Characterization of a Gene Encoding Bacteriocin of *Prevotella nigrescens*

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ชื่อวิทยานิพนธ์	การศึกษาคุณลักษณะของยีนแบคทิริโอซินของเชื้อแบคทีเรียสายพันธุ์พรีโวเทลลา ไนเกรสเซนส์
ผู้เขียน	นางเจษฎี แก้วศรีจันทร์
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บทคัดย่อ

วัตถุประสงค์

วิทยานิพนธ์นี้มีวัตถุประสงค์เพื่อศึกษาคุณลักษณะของยีนแบคทิริโอซินของเชื้อแบคทีเรียสายพันธุ์พรีโวเทลลา ไนเกรสเซนส์ และความเป็นไปได้ในการนำสารแบคทิริโอซินของเชื้อแบคทีเรียชนิดนี้มาใช้ป้องกันและรักษาโรคปริทันต์

วิธีดำเนินการวิจัย

ทำการโคลนโครโมโซมของเชื้อพรีโวเทลลา ไนเกรสเซนส์ลงในเชื้อแบคทีเรีย *Escherichia coli* ตรวจสอบความสามารถในการสร้างสารแบคทิริโอซินของโคลนที่ได้ หาลำดับนิวคลีโอไทด์ของท่อนดีเอ็นเอที่สร้างสารแบคทิริโอซิน แยกสารแบคทิริโอซินให้บริสุทธิ์โดยวิธีโครมาโตกราฟี ศึกษาความสามารถในการยับยั้งการเจริญเติบโตและความจำเพาะต่อเชื้อแบคทีเรียสายพันธุ์อื่นๆ คุณสมบัติทางชีวเคมี และความเป็นพิษต่อเนื้อเยื่อเพาะเลี้ยงของสารแบคทิริโอซิน

ผลการวิจัย

ท่อนดีเอ็นเอที่กำหนดการสร้างสารแบคทิริโอซินในเชื้อพรีโวเทลลา ไนเกรสเซนส์ อยู่บนโครโมโซมซึ่งมีความยาว 4,868 คู่เบส เรียกท่อนดีเอ็นเอนี้ว่า *nig* locus โคลนซึ่งดีเอ็นเอบางส่วนใน *nig* locus ถูกตัดออกไปไม่มีความสามารถในการสร้างสารแบคทิริโอซิน สารแบคทิริโอซิน (ให้ชื่อว่าไนเกรสซิน) มีฤทธิ์ฆ่าเชื้อแบคทีเรีย *Porphyromonas gingivalis Prevotella intermedia Tannerella forsythensis* (*Bacteroides forsythus*) และบางสปีชีส์ของ *Actinomyces* ผลจากการทำ Sodium Dodecyl Sulfate Polyacryla mide Electrophoresis (SDS-PAGE) ของสารแบคทิริโอซินที่บริสุทธิ์พบว่ามีขนาดโมเลกุลประมาณ 41 กิโลดาลตัน สารแบคทิริโอซินถูกย่อยด้วยเอ็นไซม์ proteinase K แสดงว่าสารดังกล่าวเป็นโปรตีน สารแบคทิริโอซินคงตัวที่อุณหภูมิ 100 องศาเซลเซียสเป็นเวลา 10 นาที และที่ช่วง pH ระหว่าง 6.5 และ 9.5 นอกจากนี้ยังพบว่าสารแบคทิริโอซินไม่เป็นพิษต่อเนื้อเยื่อเซลล์ปกติที่เป็นส่วนประกอบของเนื้อเยื่อปริทันต์

สรุปผลการวิจัย

วิทยานิพนธ์นี้เป็นงานวิจัยชิ้นแรกที่รายงานความสำเร็จเกี่ยวกับการโคลนยีนแบคทิริโอซินของเชื้อแบคทีเรียสายพันธุ์พรีโวเทลลา ไนเกรสเซนส์ลงใน *E. coli* สารแบคทิริโอซิน (ให้ชื่อว่าไนเกรสซิน) มีคุณสมบัติหลายประการที่น่าสนใจที่จะนำไปประยุกต์ใช้เพื่อป้องกันหรือรักษาโรคปริทันต์ได้

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Abstract

Objectives

The objectives of this work were to characterize a gene encoding bacteriocin of *Prevotella nigrescens* ATCC 25261 and to explore the possibility of using such a bacteriocin in prevention and treatment of periodontal diseases.

Methods

Chromosomal DNA of *Pr. nigrescens* ATCC 25261 strain was cloned into *Escherichia coli*. The resulting clones were tested for bacteriocin production. The DNA fragment responsible for bacteriocin production was sequenced. The bacteriocin was successfully purified using a three-step chromatographic procedure, which included one ion exchange chromatography step and two gel filtration steps. A number of bacteriocin properties were determined *in vitro*, including the antibacterial spectrum, specificity, as well as biochemical and cytotoxic properties.

Results

The bacteriocin genetic system of *Prevotella nigrescens* ATCC 25261, termed *nig* locus, was located on the chromosome and consisted of a continuous DNA segment of 4,868 base pairs. Clones bearing deletions in the *nig* locus all lacked the bacteriocin-producing ability. The *nig* locus is thus necessary for the production of a functional bacteriocin. The bacteriocin, called nigrescin, has an approximate molecular weight of 41 kDa, as judged by SDS-PAGE. Nigrescin exhibited a bactericidal mode of action, which inhibited the growth of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (*Bacteroides forsythus*), and *Actinimyces* spp. It was inactivated by proteinase K,

suggesting its proteinaceous nature and that it is in fact a bacteriocin. It was stable upon heating at 100 ^{O}C for 10 min and in pHs ranging between 6.5 and 9.5. In addition, it was not toxic to normal fibroblast cells.

Conclusion

We report the first success of cloning and expression in *E. coli* of the bacteriocin of *Pr. nigrescens* ATCC 25261. The bacteriocin, called nigrescin, has several interesting features that make it an attractive candidate in prevention and treatment of periodontal diseases.

Key words: *Prevotella nigrescens*, Gram-negative anaerobe, subgingival bacterial species, bacteriocin

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Jasadee Kaewsrichan

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Chapter 1
Introduction

Microbial Ecology of the Oral Cavity (Oral Ecosystem)

An ecosystem consists of the microbial community living in a defined habitat and the abiotic surroundings composed of physical and chemical elements. Thus, with the simplest expression, the oral ecosystem is composed of the oral microorganisms and their surroundings, the oral cavity. The development of a community within an ecosystem usually involves a succession of populations. The process begins with the colonization of the habitat by pioneer microbial populations. In the oral cavity of new-borns, streptococci (*Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus salivarius*) are the pioneer organisms (Pearce *et al.*, 1995; Carlsson *et al.*, 1970). Pioneer microorganisms fill the niche of this new environment and modify the habitat, and as a result, new populations may develop. As the process continues, the diversity and the complexity of the microbial community increase. Succession ends when no additional niche is available for new populations. At this stage, a relatively stable assemblage of bacterial populations is achieved. It is called a climax community. The concept of a stable or climax community does not imply static conditions. The stability is based upon homeostasis, which implies compensating mechanisms that act to maintain steady-state conditions by a variety of controls aimed at counteracting perturbations that would upset the steady-state. The concepts of homeostasis and bacterial succession are important in oral microbiology. Some factors, such as a high sucrose diet, may cause an irreversible breakdown in the homeostasis of the oral ecosystem, resulting in the initiation of caries (Marsh and Martin, 1992).

The microbial community of the oral cavity is highly complex and diverse. Based on both culture-dependent and culture-independent methods, about 500 bacterial species are estimated to be present in the oral cavity (Wong and Sissions, 2001; Koller *et al.*, 2000). Some of the more frequently isolated microorganisms

are listed in Table 1. Their distributions vary qualitatively and quantitatively according to the habitat. There are several habitats in the oral cavity, each characterized by different physicochemical factors. The tooth can be described as a non-shedding hard surface that offers many different sites for colonization by bacteria above (supragingival) and below (subgingival) the gingival margin. The tongue, with its papillary surface, provides sites of colonization that are protected from mechanical removal. However, in general the oral mucosa is characterized by a continuous desquamation of its epithelial cells, which allows rapid elimination of adhering bacteria. The area between the gingivae and teeth, referred to as the gingival crevice, also provides a unique colonization site (Piovano, 1999; Rodrigue *et al.*, 1989). The different anatomic structures of the oral cavity thus support the growth of a different microbial community. For example, mutans streptococci (*Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus cricetus*, and *Streptoccocus rattus*) and *Streptoccocus sanguis* are found in larger numbers on teeth, while *S. salivarius* is isolated mainly from the tongue. Moreover, *S. mutans* and *S. sanguis* appear in the oral cavity only after eruption of the teeth (Smith *et al.*, 1993).

The oral surfaces are also constantly bathed by two important physiological fluids, the saliva and the gingival crevicular fluid. The supragingival environment is bathed by saliva, while the subgingival environment (gingival crevice) is bathed mainly by the gingival crevicular fluid. These fluids are essential for the maintenance of the oral ecosystem by providing water, nutrients, adherence, and antimicrobial factors.

Factors Influencing the Oral Ecosystem

The growth of oral microorganisms is influenced by a variety of factors, such as temperature, pH, oxidation-reduction potential, the availability of nutrients and water, the anatomy of the oral structures, salivary flow, and antimicrobial substances. Each factor in a given oral habitat influences the selection of oral microorganisms and helps maintain the equilibrium among bacterial populations. The result of these selective pressures has already been

observed in the differences in the oral microbiota among the different sites of the oral cavity. All these factors are interrelated and depend on host and microbial activities as well as external factors such as diet or oral hygiene. For better clarity, these factors have been divided into four categories: physico-chemical, host-related, microbial, and external factors (Kohal *et al.*, 2003; Siqueira *et al.*, 2002; Farias *et al.*, 2001; Wong and Sissions, 2001; Sissons *et al.*, 1998).

Physico-Chemical Factors

In all *in vivo* and *in vitro* systems, the growth of microorganisms is influenced by five important physico-chemical variables. These are temperature, pH, availability of water, availability of nutrients, and oxidation-reduction potential. As the mouth is constantly bathed by saliva and crevicular fluid, water is not considered to be a limiting factor (Wong and Sissions, 2001).

Temperature

The temperature in the oral cavity is relatively constant (34 to 36 ^{O}C), which allows a wide range of microorganisms to grow. The temperature may be more variable on the mucosal and tooth supragingival surfaces. During food intake, microorganisms colonizing these sites are exposed to hot and cold meals and probably must adapt to these extreme variations of temperature. However, no data are available on the effect of this short period of temperature variation on the metabolism of oral bacteria (Engelen *et al.*, 2003; Gilbert *et al.*, 2002).

pH

In the oral cavity, the pH is maintained at near neutrality (6.7 to 7.3) by saliva. The saliva contributes to maintenance of the pH by two mechanisms (Moazzez *et al.*, 2000; David *et al.*, 1997). Firstly, the flow of saliva eliminates carbohydrates that could be metabolized by bacteria and removes acids produced by bacteria. Secondly, acidity from drinks and foods, as well as from bacterial activity, is neutralized by the buffering activity of saliva. Bicarbonate is the major salivary buffering system of saliva, but peptides, proteins, and phosphates are also involved. Increases in pH also result from bacteria that metabolize sialine and urea into ammonia. The pH is an important parameter in oral microbial ecology.

Frequent sugar intake favours the growth of aciduric bacteria such as *Lactobacillus* and *S. mutans* and predisposes to caries formation (Marsh and Martin, 1992). An increased colonization by *S. mutans* was demonstrated by simply rinsing the mouth with low-pH buffers (Svanborg, 1980). The result suggested that the low pH generated from carbohydrate metabolism, rather than carbohydrate availability, is responsible for the shift in composition of the oral microbiota *in vivo*.

The subgingival area is bathed by gingival crevicular fluid and is not controlled by the buffering salivary activity. The pH in the gingival crevice may vary between 7.5 and 8.5. An alkaline pH in the gingival crevices and periodontal pockets may exert a selective force towards the colonization of peridontopathogens (McDermid *et al.*, 1988).

Oxidation-reduction potential and anaerobiosis

Many enzymatic reactions are oxidation-reduction reactions in which one compound is oxidized and another compound is reduced. The proportion of oxidized to reduced components contributes the oxidation-reduction potential or redox potential (E_h). The E_h is greatly influenced by the presence or absence of oxygen, which is the most common electron acceptor. Aerobic bacteria need an oxidizing environment (positive E_h) for growth, while anaerobic bacteria need a reducing environment (negative E_h). The mouth is characterized by a wide range of oxidation-reduction potentials, allowing the growth of aerobic, facultative anaerobic and anaerobic bacteria (Siqueira *et al.*, 2002; Farias *et al.*, 2001). In general, the dorsum of the tongue and the buccal and palatal mucosa are aerobic environments, thus better supporting the growth of facultative anaerobic bacteria. The gingival crevice and the proximal surfaces of the teeth (surfaces between teeth) possess the lowest E_h and the highest concentration of obligatory anaerobic bacteria. The E_h values vary between +158 to +542 mV in saliva but may reach -300 mV in gingival crevices (Theilade, 1990). The E_h also varies during plaque formation, changing from positive values (+294 mV) on clean tooth surfaces to negative values (-141 mV) after 7 days. The fall in E_h during plaque formation is the result of oxygen consumption by facultative anaerobic bacteria as well as a

reduction in the ability of oxygen to diffuse through the plaque. This explains in part the increased numbers of obligatory anaerobic bacteria during plaque formation.

Nutrients

In the oral cavity, microorganisms living in the supragingival environment have access to nutrients from both endogenous (saliva) and exogenous (diet) origins. Saliva is an important source of nutrients and can sustain normal growth of microorganisms in the absence of exogenous nutrients. Among exogenous dietary components, carbohydrates and proteins have the greatest influence on the composition of the oral microbiota. The gingival crevice is not exposed to dietary components or saliva. Its principal source of nutrients is the gingival crevicular fluid. The crevicular fluid originates from plasma and is an excellent source of nutrients for fastidious microorganisms. It contains growth factors such as hemin and vitamin K required by *Porphyromonas gingivalis*, a Gram-negative rod associated with adult periodontitis (Grenier and Michaud, 1994). Many nutritional relationships also occur between microorganisms. Some microorganisms cooperate in the degradation of nutrients. Some bacteria also use nutrients and other substances produced by other microorganisms.

Table 1. Oral microbiota in humans (Marsh and Martin, 1992; Theilade, 1990)

Group	Microbial genus	Isolation frequency[1]
Gram-positive cocci		
Aerobic or facultative	*Streptococcus*	++
	Enterococcus	+
	Micrococcus	+
Obligate anaerobes	*Peptostreptococcus*	+
	Peptococcus	+
Gram-positive rod		
Aerobic or facultative	*Lactobacillus*	+
	Corynebacterium	+
	Actinomyces	++
	Bacillus	±
	Arachnia	+
	Rothia	+
Obligate anaerobes	*Eubacterium*	+
	Propionibacterium	+
	Bifidobacterium	+
	Clostridium	±
Gram-negative cocci		
Aerobic or facultative	*Neisseria/Branhamella*	+
Obligate anaerobes	*Veillonella*	++
Gram-negative rod		
Aerobic or facultative	*Campylobacter*	+
	Eikenella	+
	Actinobacillus	+
	Capnocytophaga	+
	Haemophilus	+
	Simonsiella	+
Obligate anaerobes	*Bacteroides*	++
	Fusobacterium	+
	Porphyromonas	+
	Prevotella	+
	Leptotrichia	+
	Wolinella/Selenomonas	+
Other microorganisms	*Mycoplasma*	+
	Candida	+
	Spirochetes	+
	Protozoa	+

[1] ++, isolated frequently and may constitute a high percentage of the total oral microbiota; +, isolated; ±, appears as transient

Host Factors

Host defence mechanisms

The supragingival environment of the oral cavity is controlled primarily by saliva. A large number of bacteria are removed from teeth and mucosal surfaces by the continuous flow of saliva. Saliva also contains several specific and non-specific defence factors. Secretory IgA (SIgA) is the principal specific defence factor in saliva (Carlén *et al.*, 2004;. Devine, 2003). The inhibition of bacterial adherence by SIgA is considered one of the most important defence mechanisms against mucosal bacterial invasion. SIgA binds to bacterial adhesins, thus limiting the potential interaction between bacteria and host receptors (Magnusson and Stjernstrom, 1982). In addition, SIgA may impair bacterial adherence by agglutinating bacteria, thereby facilitating their clearance by secretions (Carlén *et al.*, 2004). The non-specific defence factors include mucins, nonimmune salivary glycoproteins, lactoferrin, lysozyme, peroxidase, histatins, and cystatins (Devine, 2003). The role of mucins and other nonimmune salivary glycoproteins in bacterial adherence is complex. They may bind to bacteria and promote bacterial adherence. Conversely, some of these glycoproteins, when free in saliva, may prevent bacterial colonization by binding to their adhesins or by agglutinating bacteria in saliva. This type of aggregation may facilitate the removal of oral bacteria by swallowing (Aguilera *et al.*, 1998).

Lysozyme is a small cationic protein that is present in all major body fluids. It causes lysis of bacterial cells by hydrolyzing glycosidic linkages of peptidoglycan. The effects lead to destabilization of the cell membrane. In addition, lysozyme can aggregate oral bacterial cells and inhibit their colonization on mucosal surfaces and teeth. *In vivo*, an inverse correlation has been found between the concentration of lysozyme and the accumulation of dental plaque (Jalil *et al.*, 1992; Pollock *et al.*, 1976).

Lactoferrin is an iron-binding glycoprotein (Aguilera *et al.*, 1998). It inhibits microbial growth, probably by sequestering iron in the environment (Farnaud and Evans, 2003).

Apolactoferrin is an iron-free lactoferrin, which also possesses a direct, iron-independent bactericidal effect against various oral bacterial strains including *S. mutans* (Arnold *et al.*, 1982). It agglutinates *S. mutans*, but not other species of streptococci, *P. gingivalis*, or *Actinobacillus actinomycetemcomitans* (Souka *et al.*, 1993).

Salivary peroxidase removes toxic hydrogen peroxide produced by oral microorganisms and reduces acid production in dental plaque (Donoghue *et al.*, 1987).

Histatins (histidine-rich peptides) inhibit the growth of *Candida albicans* and *S. mutans*, as well as aggregate oral streptococci (Payne *et al.*, 1991; Oppenheim *et al.*, 1988).

Cystatins are a family of cysteine-containing phosphoproteins. These proteins are present in plasma and reach the oral cavity via the gingival crevicular fluid, and act mainly as thiol protease inhibitors and inhibit proteases produced by suspected periodontopathogens (Alugupalli and Kalfas, 1996).

Saliva does not gain access to the gingival crevice, and this area of the oral cavity is almost essentially controlled by the antimicrobial factors of plasma (Zee *et al.*, 1996). The continuous flow of gingival fluid removes non-adherent bacterial cells. Gingival fluid also contains antimicrobial substances including IgM, IgG, IgA, complement, and leukocytes (Devine, 2003). These factors are primarily protective against microbial invasion. However, the protective responses may induce inflammation and become destructive, resulting in loss of periodontal attachment (Baker, 2000).

Age

The composition of the oral microbiota varies with the age of the host. Age related changes in the oral microflora include those due to teeth eruption, changes in dietary habits, hormones, salivary flow, the immune system, or other factors.

The human oral cavity is usually sterile at birth. However, within 6 to 10 h after birth, microorganisms from the mother and a lesser extent microorganism from those present in the environment become established in the oral cavity. The

pioneer species are usually streptococci, especially *S. mitis*, *S. oralis*, and *S. salivarius* (Heydenrijk *et al.*, 2003; Nyfors *et al.*, 2003). During the first year of life, the oral microbiota contains *Streptococcus*, *Neisseria*, *Veillonella*, *Staphylococcus*, and to a lesser degree, *Actinomyces*, *Lactobacillus*, *Rothia*, *Fusobacteerium*, and *Prevotella* (Willett *et al.*, 1991). The number of black-pigmented anaerobes and spirochetes in the gingival crevice increases more extensively during adolescence. It is possible that this could be due to hormonal changes (Gusberti *et al.*, 1990). The next most important changes, which occur in the elderly, have been reported. These changes include an increased prevalence of staphylococci, lactobacilli, and *Actinomyces viscosus* after the age of 70 years and an increase in the proportion of *Candida albicans* after 80 years. However, results have indicated that the change in the oral microbiota of elderly individuals is not related to a decrease in salivary flow, an impaired immune system, or nutritional deficiencies (Percival *et al.*, 1991).

Hormonal changes

It is well documented that pregnancy and puberty are associated with an increase in gingival inflammation that is accompanied by an increase in gingival exudates (Klinger *et al.*, 1998; Dahlén, 1993; Torkko and Asikainen, 1993). It has been proposed that the exacerbation in gingival inflammation may be due to hormone-induced alterations in the microbiota of the gingival crevice (Zachariasen, 1993). Microorganisms in the subgingival area that use hormones as growth factors may be favoured during the period of hormone increase associated with puberty and pregnancy. Several studies report an increased proportion of *Prevotella intermedia* in the subgingival microbiota of pregnant woman, corresponding to increased levels of estrogens and progesterone in plasma (Bearfield *et al.*, 2002; Dahlén, 1993. *In vitro* studies also demonstrate that progesterone or estradiol can substitute for vitamin K as an essential growth factor for *Pr. intermedia* (Kornman and Loesche, 1982; Jensen *et al.*, 1981).

Bacterial Factors

Adherence

To get established in the oral cavity, microorganisms must first adhere to teeth or to mucosal surfaces. Adherence is mediated by adhesins on the surface of bacteria and by receptors on the oral surface (Liver *et al.*, 2003; Bower and Mulvey, 2002; Jenkinson, 2002; Wilson and Henderson, 1995). Microbial adhesins are found as cell wall components or are associated with cell structures, such as fimbriae, fibrils or capsules (Dorn *et al.*, 2000; Zhou *et al.*, 1998). The receptors may be salivary components or bacterial components that are bound to oral surfaces. The adherence may result from non-specific physicochemical interactions between the bacteria and the oral surfaces. However, these interactions alone cannot explain the selective attachment of bacteria to various oral surfaces. It is believed that another mechanism accounts for this selective colonization, perhaps involving specific or stereochemical interactions between bacterial adhesions and host receptors (Liver *et al.*, 2003). It is probable that the bacteria first adhere by non-specific interactions that are followed by stronger stereochemical interactions. The stereochemical interactions involved in bacterial adhesion in the oral cavity are analogous to the interaction between antigen and antibody or between an enzyme and its substrates (Bower and Mulvey, 2002). Bacteria may also colonize host surfaces by adhering to other bacteria (Rudney and Staikov, 2002). Both intra- and intergeneric (two strains from different genera) coaggregations may be important in the development of dental plaque because these allow the colonization of bacteria that are not able to adhere directly to the acquired pellicle.

Bacterial interactions

A variety of beneficial and antagonistic interactions may help in maintaining the homeostasis of the oral microbiota. Coaggregation is one example of commensalisms and synergism that occurs between microbial species. Coaggregation allows the indirect adherence of some bacteria on oral surfaces. In addition, it has been demonstrated that coaggregated cells are more resistant to phagocytosis and killing by neutrophils *in vitro* and *in vivo* (Ochiai *et al.*, 1993).

Several other examples of positive interactions are likely to occur in the oral cavity. The utilization of oxygen by facultative anaerobic bacteria reduces the oxygen concentration and the E_h to levels that allow the colonization of anaerobic bacteria. Different bacterial species may also cooperate in the utilization of substrates that they can not metabolize alone (Theilade, 1990). Studies indicate that glycoprotein degradation may involve the synergistic action of several species possessing complementary patterns of glycosidase and protease activity (Marsh and Martin, 1992; Theilade, 1990). The development of complex food chains also contributes to the diversity and stability of oral ecosystems. For example, the metabolism of carbohydrates by *Streptococcus* and *Actinomyces* generates lactate, which may be used by *Veillonella* (Babaahmady *et al.*, 1997). The utilization of lactic acid by *Veillonella* produces vitamin K, required by black-pigmented gram-negative rods, and H_2 used by *Wolinella* (Marsh and Martin, 1992; Theilade, 1990). Competitive and antagonistic mechanisms among oral resident bacteria may help to maintain the ecological balance by preventing the overgrowth of some resident bacterial species or the establishment of opportunistic and exogenous bacteria. The competition for adhesion receptors, nutritional competition, and the production of inhibitory substances are among the mechanisms involved in reducing bacterial colonization and preventing bacterial overgrowth (Jack *et al.*, 1995; Marcotte *et al.*, 1995; Parrot *et al.*, 1989; Sanders and Sanders, 1984; Donoghue and Tyler, 1975). Inhibitory substances include organic fatty acids (Donoghue and Tyler, 1975), hydrogen peroxide (Marcotte *et al.*, 1995), lactic acid (Parrot *et al.*, 1989), antibiotics (Sanders and Sanders, 1984), enzymes (Baba, 1986), and bacteriocins (Jack *et al.*, 1995; Parrot *et al.*, 1990; Tagg *et al.*, 1976; Tagg and Russell, 1981). Many *in vivo* experiments with humans and animals indicate that the production of bacteriocins confers an ecological advantage. Although the natural role of bacteriocins has not been determined, they seem to contribute to the intra- and inter-regulation of the microbiota, and appear to be involved in microbial invasion and/or defence mechanisms (Oliveira *et al.*, 1998; Rams *et al.*, 1997; Tan and Riley, 1997). For example, it was easier to implant bacteriocin-producing strains of *S. mutans* in the

oral cavity than non-bacteriocin producing strains (Hillman *et al.*, 1987; Kitamura *et al.*, 1989; van der Hoeven and Rogers, 1979).

External Factors

Oral hygiene is one of the most important factors in the maintenance of oral homeostasis and oral health. The mechanical removal of plaque by tooth brushing and flossing can almost completely prevent caries and periodontal diseases (Mathiesen *et al.*, 1996). The addition of antimicrobial agents to dentifrices, mouthwashes, and varnishes increases the effect of mechanical oral hygiene procedures (Attin *et al.*, 2003; Ihalin *et al.*, 2003).

It is well documented that frequent consumption of a high-sucrose diet enhances the development of *S. mutans* and *Lactobacillus* (Minah *et al.*, 1985; Staat *et al.*, 1975). Apart from the effect of sucrose, very few studies have addressed the effect of other dietary components on the oral microbiota. Moreover, variation in the vitamin, lipid and mineral content of the diet has no direct effect on the oral microbiota of mice (Blais and Lavoie, 1990).

Antibiotics that are given orally or systemically for the treatment of different infections may enter the oral cavity via saliva and gingival crevicular fluid and lead to an imbalance in the oral microbiota. Antibiotics may suppress some resident bacterial populations which can result in overgrowth of antibiotic-resistant bacteria, infection by opportunistic pathogens, such as *Candida*, and colonization by exogenous potential pathogens, such as yeasts and members of the *Enterobacteriaceae* (Aiello and Larson, 2003; Kollef, 2003). Many other external factors may affect the oral microbiota; these include the wearing of dentures or partial dentures, smoking, oral contraceptive usage, malnutrition, host macroenvironment, and exposure to exogenous bacterial species (Devine, 2003).

Oral Microbiota in Healthy Individuals

On teeth, microorganisms colonize in a dense mass forming dental plaque. Dental plaque consists of microbial communities organized in a complex matrix composed of microbial extracellular products and salivary compounds (Wong and Sissions, 2001; Koller *et al.*, 2000). The microbial composition of dental plaque varies according to the site and the sampling time. Dental plaque develops preferentially on surfaces that are protected from mechanical friction, such as the area between two teeth, the subgingival area, and the pits and fissures of the biting surfaces (Jr Cleland, 2001; Jones *et al.*, 2000). The predominant organisms isolated from supragingival dental plaque are Gram-positive, facultative anaerobic bacteria, particularly *Actinomyces* spp. and streptococci. Gram-negative bacteria of the groups *Veillonella*, *Haemophilus*, and *Bacteriodes* are rarely isolated and, if so, in low proportions (Theilade, 1990). In a healthy subgingival crevice, the total number of cultivable bacteria is relatively small (10^3 - 10^6 CFU/crevice) (Theilade, 1990). The subgingival plaque is also dominated by Gram-positive organisms (*Actinomyces* and streptococci). It seems that the microbiota from the subgingival crevice is an extension of supragingival plaque (Bowden *et al.*, 1979). Black-pigmented gram-negative rods including *Porphyromonas* spp. and *Prevotella* spp. are rarely isolated from a healthy gingival crevice (Marsh and Martin, 1992; Slots, 1977).

Little information is available on the microbiota of mucosal surfaces. The oral mucosa of the gingivae, palate, cheeks, and floor of the mouth are colonized with few microorganisms (0 to 25 CFU/epithelial cell). Streptococci contribute the highest proportion of the microbiota in these sites, with a predominance of *S. oralis* and *S. sanguis*. A higher bacterial density (100 CFU/epithelial cell) and diversity is found on the tongue. The predominant members of the microbiota are *Streptococcus* spp. (*S. salivarius* and *S. mitis*) and *Veillonella* spp. Black-pigmented obligatory anaerobic rods and spirochetes, which are closely associated with periodontal diseases, have been recovered in small numbers (Theilade, 1990; van der Velden *et al.*, 1986).

Oral Microbiota Associated with Oral Diseases

The indigenous microbiota plays an important role in health as well as diseases of humans and animals (Plant *et al.*, 2003; Somova and Pechurkin, 2001; Gendron *et al.*, 2000). It contributes to the development of the immune system and provides resistance to colonization by pathogenic microorganisms. However, it also constitutes a reservoir of potentially pathogenic bacteria that may infect host tissues (Bowden *et al.*, 1979; Marsh and Martin, 1992; Theilade, 1990). In the oral cavity, indigenous bacteria are often associated with the aetiology of two major oral diseases, caries and periodontal diseases. Oral diseases seem to appear after an imbalance among the indigenous microbiota, leading to the emergence of potentially pathogenic bacteria. Owing to the scope of the present study, only periodontal diseases will be described in more detail.

Periodontal Diseases

Periodontal disease is the general description applied to the inflammatory response of the gingiva and surrounding connective tissue to the bacterial or plaque accumulations on the teeth (Peros and Savitt, 1989). These inflammatory responses are divided into two general groupings: gingivitis and periodontitis. Gingivitis is extremely common, and is manifested clinically as bleeding of the gingival or gum tissues without evidence of bone loss or deep periodontal pockets (Tugnait *et al.*, 2000). Pocketing is the term given to the pathologic loss of tissue between the tooth and the gingiva, creating spaces that are filled by dental plaque. Periodontitis occurs when the plaque-induced inflammatory response in the tissue causes actual loss of collagen attachment of the tooth to the bone and deep periodontal pockets, which in some cases can extend the entire length of the tooth root (Tugnait *et al.*, 2000; Peros and Savitt, 1989). Gingivalis precedes periodontitis, although it does not always lead to this more severe condition. In fact, some experts believe that it is an entirely different disease (Prayitno *et al.*, 1993). The more common forms of periodontitis comprise at least two clinical entities, an early onset periodontitis (EOP) and an adult periodontitis (AP) (Peros and Savitt, 1989). The early onset form occurs mainly in young individuals. It is

more aggressive looking and subdivided according to whether it begins before or after puberty (Procházková *et al.*, 1997). Immune-deficiencies and a genetic link have been shown to be possible factors for all types of EOP (Baker and Roopenian, 2002; van Dyke, 1998). People with severe and widespread EOP are at high risk for tooth loss. The adult periodontitis may begin in adolescence as a slowly progressing disease that becomes clinically significant at the age of 30 years and continues throughout life. It is a chronic disease and may reflect a stable, but tenuous, stand-off between the host's defensive systems and the plaque bacteria (Persson *et al.*, 2003). It is not clear whether these entities represent multiple types of infections with two clinical manifestations, or a single mixed anaerobic infection with different levels of host containment (Peros and Savitt, 1989).

Distribution of Periodontal Diseases in Developed and Developing Populations

The previously held belief is that a higher prevalence and severity of periodontitis existed among populations of developing nations, where living standards are lower and there is less access to health care services compared to that of developed nations. However, this has not been confirmed by most studies. Anerud *et al* (1983) have compared groups of American, Norwegian and Sri Lankan young adults and found that there are strikingly similar rates of periodontal breakdown, despite the last group having much poorer oral hygiene conditions. Furthermore, Baelum and Papapanou (1996) raised very interesting issues by recalculating and comparing findings from several studies in various countries. Their analysis has shown the similarity in the disease patterns, irrespective of oral hygiene conditions and levels of access to dental care, although there are a few exceptions (Löe *et al.*, 1978a; Löe *et al.*, 1978b; Cutress *et al.*, 1982). Nevertheless, it is clear that there are no marked differences in the prevalence of severe stages of periodontitis between developed and developing populations.

A large proportion of Americans suffer from periodontal diseases. During years 1986 and 1987, surveys have showed that about half of all adults have gingivitis and 80% of the population have experienced some degree of periodontitis. About 60% of children and adolescents have at least one tooth site with gingival bleeding. Ninety-five percent of elderly persons have periodontitis, with more than one third experiencing moderate to severe periodontal diseases (attachment loss of 6 mm or greater) (http://text.nlm.nih.gov). Similarly, the oral health surveys of Thai communities conducted during a past decade indicated that prevalence of periodontal diseases among adults aged more than 30 years was very high (> 96%) (Bratthall *et al.*, 1986; Baelum *et al.*, 2003). According to these statistical records, it is realized that a large proportion of the population worldwide encounters the problem of periodontal diseases. It is, thus, important to be noted that prevention and treatment of periodontal diseases are necessary, irrespective of the economic state and age of the populations.

What Cause Periodontal Diseases?

Bacteria

It is widely accepted that periodontal diseases occur as a result of infection by a group of specific bacteria from the subgingival microbiota, particularly Gram-negative anaerobes (Newman, 1990; Slots, 1986; Dzinck *et al.*, 1985). Although this subgingival microenvironment in the periodontal pocket is very diverse and over 300 species have been isolated from different individuals with as many as 40 from a single site, only a few species have been associated with diseases (Moore and Moore, 1994). The difference in bacterial communities at diseased sites compared to those from healthy sites is indicated in Table 2. The diseased sites are dominated by anaerobes, in particular, by spirochetes and black-pigmented anaerobic species (Riviere, 1994; Peros and Savitt, 1989). Among the latter, *P. gingivalis* and *Pr. intermedia* are most often associated with EOP and AP (Genco *et al.*, 1998). Indeed, other anaerobic species such as *Tannerella forsythensis* (*Bacteriodes forsythus*), *Capnocytophaga* spp., and *Fusobacteria* spp. are also involved (Mayrand and Grenier, 1998; Peros and Savitt, 1989). Other

more acute and rapid forms of periodontal diseases may also arise due to different predisposing conditions such as hormonal changes or depressed immune systems. These diseases seem more associated with particular microbial groups. For example, localized juvenile periodontitis (LJP) is closely associated with high numbers of *A. actinomycetemcomitans* (Meyer and Fives-Taylor, 1997; Slots and Schonfeld, 1991).

Host response and periodontal tissue metabolisms

Tissue destruction in periodontitis is a complex process. It may be initiated and progressed by both direct and indirect effects of bacteria plus the effects of the altered host defence system (Baker, 2000; van Dyke, 1998; Procházková *et al.*, 1997). Because massive or even obvious bacterial invasion of the tissues is rarely encountered, direct effects of bacteria are characterized by production of enzymes (proteases, collagenase, fibrinolysin, phospholipase A) that could degrade the surrounding tissues of the superficial layers of the periodontium and elicit an inflammatory response (Holt and Bramanti, 1991; Peros and Savitt, 1989). Although overwhelmingly protective, this inflammatory response appears to be responsible for a net loss of periodontal supporting tissue, and leads to periodontal pocket formation, loosening of the teeth, and eventually to tooth loss. The monocyte/lymphocyte system is activated as a response to bacterial products and antigens. The activation of the immune system stimulates production of both catabolic cytokines and inflammatory mediators such as prostaglandin E2 (PGE2), which in turn promote the release of enzymes (including collagenases) destructive to the extracellular matrix and bone (Baker, 2000; van Dyke, 1998; Offenbacher, 1996). In addition, during the phagocytosis, polymorphonuclear neutrophils (PMNs) themselves can release lysosomal enzymes that may cause degradation of collagen and contribute to tissue damage (Baker, 2000; Peros and Savitt, 1989).

Factors that Increase the Chances for Periodontal Disease

Plaque associated periodontal diseases have been well documented (Jeffcoat, 1994). Lack of oral hygiene encourages plaque formation. Poorly contoured restorations that provide traps for debris and plaque can contribute to plaque accumulation and bacterial formation, followed by gingival inflammation and eventually periodontitis.

Smoking can cause bone loss and gum recession even in the absence of periodontal diseases. A number of studies indicate that smoking increases inflammation by triggering an over-active response of the immune system (Kamma and Nakou, 1997). It was found that smokers are 11 times more likely to harbour the bacteria that cause periodontal diseases than non-smokers. Smoking significantly affects the dental health of young people who are otherwise healthy, and is even more harmful to young people who have early onset periodontitis. Quitting of smoking decreases the severity and prevalence of periodontal diseases (Kamma and Nakou, 1997).

Certain disorders such as diabetes, Down's syndrome, and AIDS render patients more sensitive to the inflammatory process (Otsuka *et al.*, 2002; Siu *et al.*, 2000; Finegold, 1993). Degeneration of connective tissues and dryness of the mouth, problems associated with illnesses such as rheumatoid arthritis, lupus erythematosus, and Crest syndrome, may contribute to periodontal diseases (de Boer *et al.*, 2003).

Some medications such as steroids can contribute to periodontitis by increasing a person's susceptibility to bacteria. For example, taking oral contraceptives containing the synthetic progesterone desogestrel increases the risk for periodontal diseases (Klinger *et al.*, 1998; Krall *et al.*, 1997).

Periodontal diseases often occur in members of the same family. Both genetic and other factors are thought to be contributing factors. Children of parents with periodontitis are 12 times more likely to have the plaque causing bacteria and eventually periodontal diseases (Baker and Roopenian, 2002). Intimate partners and spouses of people with periodontal disease may also be at risk. In addition, researchers have found that the bacteria *P. gingivalis* may be

contagious after exposure to an infected person over a long period of time (Özmeric *et al.*, 1999). It could be therefore that up to 30% of the population may have some genetic susceptibility to periodontal diseases (Baker and Roopenian, 2002; Özmeric *et al.*, 1999).

Overall, it is realized that although certain species are consistently associated with diseased tissue not everyone infected with these species develops periodontal diseases. Several host factors including genetics, stress, smoking, some general diseases, and the overall state of oral hygiene are also important in disease incidence and progression.

Table 2. Bacterial profiles of subgingival plaques taken from three clinical forms of periodontitis (EOP, AP and LJP) and from periodontally healthy sites (Peros and Savitt, 1989).

Pathogen	Healthy sites	EOP	AP	LJP
Facultative specie				
Streptococcus spp.	+++	+	+	+
Actinomyces	+++	+	+	+
Veillinella spp.	+	+	+	+
Microaerophillic species				
A. actinomycetemcomitans	-	- to +	+	++
Capnocytophaga spp.	±	±	±	±
Anaerobic species				
Spirochetes	±	+++	+++	±
P. gingivalis	-	- to ++	- to ++	-
Pr. intermedia	±	- to ++	- to ++	±
T. forsythensis	±	- to ++	- to ++	-
Capnocytophaga spp.	-	- to ++	- to ++	-
Fusobacteria spp.	+	- to +	- to ++	±

\- not present
± may be present
\+ usually present at levels < 10% of flora
++ present at levels 10-20%
+++ present at levels > 20%
EOP early onset periodontitis
AP adult periodontitis
LJP localized juvenile periodontitis

How Serious are Periodontal Diseases?

A much less severe, but nevertheless distressing problem caused by periodontal disease is bad breath. The ultimate outcome of uncontrolled periodontal diseases is tooth loss. In accordance, normal oral activities such as chewing are hindered with eventually affecting the overall health.

As described, periodontal destruction results from the action of various toxic products released from specific pathogenic subgingival plaque bacteria, as well as from the host responses elicited against plaque bacteria and their products (Tew, 1988). The inflammatory response may result in gingival ulceration around the tooth, which can allow intact bacterial cells or their products into the systemic circulation (Kennedy *et al.*, 2003). These infections may thus influence overall health and the course of some systemic diseases (Akintoye *et al.*, 2002; Wehrmacher, 2001). There are reports describing an association between periodontal disease and heart disease (de Boer *et al.*, 2003; Wehrmacher, 2001). Experts believe that in people with periodontitis, even normal oral activities can cause tiny injuries that release bacteria into the blood stream. The bacteria that cause periodontitis may stimulate factors that cause blood clots and other proteins that contribute to a higher risk for heart disease (Wehrmacher, 2001). In rare cases, periodontal bacteria can cause an infection in the lining or valves of the heart called infective endocarditis (de Boer *et al.*, 2003). Diabetes and periodontal diseases appear to actually worsen each other. Diabetes can lead to imbalances in important immune factors, which can contribute to periodontal infections and impaired healing (Siu *et al.*, 2000). Bacteria that reproduce in the mouth can also be carried into the airways of throat and lungs, increasing the risk for respiratory diseases and worsening chronic lung conditions, such as emphysema (Travis and Potempa, 2000; Sakamoto and Sakamoto, 1988).

Since bacteria and inflammation involved in periodontal diseases may reflect systemic conditions, which are not traditionally addressed by dentists and periodontists, the way that periodontal disease is viewed and treated may change in the future (Jeffcoat, 1994).

Prevention and Treatment

That good oral hygiene and professional surveillance can prevent gingivitis has been documented (Bruakoff, 2003; Tugnait and Clerehugh, 2001). However if gingivitis occurs, it can be efficiently treated by debridement of the teeth. In some cases, short-term use of products containing chlorhexidine, fluoride, or other antimicrobial agents may be beneficial (Schwach-Abdellaoui *et al.*, 2000). These products, when used in conjunction with tooth brushing and flossing, are probably adequate to deliver any agents to subgingival sites that are 1 to 3 mm in depth. However, there may not be sufficient penetration of the agents to the pocket with depth greater than 3 mm. By a professional, subgingival debridement using irrigating devices containing an antimicrobial agent can be an additional benefit (Frame *et al.*, 2000; Shani *et al.*, 1998).

Clinical dentistry has been about 80 to 85% successful in treating periodontitis by debridement and surgical procedures (Burakoff, 2003). Although surgery is labour intensive and costly, it may be necessary when deep pockets have developed between the teeth and gums. The majority of clinical cases of periodontitis represent specific bacterial infections. Thus, elimination or suppression of these bacteria may improve the probability of long-term stability of the periodontal structures and the likelihood of maintaining a natural dentition (Schwach-Abdellaoui *et al.*, 2000; van Steenbergen *et al.*, 1993). Most studies indicated that metronidazole and tetracycline are effective on killing anaerobic bacteria (Fine *et al.*, 1998; Varjacic *et al.*, 1998). This would point to the use of these antibiotics in treatment of periodontitis. Studies on animals fed with high doses of metronidazole, however, indicate that the drug might be tumorigenic (Fine *et al.*, 1998; van Steenbergen *et al.*, 1993). This concern has caused reluctance in using metronidazole, although the FDA in the United State approved metronidazole for treatment of anaerobic infections in 1981. It was suggested that treatment of periodontitis with metronidazole in combination with the standard debridement procedures could delay surgery for several years. However, due to over prescribing and misuse of the antimicrobial agents, metronidazole and

tetracycline resistances have been reported among oral bacteria (Olsvik *et al.*, 1994).

The widespread increase in antibiotic resistance among human microbial pathogens is currently a major concern. Linked to this is the awareness of long-term development of antimicrobial resistance within the oral bacterial flora (Sefton, 1999). This not only increases the chances of treatment failures for periodontal infections but also creates an antibiotic resistant gene pool from which resistance can spread from oral bacteria to other pathogenic bacteria. For example, antibiotic resistant oral streptococci, which form early dental plaque, can exchange genes with *Streptococcus pneumoniae*, the major cause of otitis media (middle ear infection) in children, and pneumonia in elderly people. This has resulted in the generation of penicillin-resistant and in some cases multiple-drug resistant pneumococci that resist primary antibiotic treatment (Fine *et al.*, 1998; van Steenbergen *et al.*, 1993). Therefore, future strategies for controlling periodontal diseases and other microbial infections will ideally place less emphasis on the use of systemic broad-spectrum antibiotics (Yue *et al.*, 2003; Schwach-Abdellaoui *et al.*, 2000).

That the pharmaceutical industry may no longer be able to develop effective novel antimicrobial agents quickly and sufficiently has been increased concern. This concern has triggered the search for new antimicrobial compounds, such as bacteriocins. Bacteriocins have attracted a great deal of attention because of their specific killing activity (Jack *et al.* 1995). These compounds could also be considered for applications in various infectious diseases and may provide a new approach for dealing with antibiotic-resistant bacteria.

Bacteriocins and Bacteriocin Like-Substances (BLS)

Many bacterial strains, both Gram-negative and Gram-positive, produce bacteriocins or bacteriocin like-substances (BLS) (Jack *et al.* 1995). Bacteriocins are ribosomally synthesized peptides or proteins, and are characterised by their ability to inhibit closely related, and sometimes more distantly related, strains of bacteria (Jack *et al.* 1995). The use of the term BLS is recommended to denote antibacterial products not yet characterized.

Bacteriocins produced by Gram-negative bacteria were the first to be characterised in any detail. Best known of these are the colicins, which are produced by and attack strains of *Escherichia coli* (Braun *et al.*, 1994). The colicins constitute a diverse group of antibacterial proteins. They are large, complex 29-90 kDa proteins with characteristic structural domains involved in cell attachment, translocation, and bactericidal activity (Riley and Wertz, 2002; Riley and Gordon, 1999). The colicins kill closely related bacteria by various mechanisms, by for instance inhibiting the cell wall synthesis, by permeabilizing the target cell membrane, or by exhibiting RNase or DNase activity (Masaki and Ogawa, 2002). They bind to specific receptors on the outer membrane of the target cell. As a consequence, their range of activity tends to be very narrow.

Among the bacteriocins produced by Gram-positive bacteria, the ones produced by lactic acid bacteria (LAB) are perhaps the most extensively characterised. The LAB bacteriocins are, in most cases, small peptides, 3-6 kDa in size, although there are exceptions (Nes *et al.*, 1996: Joerger and Klaenhammer, 1990). Many of them are active against food-borne pathogens such as *Listeria monocytogens*, *Clostridium perfringens*, *Bacillus cereus*, and *Staphylococcus aureus* (Casaus *et al.*, 1997; Ryan *et al.*, 1996; Cintas *et al.*, 1995). Therefore, they are considered as potential natural food preservatives. The LAB bacteriocins demonstrate great heterogeneity in terms of amino acid sequence and in the types of bacteria they kill. Three classes of LAB bacteriocins have been defined by Klaenhammer (Klaenhammer, 1993), but recent results suggest that a fourth class of "complex bacteriocins" could exist. The class I bacteriocins, also known as lantibiotics, comprise small peptides (< 5 kDa) containing the unusually amino

acids lanthionine and β-methyllanthionine, and a number of dehydrated amino acids. The class II bacteriocins are small, heat-stable peptides (< 5 kDa) that do not contain modified amino acid residues. The class II bacteriocins may be subdivided into three sub-classes. These are the *Listeria*-active peptides (class IIa), the peptides requiring two components for activity (class IIb), and the *sec*-dependent secreted peptides (class IIc). The class III bacteriocins are large, heat-labile proteins (> 30 kDa). Examples of the class I, II, and III bacteriocins are nisin, pediocin AcH/PA1, and helveticin J, respectively. Most LAB bacteriocins belong to the first two classes and most of these undergo a post-translational modification, involving in cleavage of a leader peptide (Nes *et al.*, 1996; Jack *et al.*, 1995). Unusual amino acid residues found in the lantibiotics are given by additional post-translational modifications (McAuliffe *et al.*, 2001). In contrast, the non-lantibiotics (class II and class III) do not contain such modifications.

Other Gram-positive bacteria (including *Bacillus* species) have also been reported to produce bacteriocins and/or BLS (Zheng and Slavik, 1999; Hyronimus *et al.*, 1998; Novotny and Perry, 1992). However, these reports usually suffer from limited biochemical and genetic information about the bacteriocins and/or BLS, since the studies have often been carried out using culture supernatants rather than purified peptide. The only bacteriocins from *Bacillus* that have been characterized at the amino acid and DNA sequence levels are subtilin (Klein *et al.*, 1992) and subtilosin (Zheng *et al.*, 1999). These two bacteriocins are produced by *Bacillus subtilis*.

Most of the bacteriocins produced by Gram-positive bacteria are membrane active compounds that increase the permeability of the cytoplasmic membrane (Jack *et al.*, 1995). They often show a much broader spectrum of bactericidal activity than the colicins, which may be due to the lack of a requirement for a specific cell surface receptor, and/or the absence of an outer membrane to restrict their access to the cytoplasmic membrane. However, many are more restricted in their range of activity (Jack *et al.*, 1995).

Because bacteriocins mainly inhibit the growth of related species or species with the same nutritive requirements, bacteriocin production seems to be aimed at bacteria which are present in the same ecological niche as the bacteriocin producer. Studies have also shown that bacteriocin production provides the producer strain with a selective advantage over other nonproducing, isogenic bacteria (Ruiz-Barba *et al.*, 1994, Teanpaisan *et al.*, 1998). Therefore, bacteriocin biosynthesis might be expected as a high-energy-consuming process, providing an advantage to the producer strain only if the cost/benefit ratio is favourable (Riley, 1998; Dykes, 1995).

It is believed that bacteria must be able to adapt their metabolism to the changing environmental conditions. This adaptation requires the bacteria to sense the multitude of extracellular signals, followed by that they respond by controlling the expression of an adequate repertoire of genes. In accordance, bacteriocin production may be well controlled by molecular regulation systems such as induction and catabolic repression (McAuliffe *et al.*, 2001). Studies have reported that production of bacteriocins is influenced by pH, temperature, medium composition, DNA-damaging agents, and growth conditions (Diep *et al.*, 2000; de Vuyst *et al.*, 1996; Barefoot *et al.*, 1994; Fremaux *et al.*, 1993; Biswas *et al.*, 1991).

Bacteriocins are often confused in the literature with antibiotics. This would limit their use, especially in food and health-care applications. Bacteriocins can be differentiated from antibiotics on the criteria of synthesis, mode of action, antimicrobial spectrum, toxicity, and resistance mechanisms. These aspects are summarised in Table 3 and described in some detail in the text below.

Table 3. Bacteriocins vs. antibiotics

Characteristic	Bacteriocins	Antibiotics
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually adaptation affecting cell membrane composition	Usually a genetically transferable determinant affecting different sites depending the mode of action (or mutation)
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in a few cases possibly cell wall biosynthesis, and DNA or RNA degradation	Cell wall or intracellular targets, e.g., protein synthesis
Toxicity/side effects	None known	Yes

Synthesis of Bacteriocins

General features of the bacteriocin genetic organization mostly come from the characterization of LAB class II bacteriocins. Several studies showed that genes encoding bacteriocins can be located on the chromosome (Altena *et al.*, 2000; Diep *et al.*, 1996), or in a plasmid or transposon (Engelke *et al.*, 1992). Typically, they are organized in operons comprising four genes, which may or may not be located on the same transcription unit. These are the structural gene, a dedicated immunity gene, a gene encoding an ABC transporter (optional), as well as a gene encoding an accessory protein, which is essential for the externallization of the bacteriocin (Diep *et al.*, 1996; Qiao *et al.*, 1996; Engelke *et al.*, 1994; Klein and Entian, 1994; Klein *et al.*, 1993; Engelke *et al.*, 1992; Klein *et al.*, 1992; Rauch and de Vos, 1992). The structural gene usually encodes an inactive prebacteriocin. Such a prebacteriocin contains a leader peptide extending from N-terminus of the corresponding active bacteriocin. Concurrent with its export from the cell, the prebacteriocin is activated by cleavage of the leader peptide by a protease. In some cases, the protease activity has been shown to be associated with the bacteriocin-transporter protein (usually of the ABC type but occasionally

the *sec*-dependent) (Papagianni, 2003). The immunity protein is usually localized on the same transcription unit as the structural gene and generally co-transcribed, in order to ensure that the producer is not killed by its own bacteriocin (Papagianni, 2003). Because bacteriocins are encoded by one structural gene, the active sites and structure-function relationships of bacteriocins can be examined relatively easily by genetic manipulation. Molecular techniques also allow bacteriocin analogues with increased activity or with altered specificity to be constructed and evaluated. In contrast to bacteriocins, antibiotics are not ribosomally synthesized. Although several antibiotics such as vancomycin are composed of amino acids, they are enzymatically synthesized. Several peptide antibiotics are synthesized by a multiple-carrier thiotemplate mechanism, where peptide synthetases assemble amino acids to form the antibiotic molecule (Hancock and Chapple, 1999). A number of genes involve the synthesis of antibiotics. Therefore, there is relatively difficult to synthesize antibiotics by using the genetic means (Hancock and Chapple, 1999).

Bacteriocin Immunity

Bacteriocin producers are most often insensitive to their own bacteriocin. The immunity of the cell synthesizing the bacteriocin to its product is a phenomenon that distinguishes bacteriocins from antibiotics. This immunity is usually mediated via the co-production of a specific immunity protein (Papagianni, 2003). A number of bacteriocin immunity genes have been cloned and characterized. The majority of these are located within gene clusters that also encode bacteriocin structural proteins (Axelsson and Holck, 1995: Quadri *et al.*, 1995). In general, little homology has been observed among characterized bacteriocin immunity genes. The immunity of lantibiotics was initially thought to be due to one immunity gene, such as *nis*I for nisin and *spa*I for subtilin, which code for NisI and SpaI immunity proteins, respectively (McAuliffe *et al.*, 2001). Nevertheless, it appears that immunity to these bacteriocins is the result of the influence of several proteins, since the deletion of other genes result in altered host immunity (Klein and Entian, 1994). For example, non-nisin-producing nisin-

resistant strains of *Lactococcus lactis* do not have the genetic elements encoding for NisI protein, but do have sequences similar to *nis*F, *nis*E and *nis*G (Duan *et al.*, 1996). These sequences are thought to render the strains resistant to nisin. The mechanisms that govern host immunity to nisin (and other lantibiotics, i.e., class I LAB bacteriocins) thus appear to be quite complex and are not yet entirely elucidated. For the non-lantibiotic LAB bacteriocins (class II bacteriocins), the phenomenon of immunity appears to be somewhat simpler. One gene encodes the immunity protein (Kleanthous and Walker, 2001). Usually, it is a basic protein consisting of between 50 and 150 amino acid residues. It is loosely associated with the membrane (Kleanthous and Walker, 2001). The lactococin A immunity protein (LcnI) is by far the most studied one, yet the basic mechanism behind the immunity is still not understood (Venema *et al.*, 1995; Venema *et al.*, 1994; Nissen-Meyer *et al.*, 1993). Knowledge as to how the immunity protein protects the producer against its own bacteriocin may help to understand by which mechanisms the bacteriocin use to kill the sensitive bacteria.

Resistance Mechanisms

Once a new antimicrobial agent is found to be safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. Cells exhibiting resistance to several antibiotics and the transfer of resistance between organisms have been documented (Roberts, 2003; Aureli and Franciosa, 2002; Miranda and Zemelman, 2002). Antibiotic resistance is usually associated with a genetic determinant that facilitates the transfer of resistance between cells, strains, and species (Roberts, 2003; Aureli and Franciosa, 2002). Unlike most antibiotic resistance, bacteriocin resistance is in some cases thought to be the result of a physiological change in the target cell membrane (Crandall and Montville, 1998; Mazzotta *et al.*, 1997; Ming and Daeschel, 1993). For example, increasing membrane rigidity of *L. monocytogenes* by lowering C15:C17 ratio results in increased tolerance to nisin (Mazzotta *et al.*, 1997). Moreover, it was found that nisin-resistant *L. monocytogenes* have reduced amounts of phosphatidylglycerol, diphosphatidylglycerol, and bis

phosphatidylglyceryl phosphate (Ming and Daeschel, 1993). Although research shows that a change in cell membrane composition may in some cases account for resistance, some mutants produce an enzyme, nisinase, which degrades nisin (Jarvis, 1967). Gravesen *et al* (2000) have reported that *L. monocytogenes* mutants resistant to pediocin PA-1 show increased expression of gene fragments that code for β-glucoside-specific phosphoenolpyruvate-dependent phosphotransferase systems (PTS). The mechanism by which β-glucoside-specific PTS interacts with pediocin to confer resistance has not been elucidated. Although bacteriocins are not antibiotics and experimental data indicate that bacteria exhibiting nisin resistance do not show cross-resistance with antibiotics (Tchikindas *et al.*, 2000; Severina *et al.*, 1998), it is still important to understand the mechanism of bacteriocin-resistance so that it can be avoided. Resistance may be genetically encoded or the result of an adaptation. There are contradictory data regarding to what extent one obtains cross-resistance when bacteriocins from different classes are used (Crandall and Montville, 1998; Rasch and Knochel, 1998; Mazzotta *et al.*, 1997; Song and Richard, 1997).

Rationale for The Study

The microbial community of the oral cavity is highly complex and diverse (Wong and Sissions, 2001; Koller *et al.*, 2000). A consequence of a high level of bacterial diversity is a high level of opportunity for interaction between cells, and presumably intense competition between the organisms in the microbiota. Under these circumstances, the production of antimicrobial compounds (including bacteriocins) and the possession of resistance mechanisms against antimicrobial compounds produced by other organisms could be both a very effective way of securing a niche in the environment and essential for survival (Riley and Wertz, 2002; Riley and Gordon, 1999). Peptides and proteins with antibiotic action (including bacteriocins) is a resource frequently employed in nature, and can be considered a successful strategy in mediating bacterial antagonistic relationship (Devine, 2003). That the production of bacteriocins provides the producer with a selective advantage over other strains and bacteriocins are thought of as factors in

bacterial virulence have been documented (Devine, 2003; Riley and Wertz, 2002). Diversity in microbial populations may perhaps favour the evolution of bacteriocins as competitive factors (Teanpaisan *et al.*, 1998). If this assumption can be confirmed, bacteriocins would have potential applications as a possible means of controlling pathogenic bacteria in future therapy.

Prevotella nigrescens is a black pigmented, obligatory anaerobic, Gram-negative rod-shaped organism. Conrad *et al.* (1996) have considered that it might be a marker bacterium for physiologically healthy conditions in plaque as it is found in nearly all of samples taken from periodontally healthy individuals. Black-pigmented anaerobic bacteria, in particular *Porphyromonas gingivalis* and *Prevotella intermedia*, have often been isolated from and dominate the samples obtained from active periodontal sites of adult periodontitis (Teanpaisan *et al.*, 1996; Dahlén, 1993). However, in active periodontal sites, *Pr. nigrescens* was found in a much lesser extent, but it has been found and is a predominant species in the inactive periodontal sites (Teanpaisan *et al.*, 1996). It is thought that *Pr. nigrescens* might produce some antagonistic substance(s) advantageous for the producer to govern the ecological niche. Later in 1998, Teanpaisan *et al* reported the production of BLS of the *Pr. nigrescens* strains isolated clinically. The inhibitory substance was active against *P. gingivalis* and *Pr. intermedia* (Teanpaisan *et al.*, 1998). Additional results reported that mixed populations of these black-pigmented species are relatively uncommon (Teanpaisan *et al.*, 1995; Torkko and Asikainen, 1993). It may be, therefore, that BLS produced by *Pr. nigrescens* may play a protective role for the host against periodontal pathogens including *P. gingivalis* and *Pr. intermedia*. In this respect, the bacteriocin may serve as an antimicrobial agent in future therapy.

There has been recently an interest in bacteriocins, which are produced by oral microorganisms. For example, production of bacteriocins by *Streptococcus mutans* strains has been used as a typing scheme to demonstrate transmission between subjects (Balakrishnan *et al.*, 2002; Rogers, 1980). These bacteriocins, termed mutacins, have been genetically and biochemically characterized (Longo *et al.*, 2003; Krull *et al.*, 2000; Qi *et al.*, 2000). This information suggests the

possibility of using of genetically modified *S. mutans* for prevention of dental caries (Hillman, 2002). In contrast to mutacins, there are very few studies regarding bacteriocins that are produced by oral Gram-negative bacteria (Oliveira *et al.*, 1998; Farias *et al.*, 1994; Höhne *et al.*, 1993; Takada *et al.*, 1991; Hammond *et al.*, 1987; Stevens *et al.*, 1987). Only three studies are known. Takada *et al* (1991) have reported a low molecular weight bacteriocin produced by *Pr. intermedia*. The bacteriocin was active against a limited range of *Pr. intermedia* and one strain of *Fusobacterium nucleatum*. Production of a bacteriocin by *A. actinomycetemcomitans* has been reported (Stevens *et al.*, 1987). The bacteriocin appears to have a broader antagonistic activity, but little is known about its physical and chemical properties. *Tannerella forsythensis* (*Bacteroides forsythus*) has also been found to produce a bacteriocin with a broader antimicrobial activity. Again, the bacteriocin genetic system has not yet been identified (Nakamura *et al.*, 1981).

Although the production of bacteriocins is widespread and appears to be a successful strategy for mediating antagonistic relationships between different bacteria, information concerning bacteriocins produced by *Pr. nigrescens* is sparse, and there has so far been no genetic and biochemical characterization of bacteriocins from *Pr. nigrescens*. There is presently interest in the possible use of a bacteriocin for controlling periodontal pathogens. This prompted us in this study to characterize the genes involved in bacteriocin production by *Pr. nigrescens* ATCC 25261, and to explore the possibility of using such a bacteriocin in prevention and treatment of periodontal diseases. These objectives would be successful by cloning and sequencing the bacteriocin-encoding gene of *Pr. nigrescens* ATCC 25261, producing the bacteriocin in large amount followed by bacteriocin purification, and finally determining biochemical and toxic properties of the bacteriocin *in vitro*.

Chapter 2
Materials and Methods

Bacterial strains, media, and reagents

Bacterial strains and plasmid constructed and used in this work are listed in Table 4. Two competent *E. coli* strains, JM109 and BL21(DE3)pLysS, were purchased from Promega. *E. coli* cells were grown aerobically in Luria-Bertani (LB) medium (10 g/l of bacto tryptone, 5 g/l of yeast extract, 10 g/l of sodium chloride). *Pr. nigrescens* and other anaerobic bacteria were maintained on blood agar (37 g/l of brain heart, 5 g/l of yeast extract, 5 % of expired human blood, 5 mg/l of haemin, 1 mg/l of menadinone, and 2 % agar). The bacteria were grown anaerobically under an atmosphere of 80 % nitrogen, 10 % hydrogen, and 10 % carbon dioxide at 37 ^{o}C and transferred to the fresh plates every 7 to 10 days. Brain Heart Infusion (BHI) (37 g/l of brain heart, 5 g/l of yeast extract, 1 % of vitamin K, and/or 5 % of horse serum) was obtained from Merck. BHI broth, supplemented with vitamin K was used for testing the bacteriocin production of *Pr. nigrescens* ATCC 25261. *Streptococcus* and *Lactobacillus* were cultured on Rogosa medium (20 g/l D-glucose, 10 g/l trypticase, 3 g/l tryptose peptone, 5 g/l yeast extract, 2 g/l triammonium citrate, 3 g/l K_3PO_4, 3 g/l K_2HPO_4, 0.575 g/l $MgSO_4$. $7H_2O$, 0.034 g/l $FeSO_4.7H_2O$, 12 g/l $MnSO_4.4H_2O$, and 0.2 g/l L-cysteine.HCl, Difco Laboratories) in a candle jar at 37 ^{o}C. Solid and soft media were prepared by the addition of 1.5 % and 0.75 % agar, respectively (see Appendix A for more detail). Ampicillin (Sigma) at a concentration of 100 μg/ml was added in LB medium for maintaining plasmid constructs. Color screening for transformants containing pGEM-3Z with insets was carried out on LB plates containing ampicillin, IPTG (isopropyl-β-D-thiogalactoside, Amersham Biosciences) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Amersham Biosciences). IPTG was dissolved in water, filter sterilized, and added to selective plates at a final concentration of 0.5 mM. An X-gal stock solution in

N,N-dimethylformamide stored at –20 ^{O}C was added to plates at a final concentration of 80 μg/ml. Other chemical reagents were purchased from Fluka or Merck.

Purification of the whole genomic DNA

Pr. nigrescens ATCC 25261 was used in this study because the bacterium is a type strain of *Pr. nigrescens*, and it has been reported to produce a bacteriocin-like substance (Teanpaisan *et al.*, 1998). Chromosomal DNA was extracted and purified from the *Pr. nigrescens* ATCC 25261 strain by a modified method of Owen and Borman (1987). Briefly, cells were harvested directly from plates, washed in SE buffer (150 mM NaCl; 100 mM EDTA, pH 8.0) and resuspended in SET buffer (150 mM NaCl; 15 mM EDTA; 60 mM Tris HCl, pH 8.3) containing RNAase A (100 μg/ml). Sodium dodecyl sulfate (SDS) was added to a final concentration of 2 % (w/v) and incubated at 37 ^{O}C for 30 min. Proteinase K (100 μg/ml) was then added and incubated with gentle shaking for 1 h. An equal volume of chloroform was subsequently added, mixed vigorously and centrifuged at 12,000*g* for 15 min. The upper aqueous phase was aspirated and mixed with 0.5 volume of 7.5 M ammonium acetate. Chromosomal DNA was precipitated with exactly 0.54 volume of isopropanol at room temperature for 30 min, and then washed extensively with 70 % (v/v) ethanol. A preliminary result (unpublished result) has shown that the extracted chromosome was significantly contaminated with proteins. Therefore, the chromosome was further purified by using Wizard DNA Minipreps (Promega) according to the manufacturer's instruction.

Isolation of a plasmid(s) from *Pr. nigrescens* ATCC 25261

The procedure of Somkuti and Steinberg (1986) was performed to know whether or not the bacteriocin producer contains a plasmid(s). In brief, the producer cells of a 5-ml 48-h culture were collected, washed in 10 mM Tris-HCl, pH 8.2, and resuspended in 0.2 ml of 20 mM Tris-HCl, pH 8.2. Lysozyme

(7.5 mg) was then added, followed by the addition of 24 % of polyethylene glycol (PEG 20,000) and 1.25 ml of diethylpyrocarbonate (DEP). After 60-min incubation at 37 ^{O}C and centrifugation, the pellet was resuspended in 0.25 ml of 100 mM Tris-HCl, 10 mM Na_2EDTA pH 8.5. Cell lysis was achieved by the addition of 25 µl of 10 % SDS and incubation at 37 ^{O}C for 15 min. The lysate was adjusted to pH 12.2 with 5 M NaOH followed by the addition of 50 µl of 2 M Tris-HCl, pH 7 and 30 µl of 5 M NaCl. The deproteinization was carried out once with phenol, saturated with 3 % NaCl and once with chloroform. The plasmid DNA was precipitated with two volumes of cold ethanol and dissolved in 50 µl of 10 mM Tris-HCl; 10 mM Na_2EDTA, pH 8.0 and treated with RNAase (100 µg/ml).

Cloning of the bacteriocin gene

Chromosomal DNA of *Pr. nigrescens* ATCC 25261 was utilized for cloning a bacteriocin gene. pGEM-3Z (Promega) was used as the cloning vector (Fig. 1). Several restriction endonucleases (New England Biolab and Promega), which are compatible with enzymes in the polycloning region of the pGEM-3Z, were used according to supplier's recommendations to digest the DNA. In the present study, *Eco*RI, *Pst*I, and *Hin*dIII were selected for use, because they cut the DNA into beneficial fragments. According to our preliminary results on DNA typing (unpublished data), *Eco*RI was therefore the first enzyme used for this purpose. The pGEM-3Z vector was previously cut with the enzyme used for cutting the DNA, and then treated with the alkaline phosphatase. The DNA fragments were ligated with the precut-treated pGEM-3Z using T4 DNA ligase (New England Biolab), and the ligation mixture was then transformed into the competent *E. coli* (JM109) (see Appendix J for more detail). The transformants were plated on LB agar plates containing ampicillin, IPTG and X-gal, and then incubated at 37 ^{O}C for 24 h. A number of white colonies were screened for bacteriocin production using an agar overlay assay as will be described. After the bacteriocin-producing clone was obtained, size of the insert DNA was determined

by agarose gel electrophoresis following recombinant plasmids extraction and the corresponding enzyme cutting (Sambrook *et al.*, 1989; see also Appendix C for more detail).

To construct the rest of the recombinant plasmids (pGP2, pGH17, and pGH18), the following procedures were performed. The insert DNA of the first bacteriocin-producing clone we obtained was isolated by cutting the recombinant plasmid with *Eco*RI. The fragments of interest were separated using low melting point agarose gel electrophoresis and the Rapid Concert^{TM} DNA Purification Kit (Gibco). The isolated fragments were then digested with either *Pst*I or *Hin*dIII, generating smaller fragments. These smaller fragments were ligated with the *Pst*I-treated or *Hin*dIII-treated pGEM-3Z, and the ligation mixtures were transformed into the competent *E. coli* (JM109). The transformants were plated on LB agar supplemented with ampicillin, IPTG, and X-gal and incubated for 24 h at 37 ^{O}C. A number of white colonies were screened for bacteriocin production. Size determination was done by the procedure described in the previous paragraph, in cases obtaining new producing clones. However, other corresponding enzymes utilized for cloning were used instead of *Eco*RI. Plasmids' designation and their characteristics were shown in Table 4.

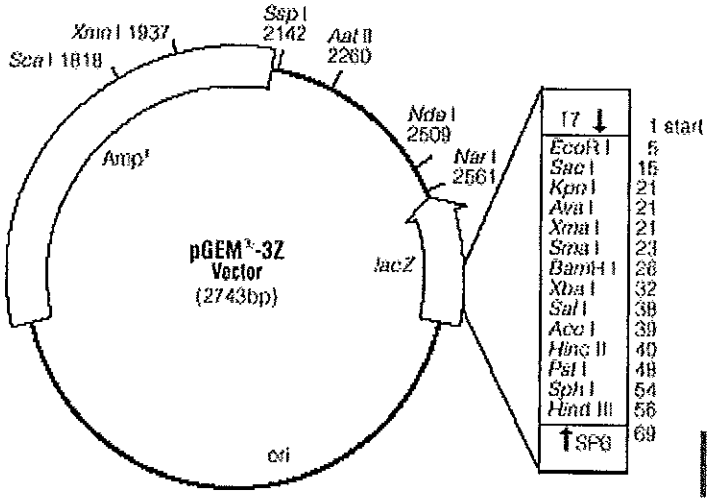


Fig. 1 Mapping of the cloning vector pGEM-3Z (Promega)

It could be summarized that the standard protocols for DNA manipulation were used, unless otherwise stated (Sambrook *et al.*, 1989). Restriction endonucleases and modified enzymes were used according to the manufacturer's instructions. Agarose was obtained from Promega. A gel with 0.8, 1.0, or 1.2 % agarose concentration was used depending on DNA length for size determination. The Rapid Concert™ DNA Purification Kit was used for elution of the DNA fragments following low melting point agarose gel electrophoresis. The eluted fragments were ligated with dephosphorylated and pre-digested plasmid vector using T4 DNA ligase. The constructed plasmids were then introduced into the competent *E. coli* by transformation (see Appendix G for more detail), followed by plating and screening for the bacteriocin-producing clones

Nucleotide sequencing

Recombinant plasmids of the bacteriocin-producing clones were extracted using Miniprep Plasmid DNA Purification Kit (Promega), according to the manufacturer's instruction (see Appendix G for more detail). The plasmids were sequenced bi-directionally. Sequencing was performed on an ABI PRISM 377 genetic analyzer using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Polymerase chain reaction (PCR) was performed using Gene Amp2400 Thermal Cycler (Perkin-Elmer). In a 50-µl volume, the PCR mixture consisted of 500 ng template DNA, 0.8 µl of Terminator Ready Reaction Mix, and 3.2 pmol of primer. PCR was done at 96 ^{O}C for 10 sec, 50-55 ^{O}C for 5 sec, and 60 ^{O}C for 4 min for a total of 25 cycles (see Appendix I for more detail). Commercial primers T7 and SP6 (Promega) were used for the first round of sequencing. Subsequently, the 3′-ends' nucleotide sequences were used to design primers for the next reactions, and so on, according to the primer walking strategy. Primers used in nucleotide sequencing were listed in Table 5. Nucleotide sequences obtained from each reaction were combined using the Navigator software (Applied Biosystems).

Table 4. Bacterial strains and plasmids used in this study

Strain/plasmid/primer	Description*	Source or reference
Strains		
Pr. nigrescens ATCC 25261	Bac^+, Original producer of nigrescin	A gift from Dr. H Shah, Queen Mary and Westfield, Medical and Dental School, London, UK.
E. coli JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17(r_K-, m_K+), *relA*1, *supE*44, Δ(*lac-proAB*), [F', *traD*36,*proAB*, *lacI*^{q}ZΔM15]	Promega
E. coli BL21(DE3)pLysS	F$^-$, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	Promega
Plasmids		
pGEM-3Z		Promega
pHAT10/11/12		Clontech
pGA17	Bac^+, pGEM-3Z with a 10-kb *Eco*RI fragment from the genomic DNA of *Pr. nigrescens* ATCC 25261 that confers bacteriocin producing ability	Present study
pGP2	Bac^+, pGEM-3Z with a 5-kb *Pst*I fragment from the 10-kb insert of pGA17	Present study
pGH17	Bac^-, pGEM-3Z with a 2.5-kb *Hin*dIII fragment from the 5-kb insert of pGP2	Present study
pGH18	Bac^-, pGEM-3Z with a 2-kb *Hin*dIII fragment from the 5-kb insert of pGP2	Present study
pG*Cla*I	Bac^-, The plasmid of pGP2, but lacks nucleotides between 2190 and 2792 bp (constructed by digesting pGP2 with *Cla* I and relegated with DNA ligase)	Present study
pH44(P2)	Bac^+, pHAT with a 5-kb *Bam*HI/ *Sph*I fragment from the 5-kb insert in pGP2	Present study

* Bac^+ and Bac^- indicated bacteriocin-producing and non-producing phenotypes, respectively

Table 5. Primers used in nucleotide sequencing

Primer's name	Sequence (5' → 3')
T7	TAATACGACTCACTATAGGG
SP6	TATTTAGGTGACACTATAG
P2-T7/2	GCTGCTGATGCAGGAAGTAT
P2-SP6/2	TGTGGTACACGCATCGTT
H17-T7/2	CCATCACCGAAACAGAGTAC
H17-SP6/2	GTTTCACGTCGTTCCGCTTT
H18-T7/2	AGCCCGCAGGACTTGTTG
H18-SP6/2	ATACGTTTCTCCAAGACGCA
P2-T7/3	TCCGGAATAGGTAAACCTG
P2-SP6/3	TTTGGCTATTGCAACGGGTT
H17-T7/3	TTCCGTTCGCCAACGCTT
H17-SP6/3	GGCGACTCGGAATACTCTTA
H18-T7/3	TTGTGCAACCGGCAAGCCT
H18-SP6/3	GGCTTGCCGGTTGCACAAT
P2-T7/4	CACACCGCACCGATTAATAA
P2-SP6/4	GTTCGCCATATCTGTTGTAGT
H17-T7/4	TAAGAGTATTCCGAGTCGCC
H17-SP6/4	GGTAGGCTGAAAATGTCAGT
H18-T7/4	GTATGGCATTCATGTCTCTG
H18-SP6/4	TGCGGGCTTATTTATCACCA
P2-H18-T7/5	TACTACTCCAGGAATGACCG
P2-H17-T7/5	AACAAGACTCCCAAGAGTCC

Computational analyses

The complete DNA sequence was translated in all possible reading frames using BLAST DNA-Protein translation program, which is available from the internet (http://www.ncbi.nlm.nih.gov). Homology comparisons were performed by using the basic logical alignment tool (BLAST) as described by Altschul *et al* (1997). BLAST searches were performed against the National Center for Biotechnology Information non-redundant protein database and the National Center for Biotechnology Information microbial genome database. For the predictions of restriction map, transcription promoter, transcription factor binding site, and protein secondary structure, sequence analysis tool "The Bioinformatics Sites for Beginner" was used, which is also available on the internet. ProtPaRam at ExPasy was used for theoretical polypeptide molecular weight calculation.

Hydrophobicity profile was calculated by the method of Kyte and Doolittle (1982), using their program freely accessed on the internet.

Construction of plasmids with mutations of genes in the *nig* locus

A. Using available restriction sites within the *nig* locus

Mutations in the *nig* locus were performed by deleting nucleotides using restriction sites available within the resulting sequence. In brief, the plasmids of the bacteriocin-producing clone were digested with one or two suitable restriction enzyme(s). The fragments of interest were separated on low melting point agarose gel, eluted from the gel using the Rapid Concert™ DNA Purification Kit, and then ligated with the precut pGEM-3Z utilizing enzyme(s) used for cutting the DNA. Following transformation of the plasmid derivatives into the competent *E. coli* (JM109), a number of white colonies were analyzed for the presence of plasmids with DNA insert. The accuracy of deletion of a gene was determined by the absence of the original restriction site(s). In the study, the restriction enzymes used for construction plasmid derivatives were *Hin*dIII, the combination of *Pst*I and *Hin*dIII, and *Cla*I used for constructing of pGH17, pGH18, and pG*Cla*I, respectively. Transformed *E. coli* cells with the plasmid derivatives were then tested for the bacteriocin production.

B. Using Exonucleases III (*Exo*III)

*Exo*III was purchased from Promega and used according to the manufacturer's instruction. It catalyzes the stepwise removal of mononucleotides starting from a 3′-OH at: 1) blunt ends, 2) recessed ends, and 3) nicks. Moreover, it will also act on 3′-overhangs of less than 4 bases. But, 3′-overhangs of $\geq$ 4 bases are protected from its activity. Therefore, either 5′- or 3′-end or both ends of the *nig* locus were selectively shortened depending on types of DNA-ends that could be generated using the available restriction sites of the pGEM-3Z. Consequently, two types of digestion could be done in the present study, the 5′-end digestion and both-ends digestion.

For the 5′-end digestion, the *nig* locus was isolated from pGP2 as the *Bam*HI/*Sph*I fragment. The reaction of *Exo*III was stopped at several time intervals. The mixed, digested-fragments were blunted by S_1 nuclease and then ligated with *Sma*I-treated pGEM-3Z. The ligated DNA mixture was transformed into the competent *E. coli* (JM109). The resulting clones were tested for the bacteriocin production. Agarose gel electrophoresis was performed to determine plasmid content and insert DNA.

For both-ends digestion, the *nig* locus was isolated from pGP2 as the *Bam*HI/*Sph*I fragment followed by S_1 nuclease treatment. *Exo*III was then added and its reaction was stopped at several time intervals. The following procedures were done in the same way as described for the 5′-end digestion.

Subcloning into the expression vector

pHAT (Clontech) was used as an expression vector (Fig. 2). To construct pH44(P2), the insert DNA was isolated from pGP2 as the *Bam*HI/*Sph*I fragment. The pHAT was linearized by digesting with *Bam*HI and then joined with the *Bam*HI/*Sph*I fragment. Subsequently, the ligation mixture was treated with S1 nuclease followed by Klenow Fragment (DNA Polymerase I) to produce the blunt DNA ends. T4 DNA ligase was then used to self-ligate the blunt ends before transforming into the competent *E. coli* (BL21(DE3)pLysS (Promega, see Appendix J for more detail). The presence of the correct insert in these constructs was confirmed by restriction analysis.

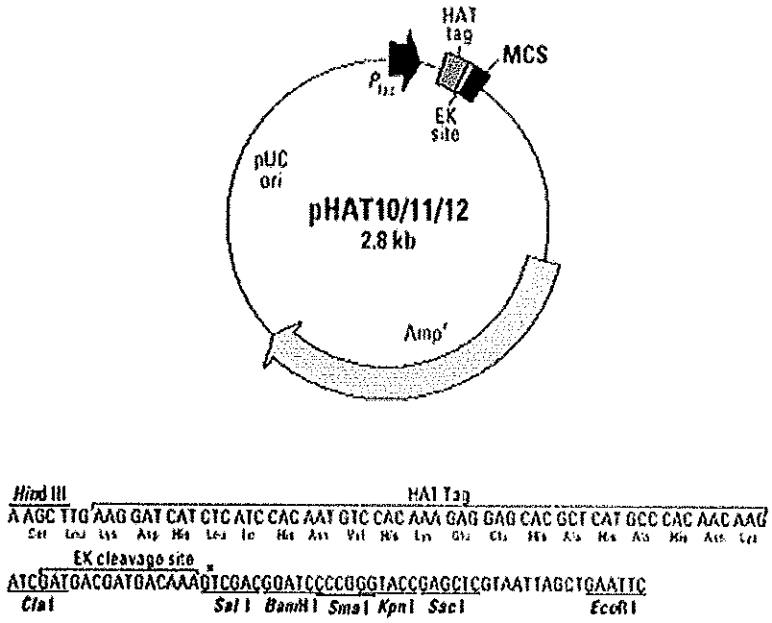


Fig. 2 Mapping of the expression vector pHAT (Clontech)

PCR amplification

To ensure that the bacteriocin-producing clones contained the insert, which originated from genomic DNA of *Pr. nigrescens* ATCC 25261, amplification of such a insert's sequence was done by PCR. Five pairs of oligonucleotide primers, annealing within the *nig* locus sequence, were used. Sequences of the primers and the length of their expected PCR products were summarized in Table 6. PCR was performed as described by Ochman *et al* (1990). Within a 50-µl volume, the PCR mixture consisted of 500 ng template DNA, 1.25 U *Taq* polymerase (Promega), 1x *Taq* buffer (supplied by the manufacturer), 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, and 0.5 µM (each) oligonucleotide primer. The reaction mixture was heated to 94 ^{O}C for 5 min before the addition of 1.25 U *Taq* polymerase. The PCR proceeded through 30 cycles of annealing at 52 ^{O}C for 30 sec, polymerization at 72 ^{O}C for 1 min, and denaturation at 94 ^{O}C for 30 sec, followed by a final polymerization step for 7 min. The

chromosome of *Pr. nigrescens* ATCC 25261 was used as the template DNA. The primers were obtained from Promega.

Table 6. Sequences of the primers used in PCR amplification and the length of their expected PCR products.

Pair No.	Primer's name	Sequence ($5' \rightarrow 3'$)	Length of predicted PCR product (bp)
1	F-1	AGTCGGTACCAAAGCCAACTGAATAATAAGG	540
	R-1	TTACGAGCTCGCAATTAAAAGTAGCCACTTG	
2	F-2	AGTCGGTACCTTCAAAGGATAGGCTACGC	360
	R-2	TTACGAGCTCATATTACACTTGACAAATCGTC	
3	F-3	AGTCGGTACCGCTATTGTGAGCTTCTTGCATG	420
	R-3	TTACGAGCTCAGAGACATGAATGCCTACG	
4	F-4	AGTCGGTACCGGCTTTTATGGAAAGTTCGGAAG	480
	R-4	TTACGAGCTCCGTATTGGCCTACACCTAT	
5	F-5	AGTCGGTACCATAGGTGTAGGCCAATACGG	1,200
	R-5	TTACGAGCTCCAATACAAAGGCTACAACGAG	

Expression of the bacteriocin-producing clone, H44(P2)

The clone H44(P2) was grown to stationary phase ($OD_{600} \geq 1.2$) at 30 ^{O}C with agitation. To induce expression, isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 3 mM was added. The induced cells were allowed to grow further for 4 h. The cells were harvested by centrifugation at 6,000 rpm for 10 min, washed with PBS pH 7.4, and collected by centrifugation. These prepared-cells were utilized for purification of the bacteriocin.

Purification of the bacteriocin

All of the purification steps were carried out at room temperature, and all of the chromatographic equipments and media were purchased from Amersham Biosciences. Two ion exchange columns, e.g., SP Sepharose FF and Q Sepharose FF, were initially selected and used in the purification procedure. The prepared cells were resuspended in 50 mM phosphate buffer pH 5.0 (buffer A) if a SP Sepharose FF was used, whereas they were dispersed in 50 mM Tris buffer pH 9.5 (buffer C) if a Q Sepharose FF was used (Q Sepharose FF was lastly replaced by QAE Sephadex A-50 in purification). The suspended cells were broken by sonication at the power output of 60 watt for 2 min. Separation of the cell lysate was performed by centrifugation at 11,000 rpm for 45 min, filtered through a 0.45 μl filter membrane, and collected for bacteriocin purification. The filtrate was applied onto either the SP Sepharose FF column or the Q Sepharose FF column. These columns were equilibrated with the appropriate buffer (buffer A or C) before used. The flow-though fraction was collected from each column for the activity assay. The bound fractions were eluted from the columns using the gradient mode by increasing concentration of NaCl in the buffers B or D (B: buffer A containing 1M NaCl; D: buffer C containing 1M NaCl). The bound fractions were collected according to the optical density (OD_{280}). Since salt appeared to inhibit growth of indicator organisms, desalting was consequently performed before testing the activity, by which the bound fractions (3 ml) were applied onto a desalting Sephadex G-25 column (i.d. 2.5 cm x 10 cm). Results indicated, however, that some parts of the sample bound to Sephadex G-25 resin, since there was a broad peak being eluted after the NaCl peak. Following purified by SP Sepharose FF, these bound fractions appeared to harbore the activity. In contrast, the bound fractions of Q Sepharose FF did not. But, the non-bound fraction from Q Sepharose FF contained the inhibitory activity. Because of being advantage, Q Sepharose FF was selected for use instead of SP Sepharose FF in the following purification. The non-bound fraction from Q Sepharose FF was consequently concentrated by lyophilization and loaded on the Sephadex G-25 column, previously equilibrated with 0.15 M NaCl containing distilled water,

which was used for elution. The bound fractions were collected and concentrated by lyophilization before loading on the Superdex 75 HR 10/30 column, equilibrated with 0.15 M NaCl containing distilled water. The fractions corresponding to the optical density peaks (OD_{280}) were collected, dialyzed, concentrated by lyophilization, and then analyzed for the inhibitory activity. Finally, the active fractions were pooled, concentrated by lyophilization, and re-purified on the same Superdex 75 column in order to increase protein purity. The pure bacteriocin was eluted as a single optical density peak (OD_{280}) after re-chromatography of the active fraction. The pure fractions were pooled, dialyzed, and dried using lyophilization. The protein powder was reconstituted with PBS and quantitatively assayed before use.

Assay of the bacteriocin activity

Detection of bacteriocin activity was performed using three analytical methods: a colony overlay assay, an agar spot assay, and a well dilution assay. The method which was chosen at any time depended on how appropriate the method was for detecting bacteriocin activity in the preparation/fraction to be tested.

For the colony overlay assay, the strains to be tested were spotted on BHI agar plates and grown for 24-48 h under optimal conditions. Five milliliters of BHI soft agar, supplemented with 1 % of vitamin K and 5 % of horse serum, were seeded with 0.05 ml of an overnight culture of the indicator bacteria, and then poured on top of the plates. After incubation at 37 ^{O}C for 24-48 h under anaerobic conditions, the plates were examined for zones of growth inhibition surrounding the colonies.

For the agar spot test, 100 µl of an overnight culture of the indicator strain were used to inoculate 10 ml of soft BHI agar, supplemented with 1 % of vitamin K and 5 % of horse serum, and poured on top of pre-made BHI agar plates. After solidification, fractions to be assayed (~ 20 µl) were separately spotted onto the surface of the plate and allowed to dry. The plate was incubated at 37 ^{O}C for 24-48 h under anaerobiosis and then checked for inhibition zones.

The well dilution assay was performed as follows: serial twofold dilutions of fractions to be tested were made in 100-µl volumes of BHI (supplemented with 1 % of vitamin K) in a 96-well plate. Each well was inoculated with 100 µl of a 300-fold diluted overnight culture of the test organism using the same BHI medium. The plates were incubated under appropriate conditions at 37 ^{O}C for 24-48 h and examined for growth inhibition. Inhibition is seen when the optical density at 600 nm in a well is less than half of that of a control well without added bacteriocin. Minimum inhibitory concentration (MIC) was used to indicate the bacteriocin activity, and defined as the lowest concentration that resulted in the absence of turbidity development after 24-48 h of incubation. Unless otherwise stated, *P. gingivalis* A244 was used as the indicator strain for assays of bacteriocin activity. Bacteria used as indicator strains for testing the bacteriocin activity were summarized in Table 7.

To determine the bacteriocin activity directly on SDS-polyacrylamide gels, the procedure of Bhunia *et al* (1987) was used with the following modifications. In brief, after SDS-PAGE, the gel was fixed by incubation in a mixture of 20 % isopropanol and 10 % acetic acid in water for 30 min at room temperature. Subsequently, the gel was washed six times with deionized water. The washed gel was placed on a pre-made BHI agar plate and laid over with 5 ml of BHI soft agar inoculated with 1 % of vitamin K, 5 % of horse serum, and 50 µl of an overnight culture of the indicator. The plate-gel combination was incubated at 37 ^{O}C overnight under an anaerobic condition prior examining a clear zone over the band of the bacteriocin.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Analysis of purified bacteriocin was carried out by SDS-PAGE on 12 % monogradient acrylamide gels at 180 V for 45 min with a Bio-Rad unit. Protein samples were mixed with an equal volume of 2x Laemmli sample buffer (100 mM Tris pH 6.0, 200 mM dithiothreitol, 20 % glycerol, 0.2 % bromophenol blue and

4 % SDS, see Appendix E for more detail). Two gels of the same samples were performed simultaneously. After electrophoresis, one gel was stained with Coomassie Brilliant Blue (Sambrook *et al.*, 1989), and another one was bioassayed using the modified method of Bhunia *et al* (1987) as described previously. The low- and high-range rainbow marker (Amersham Biosciences), RPN 755 and RPN 756 respectively, were used as protein molecular weight markers.

Determination of protein

Protein content in the samples was determined by the Bradford method (Bradford, 1976) using the D_C Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard.

Bacteriocin inhibitory spectrum

The bacteriocin was tested for its inhibitory activity by using the well dilution assay as previously described. Assays were performed in triplicate. Several indicator strains were chosen on the basis of their importance in and relatedness to the oral ecosystem (see Table 7). All test microorganisms were grown in BHI broth, supplemented with 1 % vitamin K, at 37 ^{O}C for 48 h using optimal conditions, i.e., *Streptococcus* and *Lactobacillus* were grown aerobically in a candle jar; *Actinimyces*, *Tannerella forsythensis*, *A. actinomycetemcomitans*, *Porphyromonas* spp., and *Prevotella* spp. were grown under anaerobic conditions. Sensitivity of the various indicator strains to the bacteriocin was presented using MIC. The relative sensitivity of the various indicator strains was analyzed by comparing their MIC values with the MIC value for the *P. gingivalis* A244 strain. An approximate molecular weight of 41 kDa was used for calculation of the MICs for the bacteriocin.

Specificity of the bacteriocin

The inhibitory activity of the bacteriocin toward the selected indicators was compared to that of nisin. Nisin is a commercial bacteriocin product produced by *Lactococcus lactis* (McAuliffe *et al.*, 2001). Selection of the indicator strains was based on the fact that the strains either share the same ecological niche as the original producer or are involved in incidence and progression of periodontal diseases. The experiment was carried out by the well dilution assay as previously described, and done in triplicate. The antimicrobial activity was calculated in MIC units.

Table 7. Bacteria used as the indicator strains for testing the inhibitory spectrum

Gram-positive
Streptococcus mutans ATCC 2517
S. salivalius ATCC 7073
S. sanguis ATCC 10556
Lactobacillus casei sub. *casei* ATCC 393
L. formentum ATCC 14931
Actinomyces naeslundii ATCC 12104
A. viscosus ATCC 15987
A. israellii

Gram-negative
Porphyromonas gingivalis ATCC 33277
P. gingivalis ATCC 11326
P. gingivalis 381
P. gingivalis W50
P. gingivalis A244
Prevotella intermedia ATCC 25611
Pr. nigrescens ATCC 33563
Pr. nigrescens ATCC 25261
Actinobacillus actinomytemcomitans
Tannerella forsythensis

Effect of the bacteriocin on sensitive cells

To study the effect of the bacteriocin on sensitive cells, 100 µl with different concentrations of purified bacteriocin were added to 100 µl of the indicators growing in the early exponential phase. The optical density (OD_{280}) was measured at time zero and after 24-h incubation. The number of viable cells was determined by plate counting (Mantovani and Russell, 2003).

Heat sensitivity of the bacteriocin

Thermostability of the bacteriocin was evaluated by heating the active fraction at 50, 80, and 100 ^{O}C for 10 min in a water bath. After cooling to 4 ^{O}C, the inhibitory activity was tested and compared with the non-treated control. A residual inhibitory activity was measured by the well dilution assay.

pH sensitivity

The effect of pH on the bacteriocin stability was determined according to the method of Gomori (1955). Briefly, the following buffers and their respective pH values were used: phosphate-citrate (6.5-7.5), and Tris-HCl (8.0-9.5). The bacteriocin powder was dissolved with the buffers and the solutions were incubated for 4 h at 37 ^{O}C. The mixtures were then assayed for inhibitory activity using the well dilution method. pH values below 6.5 were excluded from the experiment because of their interference with growth of the indicator strain.

Sensitivity of the bacteriocin to proteolytic enzymes

Sensitivity to protease enzymes was assayed by incubating the active fraction with either pepsin A or proteinase K (50 µg/ml final concentration in 50 mM phosphate buffer pH 7.0). A control without incubation with enzyme was used. Incubation was carried out at 37 ^{O}C for 2 hr. The effect of these enzymes was determined by assaying the residual activity using the well dilution method.

Testing of toxicity of the bacteriocin on the cultured fibroblasts

The normal gingival fibroblasts (self established cell line) were maintained routinely in Dulbecco modified Eagle's medium (DMEM, Life Technologies) supplemented with 2 mM L-glutamine, penicillin and streptomycin (100 units of each), and 10 % heat-inactivated foetal bovine serum (Life Technologies). For cytotoxic studies, approximately 10^5 tissue culture cells were seeded into a 25-ml culture flask and grown to near confluence at 37 ^{O}C in 5 % CO_2. Several bacteriocin concentrations were prepared in PBS. At the day being tested, the cells were washed twice with PBS following media withdrawal. The bacteriocin at a certain concentration was added on top of the cells and incubated for 30 min at 37 ^{O}C in 5 % CO_2. PBS was used instead of the bacteriocin solutions in PBS for the control. The cells were then washed twice with PBS and fresh culture medium was added on top. The cell morphology was observed by use of light microscope in order to see whether direct exposure to the bacteriocin has an effect on cell morphology (Hay *et al.*, 1994). The colony forming method (Davis, 1994) was used for studying toxicity of the bacteriocin. Briefly, after the test solution was removed, the cells were washed twice in PBS and fresh culture medium was added. The treated cells were allowed to recover overnight in the incubator. The cells were then trypsinized and plated with the cell number of 400 in 25-ml culture flask. The cells were allowed to grow for 1 week. The number of cells was counted by staining with 1 % crystal violet. This procedure was done in triplicate. Percent viability was calculated by dividing the average cell number in the treated culture with the number of cells in the untreated control culture and multiplying this by 100. The relationship between % viability and tested concentrations was plotted. To observe cell division, the treated cells were allowed to grow for 3 weeks. By staining with 1 % crystal violet, the dividing cells were investigated using light microscopy (Freshney, 1994).

Chapter 3
Results

Cloning of the bacteriocin gene locus (*nig* locus)

Production of a bacteriocin-like substance by *Pr. nigrescens* ATCC 25261 was previously reported (Teanpaisan *et al.*, 1998). Since bacteriocin-encoding genes can be located either on the chromosome or in a plasmid or a transposon, or on both (Altena *et al.*, 2000; Diep *et al.*, 1996; Engelke *et al.*, 1992), the first question we asked ourselves was where the gene encoding the bacteriocin was located. Therefore, it was important to know whether a plasmid(s) is present in the producer, because if it is, both genomic and plasmid DNAs must be analyzed in order to clone such a gene. However, our preliminary result showed that no plasmid was found in the producer, suggesting that all the genes needed for bacteriocin production are on the chromosome. In an attempt to express the bacteriocin-encoding gene in *E. coli*, restriction fragments from the genomic DNA of the producer were cloned into the cloning vector pGEM-3Z. In the beginning, several restriction enzymes, compatible with restriction sites within the polycloning region of the plasmid, e.g., *Eco*RI, *Pst*I, *Hin*dIII, *Bam*HI, and *Sph*I, were initially tested for digesting the chromosome. As seen in Fig. 3, all enzymes cut the DNA. But, among them, *Bam*HI, *Sph*I, and *Eco*RI produced larger DNA fragments (Fig. 3). Based on economical reason, however, *Eco*RI enzyme was the one we used first for this purpose. According to the cloning procedure as described in Materials and Methods, a number of transformants (~ 300 colonies) were tested for the bacteriocin production using the colony overlay assay. *Porphyromonas gingivalis* A244 was used as the indicator bacterium. One bacteriocin-producing clone was found and named A17. The recombinant plasmid in A17 was designated as pGA17. A17 showed a zone of inhibition in the indicator lawn, while *E. coli* (JM109) containing pGEM-3Z (without insert DNA) did not (Fig. 4). This result indicated that production of the bacteriocin by this

clone was due to the bacteriocin gene it received. Restriction analysis of pGA17 showed that such a plasmid contained a 10-kb *Eco*RI fragment insert (Fig. 5A).

We have believed that the bacteriocin-encoding gene may be smaller than 10 kb. Therefore, the 10-kb insert was further digested with other previously tested enzymes. *Pst*I was chosen for the subsequent cloning step, since it generated fragments smaller than those obtained from *Eco*RI digestion (Fig. 3). The 10-kb fragments of pGA17 were isolated and then digested with *Pst*I. The digested mixture was ligated with the *Pst*I-treated pGEM-3Z using T4 DNA ligase, and transformed into *E. coli* JM109. A number of transformants (~300 white colonies) were tested for bacteriocin-producing ability using the colony overlay assay. Results showed that a new clone produced the bacteriocin (Fig. 4). It was named P2, which contained a recombinant plasmid designated pGP2. As verified by restriction analysis, pGP2 contained a 5-kb *Pst*I fragment insert (Fig. 5A).

In an attempt to find shorter DNA fragments that still confer bacteriocin production, the 5-kb insert of pGP2 was isolated and digested with *Hin*dIII, and then cloned into the *Hin*dIII-treated pGEM-3Z. Using the procedures described for A17 and P2, another two recombinant clones were obtained, designated H17 and H18, respectively. Neither of these produced the bacteriocin. pGH17 and pGH18 were the names given for the recombinant plasmids of H17 and H18, respectively. Restriction analysis of pGH17 and pGH18 showed that the length of DNA inserts were 2.5- and 2-kb, respectively (Fig. 5B).

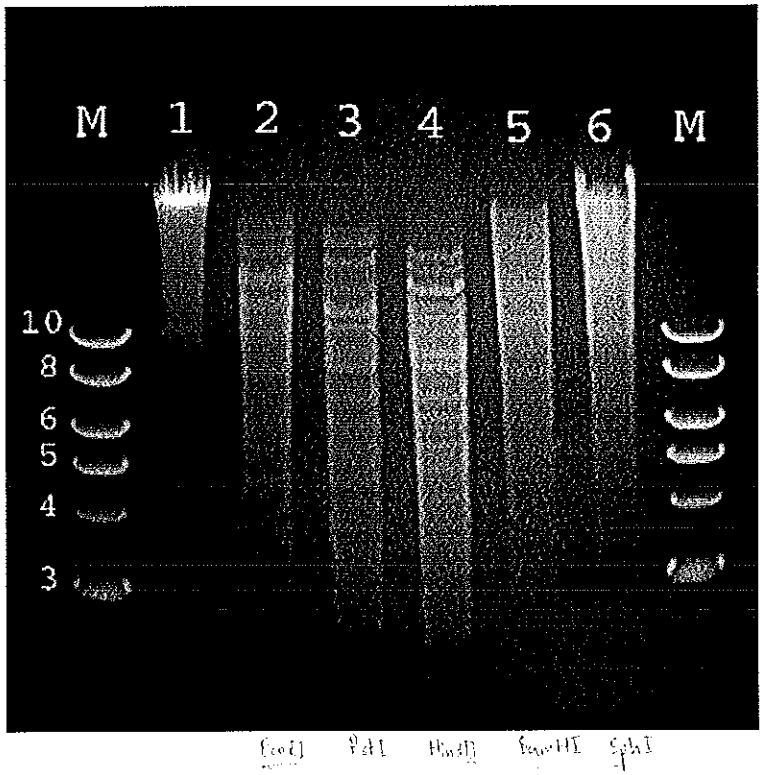


Fig. 3 Genomic DNA of *Pr. nigrescens* ATCC 25261 (~ 10 µg) was separately digested with 40 U of the selected restriction enzymes. The enzymes used were *Eco*RI (lane 2), *Pst*I (lane 3), *Hin*dIII (lane 4), *Bam*HI (lane 5), and *Sph*I (lane 6). Electrophoresis of the digested chromosome was performed on 0.8 % agarose gel. Results showed that all enzymes cut the DNA, but larger digested fragments were obtained by *Eco*RI, *Bam*HI, and *Sph*I, in comparison with those obtained from digested with *Pst*I and *Hin*dIII. Lane 1 was an undigested chromosome; Lane M was molecular size standards with sizes (in kb) indicated on the left.

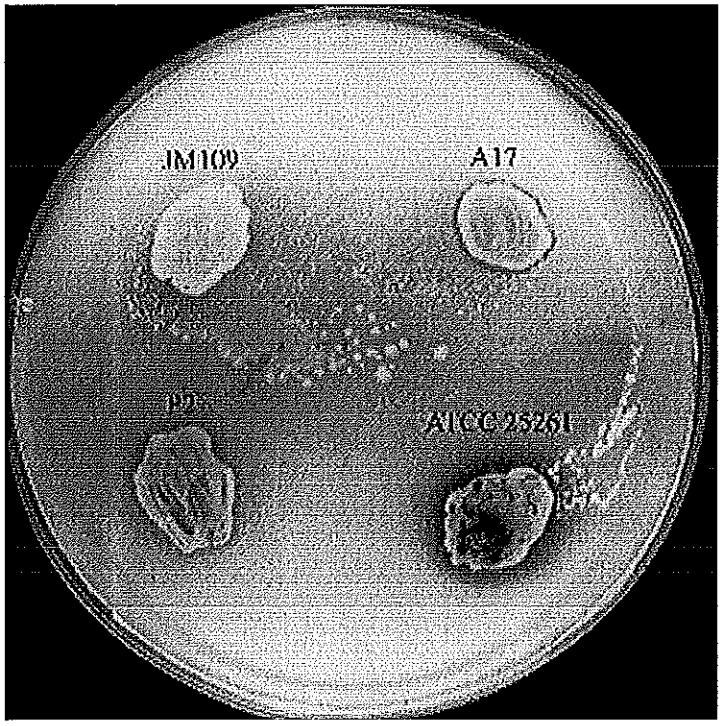


Fig. 4 The colony overlay assay of the recombinant clones. A number of transformants (~300 colonies for each cloning step) were tested for the bacteriocin production using the colony overlay assay. One bacteriocin-producing clone was obtained and named A17. In the subsequent cloning step, using *Pst*I, another producing clone was found and designated P2. *P. gingivalis* A244 was used as the indicator strain. *E. coli* (JM109) containing the pGEM-3Z vector (without insert) and *Pr. nigrescens* ATCC 25261 were used as a negative- and positive-control, respectively.

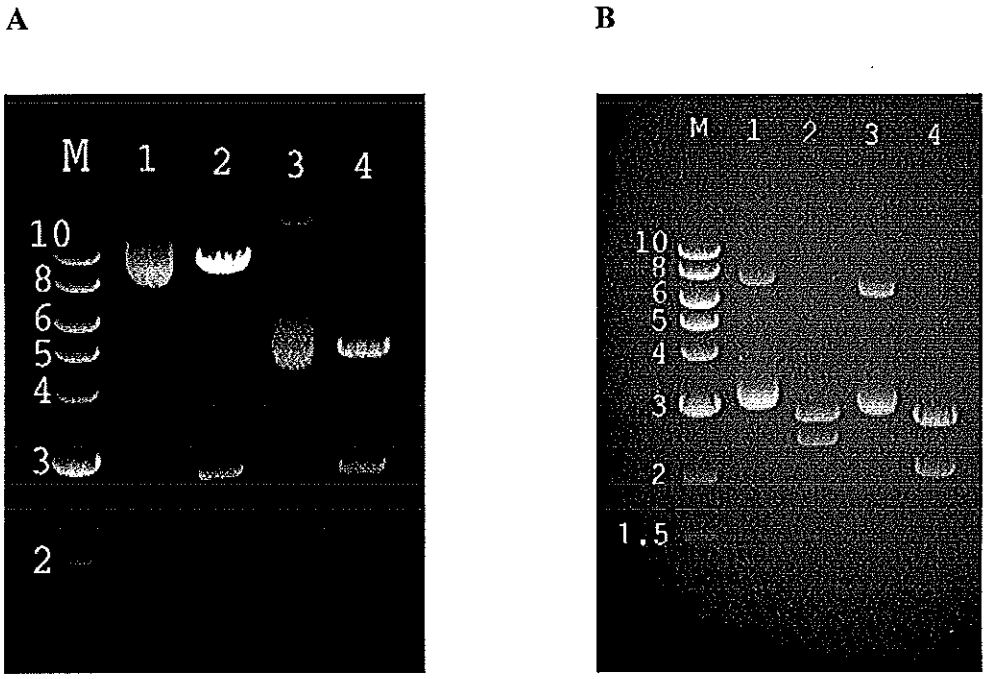


Fig. 5 Restriction analysis of pGA17, pGP2, pGH17, and pGH18 was performed. Each plasmid (~ 2 μg) was specifically digested with 10 U of certain enzymes, i.e., *Eco*RI for pGA17, *Pst*I for pGP2, and *Hin*dIII for pGH17 and pGH18. Electrophoresis of the digested plasmids was done on 1 % agarose gel.

(A) Agarose gel electrophoresis of uncut pGA17 (lane 1), uncut pGP2 (lane 3), the 10-kb insert of *Eco*RI-digested pGA17 (lane 2), and the 5-kb insert of *Pst*I-digested pGP2 (lane 4). Lane M was the standard DNA marker with sizes (in kb) indicated on the left.

(B) Agarose gel electrophoresis of uncut pGH17 (lane 1), uncut pGH18 (lane 3), and the 2.5- and 2-kb insert DNA of *Hin*dIII-digested pGH17 (lane 2) and pGH18 (lane 4), respectively. Lane M was the standard DNA marker with sizes (in kb) indicated on the left.

Nucleotide sequence analysis

The shortest chromosomal DNA that still confers the bacteriocin production was the 5-kb DNA insert of pGP2. To gain insight into the genetic requirement involved in production of the bacteriocin, this 5-kb segment was sequenced and analyzed. Since the 2-kb insert in pGH18 and the 2.5-kb segment in pGH17 are parts of the 5-kb segment, these two fragments were also subjected to DNA sequencing. DNA sequences obtained from each sequencing reaction were edited and assembled using the "Navigator" program (Applied Biosystem). The 5-kb segment of P2 consisted of 4,868 base pairs. The complete sequence (4,868 bp) was then analyzed in order to identify restriction sites; possible open reading frames (ORFs) and the molecular weights of putative gene products; putative promoter sequences, transcription factor binding sequences, and possible translation start sites; as well as sequence homology using several bioinformatics tools that are freely accessible on the internet (see Materials and Methods). The sequence's information was shown in Fig. 6. The sequence, called the *nig* locus, contains four major (possible) ORFs, designated *nigA* to *nigD* in order starting with the ORF located furthest upstream. All were oriented in the same direction. A putative promoter sequence, -35 (TGGAAA) and -10 (AAATCT), ranging between positions 122 and 132 bp, was identified upstream of *nigA*. *nigA*, ranging between positions 240 and 1331 bp, encodes a hypothetical, 363-amino acid protein with an estimated molecular mass of 41.3 kDa. Identified between the putative promoter sequence and the start codon is a possible ribosome-binding site (GAATAA) located 6 bp upstream of the predicted start codon (methionine) and the deduced amino acid sequence (Fig. 6). *nigB* gene has two possible translation start sites. *nigB*, ranging between positions 1,362 and 2,358 bp, encodes a putative protein of 331 amino acids with a calculated molecular mass of 37.1 kDa if the first methionine, at a position 1,362 bp, is used as the start codon. However, it encodes a putative 328-amino acid protein with an approximate molecular weight of 36.7 kDa if the second methionine, at a position 1,371 bp, is used as the start codon. No possible ribosome-binding site within 20 bases of the first predicted start codon could be identified. But, if translation initiation occurs at the second

methionine, a good consensus for a Shine-Dalgarno sequence (GAATGA) located 5 bp upstream of this methionine can be found (Fig. 6). Therefore, it is more likely that translation initiation occurs at the second methionine position. No possible promoter sequence could be identified upstream of *nigB*. A region of dyad symmetry that could form a stem-loop structure (ΔG = -23.1 kcal/mol), consisting of a 23-base inverted repeat separated by 7 bases, was identified downstream of the *nigB* gene. This structure has the features associated with a bidirectional *rho*-independent transcription terminator. The third ORF, *nigC*, began with the methionine residue at a position 2,943 bp. Preceding *nigC* are both a putative promoter sequence and a ribosome binding site. The promoter sequence, -35 (TGCTAT) and -10 (TTACCT) was found between positions 2,825 and 2,855 bp and the putative ribosome binging site, AGGCGA, was located 8 bp upstream of the start codon. *nigC* may encode a protein of 222 amino acid residues with a theoretical molecular weight of about 25.55 kDa (Fig. 6). The putative translation start codon of *nigD*, at a position 3,604 bp, overlaps by 5 bp the last sense codon of *nigC*. Preceding this ATG codon within *nigC* and separated from it by 4 bp is a putative RBS, GAAG. *nigD*, ranging between positions 3,604 and 4,254 bp, may encode a 216-amino acid protein with a predicted molecular weight of 24.2 kDa (Fig. 6). No putative transcription terminator was identified downstream of *nigD*.

Homology search was performed by comparing the deduced amino acid sequences of these four ORFs and those protein sequences reported in database, and achieved by using the basic logical alignment tool (BLAST). It was obvious that there is no sequence similarity between the deduced products of *nig* genes and other known bacteriocins. However, a gene product of *nigA* (NigA) was found to be identical to the ribosomal large subunit pseudouridine syntase D with 66 % identity; NigB was similar to D-alanine-D-alanine ligase with 59 % identity. NigC and NigD were similar to two hypothetical proteins of unknown function and with 50 % and 26 % identity, respectively.

*Pst*I

1 GAACACGCAATGCGAACGATACTACAAATCAGCGTCCTGGAAGTAGATATGAAGAAATAG

61 AGGTTGAAGAGCCTAAATACGAAGAATATTACGATTGGGTAGAAGTCCCTGTGGACGAAA

-35 -10

121 ATGGAAACGAAATAAAAGAGGGAGGAAAATCTGATCCACGAACTCTACCTTCGGTTCCAC

Hind III S.D. ***nigA***

181 CACAATCGCAAGCTTTACCACCTGGTCAGAATACGCCAAAGCCAACTGAATAATAAGGTA

M

241 TGATAGAGATAGCTGACGAATACTTTTATGAAGATTTAGCTGACGATCTTTTAGGCGTGA

I E I A D E Y F Y E D L A D D L L G V N

301 ACGACATTGCTGCCGATGGGGAAGAACTTTACGAACACTTCCGTGTAGAGGTTGATAAAG

D I A A D G E E L Y E H F R V E V D K G

361 GGCAAGAAGCAGTGCGGATAGACAAGTTCTTGTTTGAACGAATGCAACACTCCAGTCGTA

Q E A V R I D K F L F E R M Q H S S R N

421 ATCGTATTCAGAAAGCTGCTGATGCAGGAAGTATTTATGTGAATGGAACGCCTGTAAAAA

R I Q K A A D A G S I Y V N G T P V K S

481 GTAATTACAAGGTACGCCCCAATGATGTTATCACCTTGATGCTTGACCGTCCGAAGCACG

N Y K V R P N D V I T L M L D R P K H D

541 ATAACACTATTGTTGCAGAAGATATGCCGCTCAATGTTGTCTACGAAGACAATGAAATTA

N T I V A E D M P L N V V Y E D N E I M

601 TGGTGATAAATAAGCCCGCAGGACTTGTTGTGCACCCTGGGGCTGGTAATTTTCGTGGTA

V I N K P A G L V V H P G A G N F R G T

661 CACTCATAAATGCAATAGCTTGGCATTTGCGCGACTTAAAGAATTTCGACCCTAACGATC

L I N A I A W H L R D L K N F D P N D P

721 CTGCCGTTGGTTTGGTTCATCGTATTGATAAAGATACAAGTGGTCTACTTTTAATTGCTA

A V G L V H R I D K D T S G L L L I A K

Sty I

781 AAACACCAGATGCAAAGACATTCCTTGGTAAGCAGTTCTTCAATAAAACAACACACCGTA

T P D A K T F L G K Q F F N K T T H R S

841 GCTATGTAGCTTTAGTATGGGGGAACTTTACCGAGGACGAAGGGCGGATAGAAGGAAATA

Y V A L V W G N F T E D E G R I E G N I

901 TTGGACGCGATTCAAAGGATAGGCTACGCATGAAAGTGTTTGATACCGATTCCGGAATAG

G R D S K D R L R M K V F D T D S G I G

961 GTAAACCTGCCGTTACGCACTATAAGGTTTTAGAACGATTTGGCTATACAACCCTTATAG

K P A V T H Y K V L E R F G Y T T L I E

1021 AATGTATATTGGAAACGGGTAGGACACACCAAATTAGAGCACATATGAAGCACATCGGGC

C I L E T G R T H Q I R A H M K H I G H

*Eco*47 III

1081 ATCCGCTTTTCGGAGACGAGCGCTATGGTGGCACAGAAATATTGCGTGGACAGCGTACCA

P L F G D E R Y G G T E I L R G Q R T S

1141 GTGCATACCGAGCTTATATACAAAATTGCTTTAAATTGTGCAACCGGCAAGCCTTGCACG

A Y R A Y I Q N C F K L C N R Q A L H A

1201 CAAAGACGCTCGGTTTTGTTCACCCACGGACAGGAGAGCAAATGGACTTTACAAGCGAAC

K T L G F V H P R T G E Q M D F T S E L

1261 TTCCCGACGATTTGTCAAGTGTAATAGAAAAGTGGCGAAATTACATAAATGGTAAACAAG

P D D L S S V I E K W R N Y I N G K Q E

S.D. ***nigB***

1321 AGCTATTGTGAGCTTCTTGCATGATTAAAATAAATAATTAGAATGAAAGATATGAAACGT

L L - M K R

1381 ACAATAGCCATCGTTTGCGGTGGCGATTCTTCAGAACACGATGTTTCTCTCCGTTCTGCA

T I A I V C G G D S S E H D V S L R S A

1441 CAAGGGTTGTATTCCTTCTTCGATAAGGAACGTTACAATATTTATATAGTCGATGTAAAA

Q G L Y S F F D K E R Y N I Y I V D V K

1501 GGAACAGATTGGCATGTAAACCTCGATAACGGGCACACCGCACCGATTAATAAAAACGAT

G T D W H V N L D N G H T A P I N K N D

1561 TTCTCGTTTAATAATAATGGTCAGACAGTGTTCTTCGACTATGCGTATATAACAATACAT

F S F N N N G Q T V F F D Y A Y I T I H

1621 GGGCGACCAGGCGAGAATGGTATTATGCAAGGCTATTTTGAATTGGTAAACATTCCTTAT

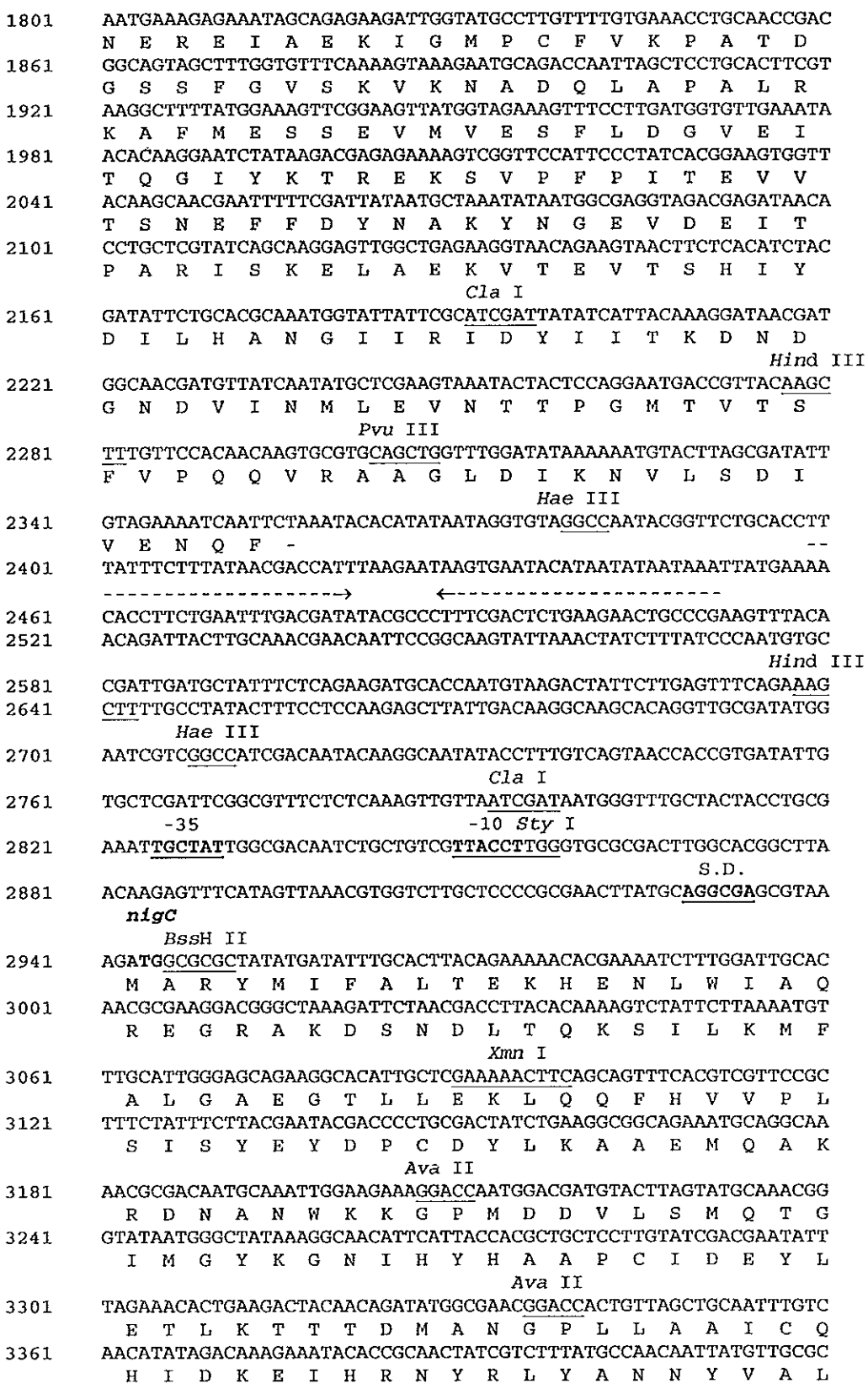
G R P G E N G I M Q G Y F E L V N I P Y

1681 TCTACTTGCGATGTGTTGGCGTCGGCACTCACTTTTGATAAGTTTGTGTTGAACCGTTAT

S T C D V L A S A L T F D K F V L N R Y

1741 TTAAAATCGTATGGCATTCATGTCTCTGAAAGTATTTTGCTGCGTCTTGGAGAAACGTAT

L K S Y G I H V S E S I L L R L G E T Y


1801 AATGAAAGAGAAATAGCAGAGAAGATTGGTATGCCTTGTTTTGTGAAACCTGCAACCGAC
N E R E I A E K I G M P C F V K P A T D
1861 GGCAGTAGCTTTGGTGTTTCAAAAGTAAAGAATGCAGACCAATTAGCTCCTGCACTTCGT
G S S F G V S K V K N A D Q L A P A L R
1921 AAGGCTTTTATGGAAAGTTCGGAAGTTATGGTAGAAAGTTTCCTTGATGGTGTTGAAATA
K A F M E S S E V M V E S F L D G V E I
1981 ACACAAGGAATCTATAAGACGAGAGAAAAGTCGGTTCCATTCCCTATCACGGAAGTGGTT
T Q G I Y K T R E K S V P F P I T E V V
2041 ACAAGCAACGAATTTTTCGATTATAATGCTAAATATAATGGCGAGGTAGACGAGATAACA
T S N E F F D Y N A K Y N G E V D E I T
2101 CCTGCTCGTATCAGCAAGGAGTTGGCTGAGAAGGTAACAGAAGTAACTTCTCACATCTAC
P A R I S K E L A E K V T E V T S H I Y
Cla I
2161 GATATTCTGCACGCAAATGGTATTATTCGCATCGATTATATCATTACAAAGGATAACGAT
D I L H A N G I I R I D Y I I T K D N D
Hind III
2221 GGCAACGATGTTATCAATATGCTCGAAGTAAATACTACTCCAGGAATGACCGTTACAAGC
G N D V I N M L E V N T T P G M T V T S
Pvu III
2281 TTTGTTCCACAACAAGTGCGTGCAGCTGGTTTGGATATAAAAAATGTACTTAGCGATATT
F V P Q Q V R A A G L D I K N V L S D I
Hae III
2341 GTAGAAAATCAATTCTAAATACACATATAATAGGTGTAGGCCAATACGGTTCTGCACCTT
V E N Q F - --
2401 TATTTCTTTATAACGACCATTTAAGAATAAGTGAATACATAATATAATAAATTATGAAAA
----------→ ←----------
2461 CACCTTCTGAATTTGACGATATACGCCCTTTCGACTCTGAAGAACTGCCCGAAGTTTACA
2521 ACAGATTACTTGCAAACGAACAATTCCGGCAAGTATTAAACTATCTTTATCCCAATGTGC
Hind III
2581 CGATTGATGCTATTTCTCAGAAGATGCACCAATGTAAGACTATTCTTGAGTTTCAGAAAG
2641 CTTTTGCCTATACTTTCCTCCAAGAGCTTATTGACAAGGCAAGCACAGGTTGCGATATGG
Hae III
2701 AATCGTCGGCCATCGACAATACAAGGCAATATACCTTTGTCAGTAACCACCGTGATATTG
Cla I
2761 TGCTCGATTCGGCGTTTCTCTCAAAGTTGTTAATCGATAATGGGTTTGCTACTACCTGCG
-35 -10 Sty I
2821 AAATTGCTATTGGCGACAATCTGCTGTCGTTACCTTGGGTGCGCGACTTGGCACGGCTTA
S.D.
2881 ACAAGAGTTTCATAGTTAAACGTGGTCTTGCTCCCCGCGAACTTATGCAGGCGAGCGTAA
nigC
BssH II
2941 AGATGGCGCGCTATATGATATTTGCACTTACAGAAAAACACGAAAATCTTTGGATTGCAC
M A R Y M I F A L T E K H E N L W I A Q
3001 AACGCGAAGGACGGGCTAAAGATTCTAACGACCTTACACAAAAGTCTATTCTTAAAATGT
R E G R A K D S N D L T Q K S I L K M F
Xmn I
3061 TTGCATTGGGAGCAGAAGGCACATTGCTCGAAAAACTTCAGCAGTTTCACGTCGTTCCGC
A L G A E G T L L E K L Q Q F H V V P L
3121 TTTCTATTTCTTACGAATACGACCCCTGCGACTATCTGAAGGCGGCAGAAATGCAGGCAA
S I S Y E Y D P C D Y L K A A E M Q A K
Ava II
3181 AACGCGACAATGCAAATTGGAAGAAAGGACCAATGGACGATGTACTTAGTATGCAAACGG
R D N A N W K K G P M D D V L S M Q T G
3241 GTATAATGGGCTATAAAGGCAACATTCATTACCACGCTGCTCCTTGTATCGACGAATATT
I M G Y K G N I H Y H A A P C I D E Y L
Ava II
3301 TAGAAACACTGAAGACTACAACAGATATGGCGAACGGACCACTGTTAGCTGCAATTTGTC
E T L K T T T D M A N G P L L A A I C Q
3361 AACATATAGACAAAGAAATACACCGCAACTATCGTCTTTATGCCAACAATTATGTTGCGC
H I D K E I H R N Y R L Y A N N Y V A L

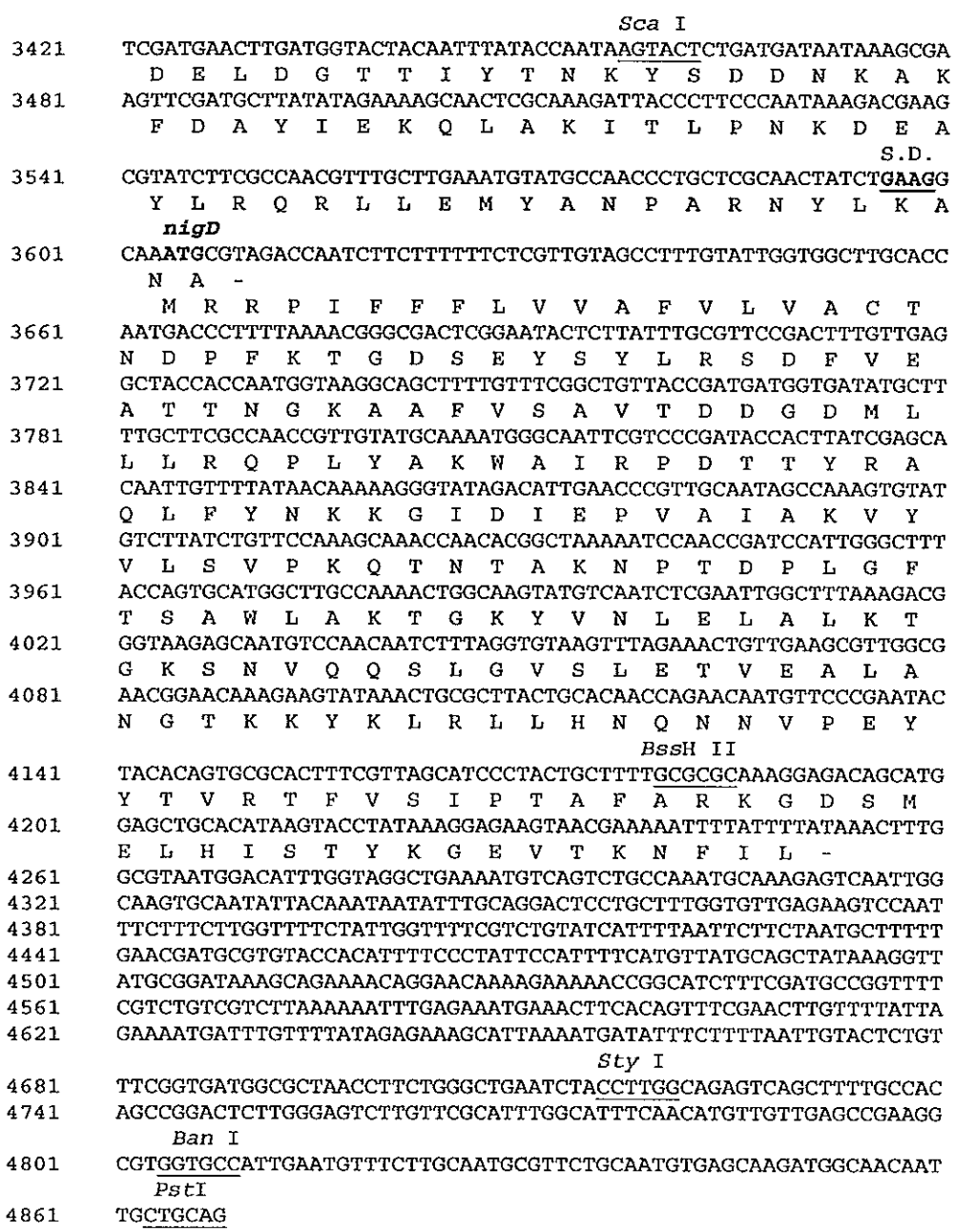


Fig. 6 The complete nucleotide sequence of the *nig* locus of pGP2 was illustrated, consisting of 4,868 bp. Four major (possible) ORFs were identified within the sequence, designated *nigA* to *nigD*, respectively. The deduced amino acid sequences are shown below the corresponding DNA sequence. The putative promoter sequences (-10 and -35), the Shine-Dalgarno (S.D.) ribosome binding sequences, and the translation start sites are underlined and/or indicated by

Legend of Fig. 6 (continued)

boldface letters. Sequence of dyad symmetry with the potential to serve as a transcription terminator is indicated by two horizontal arrows downstream of *nigB*. Stop codons are indicated by dashed lines at the ends of the protein sequences. No putative translation terminator was identified downstream of *nigD*. Restriction sites of particular enzymes are also indicated.

Hydrophobicity profile of the *nig* gene products

Hydrophobicity profiles of the *nig* gene products (NigA, NigB, NigC, and NigD) were depicted in Fig. 7. They were calculated and plotted by using a linear weight variation model of Kyte and Doolittle (1982). A span length of 13 amino acid residues was selected, because significant results were obtained, results which were easy for comparison with the others. It is accepted that the hydrophobicity profile may approximately be used for determining and/or predicting the location of proteins of interest, i.e., cytoplasmic protein, transmembrane protein, or membrane-anchored protein.

Deletion analysis of the *nig* locus

To delineate the region required for a bacteriocin positive phenotype (Bac^+), *E. coli* (JM109) was transformed with several derivatives of pGP2. The insert fragment of pGP2 was selectively deleted and shortened by using restriction enzymes and exonuclease III (*Exo*III) plus S1 nuclease, respectively.

Restriction sites within the sequence were identified and used as sites for appropriate cutting. *Hin*dIII cleaves (at positions 189, 2,277, and 2,638 bp), *Pst*I (at positions 1 and 4,862 bp), and *Cla*I (at positions 2,190 and 2,792 bp) and these enzymes generated deletions in the putative promoter sequence of *nigAB* and the 3′-end of *nigB* in pGH17, the *nigAB* and its putative promoter in pGH18, and the 3′-end of *nigB* in pG*Cla*I, respectively. The deletion mutants and their corresponding phenotypes were summarized in Fig 8. All deletions resulted in complete loss of the bacteriocin-producing ability, as neither of the derivatives displayed zones of inhibition against the indicator strain.

The 4,868-kb fragment was also shortened from either the 5′-end or both-ends of the fragment using the *Exo*III activity. When the action of *Exo*III was stopped at various time intervals and the reaction mixture treated with S1 nuclease, a range of blunted-end DNA fragments were generated. The fragments were then inserted into *Sma*I-treated pGEM-3Z using T4 DNA ligase. *E. coli* (JM109) transformed with these plasmid derivatives were tested for bacteriocin production. No bacteriocin producing clone was obtained.

The results of the deletion analysis suggest that the putative promoter of the *nigAB* and the *nigAB* gene products (NigA and NigB, respectively) are necessary for the production of a functional bacteriocin. Thus, *nigAB* may be the bacteriocin operon. However, it can also be that deletion at the 3′-end of *nigB* causes instability of the transcribed mRNA, resulting in uncompleted protein translation and ultimately in a truncated bacteriocin.

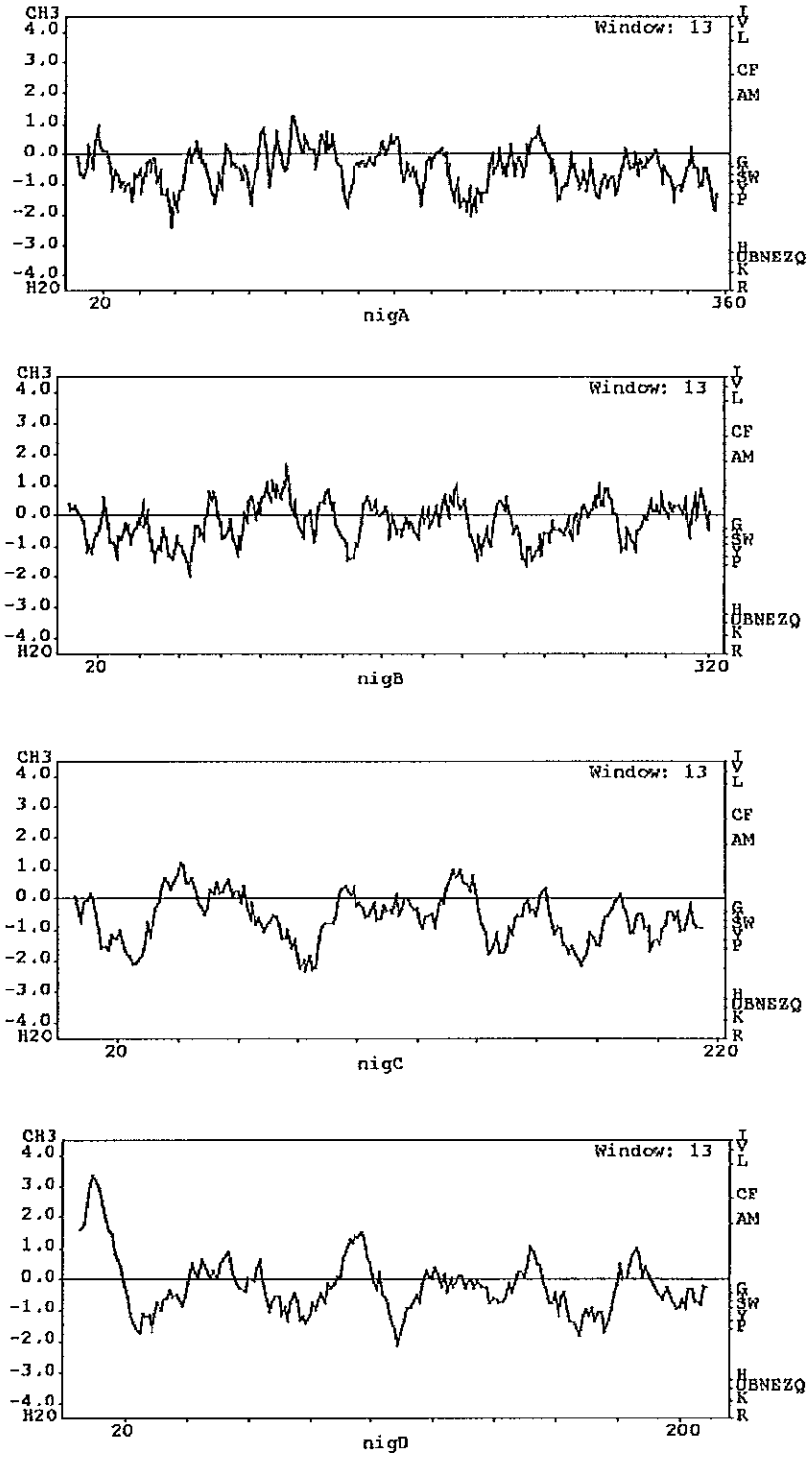
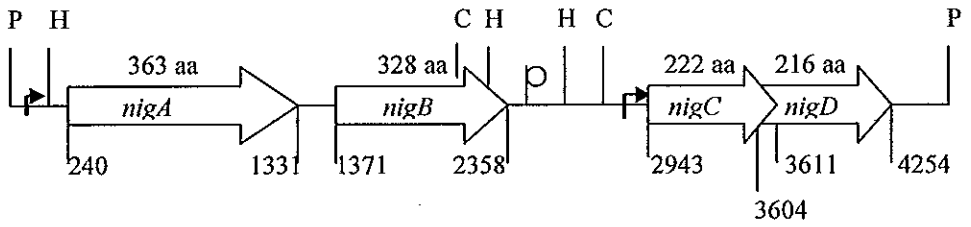


Fig. 7 Hydrophobicity plots of the *nig* gene products (NigA, NigB, NigC, and NigD) that were calculated by the method of Kyte and Doolittle (1982) with a span length of 13 amino acid residues and by using a linear weight variation model.

A



B

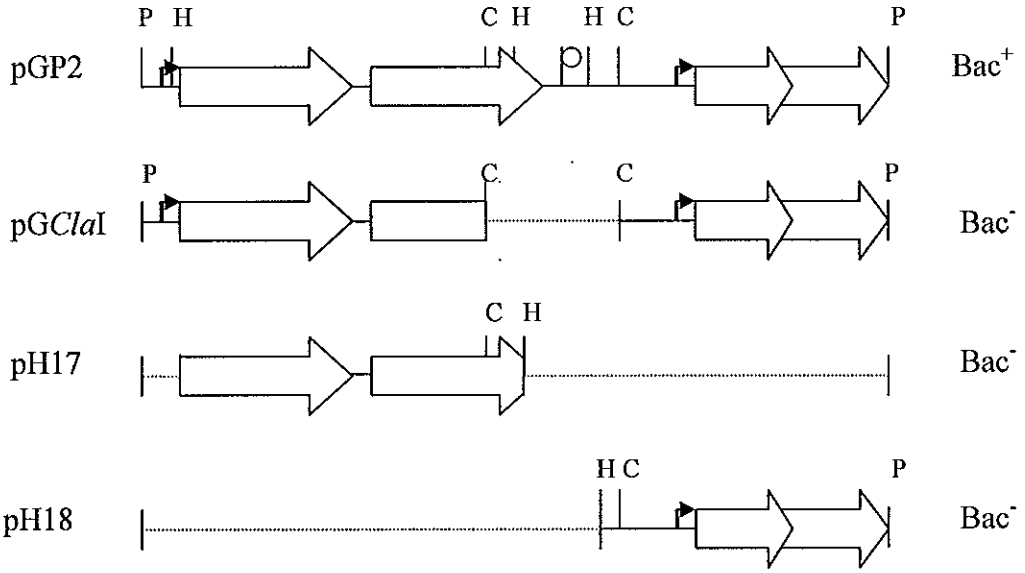


Fig. 8 (A) Schematic map of the *nig* locus. Horizontal arrows indicate putative open reading frames with nucleotide positions and predicted number of amino acids. Small-dark arrows indicate putative promoters. Stem-loop indicates a putative *rho*-independent terminator. Relevant restriction endonuclease sites are indicated as follows: P, *Pst*I; H, *Hin*dIII; C, *Cla*I.

(B) The three derivatives of pGP2 were constructed by using the specific restriction sites available within the 4,868 DNA fragment. Dashed lines indicate the regions that were removed from the fragment. The ability or inability to produce the bacteriocin (Bac^+ or Bac^-, respectively) of the *E. coli* (JM109) transformants carrying either pGP2 or its derivatives is indicated on the right. The names of the plasmid constructs are indicated on the left.

Subcloning of the *nig* locus into the expression vector

The shortest insert fragment, important in the production of bacteriocin, is the 4,868 bp-DNA segment of pGP2. To determine the bacteriocin's biochemical and cytotoxic properties, it must be produced in large amounts and this may be accomplished by producing the bacteriocin in an expression vector system. The expression vector used in the experiment was the pHAT vector (Fig. 2).

As shown in Fig. 1 and Fig. 2, the polylinker region of pGEM-3Z is not compatible with that of pHAT, an indirect procedure was therefore applied for cloning a new expression construct. The insert DNA of pGP2 was isolated as a 5-kb *Bam*HI/*Sph*I fragment. pHAT was simultaneously digested with *Bam*HI. The linearized pHAT was then ligated with the previously isolated 5-kb *Bam*HI/*Sph*I fragment. The reaction mixtures were subsequently treated with S1 nuclease followed by Klenow Fragment (DNA Polymerase I) to produce the blunt DNA ends. Two blunt ends of the linearized recombinant DNA were joined together using T4 DNA ligase. The recombinant plasmids were transformed into the competent *E. coli* BL21(DE3)pLysS (see Appendix J for more detail). A number of clones were obtained, namely the clone no. 15, 35, 37, 43, 44, 57, 70, and 96. The presence of the correct insert within these expression constructs was confirmed by restriction deletion and gel electrophoresis analysis. The correct constructs, can be cut with *Bam*HI/*Eco*RI, resulting in two DNA fragments with the length of 5 and 2.8 kb (Fig. 9). Because the *Sph*I site was modified by Klenow Fragment DNA Polymerase I, the correct constructs were not cut with *Bam*HI/*Sph*I. In contrast, cutting the original clone (pGP2) with *Bam*HI/*Eco*RI resulted in one DNA fragment with the length of ~ 8 kb. The correct expression clones were analyzed for bacteriocin production using the colony overlay assay. Results showed that except for clone no. 96, all were the bacteriocin-producing constructs (Fig. 10). However, as judged from the zone size, the best producer was clone no. 44 (Fig. 10). This expression clone, designated H44(P2), was used in the following experiments.

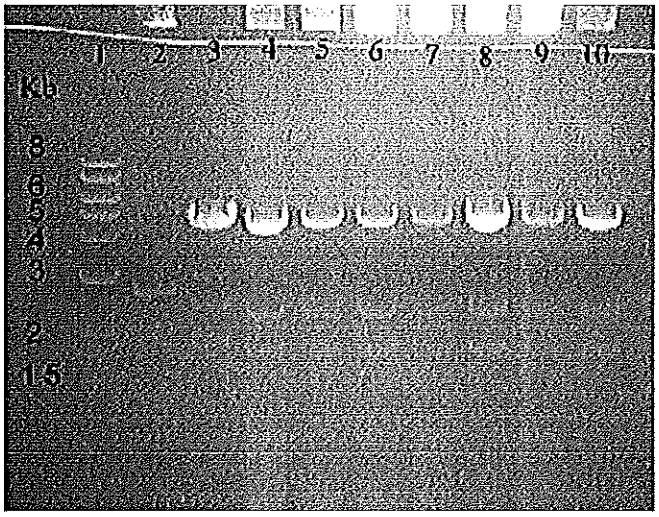


Fig. 9 Agarose gel electrophoresis (1%) of the recombinant plasmids (2 μg DNA of each) digested with *Bam*HI and *Eco*RI restriction enzymes (5 U of each). The presence of the correct inserts in the resulting constructs was confirmed by cutting the plasmids with the combination of *Bam*HI and *Eco*RI enzymes, resulting in two DNA fragments with the length approximately of 5 and 2.8 kb, respectively. All samples shown in the figure (lanes 3-10) are the correct constructs. Lane 1 indicates the size (in kb) of the standard DNA marker (New England Biolab). Lane 2 is the uncut pHAT vector. Lanes 3-10 indicate the restriction fragments of the clone no. 15, 35, 37, 43, 44, 57, 70, and 96, respectively.

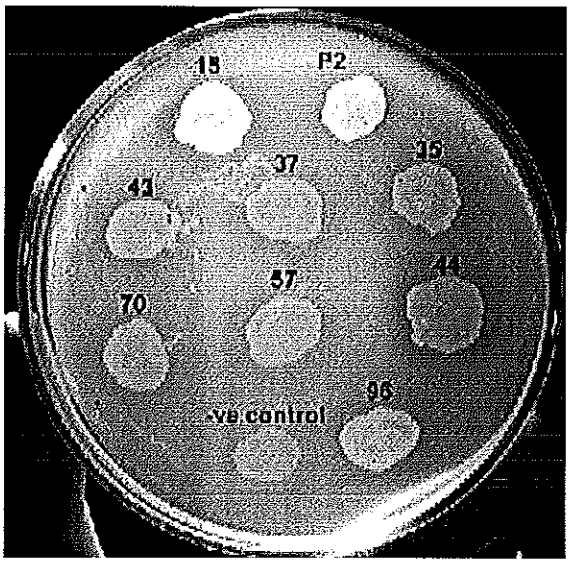


Fig. 10 The correct expression constructs described above were examined for the bacteriocin production using the colony overlay assay. *P. gingivalis* A244 was used as the indicator bacterium. P2 and *E. coli* BL21(DE3)pLysS transformed with pHAT vector (no insert) were used as a positive- and a negative-control, respectively. The numbers designate the resulting clones. IPTG at a final concentration of 1 mM was inoculated in the molten agar before pouring on top of the producers.

Proof for the ATCC 25261-derived producing *E. coli*

To ensure that the producing *E. coli*, H44(P2), received the DNA fragment of the original producer, PCR using five primer pairs (Table 6), hybridizing within the sequence of the *nig* locus, was performed. The genomic DNA of *Pr. nigrescens* ATCC 25261 was used as the template. Electrophoresis of the PCR products was performed using 1.2 % agarose gel. As expected, five PCR products with the length of 540, 360, 420, 480, and 1,200 bp were obtained (Fig. 11). Results indicate that the *nig* locus of the producing *E. coli* was obtained from the original producer, *Pr. nigrescens* ATCC 25261.

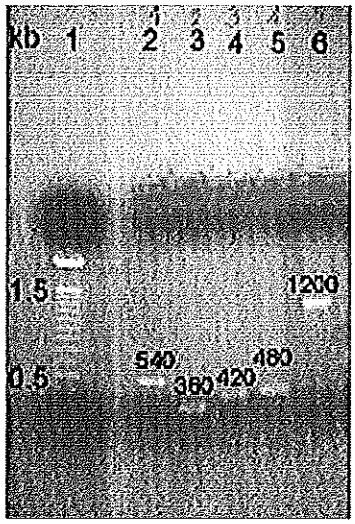


Fig. 11 Agarose gel electrophoresis (1.2 %) of PCR products obtained by using several primers, which hybridize within the sequence of the *nig* locus, and the genomic DNA of *Pr. nigrescens* ATCC 25261 as the template. The result revealed five expected PCR products with the length of 540, 360, 420, 480, and 1,200 bp, respectively, indicating that the *nig* locus of the producing *E. coli* was received from the original producer, *Pr. nigrescens* ATCC 25261.

Effect of cultivation conditions on the bacteriocin production

Preliminary results showed that cultivation conditions, such as temperature, agitation, growth rate, cell density, concentration of the induction factor (IPTG), and the induction time, influenced the bacteriocin yield produced by *E. coli*. Production of the bacteriocin by H44(P2) was found to be growth associated, i.e., more cells produce more bacteriocin. A significant increase in growth rate and cell density was achieved by culturing the H44(P2) cells at 30 ^{O}C with agitation in the range of 150-200 rpm. In contrast, cultured at 30 ^{O}C without agitation resulted in that the cells approached stationary phase more slowly. Culturing the cells at 37 ^{O}C with agitation also affected their growth, i.e., low cell density was still encountered. Optical density (OD_{600}) of the cell suspension at the time of induction should not be less than 1.2, otherwise less bacteriocin was produced. According manufacturer's instruction, IPTG at a final concentration of 1 mM was recommended for use in the induction. No bacteriocin was produced in an absence of IPTG, as the inhibitory activity was not detected and the expected bacteriocin's band, ranging between 30 and 45 kDa, was not seen (Fig. 12). However, we did not obtain high bacteriocin yield by using of the recommended concentration compared with the background expression (Fig 13). Therefore, several concentrations of IPTG, e.g., 1, 2, and 3 mM, were tested for its effect on the bacteriocin production. Results showed that IPTG at a final concentration of 3 mM was necessary for improved bacteriocin yield (Fig. 14). The culture was allowed to grow further for 4 h after induction. Growing the cells more than 4 h did not increase the bacteriocin yield, as depicted in Fig. 15.

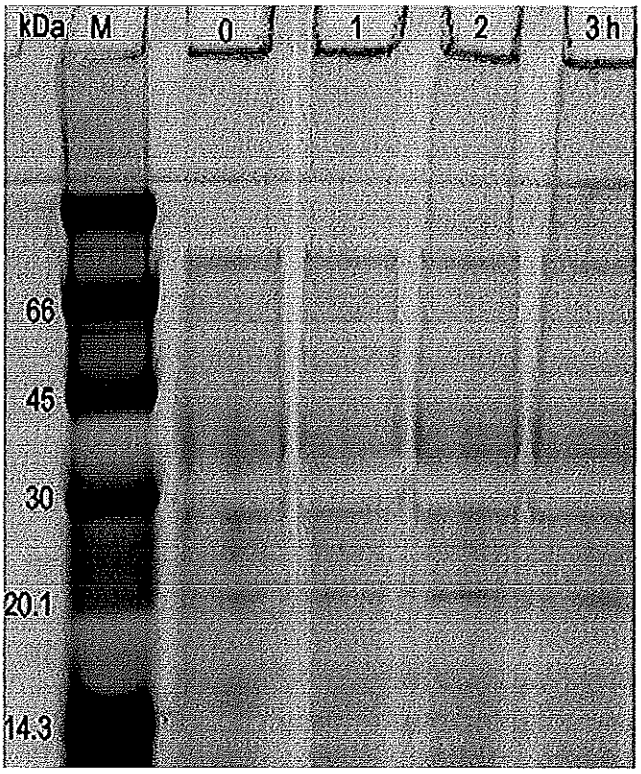


Fig. 12 The cellular protein profile of H44(P2) in an absence of IPTG. The cells were harvested when OD_{600} of the growing culture was ~ 1.2 (time = 0) and at 1, 2, 3 h thereafter. SDS-PAGE (12 % gel) of the cellular proteins (0.1 mg protein of each) was performed according to the standard method of Sambrook *et al* (1989) using the Rainbow marker (Invitrogen) as the protein molecular marker. The numbers on the left of the gel indicate the sizes (in kDa) of the standard protein marker (Lane M). No bacteriocin activity was detected in the samples, and the expected bacteriocin's band, ranging between 30 and 45 kDa, did not appear.

Fig. 13 The cellular protein profile of H44(P2) in an absence or a presence of 1 mM IPTG. In the absence of IPTG, the cells were harvested after 18-h inoculation. In the presence of IPTG, it was added after 14-h inoculation and the cells were harvested after 4-h induction. SDS-PAGE (12 % gel) of the cellular proteins (0.1 mg protein of each) was performed according to the standard method of Sambrook *et al* (1989) using the Rainbow marker (Invitrogen) as the protein molecular marker. The numbers on the left of the gel indicate the sizes (in kDa) of the standard protein marker. No difference in an expression level was observed for induced (Lane 1) and non-induced (Lane 2) cells.

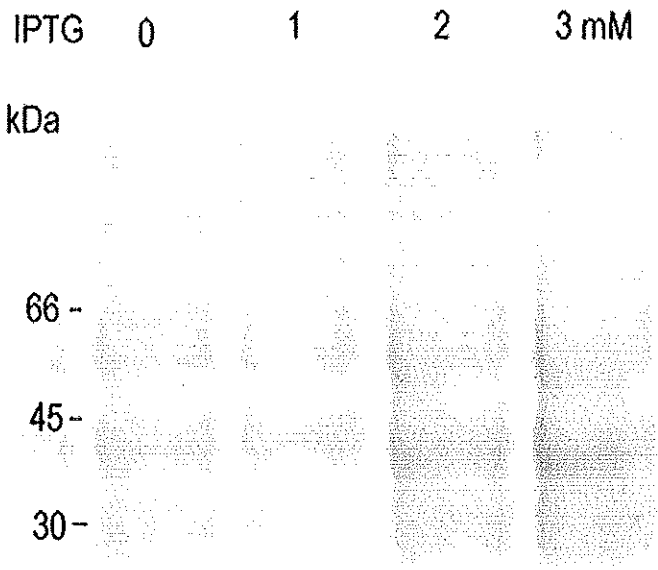


Fig. 14 The cellular protein profile of H44(P2) induced by different concentrations of IPTG (0, 1, 2, and 3 mM). The cells were grown to stationary phase (OD_{600} ~ 1.2) before induction, and harvested at 4 h after induction with certain IPTG concentrations. SDS-PAGE (12 % gel) of the cellular proteins (0.1 mg protein of each) was performed according to the standard method of Sambrook *et al* (1989) using the Rainbow marker (Invitrogen) as the protein molecular marker. The numbers on the left of the gel indicate the sizes (in kDa) of the standard protein marker. Higher expression level was observed using IPTG at a final concentration of 3 mM.

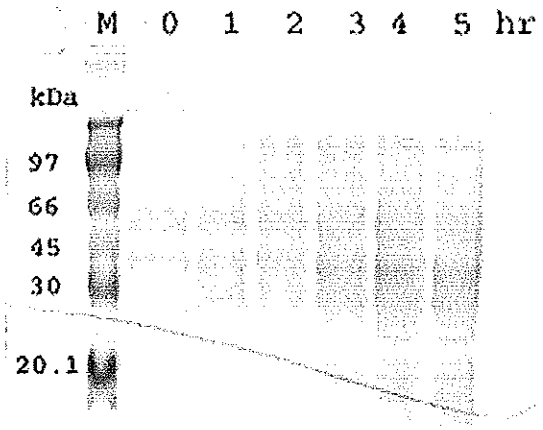


Fig. 15 The figure showed the cellular protein profile of H44(P2). The cells were grown to stationary phase (OD_{600} ~ 1.2) before the addition of IPTG (3 mM). The growing cells were harvested at the time 0, 1, 2, 3, 4, and 5 h after induction. SDS-PAGE (12 % gel) of the cellular proteins (0.1 mg protein in each lane) was performed according to the standard method of Sambrook *et al* (1989) using the Rainbow marker (Invitrogen) as the protein molecular marker. Lane M was the standard protein marker with sizes (in kDa) indicated on the left of the gel.

Purification of the bacteriocin

The bacteriocin was purified to homogeneity from a 2-liter culture of H44(P2) propagated in LB broth at 30 ^{O}C with agitation. In the beginning, we did not have information about its size, its pI, and its biochemical behaviors, because to date bacteriocins of *Pr. nigrescens* strains have not been characterized and reported. Therefore, we desired to follow the purification methods established by Nissen-Meyer *et al.*, (1992). The method used a sequence of cation exchange, hydrophobic interaction, gel filtration and/or reverse phase chromatography steps. Unfortunately, purification of the bacteriocin was not successful using this method. This might be due to that the bacteriocin has a molecular size larger than those of LAB bacteriocins, and it might thus be denatured on the hydrophobic interaction and reverse phase columns. The bacteriocin was therefore purified according the operator's experience using trial and error procedures. The bacteriocin was first purified by ion exchange chromatography and followed by two steps of gel filtration chromatography in order to avoid protein denaturation. The bacteriocin was successfully purified using step by step procedures as described in the following.

Cation exchange chromatography was tried first. The H44(P2) cells were harvested by centrifugation at 6,000 rpm for 10 min, washed with PBS, and then dispersed in 50 mM phosphate buffer pH 5.0 (buffer A). The cell suspension was subjected to sonification. The cell lysate was separated from the debris by centrifugation at 11,000 rpm for 45 min. The lysate was filtered through a 0.45 μ membrane filter before applied on the SP Sepharose FF column (cation exchanger), previously equilibrated with buffer A. No activity was detected in the flow-though fraction. The column was washed with buffer A until the optical density (OD_{280}) returned to the base line. Bound-proteins were then eluted from the column using a gradient mode with increase NaCl concentration in buffer B (1 M NaCl containing buffer A). Only one broad optical density peak was obtained. The peak began to elute at 0.15 M NaCl and was completely eluted at 1 M NaCl (Fig. 16). The whole bound fraction was collected and tested for bacteriocin activity. This fraction was unstable, since it became turbid upon storing. The

activity assay revealed that NaCl, even at 0.15 M, inhibited growth of the indicator bacterium. Therefore, the fraction was desalted before being analyzed for bacteriocin activity. A HiPrep™ (Sephadex G-25) column was used as a desalting column. The bound fraction obtained from SP Sepharose FF was applied on the HiPrep™ column. The non-retained fraction showed no activity, but two optical density peaks were observed, i.e., one sharp peak eluted before the NaCl peak (indicated in red-colored line), and another broad peak eluted after the NaCl peak (Fig. 17). Results suggest that some proteins, those eluting after the NaCl peak, were bound to the Sephadex G-25 column. Because both sharp and broad peaks were still contaminated with salt, desalting was therefore done by dialysis against a large volume of water. Dialysis membrane has pore size with molecular weight cut-off 3,500 Da. The dialyzed-fractions were dried using lyophilization, and the protein powders were dissolved with PBS before assaying for activity. Activity was not detected in the first sharp optical density peak, but it was detected in the second broad optical density peak. These results indicate that the bacteriocin binds to Sephadex G-25. There were problems encountered on dialysis, e.g., loss of protein and its time-consuming. The use of an anion exchanger may be an alternative that will eliminate the desalting step.

For anion exchange chromatography, the H44(P2) cells were re-suspended in 50 mM Tris buffer pH 9.5 (buffer C) followed by sonification. The filtered lysate was loaded on the Q Sepharose FF column (anion exchanger), previously equilibrated with buffer C. The flow-though fraction was collected for determining the activity. The column was washed with buffer C until the optical density (OD_{280}) returned to the baseline. Bound proteins were eluted from the column using a gradient mode with increasing NaCl concentration in buffer D (1 M NaCl containing buffer C). Bound fractions were collected according to the optical density peaks (OD_{280}) (Fig. 18). Dialysis of the bound fractions was performed before determining the activity. The activity was detected in the flow-though fraction.

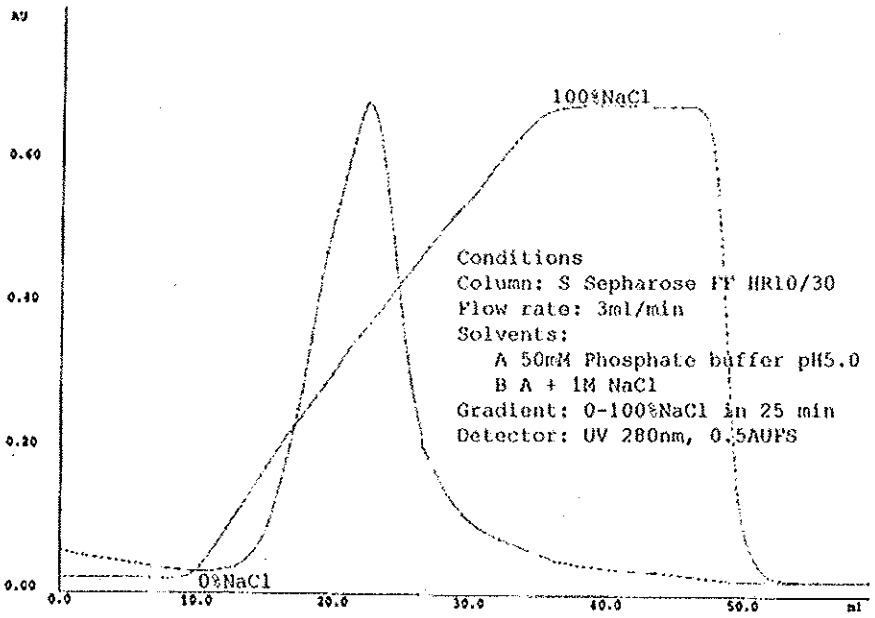


Fig. 16 The chromatogram obtained from the SP Sepharose FF column. The lysate was applied on the SP Sepharose FF column, equilibrated with buffer A. The bound fraction was eluted using a gradient mode with increase NaCl concentration in buffer B (1 M NaCl containing buffer A). The optical density peak (OD_{280}) began to elute at 0.15 M NaCl and completely eluted at 1 M NaCl. The whole bound fraction was collected, dialyzed, and assayed. Activity was detected from the bound fraction.

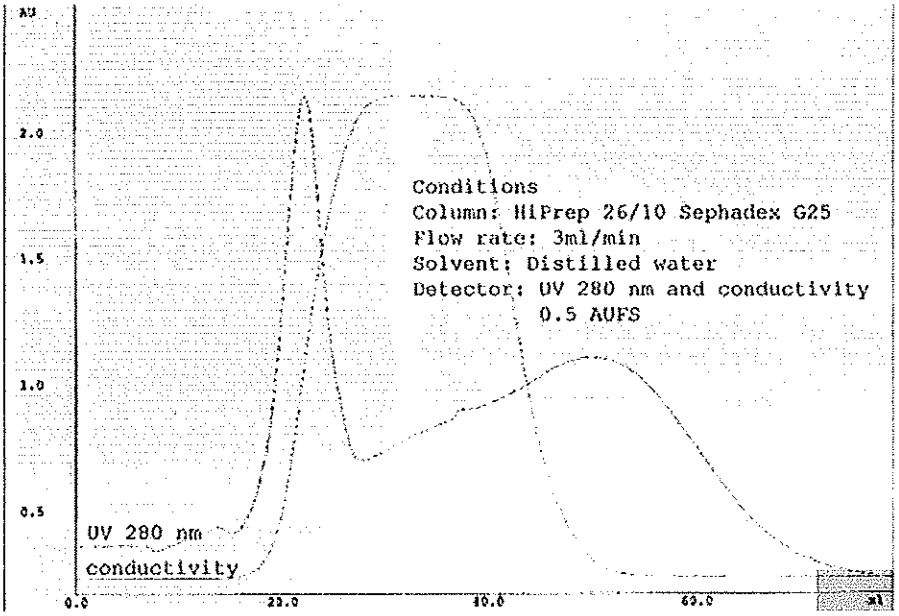


Fig. 17 The chromatogram obtained from the HiPrep™ column. The bound fraction obtained from the SP Sepharose FF column was loaded on the HiPrep™ column. Non-retained fraction showed no activity, but two optical density peaks were observed. One was the sharp peak eluted before the NaCl peak (indicated in red-colored line), and another broad one was eluted after the NaCl peak. Some proteins bound to the Sephadex G-25 column because they were eluted after the NaCl peak. After dialysis, the retained broad peak was found to contain the activity.

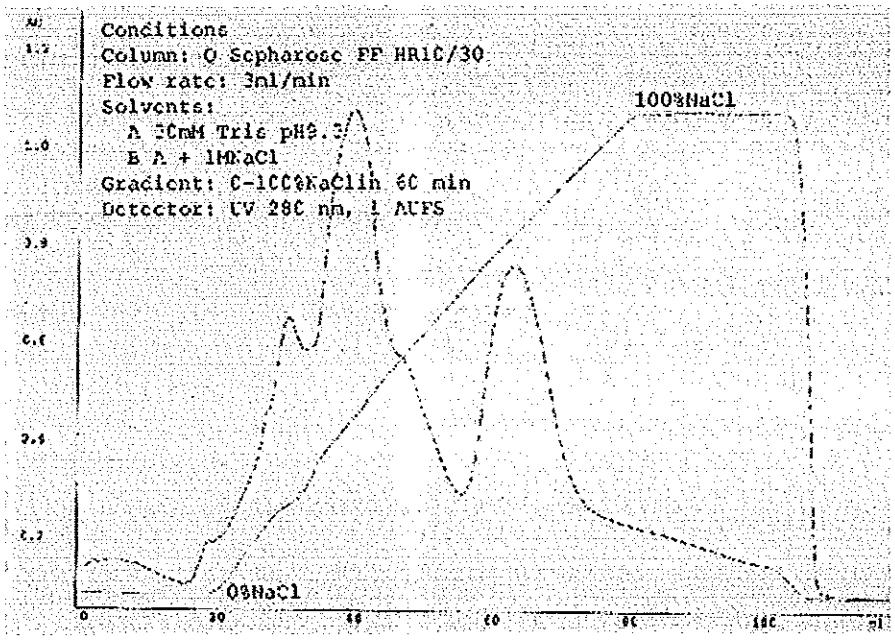


Fig. 18 The chromatogram obtained from the Q Sepharose FF column. The lysate was applied on the Q Sepharose FF column, previously equilibrated with buffer C. The flow-though fraction was collected for determining the activity. The bound fraction was eluted from the column using a gradient mode with increase NaCl concentration in buffer D (1 M NaCl containing buffer C), and collected according to the optical density (OD_{280}). The bound fractions were dialyzed and concentrated before assaying the activity. The activity was, however, detected in the flow-though fraction.

Hydrophobic interaction and reverse phase chromatography have been included and used following the anion exchange method. The active fraction obtained from the QAE Sephadex A-50 column was applied on the hydrophobic interaction and reverse phase columns. However, the activity could not be detected for all of the fractions collected. It might be that the bacteriocin, if it were a large protein, has been destroyed during these purification processes.

Gel filtration chromatography was the subsequent procedure, which was tried and used in the bacteriocin purification. Since the SephadexG-25 binds the bacteriocin, we thus included the HiPrep^{TM} column in between before using the real gel filtration chromatography. The flow-through fraction obtained from QAE Sephadex A-50 column was applied on the SephadexG-25 column, previously equilibrated with 0.15 M NaCl containing distilled water. The presence of 0.15 M NaCl has been reported to reduce ionic interaction within the column. As expected, the retained broad optical density peak (OD_{280}) obtained from the SephadexG-25 column exhibited growth inhibition of the sensitive cells (Fig. 19). These fractions were pooled and concentrated to a small volume by lyophilization. Because the bacteriocin was expected to be a large protein and it may be destroyed by hydrophobic interaction and reverse phase steps, a Superdex 75 column was therefore used in the subsequent purification step (separating limit of the Superose 75 is ranged between 5,000 and 100,000 Da). The concentrated fraction was applied on the Superdex 75 column. The activity was eluted with the optical density peak (OD_{280}). On the first run, however, the peak fraction was contaminated with a small amount of other proteins, as revealed by SDS-PAGE (see Fig. 20). As a result, this fraction was subjected to a second run on the same column, and the bacteriocin was eluted from the column as a single sharp optical density peak (OD_{280}) (Fig. 20). SDS-PAGE of the active fractions obtained from each purification step was also shown (Fig. 21). The Superdex 75 gel-filtered bacteriocin was electrophoresed on 12 % polyacrylamide gel to test the purity and to determine the size of the bacteriocin. One protein band appearing at a molecular weight of about 41 kDa was observed (Fig. 22A). This band seemed to be associated with the inhibitory activity, since a clear inhibition zone

corresponding to the protein band was detected after being overlaid with the gel containing the indicator bacterium (Fig. 22B). This result indicates that the bacteriocin has an approximate molecular weight of about 41 kDa.

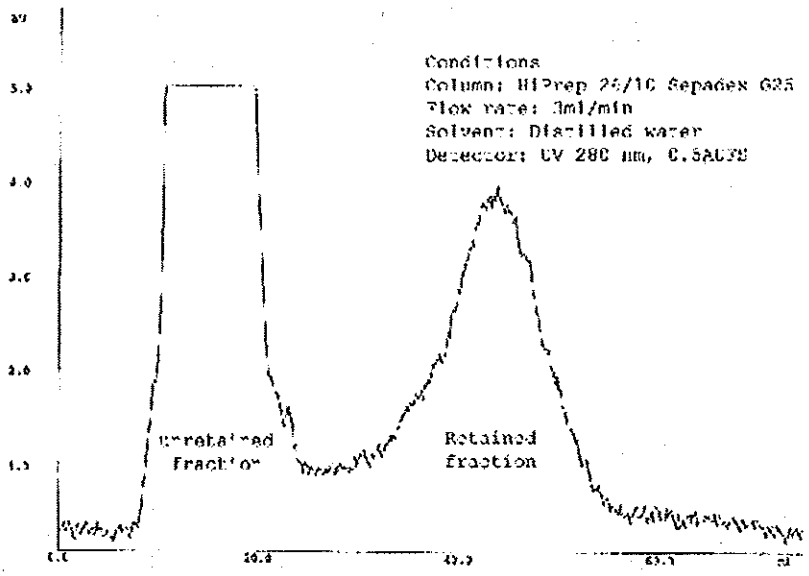


Fig. 19 The chromatogram of the HiPrep™ 26/10 Sephadex G-25 column. The flow-through fraction obtained from the QAE Sephadex A-50 column was applied on the HiPrep™ column and 0.15 M NaCl containing distilled water was used as the eluent. The activity was detected for the retained broad peak instead of the non-retained fraction. The result suggests that although the bacteriocin was expected to be a large protein, it binds to the Sephadex G-25 column.

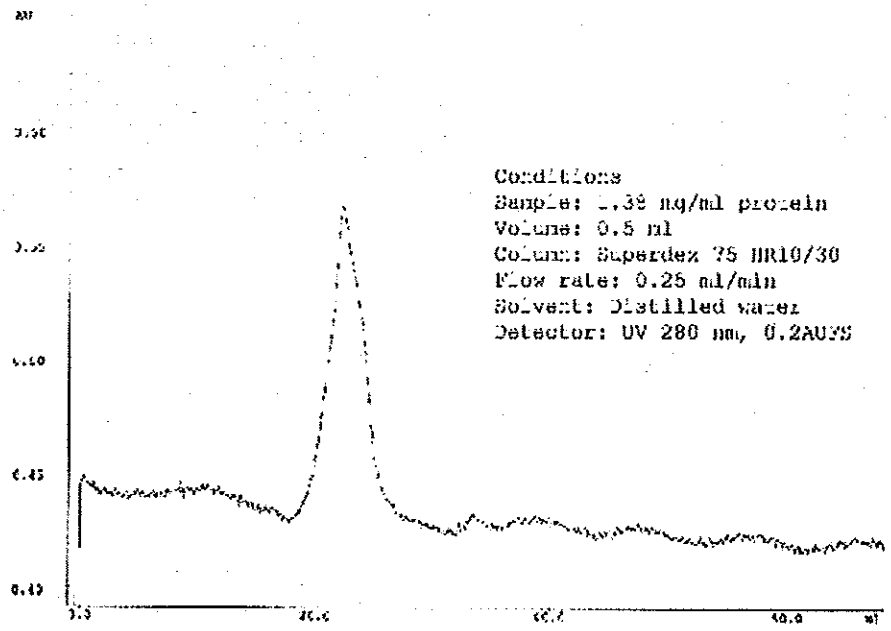


Fig. 20 The chromatogram of the second run on the Superdex 75 column. The active fractions obtained from the first run on the Superdex 75 column were concentrated by lyophilization and (0.7 mg protein) applied on the same column. The chromatogram showed the single sharp optical density peak (OD_{280}) on the second column, which co-eluted with and contained the inhibitory activity as revealed by being overlaid the SDS gel with the gel containing the indicator bacterium (see Fig. 22). The solution, 0.15 M NaCl containing distilled water, was used as the eluent.

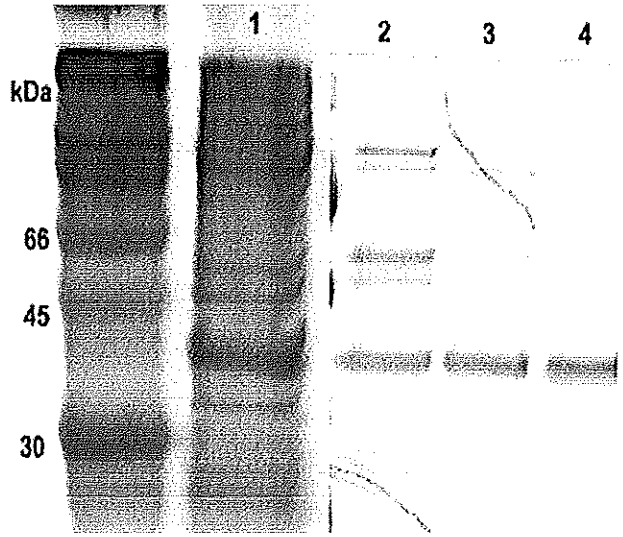


Fig. 21 SDS-PAGE (12 %) of the active fractions obtained from each step of purification as previously described: the crude lysate (2.5 mg protein, lane 1), the retained fraction of the Sephadex G-25 obtained from being applied the QAE Sephadex A-50's flow-though fraction on the column (1 mg protein, lane 2), the active fraction obtained from the first run on the Superdex 75 column (0.7 mg protein, lane 3), and the active fraction obtained from the second run on the Superdex 75 column (0.1 mg protein, lane 4).

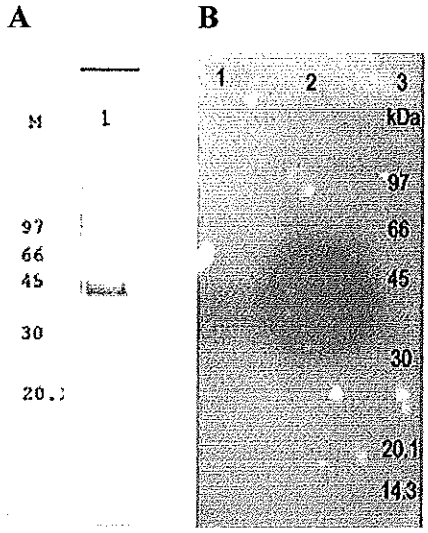


Fig. 22 The active fraction (0.1 mg protein) obtained from the second run of the Superdex 75 column was evaluated using two gels of 12 % SDS-PAGE.

(A) One gel was stained with Coomassie Brilliant Blue. The result showed that the gel filtered fraction contained one component with an approximate molecular weight of 41 kDa. Lane M indicates the size of the standard protein marker (in kDa).

(B) Another SDS-gel was bio-assayed, according to the modified method of Bhunia *et al* (1987). The washed gel was overlaid with the gel containing the indicator bacterium, and allowed to grow overnight under optimal conditions before examining a clear inhibition zone over the protein band. The protein band seemed to be associated with the antimicrobial activity (lane 2), since a clear inhibition zone corresponded to this protein band. The result suggests that the bacteriocin has an approximate molecular weight of 41 kDa. A small inhibition zone in Lane 1 was obtained by using the cellular protein of *Pr. nigrescens* ATCC 25261 (25 mg protein), and used as a positive control. Lane 3 indicates the size (in kDa) of the standard protein marker.

Effect of pH on the bacteriocin activity

In a preliminary study it was found that *P. gingivalis* A244 could not be grown in acidic pH media (pH < 6.5). The solutions with pHs lower than 6.5 were thus excluded from the experiment. To determine the effect of pH on the activity, the dialyzed bacteriocin powder was dissolved in specific pH buffers, and assayed for the residual activity. Results showed that, in buffers with pH ranging from 6.5 to 9.5, the bacteriocin activity was stable.

Heat stability of the bacteriocin

To test for heat stability, the partially purified bacteriocin (the fraction obtained from the first run on the Superdex 75 column) was exposed to heat at 50, 80, and 100 ^{O}C for 10 min. Although protein precipitation was seen on heating at 80 and 100 ^{O}C, no reduction in the activity was observed.

Proteolytic enzyme sensitivity of the bacteriocin

The purified bacteriocin was incubated with either proteinase K or pepsin A (50 µg/ml of each) in 50 mM phosphate buffer pH 7.0 at 37 ^{O}C for 2 h. Results showed that the activity was completely inactivated after being exposed to proteinase K, but full activity still remained after being exposed to pepsin A. The results suggest the proteinaceous nature of the active compound.

Antimicrobial spectrum of the bacteriocin

The antimicrobial spectrum of the bacteriocin was assayed against 18 indicator strains. Three strains belonged to the genera *Streptococcus*, two strains to *Lactobacillus*, three strains to *Actinomyces*, one strain to *Tannerella*, one strain to *Actinobacillus*, five strains to *Porphyromonas*, and three strains to *Prevotella*. The test was carried out using the well dilution assay. The results are summarized in Table 8, and indicate that the *Streptococcus* and *Lactobacillus* genera are relatively insensitive; that the *Actinomyces* spp., *Tannerella forsythensis*, and some strains of the genera *Porphyromonas* are moderately sensitive; and that the

P. gingivalis A244, *P. gingivalis* 381, and *Pr. intermedia* are highly sensitive to the bacteriocin.

Table 8. Antimicrobial activity of the bacteriocin

Organism	MIC (μM)[a]	Sensitivity[b]
Actinomyces naeslundii ATCC 12104	50.0	0.21
Actinomyces vislosus ATCC 15987	22.2	0.48
Actinomyces israellii	50.0	0.21
Streptococcus mutans ATCC 25175	NI	-
Streptococcus salivalius ATCC 7073	NI	-
Streptococcus sanguis ATCC 10556	NI	-
Lactobacillus casei sub. *casei* ATCC 393	NI	-
Lactobacillus formentum ATCC 14931	NI	-
Actinobacillus actinomycetemcomitans	NI	-
Tannerella forsythensis	21.4	0.5
Porphyromonas gingivalis ATCC 33277	18.7	0.57
Porphyromonas gingivalis ATCC 11326	16.7	0.64
Porphyromonas gingivalis 381	10.7	1.0
Porphyromonas gingivalis W50	21.4	0.5
Porphyromonas gingivalis A244	10.7	1.0
Prevotella intermedia ATCC 25611	10.7	1.0
Prevotella nigrescens ATCC 33563	21.4	0.5
Prevotella nigrescens ATCC 25261	NI	-

[a] The MIC data are expressed as the median (range) obtained from three independent repetitions for each strain, and an approximate molecular weight of the bacteriocin is 41 kDa, which was used for the MIC calculations.
[b] Ratio between the MIC for the strain tested and that for the *P. gingivalis* A244 strain.
NI not inhibited
- not determined

Effect of the bacteriocin on sensitive cells

Cultures of the sensitive cells in the exponential phase of growth were incubated for 24 h with different concentrations of the bacteriocin, ranging between 5 and 50 μM. The OD_{600} of the cultures and the number of live bacteria were then measured. Results showed that at concentrations higher than certain MICs, the OD_{600} decreased to zero. This suggests that the bacteriocin causes cell lysis and that it has a bactericidal mode of action. The activity response was found to be dose dependent, i.e., bacteriostatic activity was seen at bacteriocin concentrations lower than the MIC.

Inhibitory specificity of the bacteriocin

The inhibitory activity of the bacteriocin was compared to that of nisin. The activity was examined against various indicators. Results are summarized in Table 9 and indicate that the MICs for nisin were 6 to 20 greater than those for the bacteriocin. This suggests that the bacteriocin has a higher specific activity than nisin, especially against the strains who share the same ecological niche with the original producer.

Cytotoxicity of the bacteriocin on the gingival fibroblasts

Normal gingival fibrobalsts were used for monitoring toxicity of the bacteriocin. To determine whether the bacteriocin is toxic to the cells, a high bacteriocin concentration (84 μM or 8 times higher than the MIC for *P. gingivalis* A244) was applied to the cells for 30 min after which the cell morphology was observed. Light microscopy revealed that cell morphology was not changed (Fig. 24). Data from the colony-forming assay showed that less than 10 % of the cells were killed upon direct contact with the bacteriocin (Fig. 25). Although being in direct contact with high concentrations of the bacteriocin, the cells still functioned, as the treated cells duplicated and the cell population increased (Fig. 26). These results suggest that the bacteriocin is not toxic to the cells.

Table 9. Inhibitory activity of the bacteriocin and nisin against various indicators

Bacterial strain	MIC (μM)[1]		Ratio[2]
	The bacteriocin	Nisin	
Porphyromonas gingivalis A244	10.7	213.3	19.9
P. gingivalis ATCC 33277	18.7	106.7	5.7
P. gingivalis W50	21.4	160.0	7.5
Prevotella intermedia ATCC 25611	10.7	213.3	19.9
Pr. nigrescens ATCC 25261	NI	213.3	-
Pr. nigrescens ATCC 33563	21.4	256.0	12.0
Tannerella forsythesis	21.4	160.0	7.5
Actinomyces israellii	50.0	NI	-

[1] The bacteriocin has an approximate molecular weight of 41 kDa, which was used for calculation the MICs

[2] Ratio between the MICs for nisin and those for the bacteriocin

NI not inhibited

– not determined

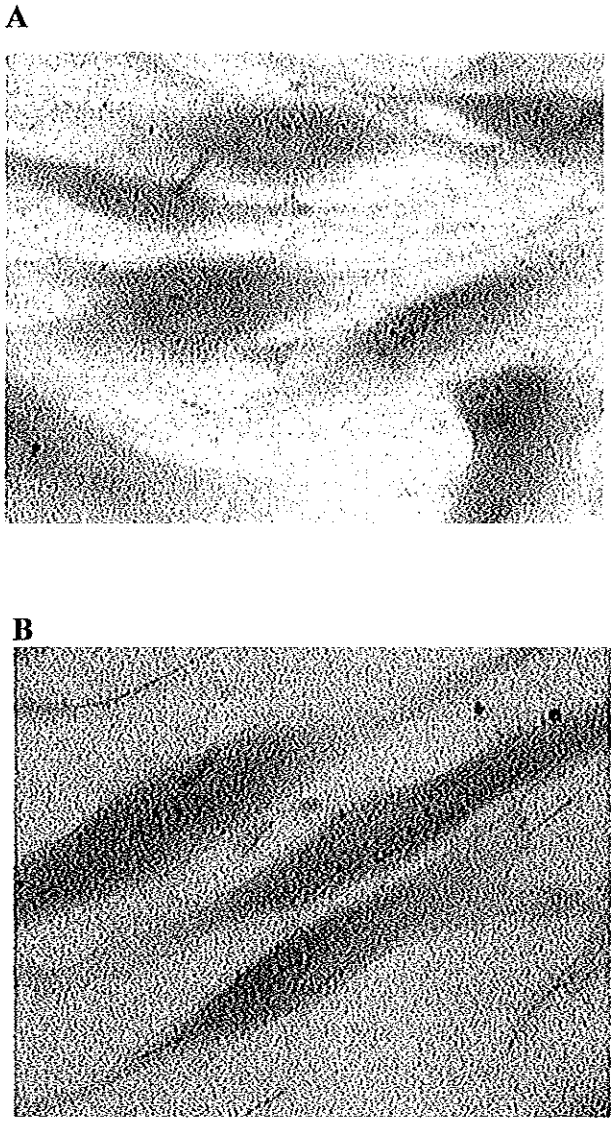


Fig. 23 Morphology of the gingival fibroblasts before (A) and after (B) being in direct contact with the bacteriocin solution at a concentration of 84 μM.

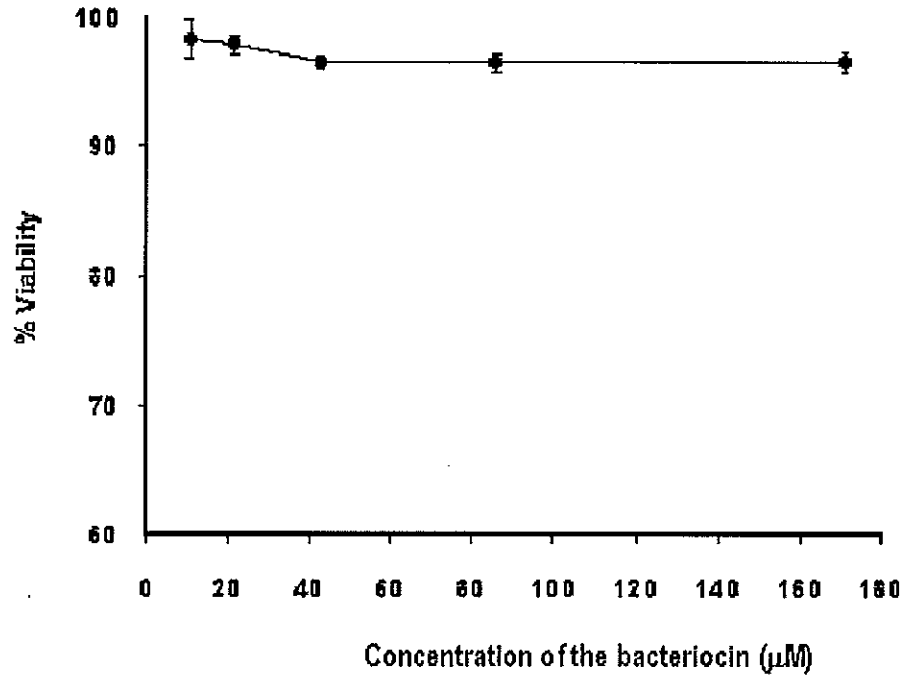


Fig. 24 The viability curve showing % survival of fibroblast cells that have been in direct contact with the bacteriocin solutions at indicated concentrations for 30 min.

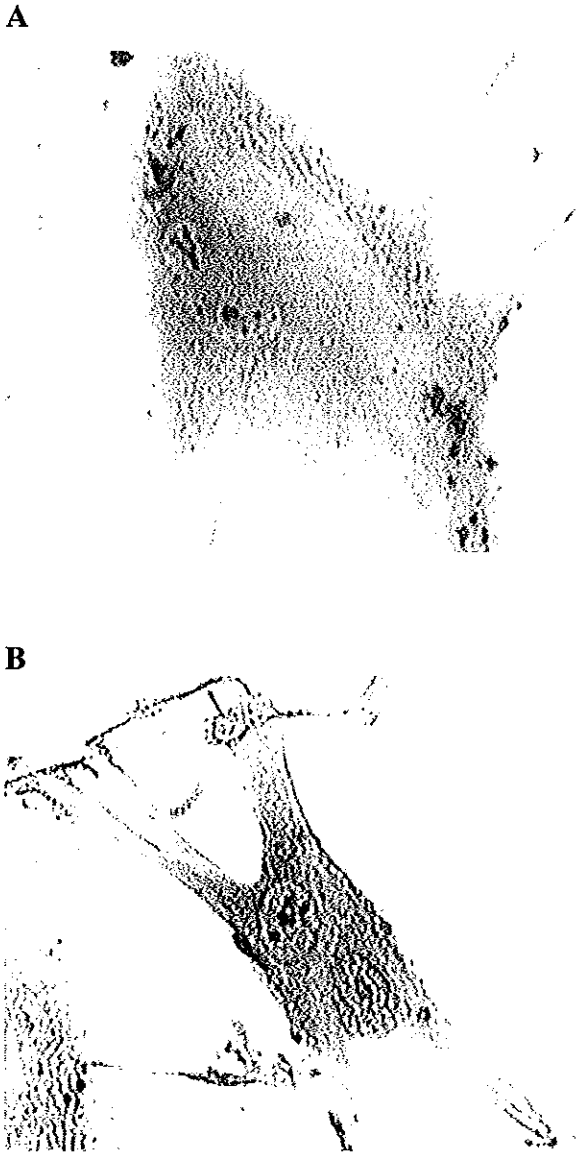


Fig. 25 The pictures show cell duplication after the cells have been in direct contact with (A) PBS pH 7.4 and (B) the bacteriocin solution at a concentration of 84 μM.

Chapter 4
Discussion

Microorganisms from diverse and complex habitats, including the oral cavity, may maintain antagonistic or synergistic relationships with one another by several mechanisms. These include production of substances, such as metabolic end products, classic antibiotics, bacteriolytic enzymes and bacteriocins that inhibit the growth of other organisms (Riley and Gordon, 1999; Riley, 1998; Jack *et al.*, 1995). Bacteriocins are ribosomally synthesized peptides or proteins exhibiting antimicrobial activity directed, in most cases, against bacteria closely related to the producer organism. Many bacterial strains have been reported to produce bacteriocins or BLS.

Recently, there has been a great deal of interest in bacteriocins produced by oral microorganisms, especially Gram-negative bacteria. However, information concerning bacteriocins produced by oral Gram-negative bacteria such as *Pr. nigrescens* is sparse. Although *Pr. nigrescens* ATCC 25261 has been shown to produce a bacteriocin (Teanpaisan *et al.*, 1998), the bacteriocin has not been biochemically and genetically characterized. The bacteriocin was active against bacterial species related to the producer and to species implicated in adult periodontitis, such as *Pr. intermedia* and *P. gingivalis* (Teanpaisan *et al.*, 1998). It is important to note that *Pr. nigrescens* strains isolated from healthy subgingival sites produce the bacteriocin, whereas those isolated from diseased sites do not produce it (Teanpaisan *et al.*, 1998). Therefore, the bacteriocin may perhaps play a protective role for the host against periodontal pathogens. The bacteriocin produced by *Pr. nigrescens* may possibly, in the future, be used for avoiding multiplication of these pathogenic bacteria. The objectives of this study were to gain insight into the genetic requirements for the production of bacteriocin in *Pr. nigrescens* ATCC 25261, and to determine the biochemical and cytotoxic properties of such a bacteriocin. For these purposes, a genomic library of *Pr.*

nigrescens ATCC 25261 was constructed and screened for a bacteriocin positive phenotype (Bac^+). The ATCC 25261 strain-derived DNA fragments present in a clone with a Bac^+ phenotype were sequenced and analyzed. Gene deletion analysis was performed in order to delineate the region required for the Bac^+ phenotype and to investigate the possible involvement of some of the genes located in that fragment. Finally, the bacteriocin produced by a Bac^+ phenotype clone was purified and its biochemical properties were studied.

The genetic determinant required for the production of bacteriocin was found to be located on the chromosome of *Pr. nigrescens* ATCC 25261. The bacteriocin genetic system consists of a continuous 4,868-kb DNA segment, termed the *nig* locus. pGP2 and pH44(P2) are the recombinant plasmids of the bacteriocin-producing clones P2 and H44(P2), respectively. Both contain the *nig* locus and confer a Bac^+ phenotype. The Bac^+ phenotype of the clones P2 and H44(P2) were, however, unstable. Plasmid analysis of Bac^- variants of the Bac^+ transformants showed that the *nig* locus was still present. The result, thus, indicates that loss of the bacteriocin producing ability was not caused by the absence of the *nig* locus. In contrast to P2 and H44(P2), a stable Bac^+ phenotype was observed for the clone A17, which contains the ATCC 25261 strain-derived 10-kb DNA segment of which the 4,868-kb DNA segment is a part. These results are difficult to explain. Nevertheless, Bac^+ phenotypic instability similar to that found in this study has been reported (O'Keeffe *et al.*, 1999).

Sequence analysis of the 4,868-kb DNA fragment revealed that it contains four major ORFs, designated *nigA* to *nigD*, respectively. All were oriented in the same direction. *nigA* encodes a putative 363-amino acid protein with an estimated molecular mass of 41.3 kDa. A putative promoter was found upstream of *nigA*, containing the typical –10 and –35 regions (Fig. 6). The *nigB* gene has two possible translation start sites, at the first and the second methionine (at positions 1,362 bp and 1,371 bp, respectively). Most probably, the gene begins at the second ATG because only this ATG is preceded by a potential ribosome-binding site, GAATGA, at a distance (5 bp) adequate to begin translation. It encodes a putative 328-amino acid protein with an approximate molecular weight of 36.7

kDa when this ATG is used as the start codon. A loop structure (ΔG = -23.1 kcal/mol), consisting of a 23-base inverted repeat separated by 7 bases, was identified downstream of *nigB*. This structure has the features associated with a bidirectional *rho*-independent transcription terminator. Since an independent promoter upstream of *nigB* could not be found and according to the evidence previously described, it is possible that the genes *nigA* and *nigB* are produced on the same transcript. The start codons of *nigA* and *nigB* are preceded by its own putative RBS, the products of *nigA* and *nigB* may thus be translated independently. In accordance, *nigAB* are expected to be the genes in an operon. Translation of *nigC* may begin by a methionine residue at position 2,943 bp. Its start codon is preceded by an identified promoter sequence and a ribosome binding site. The putative promoter sequence, -35 (TGCTAT) and -10 (TTACCT) was found between positions 2,825 and 2,855 bp and the possible ribosome binging site, AGGCGA, was located 8 bp upstream of the start codon. *nigC* may encode a protein of 222 amino acid residues with a theoretical molecular weight of about 25.55 kDa (Fig. 6). *nigD* was found downstream of *nigC*. *nigD* overlaps the end of *nigC* by 5 bp and encodes a theoretical protein of 218 amino acids with an approximate molecular weight of 24.2 kDa. Because no independent promoter could be identified upstream of *nigD* and *nigD* overlaps the 3′-end of *nigC*, it is presumed that *nigC* and *nigD* are organized in an operon and transcribed couple. As mentioned, although using the same putative promoter, n*igC* and *nigD* may be translated independently, since each proceeds by its own putative RBS. These may be clarified, however, by using a promoter replacement assay, and by determining a transcript mRNA and amino acid sequencing of the gene products. No putative transcription terminator could be identified downstream of *nigD*. Further studies examining the precise roles of these ORFs in the bacteriocin production are under way.

Sequence homology between these four ORFs and those of other known bacteriocins was not found. But this is not an unexpected result, since there is no information about bacteriocins produced by the genera *Prevotella* or other related strains in database. Homology search shows, however, that a gene product of *nigA*

(NigA) was similar to the ribosomal large subunit pseudouridine syntase D with 66 % identity, NigB was homologous to D-alanine-D-alanine ligase with 59 % identity, and NigC and NigD were similar to two hypothetical proteins of unknown function with 50 % and 26 % identity, respectively.

The ability of *E. coli* transformed with deletion derivatives of pGP2 to produce the bacteriocin was studied. Clones bearing the deletion mutant plasmids all lacked the bacteriocin-producing ability. In addition, deletion only in the 3′-end of *nigB* (in pG*Cla*I) causes the loss of bacteriocin-producing ability (Fig. 8). It might be that deletion in the 3′-end of *nigB* may result in either a non-functional mRNA for NigB and/or it may stabilize the mRNA such that neither NigA nor NigB are synthesized. The results thus indicate that NigA and/or NigB are required for bacteriocin production. It is assumed that the operon of *nigCD* may not relate to the production of bacteriocin in this strain, since the complete *nigCD* operon in pGH18 has no bacteriocin-producing ability (Fig. 8). However, more research is needed in order to acquire more knowledge about regulation of the expression of the bacteriocin-encoding gene in *Pr. nigrescens* ATCC 25261.

To ensure that the *nig* locus originates from the chromosome, PCR amplifications using several primers (Table 6) hybridizing within the *nig* locus were performed. The reactions yielded the expected PCR products (540, 360, 420, 480, and 1,200 bp), when the chromosome of *Pr. nigrescens* ATCC 25261 was used as the template (see Fig. 11). Results indicate that the *nig* locus was actually originated from the chromosome of *Pr. nigrescens* ATCC 25261. To my knowledge, these data provide the first genetic evidence of a bacteriocin produced by *Pr. nigrescens* ATCC 25261.

SDS-PAGE analysis of the bacteriocin sample obtained after the second Superdex 75 gel filtration step revealed one protein band appearing at a molecular weight of about 41 kDa. Antimicrobial activity was associated with this band, a clear inhibition zone was obtained upon overlaying the SDS gel with a gel containing the indicator bacterium. It is most likely that *nigA* is the structural gene because the molecular weight of the putative protein encoded by *nigA* is similar to that of the bacteriocin as judged by SDS-PAGE. Genetic characterization shows

that *nig*A is preceded by the putative promoter region, followed by *nigB* that has no independent promoter. Immediately downstream of *nigB* is an identified loop structure possibly acting as a *rho*-independent transcription terminator. *nigA* and *nig*B are transcriptionally coupled, as genes in bacteriocin operons. This suggests that -if one assumes that the organization of the *nig* system is similar to those of other bacteriocin gene clusters- *nigA* is the bacteriocin structural gene. However, this may be proven by determining the amino acid sequence of the bacteriocin.

In most bacteriocin operons described to date, a gene encoding the immunity protein, which protects the producing bacteria against self-toxicity, usually follows the bacteriocin structural genes (Nes *et al.*, 1996). The bacteriocin and immunity protein-encoding genes are generally co-transcribed in order to ensure that the producer strain is not killed by its own bacteriocin (Nes *et al.*, 1996). *nigB* is a good candidate to encode the immunity protein, although no homology between the putative protein encoded by *nigB* and other immunity proteins in the data bank has been found. However, this is not unexpected, as bacteriocin immunity proteins described so far show little homology with one another (Nes *et al.*, 1996). There are some indications that could reinforce the hypothesis that *nigB* may encode the immunity protein. As mentioned, *nigB* is located close to the putative structural gene (*nigA*), and a putative *rho*-independent transcription terminator was found just downstream of *nigB*. This indicates that the putative structural bacteriocin gene, *nigA*, is co-transcribed together with *nigB*. Moreover, the hydrophobicity profile of the *nigB* gene product (see Fig. 7) shows the presence of several putative transmembrane segments, indicating that it is likely to be an integral membrane protein. As in other immunity proteins, these transmembrane regions are thought to be necessary for insertion into the membrane of the bacteriocin producer strain in order to exert its function (Nes *et al.*, 1996; Fremaux *et al.*, 1993). The products of *nigCD* genes are most unlikely to be the bacteriocin and/or immunity protein, because of the difference in molecular weights between the bacteriocin and those of the *nigCD* gene products. Although genes located in the vicinity of the *nigCD* genes have not

been characterized, it is possible that *nigCD* may be the genes of another separated operon.

According to Fig. 4, bacteriocin activity of the producing-*E. coli* can be detected extracellularly. This might be caused by the release of the bacteriocin from lyzed cells. Although ABC transporters that are typically involved in export of a number of bacteriocins (Higgins, 1992; Higgins *et al.*, 1986) are missing in the *nig* locus as revealed by a homology search, it is possible that a gene for a dedicated bacteriocin exporter is present in the *Pr. nigrescens* ATCC 25261 strain, but not located in the vicinity of the bacteriocin gene cluster. The bacteriocin produced by *E. coli* must be stored within the cytoplasm, since the *E. coli* lacks the gene specifically to export such a bacteriocin. Studies have showed, however, that most bacteriocins produced by the original producer are secreted into an environment in order to exert their function (Jack *et al.*, 1995; Nes *et al.*, 1996). Judging from the zone sizes (Fig. 4), the amount of bacteriocin released from the transformed *E. coli* cells seemed to be less than that released from the original producer. For this reason, the bacteriocin was purified from the induced *E. coli* cells instead of from the cultured supernatant.

The bacteriocin, called nigrescin, can be successfully purified to homogeneity from the transformed *E. coli* cells by a three-step chromatographic procedure that includes one ion exchange chromatography step and two gel filtration steps, using Sephadex G-25 and Superdex 75 columns. The recent success in purification of peptide LAB bacteriocins makes use of a sequence of cation exchange, hydrophobic interaction, gel filtration and/or reverse phase chromatography step (see for instance Nissen-Meyer *et al.*, 1992). However, nigrescin could not be purified using these sequential chromatographic steps. The hydrophobic interaction and/or reverse phase chromatography steps might perhaps result in denaturation of nigrescin, since it is about 10 times larger than the peptide LAB bacteriocins. The method reported here is unique in that an anion exchange chromatography step is included, enabling a high recovery of active material in the flow-through fraction. Gentle purification steps -the two gel filtration chromatography steps- were used subsequently and inactivation of the

protein was thus avoided. The bacteriocin eluted from the second gel filtration column as a sharp optical density peak (OD_{280}) with an approximate molecular weight of 41 kDa as revealed by SDS-PAGE. Inhibitory activity was associated with the protein band obtained upon SDS-PAGE, since a clear inhibition zone corresponding to the protein band was detected when the SDS gel was laid over with a gel containing the indicator bacterium. The bacteriocin has a rather high molecular weight compared to the molecular weights of the peptide LAB bacteriocins. However, a numbers of bacteria -including *Bacteroides* spp. and *A. actinimycetemcomitan* found in the subgingiva- also produce high molecular weight bacteriocins (molecular weights ranging between 36 and 150 kDa; Cheung *et al.*, 1997; Farias *et al.*, 1994; Stevens *et al.*, 1987).

Addition of nigrescin to cell suspensions of the indicator strains resulted in a decrease in both the optical density (OD_{600}) and the number of viable cells, indicating a lytic and bactericidal mode of action, respectively. The bacteriocin was sensitive to proteinase K, indicating its proteinaceous nature and that it is in fact a bacteriocin. The activity was, however, not affected by pepsin A. Such a difference in susceptibility to different proteolytic enzymes has also been described for other bacteriocins (Gonzalez *et al.*, 1994; Piard *et al.*, 1990), and it was presumed that the active domains of these bacteriocins are not destroyed by some proteolytic enzymes (Ivanova *et al.*, 1998). However, a more likely explanation is that digestion with pepsin has not been performed under optimal conditions. Pepsin A requires an acidic pH (pH ~ 2) in order to function optimally, and the use of phosphate buffer pH 7.0 is thus not suitable for digesting the bacteriocin with pepsin A. The bacteriocin activity of nigrescin was maintained upon heating at 100 ^{O}C for 10 min. Therefore, nigrescin can be used in pasteurized products. It is active over a pH range from 6.5 to 9.5. Its stability in this pH range is important, since it implies that nigrescin may be active in the periodontal pocket, where the pH is between 7.5 and 8.5 depending on states of periodontal diseases. Several studies have reported the concomitantly increased pH of the periodontal pocket with more progressive and advanced periodontal lesions (McDermid *et al.*, 1988). Effect of reducing agents, such as dithiothreitol

(DTT) and β-mercaptoethanol, on the bacteriocin activity was not studied because preliminary results indicated that the reagent control was positive, suggesting their toxicity on the growth of the indicator. But it appears that nigrescin is stable to treatment with 100 mM DDT, since the activity was detected even after boiling the producer, H44(P2), in SDS-loading buffer before performing SDS-PAGE (i.e., 200 mM DTT is present in 2x SDS-loading buffer). This result is also consistent with its heat stability. Nigrescin may be denatured upon heating in the presence of SDS, but it was renatured after SDS removal. Gingival fibroblasts survived, maintained their morphology, and multiplied after being exposed to high concentrations of nigrescin, suggesting that nigrescin is not toxic to the normal cells lining the periodontium.

The criteria for selecting the indicators used in this study were based on the relationship between *Pr. nigrescens* and other oral microorganisms. *Prevotella intermedia* and *Porphyromonas gingivalis* are black-pigmented anaerobic bacteria frequently isolated from diseased periodontal sites. *Tannerella forsythensis* is one of the pathogenic species often isolated concomitantly with the black-pigmented anaerobes. *Lactobacillus, Streptococcus,* and *Actinomyces* are Gram-positive bacteria originally isolated from the supragingival region. *Lactobacillus* and *Streptococcus* cause dental caries, while *Actinomyces* are involved in bacterial interactions that are related to an incidence of periodontal diseases. Nigrescin displayed an attractive bactericidal activity, being active against *T. forsythensis, P. gingivalis,* and *Pr. intermedia*. This killing activity (bactericidal) may reflect a sophisticated mode of competition among bacteria sharing the same ecological niche. The antagonistic activity towards *Actinomyces* may be advantageous to the host in that it would decrease the number of bacteria that cause periodontal diseases. *Lactobacillus*, *Streptococcus*, and *A. actinomycetemcomitans* were, however, insensitive to the killing activity of nigrescin. In the cases of *Lactobacillus* and *Streptococcus* it is not surprising, since most bacteriocins demonstrate a narrow range of activity and often only inhibit the growth of related species. Thus, nigrescin -being produced by the Gram-negative organism *Pr. nigrescens*- would not be expected to be effective against Gram-positive bacteria

including *Lactobacillus* and *Streptococcus* strains. Interestingly, *A. actinomycetemcomitans* was also insensitive to nigrescin. This result is difficult to explain. However, production of bacteriocins by *A. actinomycetemcomitans* strains has been reported (Hammond *et al.*, 1987). It might thus be that a bacteriocin produced by *A. actinomycetemcomitans* antagonizes the action of that produced by *Pr. nigrescens*. The reason why mixed populations of *Pr. nigrescens* and *A. actinomycetemcomitans* have not been reported might be because these organisms use their own bacteriocins as weapons to dominate their habitat. In addition, *A. actinomycetemcomitans* is seldom found in diseased lesions where *P. gingivalis* is frequently isolated. This might suggest that *P. gingivalis* and *A. actinomycetemcomitans* do not compete with each other or share the same ecological niche (Asikainen and Chen, 1999; van Winkelhoff *et al.*, 1994; Rodenburg *et al.*, 1990; Wolff *et al.*, 1985). According to evidences previously described, it is not surprising that nigrescin does not affect the growth of *A. actinomycetemcomitans*.

The specificity test showed that the MICs for nisin were 6 to 20 times greater than those for nigrescin, indicating that nigrescin has a much higher specific activity towards the selected indicators. Nisin is active against both Gram-positive and Gram-negative bacteria (Jack *et al.*, 1995), although it is much less active than nigrescin against the latter (Table 8). The use of nisin for the treatment of gingivitis has been reported (Howell *et al.*, 1993). Based on our results it appears that nigrescin is more suitable than nisin for use against bacteria that cause gingivitis and other gum-diseases including periodontitis.

Nigrescin shows several interesting features that make it an attractive candidate for dental applications, especially for prevention and/or treatment of periodontal diseases.

Chapter 5
Conclusions

According to results, it can be concluded that:

1. The DNA fragment involved in the bacteriocin production of *Pr. nigrescens* ATCC 25261 was found to be located on the chromosome. The bacteriocin genetic system consists of a continuous DNA segment of 4,868 bp.
2. The 4,868 bp-segment contains four major open reading frames (ORFs), designated *nigA* to *nigD*, respectively. All ORFs are oriented in the same direction.
3. Deletion in the putative *nigAB* operon eliminates the bacteriocin-producing ability.
4. Purification of the bacteriocin, termed nigrescin, from *E. coli* transformed with the 4,868 bp-DNA fragment was achieved by using a three-step chromatographic procedure. The purification procedure consists of one ion exchange chromatography step and two gel filtration steps.
5. Nigrescin has an approximate molecular weight of 41 kDa, as judged by SDS-PAGE.
6. *nigA* seems likely to be the bacteriocin structural gene encoding nigrescin, since its gene product has a molecular weight similar to that for nigrescin as determined by SDS-PAGE, whereas *nigB* has several features suggesting that it might be the immunity gene.
7. Nigrescin is stable upon heating at 100 ^{O}C for 10 min and in pHs ranging from 6.5 to 9.5, but it is sensitive to protenase K.
8. Nigrescin inhibits the growth of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, and *Actinomyces* spp.
9. Nigrescin demonstrates a much higher specific activity towards the subgingival bacterial species than nisin.

10. Nigrescin is not toxic to normal cells lining the periodontium, even at very high concentrations.
11. Nigrescin shows several interesting features that make it an attractive candidate in prevention and treatment of periodontal diseases.

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Appendixes

A. Bacterial media

LB Medium (Luria-Bertani Medium) (Sambrook *et al.*, 1989)

Per liter:

To 950 ml of deionized H_2O, add:

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~ 0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

BHI Medium (Brain Heart Infusion Medium) (Self established)

One liter of the medium composes of

Brain heart infusion	37 g

(HiMedia Laboratory Pvt. Limited, 1998)

Yeast extract	5 g

Shake until the solutes have dissolved. Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle. Allow the medium to cool to 50 ^{O}C before adding of 1 % of vitamin K (filter sterilized), and/or 5 % of horse serum.

Blood agar (Self established)

Per liter, it composes of

Brain heart infusion	37 g
Yeast extract	5 g
Haemin	5 mg
Menadinone	1 mg
Agar	20g

Shake until the solutes have dissolved. Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle. Allow the medium to cool to 50 ^{O}C before adding of 5 % of expired human blood.

Media containing agar (Sambrook *et al.*, 1989)

Prepare liquid media according to the recipes given above. Just before autoclaving, add one of the following:

Bacto-agar (for plate) 15 g/liter

Bacto-agar (for top agar) 7 g/liter

Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar evenly throughout the solution. Be careful! The fluid may be superheated and may boil over when swirled. Allow the medium to cool to 50 ^{O}C before adding thermo-labile substances (e.g., antibiotics). To avoid producing air bubbles, mix the medium by swirling. Plates can then be poured directly from the flask; allow about 30-35 ml of medium per 90-mm plate. To remove bubbles from medium in the plate, flame the surface of the medium with a Bunsen burner before the agar hardens. Set up a color code (e.g., two red strips for LB-ampicillin plates; one black strip for LB plates, etc.) and mark the edges of the plates with the appropriate colored markers.

When the medium has hardened completely, invert the plates and store them at 4 ^{O}C until needed. The plates should be removed from storage 1-2 hours before they are used. If the plates are fresh, they will "sweat" when incubated at 37 ^{O}C. This allows bacterial colonies or bacteriophage plaques to spread across the surfaces of the plates and increases the chances of cross-contamination. This problem can be avoided by wiping off any condensation from the lids of the plates and then incubating the plates for several hours at 37 ^{O}C in an inverted position before they are used. Alternatively, the liquid can be removed by shaking the lid with a single, quick motion. To minimize the possibility for contamination, hold the open plate in an inverted position while removing the liquid from the lid.

Culture containing glycerol (Sambrook *et al.*, 1989)

Bacterial cultures growing in liquid media

To 0.85 ml of bacterial culture, add 0.15 ml of sterile glycerol (sterilized by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle). Vortex the culture to ensure that the glycerol is evenly dispersed. Transfer the culture to a labeled storage tube equipped with a screw cap and an airtight gasket. Freeze the culture in ethanol-dry ice or in liquid nitrogen, and then transfer the tube to –70 ^{O}C for long-term storage.

To recover the bacteria, scrape the frozen surface of the culture with a sterile inoculating needle onto the surface of a LB agar plate containing the appropriate antibiotics. Return the frozen culture to storage at –70 ^{O}C. Incubate the plate overnight at 37 ^{O}C.

Bacterial cultures growing on agar plates

Scrape the bacteria growing on the surface of an agar plate into 2 ml of LB medium in a sterile tube. Add an equal volume of LB medium containing 30 % sterile glycerol. Vortex the mixture to ensure that the glycerol is completely dispersed. Dispense aliquots of the glycerinated culture into sterile tubes equipped with screw caps and airtight gaskets. Freeze the cultures as described above.

This method is useful for storing copies of cDNA libraries established in plasmid vectors.

B. DNA manipulation

Preparation of organic reagents (Sambrook *et al.*, 1989)

Phenol

Most batches of commercial liquefied phenol are clear and colorless and can be used in molecular cloning without re-distillation. Occasionally, batches of liquefied phenol are pink or yellow, and these should be rejected and returned to the manufacturer. Crystalline phenol is not recommended because it must be redistilled at 160 ^{O}C to remove oxidation products, such as quinones, that cause the breakdown of phosphodiester bonds or cause cross-linking of RNA and DNA.

Caution: Phenol is highly corrosive and can cause severe burn. Wear gloves, protective clothing, and safety glasses when handing phenol. All manipulations should be carried out in a chemical hood. Any areas of skin that come into contact with phenol should be rinsed with a large volume of water and washed with soap and water. Do not use ethanol.

Equilibration of phenol

Before use, phenol must be equilibrated to a pH > 7.8 because DNA will partition into the organic phase at acid pH.

1. Liquefied phenol should be stored at –20 ^{O}C. As needed, remove the phenol from the freezer, allow it to warm to room temperature and then melt it at 68 ^{O}C. Add hydroxyquinoline to a final concentration of 0.1 %. This compound is an antioxidant, a partial inhibitor of RNAase, and a weak chelator of metal ions. In addition, its yellow color provides a convenient way to identify the organic phase.
2. To the melted phenol, add an equal volume of buffer (usually 0.5 M Tris-HCl pH 8.0 at room temperature. Stir the mixture on a magnetic stirrer for 15 minutes, and then turn off the stirrer. When the two phases have separated, aspirate as much as possible of the upper (aqueous) phase using a glass pipette attached to a vacuum line equipped with traps.

3. Add an equal volume of 0.1 M Tris-HCl (pH 8.0) to the phenol. Stir the mixture on a magnetic stirrer for 15 minutes, and then turn off the stirrer. Remove the upper aqueous phase as described in step 2. Repeat the extractions until the pH of the phenolic phase is > 7.8 (as measured with pH paper).
4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of 0.1 M Tris-HCl (pH 8.0) containing 0.2 % β-mercaptoethanol. The phenol solution may be stored in this form under 100 mM Tris-HCl pH 8.0 in a light-tight bottle at 4 ^{O}C for periods of up to 1 month.

Phenol:Chloroform:Isoamyl alcohol (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1) is frequently used to remove proteins from preparations of nucleic acid. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction.

Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol:chloroform:isoamyl alcohol mixture may be stored under 100 mM Tris-HCl pH 8.0 in a light-tight bottle at 4 ^{O}C for periods of up to 1 month.

Alkaline lysis buffers for minipreparations of plasmid DNA (Sambrook *et al.*, 1989)

Solution I

50 mM gluceose

25 mM Tris-HCl pH 8.0

10 mM EDTA pH 8.0

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/sq.in. on liqiud cycle, and stored at 4 ^{O}C.

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1 % SDS

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

C. Agarose gel electrophoresis

Ethidium bromide (10 mg/ml)

Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature.

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye, and a mask should be worn when weighing it out. After use, these solutions should be decontaminated by one of the methods described elsewhere.

Range of separation for agarose gels (Sambrook *et al.*, 1989)

Agarose (%)	Optimum range of separation Linear DNA (kb)
0.3	60-5.0
0.6	20-1.0
0.7	10-0.8
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

Electrophoresis buffers

Buffer	Working solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	1x: 0.04 M Tris-acetate 0.001 M EDTA	50x: 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA pH 8.0
Tris-phosphate (TPE)	1x: 0.09 M Tris-phosphate 0.002 M EDTA	10x: 108 g Tris base 15.5 ml 85 % phosphoric acid (1.679 g/ml) 40 ml 0.5M EDTA pH 8.0
Tris-borate[a] (TBE)	0.5x: 0.045 M Tris-borate 0.001 M EDTA	5x: 54 g tris base 27.5 g boric acid 20 ml 0.5 M EDTA pH 8.0
Alkaline[b]	1x: 50 mN NaOH 1 mM EDTA	1x: 5 ml 10 N NaOH 2 ml 0.5 M EDTA pH 8.0
Tris-glycine[c]	1x: 25 mM Tris 250 mM glycine	5x: 15.5 g Tris base 94 g glycine pH 8.3 50 ml 10 % SDS (electrophoresis grade)

[a] A precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the 5x solution in glass bottles at room temperature and discard any batches that develop a precipitate. TBE was originally used at a working strength of 1x (i.e., a 1:5 dilution of the concentrated stock) for agarose gel electrophoresis. However, a working solution of 0.5x provides more than enough buffering power, and almost all agarose gel electrophoresis is now carried out with a 1:10 dilution of the concentrated stock.

TBE is used at a working strength of 1x for polyacrylamide gel electrophoresis, twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1xTBE is required to provide adequate buffering power.

[b] Alkaline electrophoresis buffer should be freshly made.

[c] Use Tris-glycine electrophoresis buffer for SDS-polyacrylamide gel.

Gel-loading buffers (Sambrook *et al.*, 1989)

Buffer type	**6x Buffer**	**Storage temperature**
I	0.25 % bromophenol blue 0.25 % xylene cyanol FF 40 %(w/v) sucrose in water	4 ^{O}C
II	0.25 % bromophenol blue 0.25 % xylene cyanol FF 15 % Ficoll (Type 400, Pharmacia) in water	room temp.
III	0.25 % bromophenol blue 0.25 % xylene cyanol FF 30 % glycerol in water	4 ^{O}C
IV	0.25 % bromophenol blue 40 %(w/v) sucrose in water	4 ^{O}C

Gel-loading buffers (continued)

Buffer type	6x Buffer	Storage temperature
V	Alkaline loading buffer	4 °C
	300 mN NaOH	
	6 mM EDTA	
	18 % Ficoll (Type 400, Pharmacia) in water	
	0.15 % bromocresol green	
	0.25 % xylene cyanol FF	

These gel-loading buffers serve three purposes: They increase the density of the sample, ensuring that the DNA drops evenly into the well; they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels approximately 2.2-fold faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5x TBE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5 % to 1.4 %.

Which type of loading dye to use is a matter of personal preference. However, bromocresol green should be used as a tracking dye in alkaline gels because it displays a more vivid color than bromophenol blue at alkaline pH.

D. Determination of the amount of DNA or RNA

For quantitating the amount of DNA or RNA, readings should be taken at wavelengths of 260 and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA, and ~20 μg/ml for single-stranded oligonucleotides. The ratio between the reading at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. If there is contamination with protein or phenol, the OD_{260}/OD_{280} will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

E. Polyacrylamide electrophoresis

30% Acrylamide

Dissolve 29 g of acrylamide and 1 g of N.N′-methylenebisacrylamide in a total volume of 60 ml of H_2O. Heat the solution to 37 ^{O}C to dissolve the chemicals. Adjust the volume to 100 ml with H_2O. Sterile the solution by filtration through a Nalgene filter (0.45 micron pore size). Check that the pH of the acrylamide solution is 7.0 or less, and store the solution in dark bottles at room temperature.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves when handing solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide. Cheaper grades of acrylamide and bisacrylamide are often contaminated with metalions. Stock solutions of acrylamide can easily be purifies by stirring overnight with about 0.2 volume of monobed resin (MB-1, Mallinck-rodt), followed by filtration through Whatman No. 1 paper. During storage, acrylamide and bisacrylamide are slowly converted to acrylic and bisacrylic acid.

10% Ammonium persulfate

To 1 g of ammonium prsulfate, add H_2O to 10 ml. The solution may be stored for several weeks at 4 ^{O}C.

10 % Sodium dodecyl sulfate (SDS)

Dissolve 100 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68 ^{O}C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H_2O. Dispense into aliquots.

Warning: Wear a mask when weighing SDS and wipe down the weighing area and balance after use because the fine crystals of SDS disperse easily. There is no need to sterilize 10 % SDS.

2x SDS gel-loading buffer

100 mM Tris-HCl (pH 6.8)

200 mM dithiothreitol

4 % SDS (electrophoresis grade)

0.2 % bromophenol blue

20 % glycerol

2x SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1 M stock.

Effective range of separation of SDS-polyacrylamide gels

Acrylamide[a] concentration (%)	Linear range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5	57-212

[a] Molar ratio of bisacrylamide:acrylamide is 1:29

Preparing resolving gels (Sambrook *et al.*, 1989)

(for Tris-Glycine SDS-PAGE)

Components	Component volumes (ml) per gel of 10 ml				
	6%	8%	10%	12%	15%
H_2O	2.6	2.3	1.9	1.6	1.1
30 % acrylamide mix	1.0	1.3	1.7	2.0	2.5
1.5 M Tris (pH8.8)	1.3	1.3	1.3	1.3	1.3
10 % SDS	0.05	0.05	0.05	0.05	0.05
10 % ammonium, persulfate	0.05	0.05	0.05	0.05	0.05
TEMED	0.004	0.004	0.004	0.004	0.004

Preparing 5% stacking gels (Sambrook *et al.*, 1989)

(for Tris-Glycine SDS-PAGE gel electrophoresis)

Solution components	**Component volumes (ml) per gel of 4 ml**
H_2O	2.7
30 % acrylamide mix	0.67
1.5 M Tris (pH8.8)	0.5
10 % SDS	0.04
10 % ammonium, persulfate	0.04
TEMED	0.004

Tris-glycine electrophoresis buffer

25 mM Tris

250 mM glycine (electrophoresis grade) pH 8.3

0.1 % SDS

A 5x stock can be made by dissolving 15.1 g of Tris base and 94 g of glycine in 900 ml of deionized H_2O. Then, 50 ml of a 10 % (w/v) stock solution of electrophoresis grade SDS is added, and the volume is adjusted to 1000 ml with H_2O.

Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

1. Dissolve 0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No. 1 filter to remove any particulate matter.
2. Immerse the gel in at least 5 volumes of staining solution and place on a slowly rotating platform for a minimum of 4 hours at room temperature.
3. Remove the stain. Destain the gel by soaking it in the methanol/acetic solution (step 1) without the dye on a slowly rocking platform for 4-8 hrs, changing the destaining solution three or fore times.
4. After destaining, gel may be stored indefinitely in water in a sealed plastic bag without any diminution in the intensity of staining. To avoid gel swelling, store fixed gels in water containing 20 % glycerol.

5. To make a permanent record, either photograph the stained gel or dry gels as described.

F. Amino acid codons and their properties

Codons and amino acids

1st position of codon (5′ terminus)	2nd position of codon: U	2nd position of codon: C	2nd position of codon: A	2nd position of codon: G	3rd position of codon (3′ terminus)
U	UUU Phe (F)	UCU Ser (S)	UAU Tyr (Y)	UGU Cys (C)	U
	UUC Phe (F)	UCC Ser (S)	UAC Tyr (Y)	UGC Cys (C)	C
	UUA Leu (L)	UCA Ser (S)	UAA Stop	UGA Stop	A
	UUG Leu (L)	UCG Ser (S)	UAG Stop	UGG Trp (W)	G
C	CUU Leu (L)	CCU Pro (P)	CAU His (H)	CGU Arg (R)	U
	CUC Leu (L)	CCC Pro (P)	CAC His (H)	CGC Arg (R)	C
	CUA Leu (L)	CCA Pro (P)	CAA Gln (Q)	CGA Arg (R)	A
	CUG Leu (L)	CCG Pro (P)	CAG Gln (Q)	CGG Arg (R)	G
A	AUU Ile (I)	ACU Thr (T)	AAU Asn (N)	AGU Ser (S)	U
	AUC Ile (I)	ACC Thr (T)	AAC Asn (N)	AGC Ser (S)	C
	AUA Ile (I)	ACA Thr (T)	AAA Lys (K)	AGA Arg (R)	A
	AUG Met (M)	ACG Thr (T)	AAG Lys (K)	AGG Arg (R)	G
G	GUU Val (V)	GCU Ala (A)	GAU Asp (D)	GGU Gly (G)	U
	GUC Val (V)	GCC Ala (A)	GAC Asp (D)	GGC Gly (G)	C
	GUA Val (V)	GCA Ala (A)	GAA Glu (E)	GGA Gly (G)	A
	GUG Val (V)	GCG Ala (A)	GAG Glu (E)	GGG Gly (G)	G

Properties of amino acids

Amino acid	Three-letter symbol	One-letter symbol	Mass[a] (Daltons)	pK_a of side chain
Alanine	Ala	A	89.09	
Arginine	Arg	R	174.2	12.48
Asparagine	Asn	N	132.1	
Aspartic acid	Asp	D	133.1	3.86
Cysteine	Cys	C	121.12	8.33
Glutamine	Gln	Q	146.15	
Glutamic acid	Glu	E	147.13	4.25
Glycine	Gly	G	75.07	
Histidine	His	H	155.16	6.0
Isoleucine	Ile	I	131.17	
Leucine	Leu	L	131.17	
Lysine	Lys	K	146.19	10.53
Methionine	Met	M	149.21	
Phenylalanine	Phe	F	165.19	
Proline	Pro	P	115.13	
Serine	Ser	S	105.09	
Threonine	Thr	T	119.12	
Tryptophan	Trp	W	204.22	
Tyrosine	Tyr	Y	181.19	10.07
Valine	Val	V	117.15	

Weighted mean = 126.7

[a] The polymerization of amino acids into polypeptide chain results in a net loss of 18 daltons per peptide bond due to elimination of water during condensation.

G. Rapid cloning in plasmid vectors

The slowest step in cloning in plasmid is the electrophoretic purification of the desired restriction fragment of foreign DNA and the appropriate segment of plasmid DNA. In the protocol given below, ligation of plasmid and foreign DNA is carried out directly in the melted slabs of agarose recovered from the gel used for purification. The method works for both blunt-end ligation and ligation of cohesive termini, although it requires a large amount of ligase and its efficiency is about an order of magnitude lower than the standard procedure.

1. Digest the foreign DNA with the appropriate restriction enzyme(s). The amount of foreign DNA digested should be sufficient to yield approximately 0.2 μg of the target fragment. The digestion should be carried out in a volume of 20 μl or less. In a separate tube, digest 0.5 μg of the vector DNA with the appropriate restriction enzyme(s) in a total reaction volume of 20 μl or less. If the vector DNA carries identical cohesive termini, it should be treated with phosphatase as follows: When digestion with the restriction enzyme(s) is complete, add 2.5 μl of 100 mM Tris-HCl (pH 8.3), 10 mM $ZnCl_2$. Add 0.25 unit of calf intestinal alkaline phosphatase and incubate for 30 minutes at 37 ^{O}C.
2. Separate the desired fragments by electrophoresis on an agarose gel. The gel must be cast with low-melting temperature agarose and it must be poured and run in 1x TAE electrophoresis buffer containing ethidium bromide (0.5 μg/ml) rather than the conventional 0.5x TBE.
3. Examine the gel by long-wavelength ultraviolet illumination. From the relative fluorescent intensities of the desired bands, estimate the amounts of DNA that they contain. Using a razor blade cut out the desired bands in the smallest possible volume of agarose (usually 40-50 μl). Place the excised slices of gel in separate. Labeled microfuge tubes.
4. Heat the tube to 70 ^{O}C for 10-15 minutes to melt the agarose.

5. Combine aliquots of the melted gel slices in a fresh tube pre-warmed to 37 ^{O}C. The final volume of the combined aliquots should be 10 µl or less, and the molar ratio of foreign DNA:plasmid vector should be approximately 2:1. In separate tubes, set up two additional ligations as controls, one contains the plasmid vector alone and the other containe only the fragment of foreign DNA.
6. Incubate the three tubes for 5-10 minutes at 37 ^{O}C, and then add to each tube 10 µl of ice-cold 2x bacteriophage T4 DNA ligase mixture. Mix the contents of the tubes well before the agarose hardens. Incubate the reactions for 12-16 hours at 16 ^{O}C.

   2x bacteriophage T4 DNA ligase mixture is prepared as follows:

1 M Tris-HCL (pH 7.6)	1.0 µl
100 mM $MgCl_2$	1.0 µl
200 mM dithiothreitol	1.0 µl
10 mM ATP	1.0 µl
H_2O	5.5 µl
Bacteriophage T4 DNA ligase	1 Weiss unit

   Mix the components in a tube stored in an ice bath.
7. Towards the end of the ligation, remove from storage at –70 ^{O}C three tubes containing 200 µl of frozen competent *E. coli* each. As soon as the cells have thawed, place them in an ice bath. Immediately proceed to step 8.
8. Remelt the agarose in the ligation mixtures by heating them to 70 ^{O}C for 10-15 minutes.
9. Immediately add 5 µl of one of the ligation mixtures to 200 µl of competent *E. coli*. Mix the contents of the tube quickly by gentle shaking. Repeat this procedure with 5 µl taken from each of the remaining ligation mixtures. Store the transformation mixtures on ice for 30 minutes.
10. Proceed with the remainder of the transformation protocol.

Addition of linkers to blunt-ended DNA

Subcloning with synthetic linkers involves two ligation reactions. In the first reaction, blunt-ended double stranded linkers are attached to the DNA of interest (which must also be blunt-ended). Because the synthetic linkers are very small (8-12 bp), it is relatively easy to achieve the high concentration of termini of the fragment of interest. The reaction is therefore "driven by the linkers, which polymerize onto the blunt-ended termini of the target fragment.

After the synthetic linkers are attached, the ligase is destroyed by heat and the DNA is cleaved by the appropriate restriction enzyme in order to generate cohesive termini. The remnants of the linkers are then removed by gel electrophoresis or chromatography on Sepharose Cl-4B and, in a second ligation reaction, the target DNA is joined to another fragment of interest that carries compatible termini.

Synthetic linkers can be purchased in two forms that carry either a phosphate group or a hydroxy group on their $5'$ termini. Only phosphorylated molecules are substrates for bacteriophage T4 DNA ligase, and non-phosphorylated linkers must therefore be treated with bacteriophage T4 polynucleotide kinase and ATP before they can be joined to DNA. Because enzymatic phosphorylation is never completely efficient, linkers prepared in this way do not work as well as those that have been chemically phosphorylated by the manufacturer. Chemically phosphorylated linkers are therefore preferred, even though they are slightly more expensive.

Enzymatic phosphorylation of nonphosphorylated linkers

(Sambrook *et al.*, 1989)

1. Mix:

10x linker-kinase buffer	1.0 µl
non- phosphorylated linkers	0.5-2.0 µg
10 mM ATP	1.0 µl
H_2O to 10 µl	

10x linker-kinase buffer

0.66 M Tris-HCl (pH 7.6)

0.1 M $MgCl_2$

100 mM dithiothreitol

2 mg/ml bovine serum albumin (Fraction V; Sigma)

This buffer should be stored in small aliquots at –20 °C.

2. Add 2 units of bacteriophage T4 polynucleotide kinase and incubate the reaction for 1 hour at 37 °C. At the end of the phosphorylation reaction, the linkers can be used without further purification in the ligation reactions described below.

Ligation of phosphorylated linkers to blunt-ended target fragments

1. Mix in the order given

blunt-ended DNA	0.1-0.5 µg (in a volume of 7 µl or less)
phosphorylated linkers	1-2 µg (in a volume of 8 µl or less)
H_2O to 15 µl	
5 mM ATP	2 µl
10x blunt-end ligation buffer	2 µl
bacteriophage T4 DNA ligase	1 µl (1-2 Weiss units)

Incubate the reaction for 6-16 hours at 16 °C.

10x blunt-end ligation buffer

0.66 M Tris-HCl (pH 7.6)

50 mM $MgCl_2$

50 mM dithiothreitol

1 mg/ml bovine serum albumin (Fraction V; Sigma)

10 mM haxamminecobalt chloride

5 mM spermidine HCl

This buffer should be stored in small aliquots at –20 ^{O}C.

2. At the end of the incubation, inactivate the ligase by heating the reaction mixture to 65 ^{O}C for 15 minutes.

3. Cool the reaction mixture on ice, and the add:

H_2O	70 µl
Appropriate 10x restriction enzyme buffer	10 µl
Appropriate restriction enzyme	20-50 units

Mix, and then incubate the reaction for 4 hours at the optimal temperature for the restriction enzyme.

4. Add additional 10 units of the restriction enzyme, and continue incubation for another hour.

5. At the end of the incubation, add EDTA (pH 8.0) to a final concentration of 0.01 M. Extract the digested DNA once with an equal volume of phenol:chloroform, and separate the organic and aqueous phase by centrifugation at 12,000g for 30 seconds at room temperature in a micro-tube. Transfer the aqueous phase to a fresh micro-tube.

6. Remove the fragments of linkers by one of the following methods

6.1 Chromatography through a spun column (2 ml) of Sepharose Cl-4B equilibrated in 0.01 M Tris-HCL (pH 7.6), 0.1 M NaCl, 1 mM EDTA (pH 8.0). Mix the sample with 15 µl of gel-loading buffer IV, and apply the mixture directly to the column without further treatment. Start collecting fractions (~125 µl) immediately after the DNA is applied to the column. Locate the DNA of

interest by gel electrophoresis of an aliquot of each fraction. Concentrate the DNA by precipitation with 2 volume of ethanol at 0 ^{O}C.

6.2 Electrophoresis through an agarose of polyacrylamide gel of the appropriate porosity: Concentrate the DNA from the aqueous phase by precipitation with 2 volume of ethanol at 0 ^{O}C. Redissolve the DNA in 10-15 µl of TE (pH 7.6). Add 3-4 µl of the appropriate gel-loading buffer, and load the sample on the gel. After electrophoresis, recover the desired fragment of DNA from the gel.

7. Ligate the fragment of DNA carrying the newly added linkers to a fragment that carries compatible termini.

Notes

1. The spermidine and haxamminecobalt chloride [$(NH_3)_6CoCl_3$] combination increases the efficiency of ligation of linkers approximately fivefold.

2. At least three different units are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units. One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37 ^{O}C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50 % of the *Hin*dIII fragments of bacteriophage λ (5 µg) in 30 minutes at 16^{O}C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

3. Usually, the ligation reaction goes well, and there is no need to check the products of the reaction before proceeding. If necessary, however, the ligation of linkers can be checked in two ways. During ligation, phosphorylated linkers should ligate to form dimers, trimers, tetramers, and longer oligomers that can be detected as a visible smear after an aliquot of the ligation mixture is analyzed by electrophoresis through a 1.8 % agarose gel. Alternatively, a small amount of linkers that have been radiolabeled to high specific activity with [γ-^{32}P]ATP can be included in the ligation reaction. If ligation is successful, the radioactive linkers

should form a series of radioactive bands that can be resolved by electrophoresis of the ligation products through a 15 % polyacrylamide gel. These bands appear as a ladder on autoradiographs of the gel.

4. The effectiveness of the restriction enzyme digestion can be tested as follows:

4.1 Before adding the enzyme in step 3, remove 2 μl from the reaction and store at 4 ^{O}C.

4.2 Withdraw another 2-μl aliquot after adding and mixing the enzyme.

4.3 Add each of the aliquots to separate tubes containing 100 ng (in a volume of 0.5 μl or less) of a linearized plasmid that contains an internal site for the particular restriction enzyme used.

4.4 Incubate the small-scale reaction containing the enzyme along with the large-scale reaction. Store the small-scale reaction without the enzyme at 4 ^{O}C.

4.5 Analyze both small-scale reactions on a 1 % agarose gel. If the reaction works well, the plasmid DNA should be cleaved into two fragments in the sample containing the restriction enzyme.

H. Subcloning

Subcloning fragments of DNA from one type of vector to another, for example, from a recombinant bacteriophage λ to a plasmid, or from one type of plasmid to another is one of the most frequently used procedures in molecular cloning. Subcloning is a simple matter when the restriction sites at the termini of the target fragment are identical to, or compatible with, those of the new vector. The target fragment can then be ligated to the vector without enzymatic manipulation of either piece of DNA. In many cases, however, the termini of the target fragment and vector are incompatible. It is then necessary to convert one or both termini of the DNAs into forms that can be ligated easily. There are four ways that such conversion is commonly accomplished:

1. **Partial filling of incompatible recessed 3′ termini with the Klenow fragment of *E. coli* DNA polymerase I.**

   This frequently generates cohesive termini from recesses 3′ termini that are otherwise incompatible.

2. **Complete filling of incompatible recessed 3′ termini with the Klenow fragment of *E. coli* DNA polymerase I.**

   This generates blunt-ended DNA molecules that can be ligated to any other blunt-ended DNA

3. **Removal of protruding 3′ termini.**

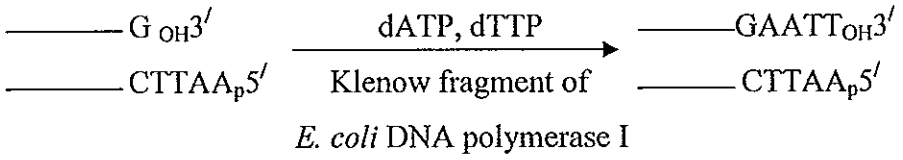
   This can be accomplished in a number of ways such as treating the DNA with nuclease S1, mung-bean nuclease, or the Klenow fragment of *E. coli* DNA polymerase I. However, the enzyme of choice for removal of protruding 3′ termini is bacteriophage T4 DNA polymerase, because of its exceptionally strong $3' \rightarrow 5'$ exonuclease activity. In the presence of high concentrations of all four dNTPs, net removal of nucleotides from the 3′ terminus ceases when the enzyme reaches the double-stranded region of the DNA molecule.

4. Addition of synthetic linkers to blunt-ended DNA.

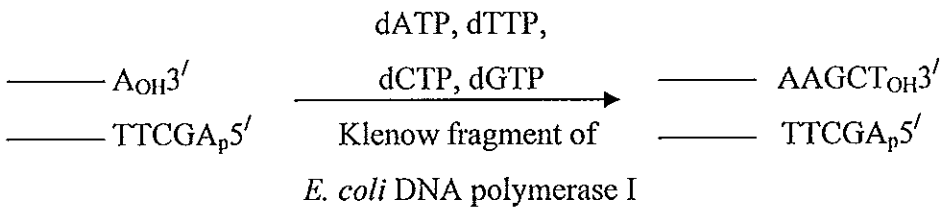
Synthetic linkers are an equimolar mixture of self-complementary, chemically synthesized oligomers that can form blunt-ended duplexes containing one or more recognition sites for restriction enzymes. Ligation of linkers to blunt-ended DNA therefore results in addition of one or more restriction sites that can be used in subcloning. The variety of synthetic linkers is now so large that it is almost always possible to tailor the termini of the target DNA and vector into forms that are ideal for the tasks at hand.

Filling recessed 3′ termini

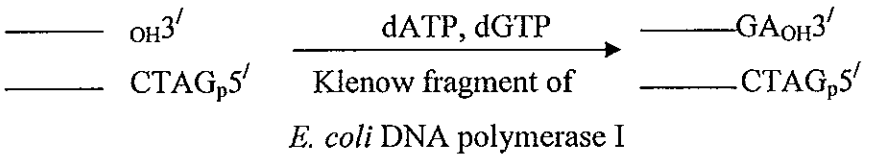
Recessed 3′ termini can be filled by the polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I in the presence of the appropriate dNTPs. Which of the four dNTPs are added to the reaction depends on (1) the sequence of the protruding 5′ termini at the end(s) of the DNA and (2) whether partial or complete filling in required. For example, to fill recessed 3′ termini created by cleavage of DNA by *Eco*RI, only dATP and dTTP need to be present in the reaction:



On the other hand, all four dNTPs are required to fill recessed termini created by *Hin*dIII:



Partial filling of recessed termini created by *Sau*3AI requires the presence of dATP and dGTP. The newly created, short-protruding termini is complementary to a partial filled terminus created by cleavage with *Xho*I:



1. In a 20-µl reaction, digest 0.2-5 µg of DNA with the appropriate restriction enzyme(s).
2. When digestion is complete, add 1 µl of a solution containing each of the desired dNTPs at a concentration of 1 mM.
3. Add 1 unit of the Klenow fragment of *E. coli* DNA polymerase I for each microgram of DNA in the reaction. Incubate the reaction for 15 minutes at room temperature.
4. Inactivate the Klenow fragment of *E. coli* DNA polymerase I and the restriction enzyme(s) present in the reaction by one of the following methods:

   4.1 Heat to 75 ^{O}C for 10 minutes

   Not all restriction enzymes are completely inactivated by this procedure. Check the manufacturer's specifications that are supplied with the enzyme.

   4.2 Add an equal volume of TE (pH 7.6) and extract the solution with phenol:chloroform. Collect the DNA by precipitation with 2 volumes of ethanol.

Notes

1. The Klenow fragment of *E. coli* DNA polymerase I works well in virtually all buffers used for digestion of DNA with restriction enzymes. There is no need to purify the DNA prior to filling recessed 3′ termini created by restriction enzymes.

2. DNA fragments that have been purified by gel electrophoresis before filing of recessed $3'$ termini should be redissolved in TE (pH 7.6). $MgCl_2$ should then be added to a final concentration of 5 mM before the appropriate dNTPs and the Klenow fragment of *E. coli* DNA polymerase I are added.
3. If desired, the DNA can be separated from unincorporated dNTPs by chromatography on, or centrifugation through, small columns of Sephadex G-50. This is not necessary when the filling fragment of DNA is to be used in ligation reactions. Bacteriophage T4 DNA ligase is not inhibited by the presence of dNTPs and works adequately in virtually all buffers used for digestion of DNA with restriction enzymes.

Removing protruding 3/ termini

Both the Klenow fragment of *E.coli* DNA polymerase I and bacteriophage T4 DNA carry a $3' \rightarrow 5'$ exonuclease activity that can be used to remove protruding nucleotides from the $3'$ termini of DNA. Both enzymes lack a $5' \rightarrow 3'$ exonuclease activity and carry a $5' \rightarrow 3'$ polymerizing activity. Despite its higher cost, the bacteriophage enzyme is generally preferred for removal of protruding $3'$ termini because its $3'$ exonuclease activity is more than 200-fold more active than that of the Klenow enzyme.

Bacteriophage T4 DNA polymerase has a pH optimum of 8-9 and displays about 50 % of maximal activity in all buffers that are commonly used for digestion of DNA with restriction enzymes. The exonuclease reaction can therefore be carried out by adding the polymerase directly to the digestion mixture together with high concentrations of the four dNTPs. The enzyme removes protruding $3'$ nucleotides until its reaches the double-stranded region of the DNA molecule, at which point further removal is balanced by incorporation of dNTPs.

1. In a 20-µl reaction, digest 0.2-5 µg of DNA with the appropriate restriction enzyme(s).

2. When digestion is complete, add 1 μl of a solution containing each of the desired dNTPs at a concentration of 2 mM.
3. Add 1-2 unit of bacteriophage T4 DNA polymerase for each microgram of DNA in the reaction. Incubate the reaction for 15 minutes at 12 ^{O}C.
4. Inactivate the bacteriophage T4 DNA polymerase and the restriction enzyme(s) present in the reaction by one of the following methods:

   4.1 Heat to 75 ^{O}C for 10 minutes

   Not all restriction enzymes are completely inactivated by this procedure. Check the manufacturer's specifications that are supplied with the enzyme.

   4.2 Add an equal volume of TE (pH 7.6) and extract the solution with phenol:chloroform. Collect the DNA by precipitation with 2 volumes of ethanol.

Notes

1. At 37 ^{O}C, the turnover number of the 3′ exonucleolytic activity of bacteriophage T4 DNA polymerase is some threefold higher than that of its polymerizing activity. At lower temperature, there is a smaller differential between the two activities, and exonucleolytic digestion is therefore carried out at 12 ^{O}C.
2. DNA fragments that have been purified by gel electrophoresis before filing of recessed 3′ termini should be redissolved in TE (pH 8.0). $MgCl_2$ should then be added to a final concentration of 5 mM before the appropriate dNTPs and the bacteriophage T4 DNA polymerase are added.
3. If desired, the DNA can be separated from unincorporated dNTPs by chromatography on, or centrifugation through, small columns of Sephadex G-50. This is not necessary when the filling fragment of DNA is to be used in ligation reactions. Bacteriophage T4 DNA ligase is not inhibited by the presence of dNTPs and works adequately in

virtually all buffers used for digestion of DNA with restriction enzymes.

I. Performing DNA sequencing

Preparing the PCR reactions

For each reaction add the following reagents to a separate tube:

Terminator Ready Reaction Mix	0.8 µl
Template (double stranded DNA)	500 ng
Primer	3.2 pmol
Deionized water	q.s.
Total volume	20 µl

Cycle sequencing on the GeneAmp 2400

Repeat the following for 25 cycles:

96 ^{O}C for 10 sec

50 ^{O}C for 5 sec

60 ^{O}C for 4 min

4 ^{O}C for α (Hold at 4 ^{O}C until ready to purify)

Purifying extension product

Unincorporated dye terminators must be completely removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with base calling.

Precipitation methods are cheaper and faster, but they remove less of the unincorporated dye terminators that can obscure data at the beginning of the sequence.

With ethanol precipitation, traces of unincorporated terminators may be seen at the beginning of the sequence data (up to base 40), but this is usually minimal.

The protocol for ethanol precipitation:

Pipet the entire contents of each extension reaction into a 1.5-ml microcentrifuge tube. Add 16 µl of deionized water and 64 µl of non-denatured 95 % ethanol (the final ethanol concentration should be 60 ± 3 %). Close the tubes and vortex briefly. Leave the tubes at room temperature for 15 min to precipitate the extension products (precipitation times shorter than 15 min will result in the loss of very short extension products, precipitation times longer than 24 h will increase the precipitation of unincorporated dye terminators). Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 min at maximum speed. Carefully aspirate the supernatants with a separate pipette tip for each sample and discard, pellets may or may not be visible. Add 250 µl of 70 % ethanol to the tubes and vortex them briefly. Place the tubes in a microcentrifuge in the same orientation as in previous step and spin for 10 min at maximum speed. Aspirate the supernatants carefully. Dry the samples in a vacuum centrifuge for 10-15 min or to dryness. Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 ^{O}C for 1 min.

Sample electrophoresis

Prepare a loading buffer by combining the 5:1 ration of deionized formamide and 25 mM EDTA (pH 8.0) with blue dextran (50 mg/ml). Resuspend each sample pellet in 6-8 µl of loading buffer, vortex and spin the samples. Heat the samples at 95 ^{O}C for 2 min to denature. Place on ice until ready to load. Load 0.75-1.5 µl of each sample into a separate lane of the gel

J. Competent *E. coli*

JM 109

JM 109 is a useful host in cloning pGEM vectors and for production of single-stranded DNA from M13 or phagemid vectors. The strain grows well and efficiently transformed by a variety of methods. Because JM 109 is *rec*A$^-$ and lacks the *E. coli* K restriction system, undesirable restriction of cloned DNA and recombination with host chromosomal DNA are prevented. The endonuclease A$^-$ mutation leads to an improved yield and quality of isolated plasmid DNA.

JM 109 can be used for blue/white color screening of the pGEM-Z and pGEM-Zf vectors. JM 109 is deficient in β-galactosidase activity due to deletions in both genemic and episomal copies of the *lac*Z gene. The deletion in the episomal (F factor) copy of the *lac*Z gene (*lac*ZΔM15) is located in the α-peptide region and, as a result, β-galactosidase activity can be complemented by addition of a functional α-peptide, and cells carrying these plasmids are able to produce functional β-galactosidase.

When plated on indicator media containing X-gal and IPTG, the host / plasmid combination will generate blue colonies. However, when the α-peptide is disrupted by cloning into the multiple cloning region of the pGEM-Z or pGEM-Zf vectors, complementation does not occur and no-β-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM-Z vector constructs remain white.

JM 109 should always be maintained on minimal plates (M-9) supplemented with 1 mM thiamine-HCl. This selects for the presence of the F$'$ episome which carries a nutritional requirement for growth (proline biosynthesis) and decreases the number of false positive.

BL21(DE3)pLysS

Bacterial strain BL21(DE3)pLysS is lysogenic for λDE3, which contains the T7 bacteriophage gene *I*, encoding T7 RNA polymerase under the control of the *lac* UV5 promoter. BL21(DE3)pLysS also contains a plasmid pLysS, which carries the gene encoding T7 lysozyme. T7 lysozyme lowers the background expression level of target genes under the control of T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. This strain can be used with protein expression vector under the control of T7 promoter.

K. Miscellaneous

X-gal (5-Bromo-4-chloro-3-indolyl-β-galactoside)

Make a stock solution by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution. Use a glass or polypropylene tube. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at –20 ^{O}C. It is not necessary to sterilize X-gal solution by filtration.

IPTG

Isopropylthio-β-D-galactoside (M.W. = 238.3) Make a solution of IPTG by dissolving 2 g of IPTG in 8 ml of distilled H_2O. Adjust the volume of the solution to 10 ml with distilled H_2O and sterilize by filtration through a 0.22-micron disposable filter. Dispense the solution into 1-ml aliquots and store them at –20 ^{O}C.

1M Dithiothreitol (DTT)

Dissolve 3.09 g of DTT in 20 ml of 0.01 M sodium acetate (pH 5.2). Sterilize by filtration. Dispense into 1-ml aliquots and store at –20 ^{O}C.

β-Mercaptoethanol

Usually obtained as a 14.4 M solution. Store in a dark bottle at 7 ^{O}C. Do not autoclave β-Mercaptoethanol or solution containing β-Mercaptoethanol.

Phosphate-buffered saline (PBS)

Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O. Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle. Store at room temperature.

Thesis-related accomplishments

1. Publication

Kaewsrichan J., Douglas C.W.I., Nissen-Meyer J., Fimland G., Teanpaisan R. 2004. Characterization of a bacteriocin produced by *Prevotella nigrescens* ATCC 25261. Letters in Applied Microbiology (accepted)

2. Presentation*

Kaewsrichan J., Nissen-Meyer J., Fimland G., Teanpaisan R. Factors affecting bacteriocin production of *Prevotella nigrescens* ATCC 25261. 82nd General Session & Exhibition of the International Association for Dental Research. March 10 – 13, 2004. Hawaii Convention Center, Honolulu, Hawaii, USA.

Teanpaisan R., Kaewsrichan J., Douglas C.W.I. Characterization of a recombinant bacteriocin from *Prevotella nigrescens* ATCC 25261. 82nd General Session & Exhibition of the International Association for Dental Research. March 10 – 13, 2004. Hawaii Convention Center, Honolulu, Hawaii, USA.

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3. Patent

Title: ท่อนยีนและโปรตีนที่ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียกรัมลบ

Thai Application Patent No. 084761

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