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### ***N*-Tetradecanoyl Homoserine Lactone, Signaling Compound for Quorum Sensing, Inhibits *Porphyromonas gingivalis* Growth**

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**Abstract:** The present study investigated the influence of synthetic *N*-acyl homoserine lactones (*N*-acyl HSLs), signaling compounds for quorum sensing, on the growth and production of proteins in periodontopathic bacteria *Porphyromonas gingivalis*. *N*-butyryl HSL, *N*-hexanoyl HSL, *N*-heptanoyl HSL, *N*-octanoyl HSL and *N*-tetradecanoyl (myristoyl) HSL were used as synthetic *N*-acyl HSLs (Sigma-Aldrich). *N*-tetradecanoyl HSL inhibited the growth of all *P. gingivalis* strains used in this study in a dose-dependent manner. When we compared the SDS-PAGE profiles of sonicated samples of *P. gingivalis* bacterial cells cultured with/without *N*-tetradecanoyl HSL, we found that protein production was changing. The growth of *P. gingivalis* was inhibited by myristoyl coenzyme A (CoA) but did not by lauroyl CoA. These results indicated that *P. gingivalis* responded to auto-produced *N*-tetradecanoyl HSL-like molecule and slowed their growth.

**Key words:** Biofilm, quorum sensing, *N*-acyl homoserine lactones (*N*-acyl HSLs), *Porphyromonas gingivalis*, periodontitis

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#### **Introduction**

Recently, dental plaque has characterized as biofilm and the formation is associated with intercellular communication; quorum sensing (QS) (McNab *et al.*, 2003). QS is widespread among gram negative and gram positive bacteria (Fuqua *et al.*, 1994; 1996; Miller and Bassler, 2001; Whitehead *et al.*, 2001). QS bacteria synthesize and secrete extracellular signaling molecules. When a critical threshold concentration of autoinducer is attained, a signal transduction cascade is triggered, resulting in the density dependent regulation of gene expression and a change in behavior of the organism for facilitating environmental adaptation (Engebrecht *et al.*, 1983; Fuqua and Greenberg, 1998). The predominant signaling molecules of gram-positive bacteria are peptides, while in gram-negative bacteria, different system of QS, which use different type of signaling molecules, has so far been described (Bassler, 1999). *Vibrio harveyi* is free-living gram-negative marine bacterium that possesses some different systems (Bassler *et al.*, 1994). The autoinducer of gram-negative bacteria has been identified as an *N*-acyl homoserine lactone (*N*-acyl HSL) (Cao and Meighen, 1989; Bassler *et al.*, 1993; Shaw *et al.*, 1997), whereas the structure of the autoinducer for one of other QS systems has been called AI-2. In the case of *V. harveyi* the hydroxybutyryl homoserine lactone is the autoinducer. Frias *et al.* (2001) suggested that the gram negative periodontal organisms do not possess *N*-acyl HSL-dependent signaling systems. However, several of these organisms such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* secrete signals functionally related to AI-2 (Fong *et al.*, 1993, 2001; Chung *et al.*, 2001; Burgess *et al.*, 2002).

*P. gingivalis* has frequently been isolated from periodontal lesions and shown to be related to the onset and progression of periodontal disease (Slots *et al.*, 1986; Slots and Listgarten, 1988; Mayrand

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and Holt, 1988; Van Winklehoff *et al.*, 1988). Although previous researchers were unable to obtain any evidence to suggest that the gram negative anaerobe *P. gingivalis* employs *N*-acyl HSL-mediated QS, they have demonstrated that this periodontopathic bacteria produces a diffusible signal molecule capable of activating the *V. harveyi* AI-2 biosensor (Chung *et al.*, 2001; Frias *et al.*, 2001; Burgess *et al.*, 2002). The objective of the present study was to investigate whether *P. gingivalis* responds to *N*-acyl HSLs. Therefore we investigated the influence of synthetic *N*-acyl HSLs on the growth and production of proteins in this organism.

## Materials and Methods

### *Bacterial Strains and Culture Conditions*

*P. gingivalis* ATCC 33277, ATCC 53977, Su63 and W50, *Prevotella intermedia* ATCC 25611, *Prevotella loescheii* ATCC 15930, *Fusobacterium nucleatum* ATCC 25586 and *A. actinomycetemcomitans* Y4 were used in this study. These strains were maintained anaerobically on blood agar plates containing trypticase soy agar (Becton Dickinson Microbiology System, Cockeysville, MD, USA) supplemented with 10% defibrinated horse blood, hemin (5  $\mu\text{g mL}^{-1}$ ; Sigma Chemical Co., St. Louis, MO, USA) and menadione (0.5  $\mu\text{g mL}^{-1}$ ; Wako Pure Chemical Industries, Osaka, Japan). The bacterial strains were cultured anaerobically in trypticase soy broth (Becton Dickinson Microbiology System) supplemented with hemin and menadione at 37.

### *Effect of Synthetic N-acyl HSLs or Acyl Coenzyme A on the Growth*

*N*-butyryl-DL-homoserine lactone (HSL), *N*-hexanoyl HSL, *N*-heptanoyl HSL, *N*-octanoyl HSL and *N*-tetradecanoyl (myristoyl) HSL were used as synthetic *N*-acyl HSLs (Sigma-Aldrich Co., Steinheim, Germany). Each *N*-acyl HSL was added to the culture broth at various concentrations (100, 10 and 1  $\mu\text{M}$ ) and its influence on the growth of tested bacteria evaluated. Growth was monitored by optical density (OD) at 660 nm. In order to confirm survival for 48 h, 100  $\mu\text{L}$  aliquots were taken from cultures with *N*-acyl HSL showing no bacterial growth, inoculated onto blood agar plates and cultured for one week under anaerobic conditions.

In order to study the effect of acyl coenzyme A (acyl CoA) on *P. gingivalis* autoinducer synthesis, *P. gingivalis* ATCC 33277 cells were cultured in the broth containing myristoyl CoA or lauroyl CoA (100 and 10  $\mu\text{M}$ ; Doosan Serdary Research Laboratories, Toronto, Canada). Growth was monitored by OD at 660 nm.

### *SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis*

*P. gingivalis* ATCC 33277 and ATCC 53977 were cultured with/without *N*-tetradecanoyl HSL for 24 h. Cells were harvested by centrifugation. Harvested cells were suspended in distilled water and disrupted by sonication. The protein content of sonicated sample was adjusted to 1 mg protein per ml. The adjusted samples were suspended in SDS-PAGE loading buffer (Laemmli, 1970) and boiled for 10 min. The samples were loaded on the gel. SDS-PAGE was done in 10 to 20% gradient micro slab gels (Daiichi Pure Chemical Co., Tokyo, Japan) and stained with Coomassie brilliant blue (CBB; Amersham Biosciences AB, Uppsala, Sweden).

## Results

### *Effects of Synthetic N-acyl HSLs on the Growth*

*N*-tetradecanoyl HSL completely inhibited the growth of all *P. gingivalis* strains tested in this study at 100  $\mu\text{M}$  (Table 1). Growth inhibition of this *N*-acyl HSL was dose-dependent (Fig. 1). *P. gingivalis* cells from cultures showing no growth formed many colonies (data not shown), indicating

that this microorganism survived at least 48 h in the broth containing *N*-tetradecanoyl HSL. Also *N*-tetradecanoyl HSL slightly inhibited the growth of *P. intermedia* and *F. nucleatum* at 100  $\mu$ M (Table 3). In this study, no synthetic *N*-acyl HSL affected the growth of any other oral bacteria tested.

*Effects of Acyl CoA on the P. Gingivalis Growth*

Myristoyl CoA inhibited the growth of *P. gingivalis* in a dose- dependent manner (Table 2). However lauroyl CoA showed no inhibitory effect on the growth of *P. gingivalis*.

*Effect of N-tetradecanoyl HSL on the Protein Expression*

When we compared the SDS-PAGE profiles of sonicated samples of *P. gingivalis* bacterial cells cultured with or without *N*-tetradecanoyl HSL. The effects of *N*-tetradecanoyl HSL on the protein expression of *P. gingivalis* ATCC 33277 and ATCC 53977 are shown in Fig. 2. We found that SDS-PAGE profile of *P. gingivalis* ATCC 33277 obviously changed by addition of *N*-tetradecanoyl HSL. In ATCC 53977, some protein bands disappeared by addition of this synthetic homoserine lactone. We confirmed that the alterations of the protein profile were not due to contamination by gram staining and culture as black-pigmented gram-negative coccobacillus.

Table 1: Effects of *N*-acyl HSLs on the growth of *P. gingivalis* strains

<i>N</i> -acyl HSLs	<i>P. gingivalis</i> strains			
	ATCC 3327	ATCC 53977	W 50	Su 64
<i>N</i> -tetradecanoyl HSL				
100 $\mu$ M	-	-	-	-
10 $\mu$ M	+	±	+	±
1 $\mu$ M	++	+	++	++
<i>N</i> -butyryl HSL 100 $\mu$ M	++	+	++	++
<i>N</i> -hexanoyl HSL 100 $\mu$ M	++	+	++	++
<i>N</i> -heptanoyl HSL 100 $\mu$ M	++	+	++	++
<i>N</i> -octanoyl HSL 100 $\mu$ M	+	±	++	+
Control	++	+	++	++

Growth was monitored by Optical Density (OD) at 660 nm, ++:  $0.3 \leq OD_{660}$ , +:  $0.15 \leq OD_{660} < 0.3$ , ±:  $0.08 \leq OD_{660} < 0.15$ , -:  $OD_{660} < 0.08$

Table 2: Effects of acyl CoA on the growth of *P. gingivalis* ATCC 33277

Acyl CoA	Concentration ( $\mu$ M)	Culture time	
		24 h	48 h
Myristoyl CoA	100	-	-
	10	-	+
Lauroyl CoA	100	+	+
	10	++	++
Control	-	++	++

Growth was monitored by Optical Density (OD) at 660 nm, ++:  $0.3 \leq OD_{660}$ , +:  $0.15 \leq OD_{660} < 0.3$ , ±:  $0.08 \leq OD_{660} < 0.15$ , -:  $OD_{660} < 0.08$

Table 3: Effects of *N*-acyl HSL on the growth of oral bacteria

<i>N</i> -acyl HSLs ( $\mu$ M)	<i>P. intermedia</i>	<i>P. loescheii</i>	<i>F. nucleatum</i>	<i>A. actinomycetem</i>
	ATCC 25611	ATCC 15930	ATCC 25586	-comitans Y 4
<i>N</i> -tetradecanoyl HSL				
100	+	+	±	+
10	+	+	+	+
1	++	+	+	+
<i>N</i> -butyryl HSL 100	++	+	+	+
<i>N</i> -hexanoyl HSL 100	++	+	+	+
<i>N</i> -heptanoyl HSL 100	++	+	+	+
<i>N</i> -octanoyl HSL 100	++	+	+	+
Control	++	+	+	+

Growth was monitored by Optical Density (OD) at 660 nm, ++:  $0.3 \leq OD_{660}$ , +:  $0.15 \leq OD_{660} < 0.3$ , ±:  $0.08 \leq OD_{660} < 0.15$ , -:  $OD_{660} < 0.08$

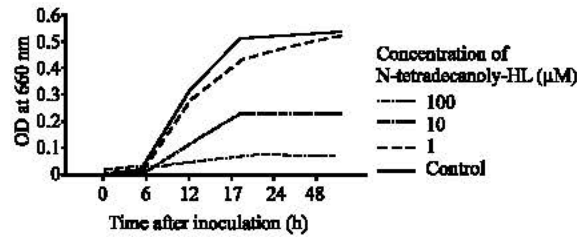


Fig. 1: Inhibitory effect of *N*-tetradecanoly HSL on the growth of *P. gingivalis* ATCC 33277

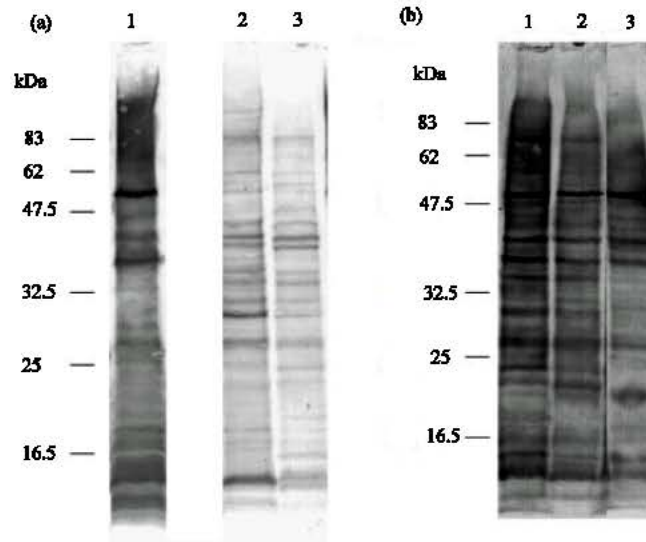


Fig. 2: SDS-PAGE profile of *P. gingivalis* sonicated samples (CBB staining), a) ATCC 33277, b) ATCC 53977. Lanes, 1 Sonicated sample of *P. gingivalis* intact cells, 2 Sonicated sample of *P. gingivalis* cells cultured with 10 μM *N*-tetradecanoly HSL, 3 Sonicated sample of *P. gingivalis* cells cultured with 1 μM *N*-tetradecanoly HSL

## Discussion

Since bacteria within the biofilms reach a high density, it has been suggested that QS might play a key role in bacterium-bacterium communication and, therefore, in the formation of biofilm (Schripsema *et al.*, 1996; Fong *et al.*, 2001). QS mechanisms control the production of virulence factors in some species of bacteria (Passador *et al.*, 1993; De Kievit and Iglewski, 2000; Winzer and Williams, 2001). *P. gingivalis* has been long considered one of the main periodontopathic bacteria, playing an important role in bone and tissue destruction (Holt *et al.*, 1999). It is absent in health and during disease reaches an important portion of the total population and has the capability of producing a large number of virulence factors. In the periodontal pocket, *P. gingivalis* is found predominantly as a component of complex biofilm containing multiple bacterial species. To facilitate adaptation to life within the oral cavity, *P. gingivalis* must be capable of sensing and responding to prevailing environmental conditions, including variations in temperature, oxygen tension, pH, nutrient availability and the presence of other cells (Xie *et al.*, 1997; Lamont and Jenkinson, 1998).

*N*-acyl HSL dependent QS systems exist in many gram negative bacteria (Fuqua *et al.*, 1994; 1996; Miller and Bassler, 2001; Whitehead *et al.*, 2001), however several investigators (Chung *et al.*,

2001; Frias *et al.*, 2001; Burgess *et al.*, 2002) suggested that periodontal bacteria including *P. gingivalis* do not possess *N*-acyl HSL dependent signaling circuits. In their reports, those bacteria were tested for production of extracellular autoinducer-like activities that stimulates the expression the genes in biosensors of *V. harveyi* or *Chromobacterium violaceum*. It suggests that *P. gingivalis* does not employ *N*-acyl HSLs which are able to stimulates the biosensors. The objective of the present study was to investigate whether *P. gingivalis* possesses *N*-acyl HSL dependent QS system by the assay using synthetic *N*-acyl HSLs. *N*-tetradecanoyl HSL inhibited the growth and affected on protein production in *P. gingivalis*. The growth of *P. gingivalis* was inhibited by myristoyl CoA but did not by lauroyl CoA, suggesting that *P. gingivalis* responds to auto-produced *N*-tetradecanoyl (myristoyl) HSL-like molecule(s) and slows their growth.

The growth of some strains of *Rhizobium leguminosarum* bv. *viciae* is inhibited by *N*-(3-hydroxy-7-*cis* tetradecanoyl) HSL, which was previously known as the *small* bacteriocin (Van Brussel *et al.*, 1985; Schripsema *et al.*, 1996; Thorne and Williams, 1999; Wilkinson *et al.*, 2002). The *cinRI* locus is at the top of a regulatory network QS loci in *R. leguminosarum* bv. *viciae* (Lithgow *et al.*, 2000). *CinI*, LuxI homologue, produces the *N*-(3-hydroxy-7-*cis* tetradecanoyl) HSL and *CinR* is a LuxR-type regulator. *CerI*, which is also LuxI homologue, in *Rhodobacter sphaeroides* synthesizes *N*-(7-*cis*-tetradecanoyl) HSL (Puskus *et al.*, 1997). Laue *et al.* (2000) demonstrated that *Pseudomonas fluorescens* F113 makes at least three different *N*-acyl HSLs including *N*-(3-hydroxy-7-*cis* tetradecanoyl) HSL and identified a gene *hdtS* which does not belong the LuxI or LuxM family of *N*-acyl HSL synthases. Interrogation of the *P. gingivalis* W83 genome sequence database (www.tigr.org) for homologues of *N*-acyl HSL synthases belonging to either the LuxI or LuxM family failed to identify any candidates. However, one open reading frame (ORF) with significant homology with *HdtS* amino acid sequence was identified (TIGR locus: PG1249, 1-acyl-sn-glycerol-3-phosphate acetyltransferase, putative). The ORF showed 25% identity and 48% amino acid similarity with the *HdtS* over 201 amino acids. The ORF possesses two motifs which are conserved in the lysophosphatidic acid acyltransferases (West *et al.*, 1993; Shih *et al.*, 1999). It is possible that the ORF is an acyltransferase which transfers acyl-chains onto a substrate to generate some *N*-acyl HSLs. Further study is required to define its function for *N*-acyl HSL formation. *N*-octanoyl HSL showed weak inhibitory effect on growth of *P. gingivalis* (Table 1). Wilkinson *et al.* (2002) suggested that the growth inhibition of *N*-(3-hydroxy-7-*cis* tetradecanoyl) HSL in *R. leguminosarum* required the presence of other *N*-acyl HSLs such as *N*-octanoyl HSL. In the case of *P. gingivalis*, *N*-octanoyl HSL may be associated with the growth inhibition of *N*-acyl HSLs.

The present study suggests that *P. gingivalis* produces *N*-tetradecanoyl HSL-like signaling molecule and responds to this *N*-acyl HSL as if there had been an increase in cell density and changed protein production to become more fit for habitation in the worse environment.

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