In vitro effects of *N*-acetyl cysteine alone and in combination with antibiotics on *Prevotella intermedia*

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N-acetyl cysteine (NAC) is an antioxidant that possesses anti-inflammatory activities in tissues. In the field of dentistry, NAC was demonstrated to prevent the expression of LPS-induced inflammatory mediators in phagocytic cells and gingival fibroblasts during the inflammatory process, but the effect of NAC on oral pathogens has been rarely studied. Here, we examined the effect of NAC against planktonic and biofilm cells of *Prevotella intermedia*, a major oral pathogen. NAC showed antibacterial activity against the planktonic *P. intermedia* with MIC value of 3 mg/ml and significantly decreased biofilm formation by the bacterium even at sub MIC. NAC did not affect the antibiotic susceptibility of planktonic *P. intermedia*, showing indifference (fractional inhibitory concentration index of 0.5–4) results against the bacterium in combination with ampicillin, ciprofloxacin, tetracycline or metronidazole. On the other hand, viability of the pre-established bacterial biofilm exposed to the antibiotics except metronidazole was increased in the presence of NAC. Collectively, NAC may be used for prevention of the biofilm formation by *P. intermedia* rather than eradication of the pre-established bacterial biofilm. Further studies are required to explore antibacterial and anti-biofilm activity of NAC against mixed population of oral bacteria and its modulatory effect on antibiotics used for oral infectious diseases.

Keywords: *Prevotella intermedia*, *N*-acetyl cysteine, biofilm, modulator of antibiotic activity, oral pathogen

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Introduction

Prevotella intermedia has long been known to be associated with oral diseases such as periodontal diseases (Ashimoto *et al.*, 1996; Maeda *et al.*, 1998; Mombelli *et al.*, 2000), periapical periodontitis (Gomes *et al.*, 1994, 1996; Jacinto *et al.*, 2003) and noma (an acute gangrenous disease) (Falkler *et al.*, 1999; Bolivar *et al.*, 2012). It has been suggested that dental plaque biofilm may act as a reservoir of respiratory pathogens, especially in patients with periodontal disease (Scannapieco *et al.*, 1998). In fact, *P. intermedia* colonizes in the respiratory tract and is associated with cystic fibrosis and chronic bronchitis (Shinzato and Saito, 1994; Brook and Frazier, 2003; Ulrich *et al.*, 2010). One reason for difficulty in controlling *P. intermedia* is that the bacterium has resistance to many antibiotics including penicillins, cephalosporins, and tetracyclines (Andrés *et al.*, 1998; Fosse *et al.*, 2002). Moreover, *P. intermedia* cells produce biofilms in which the bacterial cells become more resistant to antibiotics (Takahashi *et al.*, 2006).

N-acetyl cysteine (NAC) is a potent thiol-containing antioxidant which serves as a precursor of glutathione synthesis (Gokcimen *et al.*, 2007; Kaplan *et al.*, 2008). Stimulation of glutathione synthesis following administration of NAC results in greater availability of glutathione for the detoxification of oxygen-derived free radicals (Ross, 1988). NAC is widely used in medical treatments of chronic bronchitis (Stey *et al.*, 2000; Quah *et al.*, 2012). The positive effects of NAC treatment have primarily been attributed to its mucus-dissolving properties by disrupting disulfide bonds in the mucus and its ability to decrease biofilm formation, resulting in significant reductions in bacterial infections (Olofsson *et al.*, 2003). In the context of oral diseases, NAC has shown to prevent the expression of LPS-induced inflammatory mediators (IL-1β, -6, and -8) in phagocytic cells and gingival fibroblasts during the inflammatory process (Hsu and Wen, 2002; Kim *et al.*, 2007; Toker *et al.*, 2009). Recently, it was shown that NAC inhibits the growth of *Enterococcus faecalis*, which is often associated with endodontic treatment failures (Rôças *et al.*, 2004), and eradicates its biofilm (Quah *et al.*, 2012). Moreover, it has been proposed that NAC can be a substitute for ibuprofen for post-endodontic pain (Ehsani *et al.*, 2012). However, the effect of NAC on other oral pathognes has not been studied. In consideration of the above, the present investigation was undertaken to study the effect of NAC on the growth and biofilm formation of *P. intermedia*. As NAC is known to be an important modulator of antibiotic activity against several bacterial strains (Goswami and Jawali, 2010), we also examined the effect of NAC on the antibiotic susceptibility of *P. intermedia*.

Materials and Methods

Bacterial strain and culture condition

P. intermedia ATCC49046 was obtained from the American Type Culture Collection. The bacterium was grown on either brucella agar (Difco Laboratories) containing 5% laked sheep blood, 5 μg/ml of hemin, and 1 μg/ml of vitamin K_1 or brucella broth (Difco) containing 5 μg/ml of hemin and 1 μg/ml of vitamin K_1 (B-HK) at 37°C under an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. In some experiments, bacterial suspension was subcultured in the media which were adjusted pH to 5.0 or 6.0 by addition of concentrated HCl.

Chemicals

NAC (Sigma Chemical Co.) was dissolved in distilled water at a concentration of 50 mg/ml. Ampicillin, ciprofloxacin, metronidazole, and tetracycline (Sigma) were also dissolved in distilled water to make a 50 mg/ml solution.

Assessment of minimum inhibitory concentrations (MICs) and planktonic bacterial growth

MICs of NAC and antibiotics were determined by agar dilution method as described in our previous study (Moon *et al.*, 2011). The MIC was defined as the lowest concentration that inhibited the bacterial growth on brucella blood agar according to CLSI guidelines (CLSI, 2007). To examine the effect of NAC on the planktonic bacterial growth, a 24-h culture of *P. intermedia* was adjusted to an optical density of 0.15–0.2 at 600 nm (OD_{600}), dispensed (100 μl/well) into a polystyrene 96-well plate containing various concentrations of NAC in B-HK (100 μl). Several identical microtiter plates were prepared for absorbance measurement, viable count enumeration and pH measurements. Then, the plates were incubated at 37°C anaerobically. At various time-points between 0 and 48 h, OD_{600} and culture pH were recorded, and at the time-points, the culture aliquots were serially diluted and plated on brucella blood agar to enumerate the number of viable bacterial cells. Bacterial doubling times were determined by dividing the time interval considered with the number of rounds of replication (n), which was calculated as follows: $n = \ln(CFU_2/CFU_1)/\ln2$, where CFU_1 and CFU_2 are the numbers of CFU obtained at the beginning and end of the time interval, respectively (Chong *et al.*, 2008; Moon *et al.*, 2013).

Biofilm formation and quantification of biofilm biomass

Biofilm formation assay was performed as described previously (Honma *et al.*, 2009; Moon *et al.*, 2013) with some modifications. Briefly, *P. intermedia* was grown to early exponential phase and then adjusted to an OD_{600} of approximately 0.3. The bacterial suspension was dispensed (200 μl/well) into triplicate wells of 48-well plates containing various concentrations of NAC in B-HK (200 μl) and incubated at 37°C anaerobically. Heat-killed bacterial cells that were initially killed by exposure to 100°C for 10 min were included as controls. After 24 h of incubation, planktonic and loosely bound bacterial cells were removed by aspirating the spent media, and then the remaining biofilm cells were stained with 0.1% crystal violet for 10 min. The plates were washed three times with distilled water, air dried, and then the bound dye was solubilized in 400 μl of 95% ethanol, and OD_{600} was recorded.

Scanning electron microscopy (SEM)

Biofilms of *P. intermedia* ATCC49046 were developed with or without NAC on a polystyrene 48-well plate as described above. After washing with physiological saline, the remaining biofilm cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h, postfixed with 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4°C, and dehydrated through a graded ethanol series. The samples were dried by critical point drying, coated with gold using a sputter-coater (IB-3, Eiko), and observed by a Hitachi S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc.). The SEM images were analyzed using ImageJ (NIH) for cell length measurement. Mean cell length was determined from 15 to 20 randomly chosen cells per SEM image.

Viscosity of spent culture media

P. intermedia ATCC 49046 cells were grown in B-HK containing NAC at sub-MICs. After a 24-h incubation, the culture medium was centrifuged to remove cells, and then the viscosity of the spent culture medium was measured at 25°C using an SV-10 vibroviscometer (A&D Company Ltd).

Measurement of fractional inhibitory concentration index (FICI)

The effect of NAC on the antibiotic susceptibility of planktonic *P. intermedia* cells was assessed by measuring FICI as described previously (Zhao and Liu, 2010) with some modifications. Briefly, a two-dimensional checkerboard in a 96-well microtiter plate with 7 doubling dilutions of NAC and 11 doubling dilutions of each antibiotic in aliquots of 100 μl of B-HK was made. After doubling dilutions, each well of the plate was inoculated with the culture of *P. intermedia* to yield the appropriate density ($1–2 \times 10^8$ cells/ml) in a 200 μl final volume and incubated for 48 h at 37°C anaerobically. FICI was calculated for each combination using the following formula: FICA + FICB = FICI, where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB = MIC of drug B in combination/MIC of drug B alone (Zhao and Liu, 2010). The FICI was interpreted as follows: FICI ≤ 0.5 = synergy; FICI > 4.0 = antagonism; and FICI > 0.5 but ≤ 4) = indifference (Zhao and Liu, 2010; Barbee *et al.*, 2014).

Determination of viability of pre-established biofilm

Biofilms of *P. intermedia* ATCC49046 were developed without test agents on a polystyrene 96-well plate as described above. Then the pre-established biofilms were washed once with physiological saline, and the supernatant saline containing non-adherent cells was removed by gentle pipette aspiration without disturbing the established biofilms at the bottom of the plates. Then, 200 μl of B-HK containing

test agents at various concentrations were dispensed in triplicate into each well of the 96-well plate. The plate incubated for 24 h was washed twice with physiological saline, and the biofilm cells were dispersed in 200 μl of physiological saline by pipetting and scraping the bottom of the wells using a pipette tip, as described previously (Simmons and Dybvig, 2007; Lemos *et al.*, 2010). The viability of the biofilm cell suspension was determined by a commercial LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen) according to the manufacturer's instructions. Briefly, the bacterial suspension was mixed with 1 μl of a mixture of SYTO 9 and propidium iodide (1:1), and incubated in the dark for 15 min at room temperature. Subsequently, 200 μl of each sample suspension were pipetted into a black 96-well microplate (Corning Costar) and analyzed. The fluorescence emission was measured at 535 nm for SYTO 9 (green emission) and 625 nm for propidium iodide (red emission). Both dyes were excited at 485 nm. The percentage of live cells in the suspension was plotted as a function of the ratio between the green and red fluorescence intensities.

Confocal laser scanning microscopy (CLSM)

P. intermedia biofilms were established on glass-bottom confocal dish (SPL Lifesciences) as described above. Following exposure to 1.5 mg/ml NAC, 10 μg/ml ampicillin or NAC + ampicillin for 24 h, the biofilms were stained with LIVE/DEAD *Bac*Light Bacterial Viability Kit as described above and examined under a confocal laser scanning microscope (Nikon D-Eclipse C1si, Nikon).

Statistical analyses

Statistical analyses were performed in accordance with the results of Shapiro-Wilk test of normal distribution. The data were further analyzed by use of 1-way ANOVA, followed by the Tukey's honestly significant difference (HSD) multiple comparison post hoc test. All values were expressed as mean ± SD. All statistical analyses were performed using IBM SPSS version 22 statistical software (IBM SPSS).
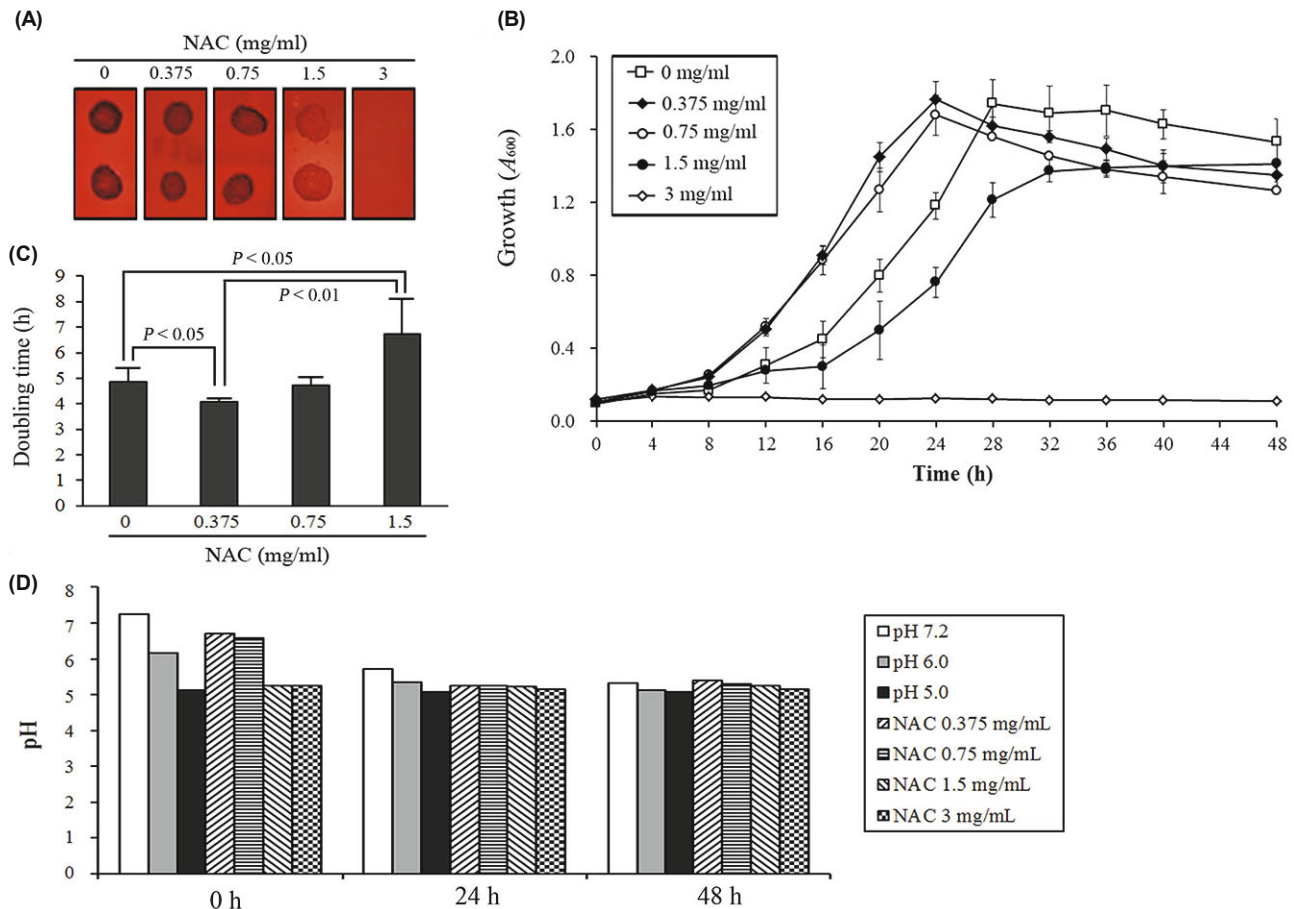

Fig. 1. Effects of NAC on the growth of *P. intermedia* ATCC49046. (A) MIC determination of NAC by agar dilution method. The bacterial cells were spot-inoculated (10^5 CFU/spot) onto brucella blood agar plates containing NAC at various concentrations and incubated at 37°C for 3 days anaerobically. The MIC was defined as the lowest concentration that inhibited the bacterial growth on the plate. (B) Bacterial growth kinetics. The growth of *P. intermedia* was expressed as optical density at 600 nm (shown as OD_{600}) for the cultures in 96-well plates for 48 h at 37°C with and without NAC. OD_{600} of the bacterial cell cultures was measured every 4 h. Results are expressed as mean ± SD of two independent experiments performed in triplicate. (C) Doubling times (h) of *P. intermedia* ATCC49046 grown in the presence and absence of NAC. (D) Culture pH during the bacterial growth.

Results

Effect of NAC on the growth of *P. intermedia*

By agar dilution method, MIC of NAC against *P. intermedia* ATCC49046 was determined to be 3 mg/ml (Fig. 1A). Effect of NAC on the bacterial growth was further monitored in B-HK by determination of OD_{600} and the bacterial doubling time. As shown in Fig. 1B, the bacterial growth was completely inhibited in the presence of NAC at 3 mg/ml. In the presence of 1.5 mg/ml NAC ($1/2 \times$ MIC), the doubling time of the bacterial cells significantly increased (Fig. 1C). On the other hand, the replication of *P. intermedia* was faster when the bacterial cells were exposed to NAC at concentrations of $1/8$–$1/4 \times$ MIC, with the doubling times of 4.9 ± 0.46 h for control, and 4.1 ± 0.13 h ($1/8\times$ MIC; 0.375 mg/ml) and 4.2 ± 0.17 h ($1/4 \times$ MIC; 0.75 mg/ml) for the bacterial cells exposed to NAC. Since the addition of NAC

(0.375–3 mg/ml) lowered the initial pH of B-HK from 7.2 down to 5.23, we also measured the culture pH during the bacterial growth (Fig. 1D). The bacterial cells grown in media with initial pH values between 6.0 and 7.2 (pH 6.0 and pH 7.2 media and the media [initially pH 7.2] containing 0.375 and 0.75 mg/ml NAC) decreased the culture pH down to 5.1–5.3 after 48-h incubation. The bacterial cells grown in media with initial pH values below 5.3 (pH 5.0 medium and the media with 1.5 and 3 mg/ml NAC) maintained the culture pH within 0.1 unit of the initial pH during 48 h of incubation.

Effect of NAC on the biofilm formation by *P. intermedia*

We investigated the effect of NAC on formation of *P. intermedia* biofilm by the crystal violet staining method. *P. intermedia* cells were consistently able to form biofilm on the polystyrene surface of the 48-well plates over the 24-h incubation period. Biofilm formation by *P. intermedia* was not reduced in the presence of NAC at $1/8$–$1/4 \times$ MIC. However, the biofilm formation was significantly affected by NAC at $1/2$–$1 \times$ MIC (Fig. 2A). A small amount of biofilm was formed by heat-killed *P. intermedia* cells. In pH 5 medium, the amount of the biofilm biomass reduced by 30%, but the amount was still significantly higher compared to that formed in the presence of 3 mg/ml NAC. The ultrastructure of the bacterial cells in the biofilms was observed under a scanning electron microscope (Fig. 2B). In the absence of NAC, *P. intermedia* cells formed a uniform three-dimensional structure and exhibited normal rod-shaped morphology with a cell length of 1.51 ± 0.20 μm. In the medium artificially adjusted initial pH to 5.0, the bacterial cells were observed to be shorter (0.99 ± 0.32 μm) than the cells in the medium at pH 7.2 without NAC ($P < 0.001$). The biofilm cells grown with 1.5 mg/ml NAC appeared irregular in shape, but their average length remained unaltered (1.41 ± 0.33μm, $P > 0.05$). In the biofilms exposed to 3 mg/ml NAC, very few *P. intermedia* cells were observed attached on the plate and appeared shorter and more irregular in shape than the bacterial cells in pH 5.0 medium. Some small pieces of cell debris were also found.

Effect of NAC on the production of extracellular polymeric substances (EPS) by *P. intermedia*

EPS is the primary matrix material of biofilm, and viscosity of culture supernatant is an indication of the relative amount of
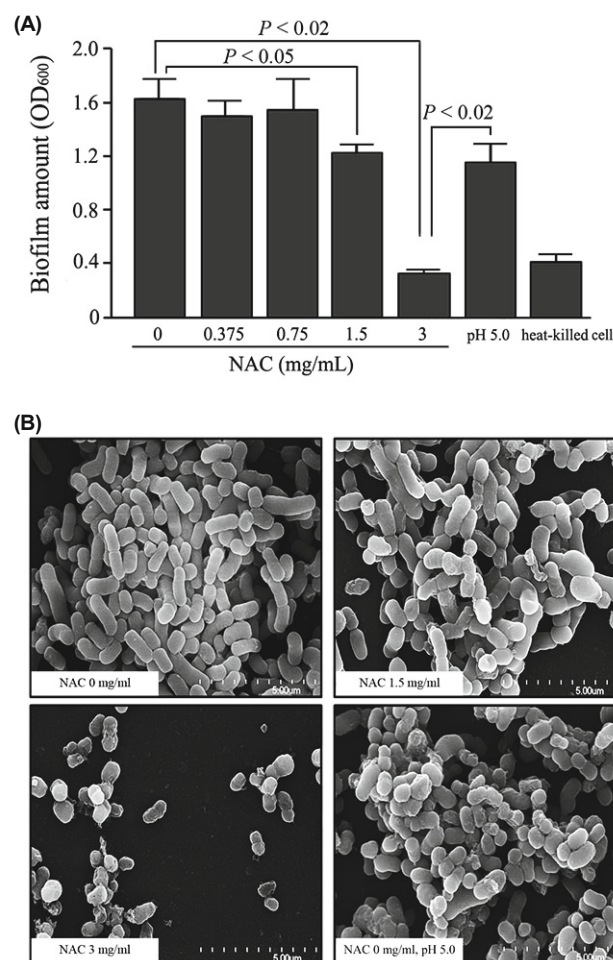

(A)

(B)

Fig. 2. Effects of NAC on biofilm biomass and ultrastructure of *P. intermedia* ATCC49046. (A) Analyses of the biofilm biomass of *P. intermedia* ATCC49046. *P. intermedia* cells grown to an OD_{600} of approximately 0.3 in B-HK broth were further incubated in 48-well plates with or without NAC for 24 h. The biofilm biomass was quantitated by crystal violet staining method. Data are mean ± SD of three independent experiments. (B) Scanning electron microscope (SEM) images of *P. intermedia* cells in the biofilm after a 24-h of incubation.

Table 1. Minimum inhibitory concentrations (MICs) of antibiotics against *P. intermedia* ATCC49046 and fractional inhibitory concentration index (FICI) for combination of NAC with antibiotics

	FICI *	MICs of antibiotics		MICs of NAC	
		without NAC	with NAC	without antibiotics	with antibiotics
Ampicillin	0.63	0.25	0.0313	3.0	1.5
Ciprofloxacin	2.0	1.5	1.5	3.0	3.0
Metronidazole	1.0	3.5	1.75	3.0	1.5
Tetracycline	2.0	0.5	0.5	3.0	3.0

* FICI was calculated for each combination using the following formula: FICA + FICB = FICI, where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB = MIC of drug B in combination/MIC of drug B alone.
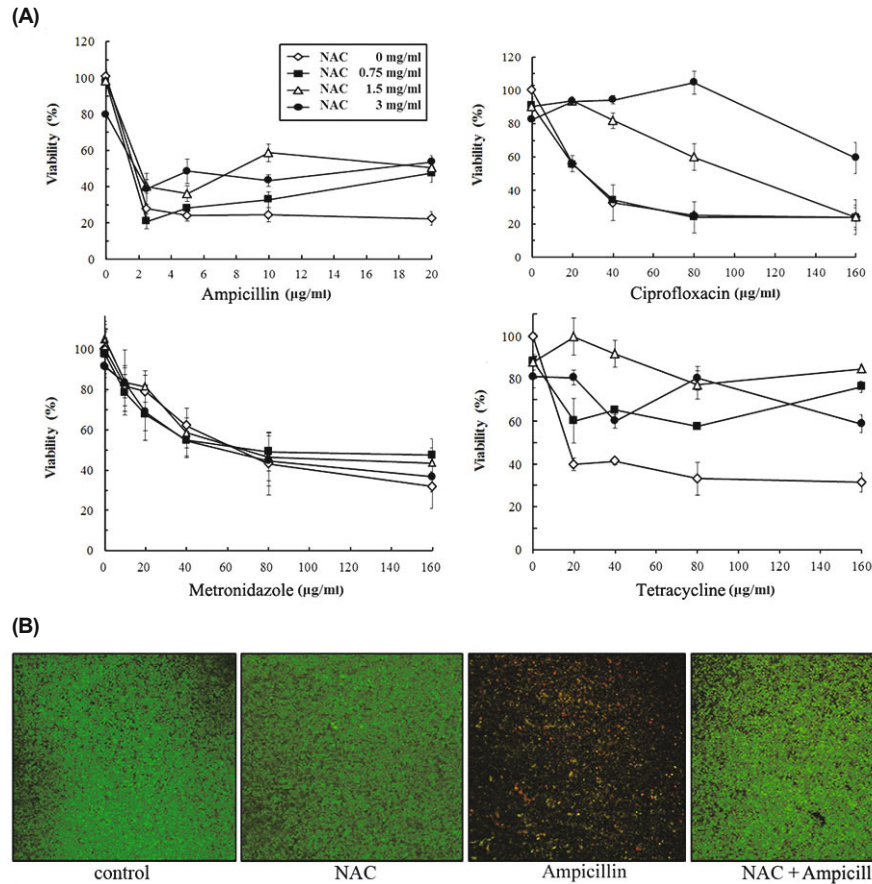
(A)

(B)

Fig. 3. **Effect of NAC on the antibiotic susceptibility of *P. intermedia*.** (A) Viability of the pre-established *P. intermedia* biofilms exposed to antibiotics with or without NAC. The biofilm viability was determined by a commercial LIVE/DEAD *Bac*Light bacterial viability kit. (B) CLSM images of the *P. intermedia* biofilms. The bacterial biofilms were exposed to 1.5 mg/ml NAC, 10 μg/ml ampicillin or both for 24 h. Live and dead cells are visualized in green and red, respectively. Data are mean ± SD of three independent experiments. Magnification power is 600 ×.

EPS production in the culture (Denny *et al.*, 1988; Donlan, 2002). To identify whether NAC affects EPS production by *P. intermedia*, we measured the viscosity of the spent culture medium of the bacterium. It was demonstrated that NAC (0.75–3 mg/ml) did not significantly affect the viscosity of the bacterial spent culture medium (data not shown).

Effect of NAC on the antibiotic susceptibility of *P. intermedia*

The effect of NAC on the antibiotic susceptibility of planktonic *P. intermedia* cells was assessed by FICI measurement. The bacterial cells had FICI of between 0.5 and 4.0 for the combination of NAC with ampicillin, ciprofloxacin, metronidazole or tetracycline (Table 1). We also examined the effect of NAC on the antibiotic susceptibility of *P. intermedia* cells in already established biofilm on the 96-well plate. As shown in Fig. 3A, NAC up to 3 mg/ml did not significantly affect the viability of the pre-established biofilm. The biofilm viability was significantly decreased by single antibiotic: ampicillin (> 2 μg/ml), ciprofloxacin, metronidazole, and tetracycline (> 20 μg/ml). In the presence of NAC ranging 0.75–3 mg/ml, the anti-biofilm effect of metronidazole was not affected by NAC, but the effect of the other three antibiotics was decreased.

The viability of the pre-established *P. intermedia* biofilms exposed to 1.5 mg/ml NAC, 10 μg/ml ampicillin or NAC-ampicillin combination was further examined by CLSM. The biofilms were stained with LIVE/DEAD *Bac*Light Bac-

terial Viability Kit consisting of green-fluorescent SYTO 9, which stains live bacteria, and red-fluorescent propidium iodide, which only penetrates non-viable bacterial cells with damaged/perturbed cell membranes (Alkawareek *et al.*, 2012). While the pre-established biofilms exposed to NAC and NAC-ampicillin combination were shown to be predominantly green (viable) as well as unexposed control biofilm, the fraction of red color (non-viable) increased in the ampicillin-exposed biofilm (Fig. 3B).

Discussion

NAC is a non-antibiotic compound that possesses antibacterial properties (Olofsson *et al.*, 2003; Zhao and Liu, 2010). The growth of various aerobic and facultative bacteria, such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Bacillus cereus*, *Pseudomonas mendocina*, *Bacillus megaterium*, and *Staphylococcus warneri*, was completely inhibited in the presence of 0.4–2 mg/ml NAC (Olofsson *et al.*, 2003; Goswami and Jawali, 2010). The antibacterial activity of NAC is likely to be achieved by inhibition of amino acid (cysteine) utilization in bacteria or reaction of its sulfhydryl group with bacterial cell proteins. In the present study, NAC showed a growth inhibitory effect (MIC 3 mg/ml) against planktonic *P. intermedia*, while the replication of the bacterium was faster in the presence of NAC at concentrations of 0.375 and 0.75 mg/ml (Fig. 1).

Strictly anaerobic bacteria such as those found in diseased periodontal sites may be described as bacteria that grow in highly reduced conditions (negative *Eh* values) or absence of free oxygen (Leke *et al.*, 1999). To meet this requirement, reducing agents such as sodium thioglycollate and L-cysteine HCl are often supplemented to medium to prevent the accumulation of peroxides which are lethal to some anaerobes. Leke *et al.* (1999) reported that, depending on the types of culture media, *Eh* values of the uninoculated media vary from -62 mV to -107 mV under anaerobic atmosphere and that maximum growth of *Porphyromonas gingivalis* was observed in the presence of 3 mM cysteine (initial *Eh* ranging from -137 mV to -156 mV). Therefore, it is conceivable that the presence of the thiol reducing agent NAC ranging from 0.375 to 0.75 mg/ml (corresponding to 2.3–4.6 mM) may lower redox potential of the culture medium, resulting in slight stimulation of *P. intermedia* growth.

Meanwhile, *P. intermedia* consumes not only nitrogenous compounds but also carbohydrates as additional energy sources for the bacterium (Takahashi *et al.*, 1997; Takahashi and Yamada, 2000). Takahashi and Yamada (2000) reported that the addition of 0.5% glucose to culture media increased the growth rate and the cell yield of *P. intermedia*, and that the glucose contained in the media was consumed by the growing bacterial cells and thus the culture pH was lowered to 5.3 after 48 h. Similarly, we also observed that, in brucella media which originally contain dextrose, the bacterial cells decreased pH steadily until the pH reached about 5.1–5.3 when initial pH value was 6.0–7.2 (Fig. 2B). On the other hand, the bacterial cells grown in the media with initial pH values below 5.3 maintained the culture pH within 0.1 unit of the initial pH. In our preliminary experiment, pH 5.0 medium partially limited *P. intermedia* growth, showing the maximum OD_{600} value of approximately 1.2, while the bacterial growth in pH 6.0 medium reached OD_{600} value of approximately 1.6, which was similar to that observed in neutral condition (pH 7.2) (data not shown). This is consistent with another previous observation of the reduced growth yield of *P. intermedia* in acidic media (pH 5.0–5.5) (Takahashi *et al.*, 1997), although the bacterium has been reported to be able to grow over a wide range of pH (5.0–8.0) (Takahashi and Schachtele, 1990; Takahashi *et al.*, 1997). It seems that acidic pH below 6.0 affected fermentation pattern of *P. intermedia* and consequently reduced the bacterial growth, suggesting that pH is the most important factor determining glucose fermentation (Koussémon *et al.*, 2003; Rao *et al.*, 2004). Notably, the growth of *P. intermedia* was completely inhibited in the medium supplemented with 3 mg/ml NAC, despite the fact that its pH during the culture was almost same as the culture pH values of the medium containing 1.5 mg/ml NAC and pH 5.0 medium, both of which did not inhibit the bacterial growth completely. Besides, the SEM images revealed that morphological change of the bacterial cells exposed to NAC was different from that of the cells grown in pH 5.0 medium. All of our observations may imply that NAC exerts antibacterial effect against *P. intermedia* in which the mechanism of its action cannot be fully explained by a pH-lowering effect.

NAC has shown to decrease biofilm formation by a variety of bacteria and reduce the production of EPS (Pérez-Giraldo

et al., 1997; Marchese *et al.*, 2003; Olofsson *et al.*, 2003; Schwandt *et al.*, 2004). In some bacteria such as *Pseudomonas aeruginosa* and slime-producing uropathogenic *Escherichia coli* strains, EPS production and biofilm formation were significantly decreased by NAC at concentrations as low as 0.5 mg/ml (Marchese *et al.*, 2003; Zhao and Liu, 2010). Zhao and Liu (2010) reported that among 20 clinical isolates of *P. aeruginosa*, MICs of NAC for 18 isolates were 10 to 40 mg/ml and MICs for another 2 isolates were > 40 mg/ml. In their study, however, even mature biofilm of *P. aeruginosa* PAO1 strain could be detached by 0.5 mg/ml NAC and was completely disrupted by 10 mg/ml NAC. In the present study, the amount of biofilm formed by *P. intermedia* ATCC49046 was decreased in the presence of 1.5 and 3 mg/ml NAC (1/2 and 1 × MIC). As shown in Fig. 2A, even dead cells of *P. intermedia* were able to attach to some extent to the polystyrene surface probably *via* physicochemical adhesion (Carvalho, 2013). It was also observed that the growth of *P. intermedia* in the presence of 3 mg/ml NAC was completely inhibited, and *P. intermedia* cells grown to early exponential phase yet formed a small amount of biofilm when exposed to 3 mg/ml NAC. These results suggest that this *P. intermedia* biofilm in a small quantity may have consisted mostly of dead cells. On the other hand, the EPS production, reflected by viscosity of culture media, was not significantly affected by NAC. In addition, the pre-established *P. intermedia* biofilm was not disrupted by NAC up to 12 mg/ml (data not shown). Therefore, it is quite reasonable to speculate that the effect of NAC to reduce biofilm formation of *P. intermedia* is mostly due to its inhibitory effect on the bacterial cell growth thereby total number of the bacterial cells decreased, rather than reduction in EPS production or disruption of the preformed biofilm. Taking into account our findings given above, use of NAC may be effective to prevent biofilm formation by inhibiting growth of *P. intermedia* rather than eradication of the pre-established bacterial biofilm.

NAC is a thiolic antioxidant produced by the body, which serves as a precursor of glutathione synthesis (Gokcimen *et al.*, 2007; Kaplan *et al.*, 2008). Glutathione is a sulfur-containing nucleophilic substance found in high concentrations in the kidney and plays a central role in protecting cells from oxygen-derived free radical injury and other activated toxic compounds (Kaplan *et al.*, 2008). It has been found that some antibiotics such as ciprofloxacin stimulate the induction of reactive oxygen species (ROS) in several bacterial species (Becerra and Albesa, 2002; Albesa *et al.*, 2004), and both NAC and glutathione affect antibiotic susceptibility of a number of bacteria (Goswami and Jawali, 2007, 2010; Goswami *et al.*, 2007). However, such modulatory effects do not seem necessarily related to the antioxidant properties. As an example, glutathione has shown to provide *E. coli* for substantial protection against ciprofloxacin and other fluoroquinolones while other antioxidants, such as histidine, mannitol, and sodium pyruvate, did not alter the ciprofloxacin sensitivity (Goswami *et al.*, 2006). Moreover, streptomycin sensitivity of *E. coli* is also reduced by glutathione but the protection is not due to the antioxidant-mediated scavenging of ROS (Goswami *et al.*, 2007). More recently, it was reported that NAC reduced the antibacterial activity of

aminoglycosides, fluoroquinolones, and erythromycin against several bacteria, including opportunistic respiratory pathogens *Klebsiella* and *Pseudomonas* (Goswami and Jawali, 2010). On the other hand, the antibacterial efficacy of β-lactams was increased in the presence of either NAC or glutathione (Goswami and Jawali, 2007, 2010). It has been supposed that, considering that β-lactams act at the transpeptidation step of cell wall synthesis, the glutathione-mediated augmentation of β-lactam antibacterial activity could be due to the competition between glutathione uptake process and gamma-glutamyl transepeptidase-mediated cell wall synthesis (Goswami and Jawali, 2007).

  Here, we examined the effect of NAC on the antibiotic susceptibility of planktonic and biofilm *P. intermedia* cells by measuring FICI and biofilm viability. The antibiotic susceptibility of planktonic *P. intermedia* cells was not affected by NAC, while modulatory effects of NAC on antibiotic activity against the bacterial biofilms were observed. Unexpectedly, efficacy of ampicillin, a β-lactam antibiotic, was decreased in the presence of NAC. Moreover, antibacterial activity of tetracycline against *P. intermedia* biofilm was also decreased unlike previous observations in which the presence of NAC did not alter the MIC of tetracycline against several other bacteria (Goswami and Jawali, 2010). The underlying mechanism of this alteration in antibiotic susceptibility of *P. intermedia* remains unrevealed; however, our findings support the previous study (Goswami and Jawali, 2010) showing that administration of NAC during antibiotic therapy could modulate the outcome of the therapeutic process, depending on the target bacterial pathogen and antibiotic being used for the therapy. Our results also indicate that NAC exerts different antibiotic modulatory effects on the planktonic cells and the biofilm even in the same strain. It is noteworthy that, in a range of pH 5.0–7.2, antibiotics used in the present study showed similar antibacterial effects against *P. intermedia*, except ciprofloxacin which showed slightly reduced antibacterial activity against both the planktonic and the biofilm bacterial cells in acidic pH (data not shown). Drug compound or active ingredient of ciprofloxacine is not significantly affected by acidic pH (Ali, 2014), but it has been found that the antibacterial activity of ciprofloxacin against some bacteria such as *E. coli* and *P. aeruginosa* was affected by human urine and in particular by its pH (Kamberi *et al.*, 1999). Therefore, we cannot entirely exclude the possibility that pH-lowering effect of NAC is associated with the reduction in ciprofloxacin susceptibility of *P. intermedia* biofilm.

  The treatment efficacy of NAC was demonstrated in clinical trials and experimental models of various respiratory conditions, such as chronic obstructive pulmonary disease, cirrhosis, portal hypertension, and acetaminophen poisoning (Zafarullah *et al.*, 2003; Vercelino *et al.*, 2008). In the periodontal field, the benefit of NAC was also demonstrated by several studies in which it prevented the expression of LPS-induced inflammatory mediators during the inflammatory process (Hsu and Wen, 2002; Kim *et al.*, 2007) and bone resorption (Lee *et al.*, 2005). In addition, it has been proposed that NAC can be a substitute for ibuprofen for post-endodontic pain (Ehsani *et al.*, 2012) and an alternative controlling agent against *Enterococcus faecalis* biofilm (Quah *et al.*,

2012). Although the combined use of NAC with antibiotics will require careful studies, but its clinical usefulness as a controlling agent for oral infectious diseases would not be limited. Further studies are also needed to explore antibacterial and anti-biofilm activity of NAC against mixed population of oral pathogens and the mechanism underlying the modulatory effect of NAC on antibiotic susceptibility against biofilm as well as planktonic cells of diverse bacterial pathogens.

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