassed solution.

Addition of Stacking Gel

The water was poured off the separating gel by turning the gel upside down. A paper towel was used to remove the remaining water by capillary action. The stacking gel was added to the top of the separating gel with a pipetter. Once all of the stacking gel was loaded between the glass plates, the plastic comb was placed to create spaces for the loading wells.

Sample Preparation

The 6X sample buffer was pre-warmed to 37° C. Fifteen microliter aliquots of samples, which were in 1mL tubes, were removed from the refrigerator and warmed to room temperature. Tubes were labeled 1-9. Loading buffer 6X SDS (3 μL) was warmed to 37° C and then added to each of the tubes and mixed with a pipette tip. All 18 μL samples were boiled for 5 minutes. The samples were cooled and spun down in the microcentrifuge.

Electrophoresis

Fifty milliliters of 10X Tris glycine/SDS buffer (1 L) were mixed with 450 mL dH20. After the stacking gel was formed and the comb removed, the wells were rinsed with dH_2O, and 1X Tris glycine/SDS buffer was poured into the electrophoresis bath, until it was full. The samples were loaded into their respective wells in the stacking gel. Five microliters of a molecular weight standard were placed into Well/Lane 1. Electrophoresis was performed at

200 volts for 36 minutes. The gel was removed from the assembly and fixed (50% methanol, 10% acetic acid, 40% diH_2O) for 2 hours with gentle shaking. The gel was stained (50% methanol, 0.05% Coomasie brilliant blue, 10% acetic acid, 40% diH_2O) for 4 hours. The stained gel was destained (7% Acetic acid, 5% methanol, 88% diH_2O) overnight with gentle shaking.

Nickel-Affinity Blot

A polyvinylideneflouride (PVDF) membrane (Millipore, Bedford, Massachusetts) was cut to the size of the SDS gel, soaked with methanol, briefly rinsed with dH_2O, and immersed in transfer buffer (1 X Tris-Gly electrophoresis buffer containing 15% methanol). The gel was gently shaken for 10 minutes. The protein was transferred from gel to PVDF (Millipore) membrane in a (Bio-Rad, Hercules, CA) liquid transfer tank, containing transfer buffer, under at 100V for 1hour. The membrane was fixed in 0.05% glutaraldehyde-PBS for 30 minutes, with gentle shaking. The membrane was rinsed with dH_2O, and washed in PBS for 10 minutes, followed by blocking (to use general protein to bind to the membrane so that non specific binding of antibodies to the membrane may be reduced-reduce the background) in PBS containing 2% milk, at 37° C, 30 minutes, and finally rinsed with dH_2O water. A 1:1000 dilution of the Ni-HRP (Nickel-Horseradish peroxidase, Novagen®) in PBS containing 0.05% milk was poured into a plastic bag containing the membrane. The bag was sealed and gently shaken at room temperature overnight. The membrane was washed 3 times with PBS containing 1% milk

5 minutes each time. The membrane was developed with Opti-4CN (Bio-Rad) and rinsed with dH_2O to stop the reaction.

Large Scale Enterokinase Treatment

Enterokinase treatment was performed as decribed on page 45. After the cleavage reaction, released HBD-3 peptide was isolated with HPLC.

HPLC Isolation of HBD-3 Peptide

In high performance liquid chromatography (HPLC) a solvent (mobile phase) is continuously applied to the column (stationary phase). The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster, and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions.

Isolation of hBD-3 fusion protein after Ni-affinity chromatography and final purification of hBD-3 peptide was performed using an analytical reverse phase (RP-C_{18}) column (Waters, Milford, Massachusetts) HPLC according to Harder et al. (2001) and as described for hBD-2 (Harder et al., 2000).

Solvent A and Solvent B were prepared as shown in table 5. The column was not allowed to run dry.

TABLE 5.--HPLC Reagents

Reagents	(Solvent A)	(Solvent B)
Acetonitrile	20 mL	980 mL
TFA	1 mL	850 μL
dH_2O	980 mL	20 mL

The pump injector and detector were purged. The column was equilibrated until a stable base line was reached. The HPLC program used for HBD-3 isolation is shown in Table 6.

TABLE 6.--hBD-3 HPLC Program

Time (minutes)	Flow (mL/minutes)	%A	%B
0	1	100	0
25	1	50	50
26	1	0	100
30	1	0	100
31	1	100	0
35	1	100	0

Injection volume was 200 µL each time, and the running time was 35 minutes. Glass tubes were used to collect the elution peaks. The tubes containing the eluant were placed in a lyophilizer. All liquid phase was removed under vacuum including the volatile organic solvents, acetonitrile and TFA, leaving only a white crystalline peptide. The lyophilized peaks were stored at -70° C,

while waiting for quantification (by the Bradford protein assay) and minimal inhibitory concentration activity screening.

MALDI-MS (TOF)

This type of mass spectroscopy uses a nitrogen ultraviolet (UV) laser (337 nm) (Perkin Elmer, Shelton, Connecticut) to generate ions from high mass peptides and proteins. The key to this technique is that in the presence of matrix-like alpha-cyano-4-hydroxy cinnamic acid (CHCA), large molecules like peptides ionize instead of decomposing. Although the mechanism is uncertain, it may involve absorption of UV light by the matrix, followed by transfer of this energy to the peptide, which then ionizes, into the gas phase as a result of the relatively large amount of energy absorbed. To get the resulting ions to move down the flight tube in the mass spectrometer they are accelerated in an electric field (25 kV). The ions travel down a linear flight path and their mass/charge (*m/z*) ratio is determined by the time it takes for them to reach the detector. Hence the instrument is called a time of flight instrument. The relationship that allows the (*m/z*) ratio to be determined is E = ½(m/z) v2. In this equation E is the energy imparted on the charged ions as a result of the voltage that is applied by the instrument and v is the velocity of the ions down the flight path. Because all of the ions are exposed to the same electric field, all similarly charged ions will have similar energies. Therefore based on the above equation, ions that have larger mass must

have lower velocities and hence will require longer times to reach the detector (Lensey 2001).

Quantification of Target Protein

For a 1 mg/mL concentration of protein, measured at a given absorbance wavelength, there exists an extinction coefficient, related to its amino acid sequence. Using software available on the internet site http://parischem.yale.edu/extinct.html, the extinction coefficient for rhBD-3 was determined to be 0.6355. The absorbance of an unknown concentration of target protein was measured, this number was divided by the extinction coefficient of rhBD-3 and the concentration of target protein was determined.

Susceptibility Against HBD-3, Colony Killing Assays

Microbicidal assays for hBD-3 were conducted against various Gram-positive and Gram-negative oral bacterial species. These species included the Gram-positive: *Streptococcus sanguis*, the Gram-negative species: *Actinobacillus Actinomycetemcomitans; Porphyromonas gingivalis*, *Fusobacterium nucleatum.*

Actinobacillus Actinomycetemcomitans strain *JP2* (*A.a.JP2*) is implicated in localized juvenile periodontitis (LJP). It was a kind gift from Dr. Y. Han (CWRU). The bacteria were grown anaerobically in sterile trypticase soy broth (3%) supplemented with 2% yeast extract and 0.04% sodium bicarbonate. Agar plates were made with liquid medium plus 1.5% agar.

Streptococcus sanguis (*S.s.* ATCC 10556) was grown under the same conditions and in the same media with *A.a.JP2.*

F.n. ATCC 25586 was grown in mycoplasma broth medium. Mycoplasm broth base 21 g/L hemin 5 μg/L and menadione 1 μg/L.

P.g. ATCC 33277 was grown in ETSB medium prepared as described by Syed et al. 1980.

Both *F.n.* and *P.g.* were maintained on blood agar plates. The plates were made from *P.g.* liquid medium plus 3% sheep blood and1.5% agar. The reagents listed in Table 7 are per 1 L. The pH was adjusted to a pH 7.1.

TABLE 7.--Recipe for Modified Trypticase Soy Broth (ETSB) for *P.g.*

Ingredients	Amount
Trypticase Soy Broth (BBL)	30 g
Yeast Extract (Difco)	1 g
Glucose (dextrose)	1 g
Potassium nitrate	0.5 g
Sodium lactate (60%) (Lactic acid) (Sigma L-1375)	1 mL
Sodium succinate (Succinic acid)	0.5 g
Sodium Fumerate (Fumaric acid) (Sigma F-1506)	1 g
Distilled water	950 mL

This basal medium was autoclaved, then the filter-sterilized supplements from Table 8 were added. The bacteria were grown in an anaerobic chamber (5% H_2 / 5% CO_2 / 85% N_2).

TABLE 8.--Supplements to Modified Trypticase Soy Broth

Sodium Carbonate, anhydrous	0.4 g
Cysteine HCl (Sigma C-7880)	0.4 g
Hemin (Sigma H-2250) Stock Solution (50mg of Hemin in 1.0mL of 1.0N NaOH & 99.0mL dH_2O)	10 mL
Vitamin K (Sigma M-5625) Stock solution 250mg of Vitamin K (menadione) in 50mL of 95% ethanol.	0.2 mL
Distilled Water	40 mL
Sheep Blood (for are good up to 2 weeks) Let media cool to 50-55°C prior to adding blood, to avoid hemolization.	20 mL

Reference for original ETSB recipe: Syed. (1980).

hBD-3 Microbicidal Activity Test

A.a. and *S.s.* were inoculated at a ratio of 1:1,000. *F.n.* and *P.g.* were inoculated at a ratio of 1:20. These cultures were inoculated to 10 mM phosphate buffer (containing 1% TSB), pH 7.4, and grown in an anaerobic chamber at 37° C overnight. The cells were collected by centrifugation at 6000 rpm, at 4° C, for 10 minutes. The supernatant was aspirated. The cells were washed with pre-reduced buffer. The cells were again centrifuged and resuspended with pre-reduced buffer. The absorbance was detected and the colony forming units were calculated as shown in Table 9.

TABLE 9.--Calculation of Colony Forming Units

Bacteria	Calculation of colony forming units
A.a.	$OD_{600} = 6.05 \times 10^8$ CFU/mL
S.s.	$OD_{600} = 2.0 \times 10^8$ CFU/mL
F.n.	$OD_{600} = 3.8 \times 10^8$ CFU/mL
P.g.	$OD_{600} = 3.8 \times 10^8$ CFU/mL

The cells were diluted to $4x10^6$ CFU/mL with reduced buffer. In microcentrifuge tubes, four different amounts of purified hBD-3 were added and used 0.01% acetic acid to make up the solvent difference. Pre-reduced phosphate buffer-TSB was used to make the total volume 100 µL, containing 50 µL cells each tube, which was anaerobically cultured at 37° C for 3 hours, followed by 10 fold serial dilution. One hundred milliliters was plated on blood agar plates (*F.n.* and *P.g.*). *A.a* and *S.s.* colonies were counted on Day 2. *F.n.* and *P.g.* colonies were counted on Day 7.

RESULTS

Transformation of *E. coli* BL21(DE3)

Single colonies of transformed bacteria expressing the BL21(DE3) plasmid were picked from kanamycin (30μg/mL) containing agar plates and inoculated to a nutritional broth. These transformed bacteria were then grown to a greater concentration and stored in glycerol at -70º C, for later use, as described in Materials and Methods section entitled Transformation.

Plasmid DNA Mini Preparation of pET-hBD-3 plasmid.

Positive clone expansion was then performed, as described on in the Materials and Methods section entitled Mini-preparation of pET-30-hBD-3 plasmid. Each of the 100 mL agar plates, that were streaked, yielded transformed BL21(DE3) E. coli. The number of colonies of BL21(DE)3 *E. coli* yielded are listed in Table 10.

TABLE 10.--Colony Yield From Plasmid Mini Preparation

PLATE	Volume of SOC Medium	Volume of BL21(DE)3 cells + plasmid	Volume of Ampicillin (Amp) or Kanamycin (Kan)	Number of Isolated Colonies After 24hr
1	50 μL	50 μL of test plasmid	Amp 50 μg/mL	50
2	80 μL	20 μL of test plasmid	Amp 50 μg/mL	14
3	90 μL	100 μL of test plasmid	Amp50 μg/mL	6
4	50 μL	50 μL BL21(DE)3 cells 20 ng pET-hBD-3 plasmid	Kan 30 μg/mL	13
5	80 μL	20 μL BL21(DE)3 cells, 20 ng pET-hBD-3 plasmid	Kan 30 μg/mL	2
6	90 μL	20 μL BL21(DE)3 cells, 20 ng pET-hBD-3 plasmid	Kan 30 μg/mL	1
7	50 μL	50 μL BL21(DE)3 cells, 2 ng pET-hBD-3 plasmid	Kan 30 μg/mL	6
8	80 μL	20 μL BL21(DE)3 cells, 2 ng pET-hBD-3 plasmid	Kan 30 μg/mL	1
9	90μL	10 μL BL21(DE)3 cells, 2 ng pET-hBD-3 plasmid	Kan 30 μg/mL	2

The cells were lysed and spun down using the spin prep filter (Novagen®) to collect the plasmid. The DNA yield was determined spectrophotometrically, by measuring the absorbance of a dilution of the purified plasmid at 600 nm. The results are listed in Table 11.

TABLE 11.--Spectrophotometric Quantification of DNA Yield

Clone	OD_{260}	OD_{280}	OD_{260}/OD_{280}	Concentration (μg/μL)
1	0.031	0.017	1.82	0.15
2	0.026	0.015	1.73	0.13
3	0.024	0.013	1.85	0.12
4	0.021	0.014	1.5	0.11
5	0.028	0.016	1.75	0.14
6	0.026	0.014	1.86	0.13

Note: all the samples were at 1:100 dilutions.

Table 11 shows the quantity of DNA yield for the six clones. Aliquots of this plasmid were evaluated in a 1% agarose gel, with a 0.5X Tris acetic acid (TAE) EDTA buffer, electrophoresis for 50 minutes at 50 V. Three of the samples were electrophoresed and showed an identical band. The result of this gel is shown in Figure 5. On the left of this gel is a DNA marker. The right three lanes reveal an upper row of bands, which are likely circular DNA. Further down the gel there are three more faint bands of super coiled DNA, circumscribed by a red oval.

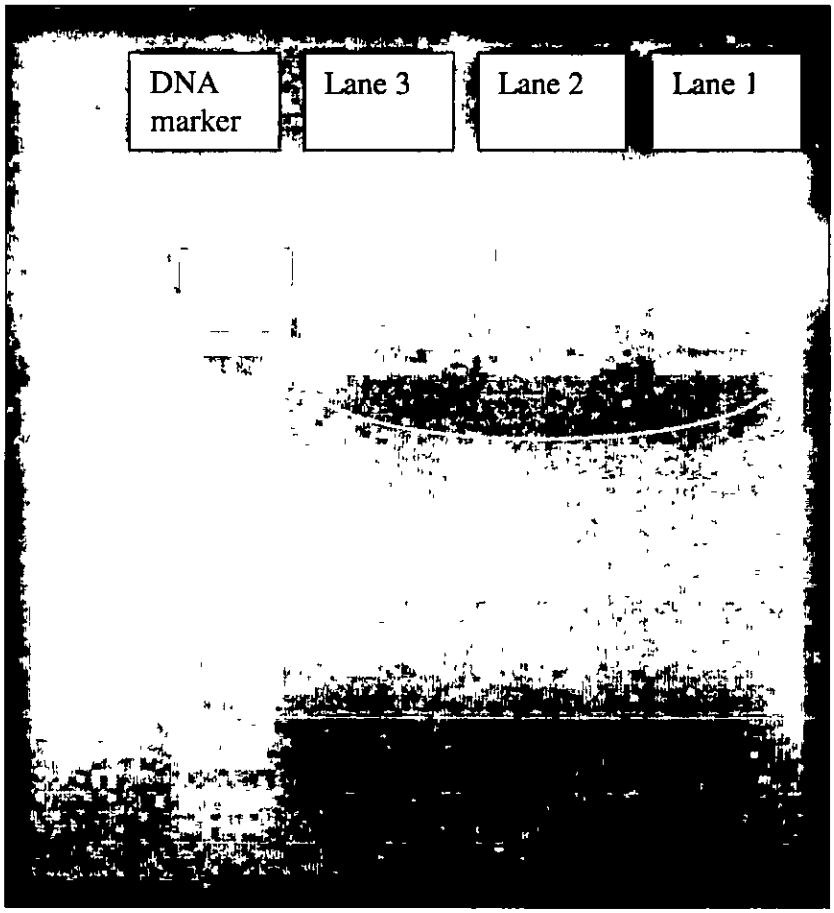


Figure 5. pET-30c-HBD3 plasmid electrophoresis. 1% agarose in 0.5 X TAE buffer, 50 Volts, 50 minutes. The red oval denotes three bands of tightly coiled DNA.

Small Scale Expression

Small scale expression was performed as described in the Materials and Methods section entitled Small Scale Expression. Transformed cells were prepared, stored at -70º C, thawed and used to inoculate a broth, and left to culture overnight. The following day OD_{600} was measured and recorded, at t = 0, Table 12.

TABLE12.-- Determination of Cell Density, Small Scale

	$OD_{600\ nm}$					
Time (minutes)	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6
0	0.100	0.98	0.102	0.105	0.94	0.99
70	0.439	0.444	0.441	0.436	0.432	0.437
80	0.538	0.537	0.542	0.546	0.532	0.541
90	0.669	0.665	0.671	0.672	0.659	0.665

The overnight culture was incubated at 37°C, with shaking at 250 rpm, until OD_{600} reached 0.4–0.8. The OD_{600} was measured and recorded, again at t = 70 minutes. Then the entire culture was added to 100 mL LB broth with 30 μg/mL kanamycin. The 100 mL culture was shaken at the desired temperature until the OD_{600} was approximately 0.6-1.0 in LB broth with 30-mg/mL kanamycin, at 37°C. The OD_{600} was monitored during incubation by removing aliquots aseptically, in the hood. The results of spectrophotometric determination are shown in Table 13.

When the OD_{600} was found to be above 0.6, the 100 mL culture was split into 2 x 50 mL cultures. IPTG was added to 1 mM (500 µL of sterile 100 mM IPTG) to one of the 50 mL Falcon tube cultures. The other culture served as the uninduced control. These cultures were incubated with shaking at 37° C, for 4 hours, centrifuged, washed, centrifuged, washed and sonicated to lyse the cells and harvest the fusion protein. The resulting cell lysate was collected. An S•Tag™ Assay was then performed to identify if the S•Tag that is present in hBD-3 fusion protein was present in the cell lysate of the transformed cells that were produced during small scale expression.

S-Tag Assay

S•Tag™ Assay was performed as described in the Materials and *Methods* section entitled S-Tag Assay. This protein tagging/detection system uses the addition of the protein from lysed cells as the substrate for an enzyme cleaving reaction. The cells produced in small-scale expression were sonicated. These sonicate contained nonspecific protein, as well as, the hBD-3 fusion protein, which was genetically engineered to contain an S•Tag™. The sonicate containing the hBD-3 fusion protein (which contains an S•Tag™) was added to a buffer containing a ribonuclease S-protein. As was described in the Methods section entitled S-Tag Assay, the specific binding of the S•Tag to the ribonuclease S-protein is high (K_d = 10^{-9}M). Neither the S•Tag sequence nor the S-protein alone has enzymatic activity, but the two components together form active ribonuclease. Therefore, the assay of ribonuclease activity following the addition of purified S-protein provides a direct measurement of the concentration of S•Tag fusion protein. After a 5-minute incubation, the reaction was stopped by adding trichloroacetic acid (TCA). The resulting precipitate was removed by centrifugation. Activity was measured by reading the absorbance of the supernatant at 280nm, which increases as the poly(C) is broken down into acid-soluble nucleotides by the enzyme. The results are listed in Table 13.

TABLE 13.--S-Tag Assay for Small Scale Expression

	Sterile H_20	S-Tag Standard	Sample	10X assay buffer	S-protein	$0D_{280nm}$
Control	175 µL	0 µL	0 µL	20 µL	5 µL	0
Standard	174 µL	1 µL	1 µL	20 µL	5 µL	0.522
Lysate (natural)	174 µL	0 µL	1 µL	20 µL	5 µL	2.860
	174 µL	0 µL	1 µL	20 µL	5 µL	2.828
	174 µL	0 µL	1 µL	20 µL	5 µL	2.860
Pellet/ (denatured)	174 µL	0 µL	1 µL	20 µL	5 µL	2.609
	174 µL	0 µL	1 µL	20 µL	5 µL	2.627
	174 µL	0 µL	1 µL	20 µL	5 µL	2.622

These spectrophotometric results show that there is protein that has an absorbance similar to what we would expect for HBD-3 fusion protein. These results also indicate that after IPTG induction we found S•Tag both in the cell lysate (natural) and the pellet (denatured).

Radial Diffusion Assay

Following S•Tag assay, the protein generated in the small scale expression was run through a nickel affinity column to isolate the target fusion protein. A radial diffusion assay was performed to confirm microbicidal activity, prior to investing more time in large scale production, identification, and isolation. The results of this radial diffusion assay are shown in Figure 6. It can be seen in Figure 6 that the bacterial lawn was inhibited by the fusion protein.

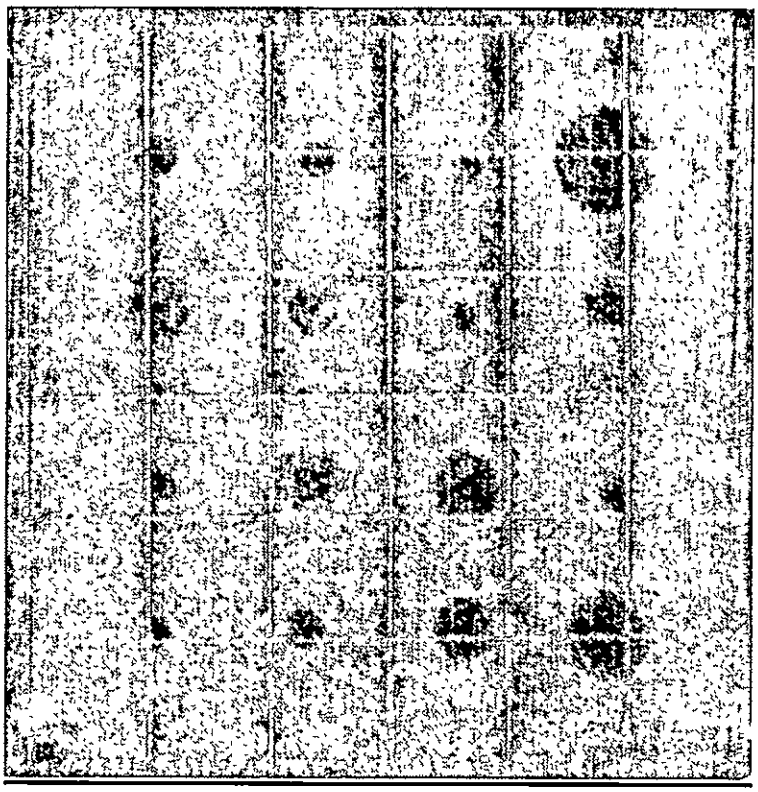


Figure 6. Radial Diffusion Assay of Fusion Protein. Note the circular areas demonstrating antimicrobial activity.

Large Scale Expression

Thirty milliliters of overnight culture was added to 1 L of LB broth as described in the Materials and Methods section entitled Large Scale Expression. The OD_{600} was monitored during incubation by removing aliquots aseptically, in the hood. The results of spectrophotometric determination are shown in Table 14.

TABLE 14.--Determination of Cell Density Large Scale

Time (mins)	0	70	80	90
OD_{600}nm	0.100	0.206	0.433	0.60

IPTG was added (10 mL of 100 mM IPTG) to the 1 L culture to yield a concentration of 1 mM IPTG. The culture was incubated at 37° C, 240 rpm, 4 hours. The culture was then loaded into four 250 mL tubes with caps and centrifuged at 8000 rpm, for 10 minutes, at 4° C. The supernatant was aspirated off and the cells were washed with ice-cold 20 mL 1X Ni-NTA binding buffer, pH 8.0 (convenient for the Ni-affinity chromatography). The nickel of this Ni-NTA binding buffer binds to the Histidine•Tag on the hBD-3 fusion protein.

HBD-3 Isolation and Verification

Nickel Affinity Chromatography

The fusion protein was run through the nickel affinity column. The fusion protein, which has an affinity for nickel, bound to the column; while non specific protein was washed though the column. After the nonspecific protein was sufficiently washed through the column, the concentration of imidazole was increased. The imidazole bound the nickel in the column, more competitively, than the histidine tags of the fusion protein. This allowed the fusion protein to be eluted separately through the column and collected. The washes and elutions from this column were collected. Aliquots of these collections were placed in a cuvette and evaluated spectrophotometrically

(OD_{280}). The results are listed in Tables 15 and 16. The wash buffer and the elution buffer were measured as the negative control or blank, respectively. The results of spectrophotometry listed in Table 15 were measured against the wash buffer as the negative control. Absorbance readings greater than zero indicate some degree of protein present in the washed sample. The washed sample contains the nonspecific cell protein from the lysed transformed bacteria that does not bind the nickel affinity column. Table 15 shows a decreasing absorbance after each washing of the column, as we would expect to see, if the largest amount of nonspecific cell protein was washed through the column initially and less and less residual nonspecific protein remains in the column to be washed out.

TABLE 15.--Results of Nickel Affinity Chromatography/Wash

	Cell Lysate	Flow through	1st Wash	2nd Wash	3rd Wash
OD_{280}	4.00	3.815	2.760	0.330	0.225

Table 16 and Figure 7 illustrate that, as the concentration of imidazole was increased in the elution buffer in relation to the wash buffer (the imidazole competitively bound to the nickel in the column and) hBD-3 protein was eluted (most greatly in the first and second elutions and then in decreasing amounts with each elution as there was less residual hBD-3 remaining in the column with each successive elution).

TABLE 16.--Results of Nickel Affinity Chromatography/Eluate

	1st Elution	2nd Elution	3rd Elution	4th Elution	5th Elution
OD_{280}	1.549	2.966	0.958	0.288	0.132

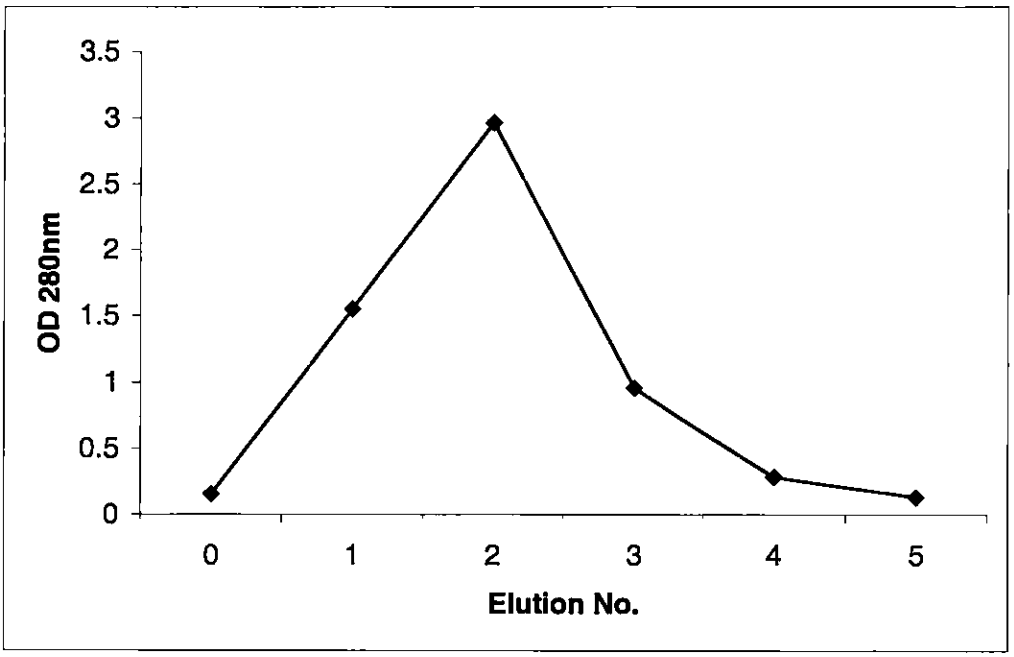


Figure 7. Nickel Chromatography/Elution of Fusion Protein

The results of Table 16 have been plotted on a graph, in Figure 7. In summary, these optical density measurements show that the protein produced has an affinity for nickel, and that it can be eluted by high concentrations of imidazole.

SDS PAGE Analysis

SDS PAGE analysis was performed to show the size of the fusion protein that passed through the nickel affinity column and also to give an impression of how much protein was produced, and understand what was the

efficiency of the Ni-affinity chromatography. The results of the gel are shown in Figure 8.

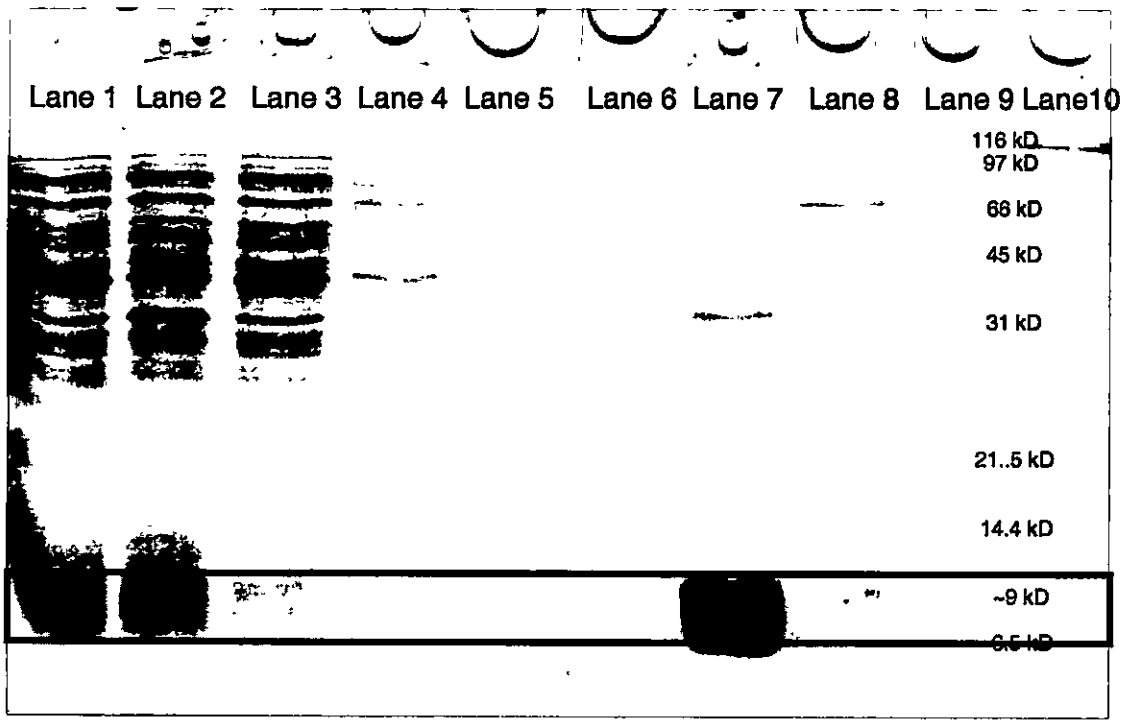


Figure 8. SDS PAGE/large scale. These samples were collected after cell lysis and Ni affinity chromatography. Lane 1 contained cell lysate, Lane 2 contained pellet, Lane 3 contained Flow through, Lanes 4 & 5 contained the first & second washed respectively, Lanes 6-9 contained the elutions 1-4, respectively, and Lane 10 contained the molecular weight marker. The target fusion protein is reported to be ~9kD, note the dense bands circumscribed by the red box.

In Figure 8, Lanes 1, 2, 3, 6, 7, and 8, each show a dense target band, which is indicative of a large amount of protein. Lane 1 contains the cell lysate, which was yielded after IPTG induction of the fusion protein, cell lysis, and centrifugation. Lane 2 contains the insoluble pellet, which was yielded after

lysis and centrifugation. Lane 3 contains the flow through, the initial run of all the protein through the nickel-NTA column. Here we see that the target band in Lane 3, 4, & 5 are lighter than that of the cell lysate, in Lane 1, or the pellet in Lane 2. This is because the nickel column should bind the recombinant HBD-3 fusion protein. Only a fraction of the total protein, the nonspecific protein that lacks nickel affinity, would be washed through the column and be collected. Lanes 6, 7, and 8 shows that the target band of protein has reappeared and is enriched. This protein must posseses an affininity for the nickel-NTA column, for it not to have been washed through the column. Once the concentration of imidazole was raised high enough, interfering with Nickel binding, the target protein started eluting through the column. The gel shows that the BL21(DE3) E. coli that were transformed with Dr. Jurgen Harder's plasmid have produced a protein that binds to nickel as the target protein. Recombinant hBD-3 fusion protein should bind to nickel, due to its His•Tag resin. By relating this target band to the protein marker, this gel also shows that the size of the protein is the size that we would expect it to be. It also shows that the target protein is the major band in the cell lysate, which indicates high expression efficiency, during induction with IPTG.

Nickel Affinity Gel

Another SDS PAGE gel, identical with that shown in figure 8, was transferred to a PVDF (Millipore) membrane, the membrane used for the nickel affinity blot. After incubation at room temperature and treatment with

nickel conjugated Horse radish peroxidase (Ni^{2+}-HRP). The horseradish peroxidase binds nickel (Ni^{2+}-HRP) that is associated with the hBD-3 fusion protein from the Ni-NTA buffer. Bands are evident in Lanes 1 & 2 & 7. These lanes contained the target protein with nickel affinity. The gel, in Figure 9, shows that the HRP bound to the protein that had nickel affinity. The results showed that Lane 7, the second elution, had the majority of Nickel binding protein.

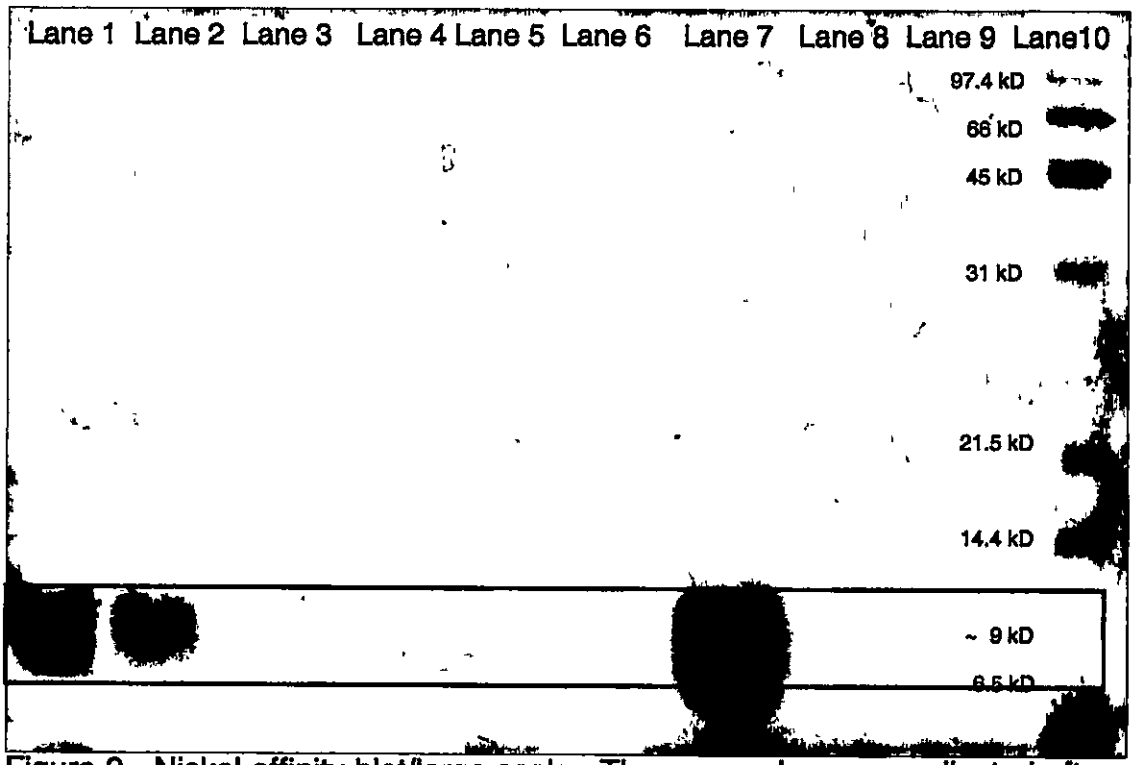


Figure 9. Nickel affinity blot/large scale. These samples were collected after cell lysis and Ni affinity chromatography. Lane 1 contained cell lysate, Lane 2 contained pellet, Lane 3 contained Flow through, Lanes 4 & 5 contained the first & second washed respectively, Lanes 6-9 contained the elutions 1-4, respectively, and Lane 10 contained the molecular weight marker. The target fusion protein is reported to be ~9kD, note the dense bands circumscribed by the red box.

RP-HPLC Isolation of HBD-3 Fusion Protein

Upon confirmation that the transformed BL21(DE3) *E. coli* had produced a protein that was of similar size of HBD-3 fusion protein and possessed nickel affinity; high performance liquid chromatography was performed, as described in the Methods section entitled HPLC isolation of HBD-3 peptide. Figure 10 shows a representative sample of one of 20 HPLC runs.

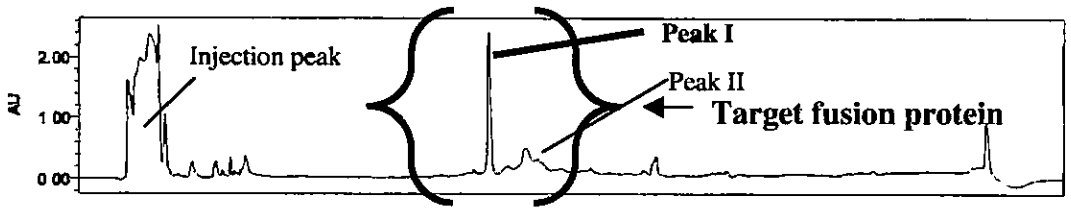


Figure 10. A representative pre-enzyme treatment HPLC.

The target protein peaks were between 14 minutes and 20 minutes. The protein that elicited these peaks was collected.

rEK Cleavage/Rrelease of HBD-3 peptide

As described in the Materials and Methods section entitled, Large Scale Enterokinase Treatment, the protein that was collected from HPLC purification was treated with an enterokinase enzyme purchased from Novagen. This enterokinase enzyme cleaves the S•Tag and His•Tag and other non HBD-3 components of the fusion protein leaving only HBD-3 peptide.

An aliquot of the untreated HBD-3 fusion protein and an aliquot of the treated HBD-3 fusion protein were run through an SDS-PAGE gel. The results of this gel are shown in Figure 11.

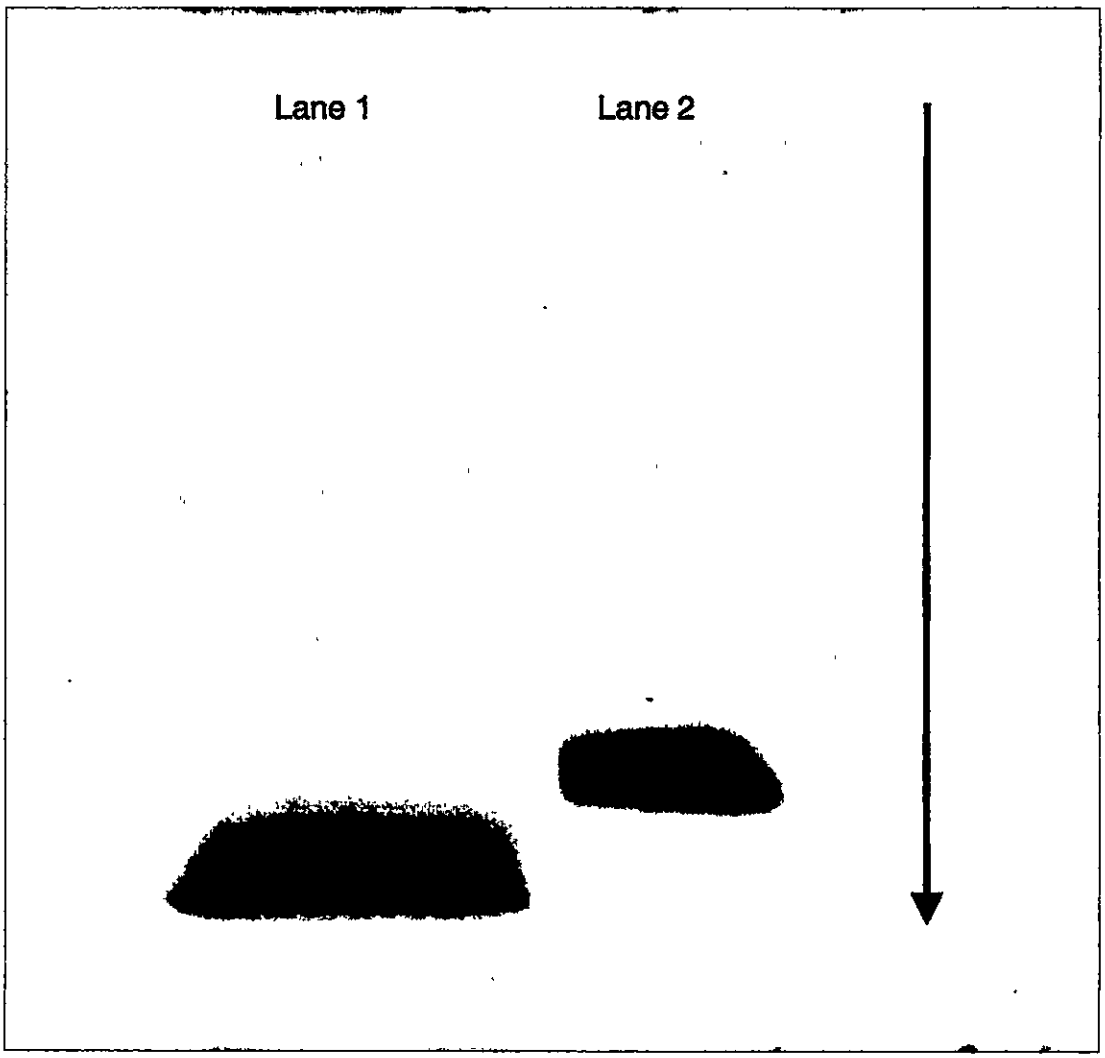


Figure 11. Enterokinase treated vs. untreated fusion protein/ SDS PAGE. Lane 1 contains the enterokinase treated fusion protein. Lane 2 contains untreated fusion protein. A black arrow indicates the direction of migration.

Figure 11 shows that the protein in Lane 1, which was treated with enterokinase (cleaving the engineered S•Tag and His•Tag and leaving only the HBD-3 protein) traveled further along the gel than the untreated HBD-3 fusion protein, because of its smaller molecular mass. This gel suggests that the enterokinase was effective in cleaving hBD-3 free from the rest of the fusion protein. This smaller peptide moved faster through the gel.

A parallel SDS PAGE gel, as that shown in Figure 11, was transferred to a PVDF membrane, as described in the Materials and Methods section entitled Nickel Affinity Blot. After incubation at room temperature the fusion protein was treated with nickel affinity horseradish peroxidase (Ni-HRP). The horse radish peroxidase binds nickel. The results are shown in Figure 12. The more evident band is Lane 2, indicated by a red arrow. This lane contains untreated hBD-3 fusion protein, its remaining tags form the complex Ni-HRP. The target protein with nickel affinity tags bound to the Ni-HRP stains brown. The band in Lane 1 is less evident, because the protein in that lane had its tags cleaved; it is circumscribed by a black oval. Some mild staining did occur and this may be due to nonspecific binding. In both lanes a second set of bands are noticible, these may be due to dimerization. This is a second confirmation that the enzyme cleavage has been effective.

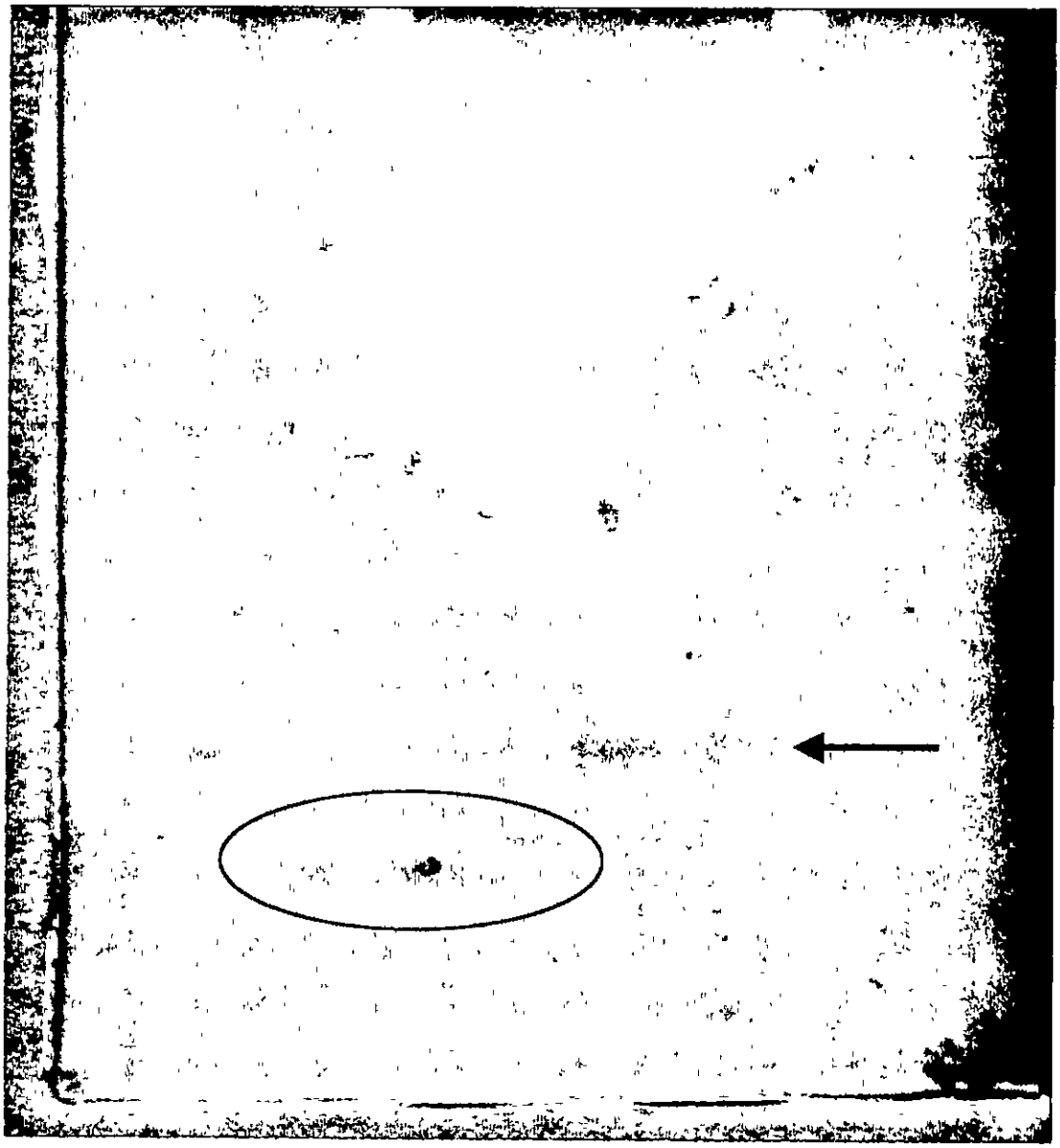


Figure 12. Enterokinase treated vs. untreated fusion protein nickel affinity blot. Lane 1 contains the enterokinase treated fusion protein. Lane 2 contains untreated fusion protein. A red arrow indicates the untreated protein band. A black oval circumbscribes minor nonspecific inding to the treated protein.

With confidence that the enterokinase enzyme cleavage has been shown to be effective, the complete stock of HBD-3 fusion protein was treated

with enterokinase enzyme, as decribed in the Materials and Methods section entitled Large scale cleavage. The complete supply of peptide produced (in the Materials and Methods section entitled Large Scale Expression) was cleaved with Recombinant Enterokinase (rEK) from Novagen® (as described in the Materials and Methods section entitled Large Scale Enterokinase Treatment). This peptide was further purified by HPLC. Figure 14 is a representative sample of one of 40 HPLC runs. The peaks from 15-22 minutes were collected, separately. For example, peptide that was eluted concurrently with the peak at 15 minutes was collected separately, from that collected between 17 and 19 minutes, was separated from the peak at 22 minutes.

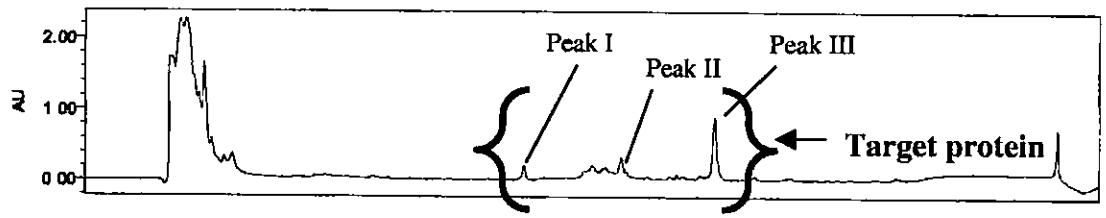


Figure 13. A representative post-enzyme treatment HPLC

After HPLC purification, the peptide was pooled and an aliquot was sent for mass spectroscopy. The results of the mass spectroscopy are shown in Figure 14. These results confirm that the structure of the peptide that we produced exactly match the previously documented size of the HBD-3 peptide generated by Harder et al. 2001.

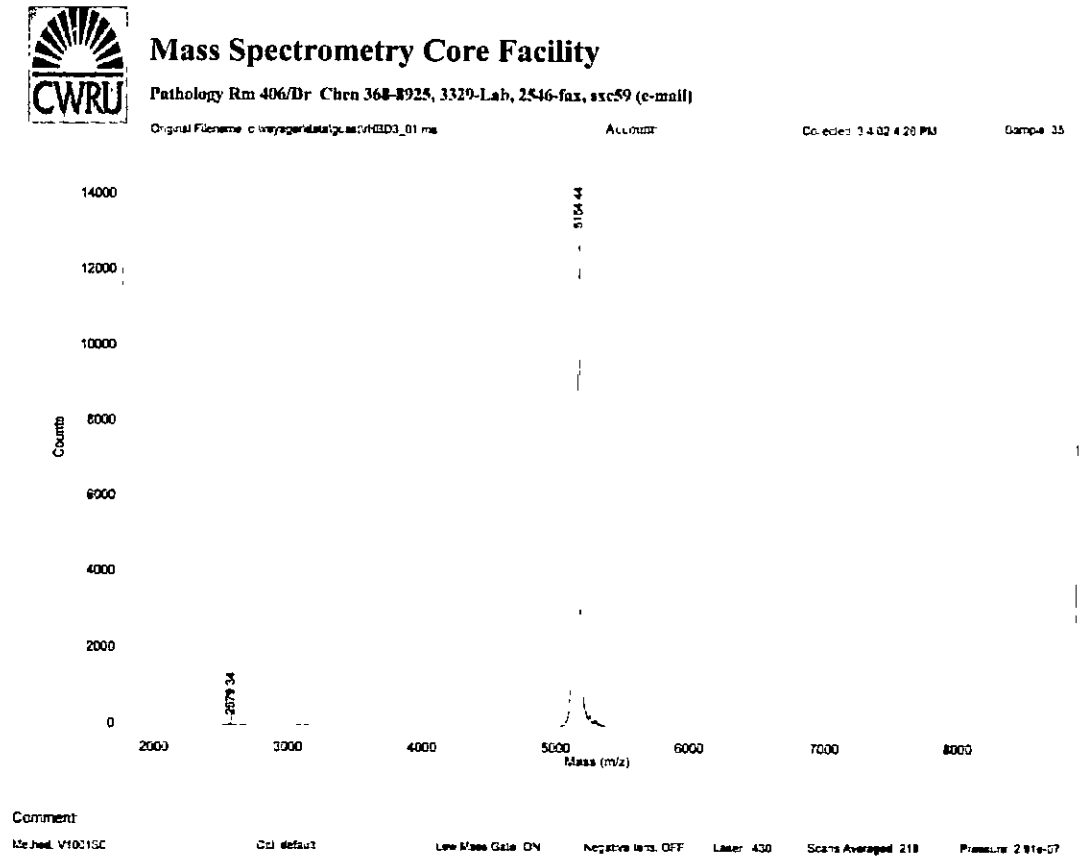


Figure 14. Mass spectroscopy-Maldi TOF of rhBD-3, post enzyme treatment. Note the isolated peak at 5154.44 as described by Harder et al. (2001).

Susceptibility Against HBD-3 / Colony Killing Assays

The activity of the produced and purified rHBD-3 peptide was tested in colony killing assays, as described in the Materials and Methods section entitled Susceptibility Against HBD-3/Colony Killing Assays. The bacteria tested were *A.a. (JP2)*, *F.n.* (ATCC 25586), *S.s.* (ATCC 10556), and *P.g.* (ATCC 33277). Figures 15-17 reveals that HBD-3 peptide was active against *A.a. (JP2), S.s. and P.g.* in a dose dependent fashion.

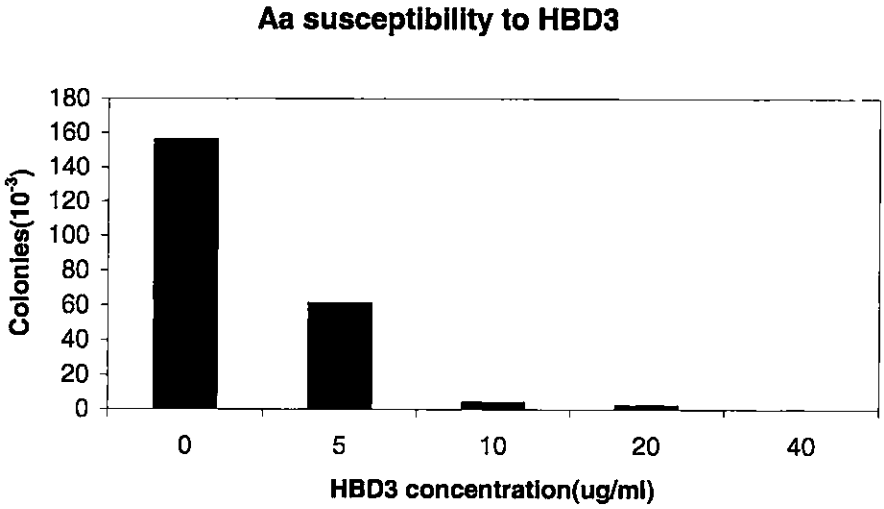


Figure 15. *A.a. (JP2)* susceptibility to HBD-3.

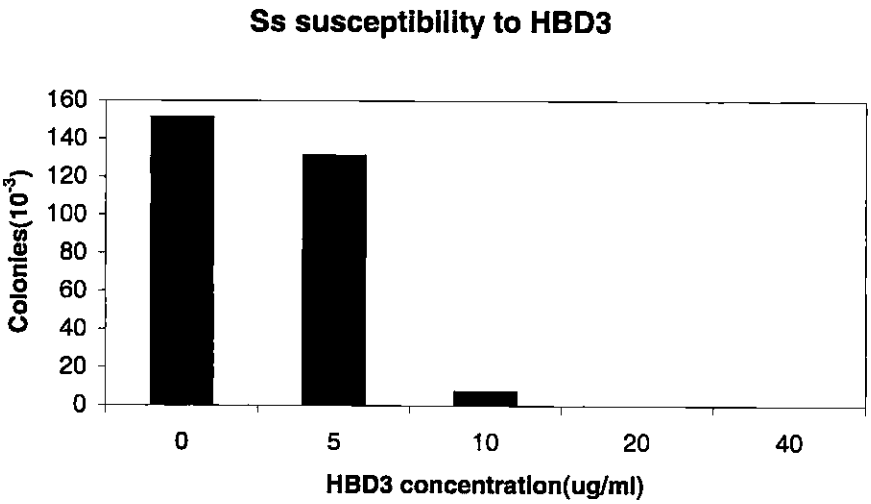


Figure 16. *S.s.* susceptibility to HBD-3

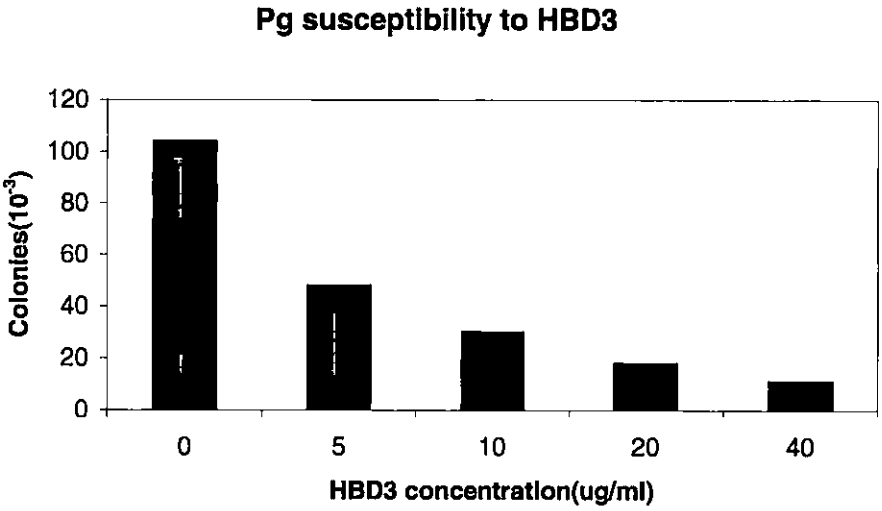


Figure 17. *P.g.* susceptibility to HBD-3

However, regarding *F.n.*, Figure 19 shows that HBD-3 peptide was not dose dependently active.

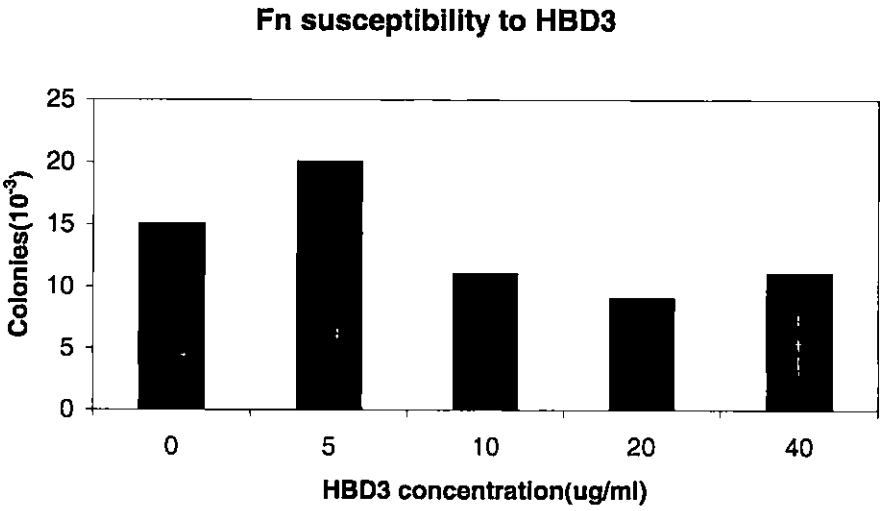


Figure 18. *F.n.* susceptibility to HBD-3

DISCUSSION

Innate Immunity-Under appreciated

Our immune system is categorized into innate and adaptive/acquired immunity. The scientific community has under appreciated the innate immune system for some time, in general. For more than half a century, immunological research has been heavily oriented towards the adaptive/acquired immune response. The major role of specific or acquired immunity has been underscored by the rapidly lethal character of severe immune deficiency diseases (Athanassakis 2001). Acquired immunity has been thought of as the second line of defense, poised to combat those pathogens that can overcome the innate immune system and protect us from re-infection by the same organism.

Natural or innate immunity has been known since the first description of an inflammatory reaction attributed to Celsus (Milligan and Celsus 1831). Bordet elucidated the complement system in the early 20th century (Jules 1990), which can be combined with acquired immunity or function independently. The innate immune system has been thought of as the first line of defense, a barrier to infection. The literature supports the innate immune system's role in host defense during the early stages of infection, such as the complement pathway, natural killer (NK) cells, and the epithelial

barriers of the body. Recently, however, other features of the innate immune system have been recognized, they include cytokine production by phagocytes, NK cell activation through Type I interferons, and antibacterial peptide activity by epithelial cells and neutrophils (Medzhitov and Janeway Jr. 1998).

The innate immune system also appears to actively stimulate T lymphocytes of the acquired immune system (Liu et al. 1998). Recognition of microbial structures by host cells relies on diverse families of genome-encoded receptors that allow detection of infectious particles and provide signals that activate the defense mechanisms. The role of the innate immune system appears to be much more responsive to host pathogens through recognition of pathogen associated molecular patterns (PAMPs) by Toll-like receptors, evolutionarily ancient cell surface receptors that originated in drosophilae. This results in activation of signaling pathways with induction of genes and cytokines that interact with adaptive immunity. (Janeway and Medzhitov 2002).

Oral Expression of Defensins and Oral Bacteria

Expression of β-defensin within the gingiva shows a pattern of expression consistent with function as a microbial barrier in the stratified epithelial tissues (Dale et al. 2001). The expression of hBD-1 and hBD-2 are greatest in the upper portion of the tissue, and particularly strong at the gingival margin. The β-defensins hBD-1 and hBD-2 are expressed in areas

of differentiation and inflammation, but are expressed poorly or not at all in the junctional epithelium (Dale et al. 2001). α-defensins and LL-37 are present in high amounts in neutrophils that migrate through the junctional epithelium to the gingival sulcus (Dale et al. 2001).

Pertinent to periodontal disease is the breakdown of the junctional epithelium, the seal between the periodontium and the tooth. α-defensins from neutrophils have been shown to be present at the junctional epithelium in these patients. However, human β-defensin-1 and 2 have been shown to be absent from this critical area of the mouth (Dale et al 2001). Since undifferentiated epithelium is unlikely to produce β-defensins it is not unexpected that cells at the junctional epithelium lack β-defensin expression (Krisanaprakornkit et al 2000). Diabetic patients and others with defective neutrophil chemotaxis (Naghibi 1987) exhibit increased periodontal disease. This may be due to the lack of α-defensin carrying neutrophils at the junctional epithelium, when left untreated. Without functional neutrophils percolating into the periodontium or β-defensin production at the DEJ there is little innate defense in this initial confrontational site to defend against bacterial challenges. Arming the junctional epithelium with β-defensins, which could be induced someday through gene transfection, may prove to be beneficial.

Bacterial Strategies for Overcoming Innate Immunity

Bacteria rely on their ability to circumvent host defense mechanisms, in order to survive. Some, like the oral anaerobe *Porphyromonas gingivalis* (*P.g.*), act as a stealth organisms at the epithelial border (Lamont and Jenkinson 1998) and *P.g.'s* LPS does not induce innate responses in vascular endothelium (Darveau et al. 1998). *P.g.* has been shown to invade human oral epithelial cells (Lamont et al. 1995; Weinberg et al. 1994). Stealth-like characteristics of *P.g.* i.e., not inducing IL-8, along with its invasive capability provide this organism with unique characteristics to colonize the periodontium. However other periodontal pathogenic organisms do induce inflammatory activities, which ultimately lead to disruption of the periodontal epithelium (McGraw et al. 1999).

Attachment, Colonization and Virulence factors

Microbial attachment and invasion of the epithelial cell layer are important virulence factors. Skin and mucous membranes keep bacteria from invading the body. Mucosal surfaces are protected by active removal of bacteria. For example, the acid environment in the stomach, the ciliary movement in the upper respiratory tract and the continuous flushing with urine of the lower urinary tract. Motility and attachment factors (adhesins) found in many pathogenic bacteria are essential for adhering to cellular surfaces and withstanding mechanical removal (Hornef et al. 2002). For example, lack of expression of toxin-coregulated pili (TCP) in *Vibrio cholerae*, significantly

reduces the severity of bacteria-induced diarrhea in humans (Tacket et al. 1998). Alternatively, the secretion of bacterial toxins impairs protective functions and facilitates colonization. *Bordetella pertussis*, which causes whooping cough, paralyzes the ciliary clearance function of the respiratory tract via the release of cell wall constituents that induce nitric oxide–mediated ciliostasis (Flak and Goldman 1999).

An extensive commensal microflora is another way of protecting the host's mucosal surfaces. For example, in patients undergoing antibiotic treatment, which inhibits all flora, infection with the opportunistic pathogen *Clostridium difficile* causes pseudomembranous colitis, and results in disturbance of the enteric microflora. Therefore, colonization by pathogens in the presence of a resident flora demands strategies that enable invading microbes to displace commensal organisms from their niche (Hornef et al. 2002).

Evasion of Innate Immunity by Bacteria at Mucosal Surfaces

Mucosal membranes secrete antimicrobial factors that combat bacteria. Bacteria counter immunoglobulins of adaptive immunity by proteolytically degrading them. In difference to immunoglobulins, i.e. antibodies, which can be proteolytically degraded by certain bacteria, locally secreted defensins are relatively resistant to enzymatic degradation (Hornef et al. 2002). However, degradation of at least some antimicrobial peptides by bacterial proteases has been reported, and active transport of peptides out of

the bacterial cytoplasm also occurs (Guina 2000). Since antimicrobial peptides function electrostatically by binding to anionic sites in bacterial membranes, some bacteria degrade extracellular matrix and the resulting fragments bind to antimicrobial peptides and abolish their efficacy (Schmidtchen et al. 2001). Some bacteria can make themselves less susceptible to antimicrobial peptides by the addition of positively charged residues on their membrane surfaces. For example, *Staphylococcus aureus*, the dominant causative agent of wound infections, modifies its membrane lipid, phosphatidyl glycerol with lysine, and adds D-alanine to teichoic acid. These changes reduce the net negative charge of the membrane, thereby avoiding antimicrobial peptide effects (Peschel and Collins 2001). Adaptation to antimicrobial peptides seems to play a critical role in microbial virulence, *S. aureus* that was unable to modify phosphatidylglycerol and reduce its negatively charged surface membrane showed significantly less infectivity to mice (Peschel et al. 2000). Therefore, bacteria have evolved to adapt to antimicrobial peptides, and these mechanisms appear to be important in the expression of virulence. However, high concentrations of antimicrobial peptides at vulnerable body sites *in vivo* do nevertheless, impede microbial colonization and growth (Hornef et al. 2002).

Epithelial Cell Recognition of Bacterial Products

Epithelial cells have the ability to sense the presence of microbial organisms and respond specifically through the identification of conserved

components of these microbes. Bacteria might avoid innate recognition by steric shielding or modification of the exposed bacterial envelopes (Janeway and Medzhitov 2002). Host-like bacterial capsular structures that imitate host membranes are important virulence factors (Guo et al 1997). Various LPS species from different commensal as well as pathogenic bacteria show some variance in their capacity to induce cytokine synthesis. Alterations in the structure of *Salmonella* LPS decrease its likelihood to provoke innate immune responses (Guo et al. 1997). This is particularly true with the LPS of *P.g.* (Darveau et al. 1998). However, most LPS and peptidoglycans are essential to bacterial cell functions. Structural and major modifications might decrease the viability and fitness of bacterial intruders (Janeway and Medzhitov 2002). In normal situations, bacterial flagellin, an important virulence factor for many bacteria (for example *V. cholerae*), is recognized by toll-like receptor-5. However, some bacteria, such as *Salmonella enterica serovar Typhimurium* lack flagellar expression yet remain virulent. This is an example of a bacterium that may have evolved so that it would not elicit an innate immune system response (Schmitt et al. 2001).

Invasion

In contrast to the avoidance of immune recognition, some microbial pathogens enhance immune-activation and pro-inflammatory responses by producing maximally stimulatory PAMPS. *Salmonella dysenteriae* causes bacillary dysentery in humans. *S. dysenteriae* contains the msbB gene,

whose product is involved in the biosynthetic pathway of lipid A, the immunostimulatory part of the LPS molecule that mediates inflammation. Epithelial cell activation induces intestinal leukocyte infiltration followed by disruption of the enteric mucosal layer, which facilitates bacterial invasion (D'Hauteville et al. 2002). Deletion of the msbB gene in *E. coli* leads to the production of hypoacylated lipid A with strongly decreased pro-inflammatory activity. Therefore, inflammation during the early stages of infection might be advantageous. On the other hand, long-term microbial colonization benefits if cellular stimulation and activation of host defenses are avoided. This is illustrated by *Helicobacter pylori*, which colonizes the human gastric mucosa and causes chronic infections. *H. pylori* activates stomach epithelial cells in a process that is mainly dependent on proteins encoded by the CagA pathogenicity island. After prolonged colonization, part of the *Helicobacter* population in the stomach tends to delete cag genes. This may reflect a need to reduce the inflammatory response as soon as microbial colonization is established (Fischer et al. 2001); which allows for a more commensal living arrangement.

Cytokine Secretion in Response to Bacteria

The innate immune system's activation of the adaptive response occurs through cytokine secretion, antigenic processing and presentation, as well as proliferation and differentiation of effector cells. The production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1

(IL-1β), IL-8 and IL-12 by host cells upon sensing bacterial products is crucial in the innate and adaptive immune responses to infection. These cytokines play a role in enhancing the bactericidal capacity of phagocytes, recruiting additional innate cell populations to sites of infection, inducing dendritic cell maturation and directing the ensuing specific immune response to the invading microbes. Some bacterial pathogens have evolved mechanisms for modulating cytokine production by host cells, which modifies the host's subsequent immune response (Hornef et al. 2002).

How Do Defensins and Bacteria Interact?

In higher organisms, a variety of host defense mechanisms control resident microflora and usually prevent invasive microbial disease. Antimicrobial peptides belonging to the cathelicidin and defensin gene families are of particular importance to the mammalian immune defense system. Current evidence points to cathelicidins and defensins as being multifunctional; i.e., they act as natural antibiotics and as signaling molecules that activate host cell processes involved in immune defense and repair. The lack of normal expression of these peptides has recently been associated with acquired immune deficiency syndrome (AIDS) (Biragyn, Ruffini et al. 2002). β-defensins bind TLR-4 on dendritic cells, resulting in their maturation, which subsequently results in activation of T cells. Moreover, defensins have considerable immunological adjuvant activity and treatment of beta-defensins

or selected chemokines to an idiotypic lymphoma antigen has yielded potent antitumor vaccines (Biragyn, Belyakov et al. 2002).

Zasloff (2002) proposes that microbial cell membranes are the microbe's Achilles heel. The unique outside surface of the bacterial cell membrane contains many lipids with negatively charged phospholipid head groups (phosphatidyl glycerol). This is in contrast to plant and animal membrane outside surfaces, which contains lipids with no net charge (phosphatidyl choline). Almost all of the active antimicrobial peptides have a structure, composed of hydrophilic, hydrophobic and cationic amino acids arranged in a molecule that can organize into an amphipathic structure with net cationic charge. The antimicrobial peptides are hypothesized to congregate, internalize and aggregate into the microbial cell membrane, resulting in membrane disruption and bacterial death.

Why haven't most microbes become resistant to these evolutionarily ancient antimicrobial peptides? A microbe would have to redesign its membrane, and change the composition or organization of its lipids to become resistant to all antimicrobial peptides. This is asking a lot of the bacterium. For example, resistance-enhancing changes to LPS structure in *Salmonella* are tightly regulated by the PhoP-PhoQ and PmrA-PmrB two-component signal transduction systems, which are central regulators of bacterial virulence. Therefore, decreasing susceptibility to antimicrobial peptides may result in a decrease of the bacteria's virulence (Hornef et al.

2002). Destruction of the antimicrobial peptides are also difficult because they are composed of short sequences of amino acids lacking unique epitopes that could serve as a recognition site for a protease required the selective destruction by an antibiotic. In addition, multicellular organisms attack microbes with multiple peptides of different structural types; thus destruction of one antimicrobial peptide wouldn't be enough to defend the microbe against a multiple offensive threat (Zasloff 2002).

Bacteria/Host interactions: Symbiosis, Commensalism

Humans have evolved in the continuous presence of various microbes. Microbial organisms have evolved with their human hosts to avoid or overcome protective host barriers; and some have evolved non-pathogenic or even beneficial relationships with their human host. Symbiotic relationships are mutually beneficial for both partners, whereas pathogenic relationships benefit one partner at the expense of the other. Commensal relationships are generally defined as those that benefit one partner without necessarily being detrimental to the other. The term commensal derives from the Latin commensalis and literally means "at table together" (Wostmann et al. 1983). As an example of commensalisms, carbohydrates form the bulk of most human and animal diets and thus are critical nutrients for both host and bacteria. Mammals are able to absorb simple sugars such as glucose and galactose in the proximal part of the small intestine. By recruiting a flora that can hydrolyze complex carbohydrates, mammals avoid the need to evolve an

array of glycosylhydrolases that would be required to break down the wide variety of linkages that occur in dietary polysaccharides. The host also acquires flexibility in its capacity to adapt to shifts in the diet. This material includes polysaccharides from components of plant cell walls and undigested starch. The host's problem of gaining nutritional benefit from these polysaccharides has been solved, in part, by adopting a microbial population that has the ability to degrade these biomolecules (Moore and Holdeman 1974). The microbes, in turn, gain access to abundant, readily fermentable carbon sources that would otherwise be worthless to the host. Bacteroides, in the gut, can degrade and ferment a wide variety of polysaccharides, including xylan psyllium hydrocolloid, and numerous other plant polysaccharides. *Bacteroides thetaiotaomicron (B. thetaiotaomicron)* is a prominent, member of the normal mouse and human distal intestinal microflora. *B. thetaiotaomicron* can hydrolyze certain disaccharides (e.g., sucrose, lactose, and maltose) to their constituent monosaccharides and also degrade starch to glucose monomers (Salyers et al. 2000). *Bacteroides* species have also evolved the capacity to degrade a variety of host-derived glycoconjugates (glycans) including chondroitin sulfate, mucin hyaluronate and heparin. The chondroitin sulfate and hyaluronate degradation pathways in *B. thetaiotaomicron* have features resembling those of the starch utilization system. *B. thetaiotaomicron* degrades chondroitin sulfate and hyaluronic acid to disaccharides via the action of two chondroitin lyases (Salyers and O'Brien 1980).

It has been observed from comparisons of normal and germ free mice, that the presence of a microflora increases intestinal epithelial turnover and also deconjugates and dehydroxylates bile acids metabolizes bilirubin reducing cholesterol to coprostanol and degrading mucus glycoproteins produced by the intestinal epithelium (Savage et al. 1981).

Bacteria and Host Equilibrium

Lactobacillus species have been associated with beneficial effects such as promotion of gut maturation and integrity, antagonisms against pathogens, immune modulation, the maintenance of intestinal immune homeostasis and prevention of inflammation (Schiffrin and Blum 2002). NK cells produce gamma interferon (IFN-gamma) and other cytokines necessary to control certain bacterial, parasitic, and viral infections. A balance between receptors mediating activating and inhibitory signals controls NK cell-mediated effector functions. Several studies have shown that commensal *lactobacilli* isolated from the human gastrointestinal tract activate human mononuclear cells and are potent inducers of IFN-gamma and monocyte-derived interleukin 12 (IL-12). NK cell activation was shown for *Lactobacillus johnsonii. Staphylococcus aureus* and *L. johnsonii* have been shown to mediate activation of human peripheral blood NK cells and secretion of IFN-gamma upon contact with monocytes. *S. aureus* and *L. johnsonii* preactivated monocytes retain their capacity to induce NK cell activation. In contrast, cytokine-primed monocytes do not induce NK cell activation unless

S. aureus and *L. johnsonii* are present (Haller et al. 2002). These bacteria are harmless and even beneficial under normal circumstances, but may cause local or systemic inflammatory disease if the integrity of the hosts' surface is disturbed.

β-defensin Function

Recall that hBD-1 is constitutively expressed and that hBD-2 is inducible by *F.n.* (Krisanaprakornkit et al. 2000). Challenge of normal human gingival epithelial cells (NHOECs) by *P.g.* (the bacterium which is most strongly associated with periodontal disease) results in no hBD-2 mRNA induction. It is very advantageous for a successful pathogen, like *P.g.*, to not trigger the production of the potent gram-negative antimicrobial hBD-2 (Weinberg et al. 1998; Krisanaprakornkit et al. 2000; Krisanaprakornkit et al.1998). hBD-2 is expressed only in the presence of infection or inflammation in most tissues (including skin, trachea, gut epithelium), but interestingly it is expressed in normal uninflamed gingival tissue due to constant stimulation by oral bacteria. Normal oral gingival epithelial cells do not express hBD-2 in cell culture unless stimulated. Bacterial products, proinflammatory cytokines and phorbol ester, a general cell activator, are all effective stimulants of hBD-2 in NHOEC's. Cell wall preparations of the commensal organism, *F.n.*, are very effective in up regulating hBD-2 mRNA expression in NHOEC's.

Because *F.n.* pretreatment of NHOEC's suppressed *P.g.* invasion through induction of hBD-2, Weinberg et. al, investigated if these organisms differed in their sensitivities to β-defensins. *P.g.* was shown to be killed by rhBD-1 and –2 at low micromolar concentrations, while *F.n.* was not. Moreover, even at concentrations, over 100 μg/mL, neither rhBD-1 nor hBD-2 killed *F.n.* It appears as though *F.n.* has an adaptive advantage over *P.g.* by inducing an antimicrobial agent to be produced from NHOEC's to which it is resistant, while competitive bacteria are susceptible.

The purpose of this study was to produce hBD-3 and test its microbicidal activity against *A.a.*, *S.s.*, *P.g.*, and *F.n.* Similar to rhBD-1 and rhBD-2, rhBD-3 was effective in killing *P.g.* and the other bacterial species, but ineffective against *F.n.* It has been shown that high salt concentration may affect the potency of hBD-2 and to a lesser extent, hBD-3. hBD-3 was found to be effective at killing *Staphylococcus aureus* (*S.a.*) in an hypertonic environment, such as the skin (Harder 2001), an environment in which hBD-2 was ineffective at killing *S.a* (Schröder 1999). The saliva of the oral cavity is hypotonic in contrast to isotonic mucosal secretions and serum (Rozenfarb 1997). Therefore, our results suggest that hBD-2 and hBD-3 can effectively kill oral bacteria in the oral cavity. However, due to the hypertonicity of salty secretions on the skin, hBD-3 may be more effective against *Staphylococcus aureus* than is hBD-2.

Interestingly, *F.n.* was also found to be resistant to human α-defensins (Miyasaki et al. 1998). Coupled with our recent findings that *F.n.* is also resistant to human β-defensins; the collective information may be suggesting that *F.n.* has evolved to coexist with the human host in a symbiotic fashion. *F.n.* is known to induce hBD-2 expression in NHOECs. *P.g.*, which is known to invade nascent oral epithelial cells, is highly sensitive to hBD-2 while not inducing its expression in oral epithelial cells. Therefore, the protection elicited against *P.g.* invasion in *F.n.* challenged epithelial cells, may be due to hBD-2 expression. Our data indicates that *P.g.* is sensitive to hBD-3, while *F.n.* is not. Our data indicates that hBD-3 dose dependently kills *P.g.* and is ineffective against *F.n.* This observation strengthens the story that *F.n.* acts as a commensal with the human epithelial cell, and that this relationship may contribute to homeostasis and health in body sites constantly challenged with bacteria.

<u>*F.n.* Symbiosis</u>

F.n. probably co-evolved with human hosts in the oral cavity as a symbiotic or commensal organism. *F.n.* may suppress *P.g.* by displacing it from it's periodontal niche by inducing β-defensin responses through chronically stimulating gingival epithelium. β-defensins once released into intercellular spaces, may alert the adaptive immune system to trouble brewing (Biragyn et al. 2002). *F.n.*, like the pathogenic bacteria described above, may express factors that directly or indirectly circumvent innate immune defense,

such as degradation of extracellular matrix, whose resulting fragments bind to antimicrobial peptides and abolish their efficacy (Schmidtchen et al. 2001). Alternatively, *F.n.* may secrete a proteolysin that effectively lyses rhBD-1, 2,and 3. If, as is the case for hBD-2, *F.n.* does induce hBD-3, it may be due to an evolutionarily conserved altering of *F.n.*'s bacterial cell envelope. This may result in a decreased, yet enhanced resistance to β-defensins. Future studies will undoubtedly reveal further details of the intriguing relationship between *F.n.* and the innate immune system, and will hopefully provide us with the knowledge to improve the prevention and treatment of periodontal diseases in the near future.

Final Thoughts

How can we take advantage of this science? Hopefully the factor in *F.n.'s* envelope that upregulates the β-defensins could be isolated, purified and produced recombinantly. This would allow clinicians to use this factor on implants that are placed in the mouth and other body sites or surgical instruments, or introduced into mouthwash and applied locally by periodontists in the periodontal pocket; all intended to enhance our native immune response.

CONCLUSIONS

The technique devised by Weinberg to generate recombinant hBD-3 was successful. This of inducing the production of defensins could be applied to other antimicrobial peptides. The plasmid was successfully transformed into the BL21(DE3) *E. coli* bacteria. The BL21(DE3) *E. coli* exhibited the antibiotic resistance that the plasmid conferred. S•Tag assay confirmed that a protein had been produced that contained the S•Tag that the rhBD-3 fusion protein was engineered to have. Nickel affinity chromatography successfully demonstrated that the protein produced, contained the His•Tag that the rhBD-3 fusion protein was engineered to have. The target fusion protein was successfully cleaved, as was confirmed by SDS-PAGE analysis and nickel affinity blotting. The radial diffusion assays successfully confirmed that the target protein had microbicidal activity against MP35 *E. coli*. The target protein was successfully isolated via C_{18} RP-HPLC. Upon analysis with MALDI-TOF MS the molecular mass of our target protein was confirmed to be identical to that of rhBD-3 as described by Harder et al. (2001). rhBD-3 was found to be microbicidal in a dose dependent manner against *A.a(JP2)*, *S.s.*, and *P.g.* This was not the case with *F.n.*, which was found to be resistant to the effects of rhBD-3.

F.n. is ubiquitous in man. Humans produce β-defensins in every epithelial tissue studied. Our findings indicate that *F.n.* is not susceptible to the antimicrobial properties of human β-defensin-3; however, the very successful periodontal pathogen *P.g.* is killed by human β-defensin-3. We know that *F.n.* induces the expression of hBD-2, and that hBD-2 is lethal to *P.g.* As of yet we do not know whether *F.n.* induces expression of hBD-3. It appears as though *F.n.* has adapted through evolution to induce the expression of hBD-2, which kills *P.g.* This is a situation beneficial to *F.n.* because it has less competition in its oral environment of the periodontium, and it appears beneficial to the human host due to the pathogenic nature associated with *P.g.* This study adds to the story of *F.n.*, the fact that this is another β-defensin that *F.n.* is resistant to and *P.g.* is not.

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