

# Ultrastructural effects and antibiofilm activity of LFchimera against *Burkholderia pseudomallei*

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Received: 14 July 2015 / Accepted: 28 November 2015 / Published online: 11 January 2016  
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**Abstract** Lactoferrin chimera (LFchimera), a hybrid peptide containing the two antimicrobial stretches of the innate immunity factor bovine lactoferrin, viz. LFamp-in265-284 and LFCin17-30, has strikingly high antimicrobial activity against the category B pathogen *Burkholderia pseudomallei*. The action mechanisms of LFchimera against *B. pseudomallei* is not fully understood. The aim of this study was to further investigate the effect of treated *B. pseudomallei* with LFchimera using (immune) electron microscopy. The effects of LFchimera on biofilm formation and against preformed biofilm of *B. pseudomallei* were also determined. After exposure to LFchimera, transmission electron microscopy revealed swelling of the

periplasmic space of *B. pseudomallei* and a highly inhomogeneous electron density in the intracellular DNA region. Localization of LFchimera in *B. pseudomallei* using immunoelectron microscopy showed gold particles in intracellular structures without accumulation on the membranes. LFchimera also possessed stronger bactericidal activity than ceftazidime against *B. pseudomallei* grown in biofilm. Moreover, limited exposure of *B. pseudomallei* to LFchimera at subcidal concentration could reduce biofilm formation. Altogether, the results indicate that LFchimera possesses antibacterial and antibiofilm activities and can modulate *B. pseudomallei* colonization. Therefore, the efficacy of LFchimera merits further development of this agent for the therapy of melioidosis.

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**Keywords** Antimicrobial peptide · Biofilm · *Burkholderia pseudomallei* · LFchimera · (Immune) electron microscopy · Melioidosis

## Introduction

The environmental saprophytic bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis, a fatal infectious disease of both humans and animals. It is a Gram negative, motile, non-spore-forming bacillus which can be isolated from soil and water in Southeast Asia and Northern Australia (Wiersinga et al. 2012). The clinical manifestations of melioidosis is variable and ranges from localized and chronic infection to acute fulminant sepsis (Limmathurotsakul and Peacock 2011). *Burkholderia pseudomallei* is classified by the Centers for Disease Control and Prevention as a category B warfare agent (Rotz et al. 2002). The treatment of melioidosis is quite difficult because *B. pseudomallei* is intrinsically resistant to a

diverse group of antibiotics with those regularly used for the practical treatment of sepsis (Dance 2014). Currently, the most commonly used antimicrobials for treating melioidosis are ceftazidime (CAZ), imipenem, amoxicillin-clavulanate, doxycycline, and trimethoprim-sulfamethoxazole, but there are several reports on in vitro and in vivo resistance to these drugs among *B. pseudomallei* isolates (White 2003; Cheng and Currie 2005; Limmathurotsakul and Peacock 2011). Moreover, *B. pseudomallei* are even more resistant to antibiotics when switched to the biofilm mode of growth (Sawasdidoln et al. 2010; Pibalpakdee et al. 2012; Bandeira Tde et al. 2013), which emphasizes the importance of treating melioidosis with antimicrobials that are effective against *B. pseudomallei* biofilms and less prone to develop resistance. Therefore, the development of novel anti-infective agents against *B. pseudomallei* both in planktonic and biofilm form is increasingly mandatory.

Lactoferricin (LFcin) and lactoferrampin (LFampin), two antimicrobial domains stretches of the innate immunity factor bLF, have been used for the development of series of antimicrobial peptides with broad spectrum activity against a range of Gram-positive and Gram-negative bacteria as well as yeast, like *Candida albicans* (Bellamy et al. 1992; Van Der Kraan et al. 2004; Valenti and Antonini 2005; van der Kraan et al. 2005; Van Der Kraan et al. 2006; Haney et al. 2007). A hybrid peptide (LFchimera) containing both LFampin265-284 and LFcin17-30 has strikingly higher antimicrobial activities than the individual peptides (Bolscher et al. 2009; Leon-Sicaireos et al. 2009; Flores-Villasenor et al. 2010; Bolscher et al. 2012; Flores-Villasenor et al. 2012a, b; Puknun et al. 2013). By screening for the susceptibility of *B. pseudomallei* to LF-peptides and LFchimera, we have shown previously that this LFchimera possesses stronger killing activity against *B. pseudomallei* than its constituent peptides and the preferential antibiotic CAZ (Puknun et al. 2013). Moreover, we have found that the killing activity of LFchimera is similar for both *B. pseudomallei* and *B. thailandensis* and thought to be mediated by disruption of the plasma membrane and subsequently leakage of intracellular nucleotides leading to cell death (Kanthawong et al. 2014). However, the detailed

mechanism by which the LFchimera kills *B. pseudomallei* is not clearly understood. Localization of LFchimera in *B. pseudomallei* as well as its effect on morphology analyzed by immunoelectron microscopy may help to clarify the mechanisms of cell death. In addition, the effect of LFchimera in inhibiting *B. pseudomallei* biofilm formation and against preformed biofilm has never been reported. Therefore, this study aims to further elucidate the mechanism of action of LFchimera by analyzing its effect on the above mentioned processes.

## Materials and methods

### Peptide synthesis, purification and labeling

LFchimera (Table 1) and fluorescent labeled variants were synthesized using Fmoc-protected amino acids (Orpegen Pharma GmbH, Heidelberg, Germany) using a Syro II peptide synthesizer (Biotage, Uppsala, Sweden) and purified with an Ultimate 3000 RP-HPLC (Thermo Scientific, MA) to a purity of at least 95 % as previously described (Bolscher et al. 2012; Puknun et al. 2013). The authenticity of the peptides was confirmed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) on a Microflex LRF mass spectrometer equipped with an additional gridless reflection (Bruker Daltonik, Bremen, Germany) as described previously (Bolscher et al. 2012).

### Bacterial strains and growth conditions

Seven isolates of *B. pseudomallei* were used in this study (Table 2). *Burkholderia pseudomallei* were maintained on nutrient agar (NA) (Criterion, Hardy Diagnostics, CA) except for *B. pseudomallei* M10, SRM117 and PPKM which were grown on Luria-Bertani (LB) agar (Criterion, Hardy Diagnostics, CA) containing 15, 50 and 60 µg/ml tetracycline. A single colony of each bacteria initially grown on NA or LB was inoculated into brain-heart infusion (BHI) broth (Criterion, Hardy Diagnostics, CA) or

**Table 1** Sequences and characteristics of LFchimera

Peptide <sup>a</sup>	Sequence	Mol wt	Charge <sup>b</sup>
LFchimera	FKCRRWQWRMKKLG—K DLIWKLLSKAQEKFGKNKSR	4,422	12+

<sup>a</sup> The purity of peptides was at least 95 % and the authenticity of the peptides was confirmed by ion trap mass spectrometry

<sup>b</sup> Calculated net charge at neutral pH

**Table 2** Killing activity of LFchimera against *Burkholderia pseudomallei* isolates

Isolate	Relevant characteristics	Killing activity of LFchimera (%)			
		1 $\mu$ M	5 $\mu$ M	10 $\mu$ M	20 $\mu$ M
1026b	Clinical isolate from blood, susceptible to CAZ (DeShazer et al. 1997)	40	75	96	98
SRM117	O-side chain LPS-defective mutant from a clinical isolated 1026b wild type (DeShazer et al. 1998)	30	57	87	98
NF10/38	Clinical isolate from blood, susceptible to CAZ (Tunpiboosak et al. 2010)	16	44	97	99
PPKM	<i>ppk</i> mutant from a clinical isolated NF10/38 wild type (Tunpiboosak et al., 2010)	34	53	98	100
H777	Wild type, clinical isolate from blood, susceptible to CAZ (Taweechaisupapong et al. 2005)	8	34	78	85
M10	Biofilm-defective mutant from a clinical isolated H777 wild type, susceptible to CAZ (Taweechaisupapong et al. 2005)	12	31	58	79
979b	Clinical isolate from blood, CAZ resistant (Kanthawong et al. 2009)	11	37	51	74

CAZ ceftazidime

Modified Vogel and Bonner's medium (MVBM) (Lam et al. 1980), incubated overnight at 37 °C in a 200 rpm shaker-incubator and used as inoculum in all experiments.

### Killing effects of LFchimera against planktonic bacteria

The killing activities of LFchimera against *B. pseudomallei* were determined by colony culturing assays as described previously (Puknun et al. 2013). Briefly, bacterial cells were washed three times and were re-suspended (approximately  $10^5$  CFU/ml) in 1 mM potassium phosphate buffer (PPB), pH 7.0. The bacterial suspension was then added to an equal volume of the test agents to reach a final concentration of 1, 5, 10 and 20  $\mu$ M. A bacterial suspension in PPB without peptide served as a control. Following incubation at 37 °C for 60 min, the incubation mixture was serially diluted in physiological saline and plated in triplicate on NA. Colonies were counted after 24 h of incubation at 37 °C. The percentage killing effects were calculated using the formula  $[1 - (\text{CFU sample}/\text{CFU control})] \times 100 \%$ . Each assay was performed on two separate occasions, with triplicate determinations each time.

### Localization of LFchimera using fluorescence microscopy and immunoelectron microscopy

The localization of LFchimera in *B. pseudomallei* was studied by incubating *B. pseudomallei* (approximately  $10^7$  CFU/ml) in 1 mM PPB with 10  $\mu$ M FITC-LFchimera for 15 min. Subsequently, bacteria were examined by a Leica fluorescence microscope (Wetzlar, Germany). Images were recorded using a cooled CCD camera (U4000, Apogee Instruments, CA). Bacterial suspension in PPB without peptide served as a control.

For transmission immunoelectron microscopy (TEM), *B. pseudomallei* cells were re-suspended in 1 mM PPB at

$10^7$  CFU/ml and incubated with 10  $\mu$ M FITC-LFchimera for 30 min. Bacteria were harvested by centrifugation and fixed in 4 % paraformaldehyde solution in 0.1 M phosphate buffer pH 7.4 overnight in the refrigerator and then stored in 1 % paraformaldehyde fixative in the same buffer. Cryosections were prepared and incubated with ultra small gold-labelled mouse anti-FITC antibodies (Aurion, The Netherlands). The signal was enhanced with R-gent SE-EM (Aurion, The Netherlands) and sections were viewed in a FEI Technai-12 microscope (Technai Transmission Electron Microscopic, OR) equipped with a Lab6 filament, compu-stage, eagle  $4 \times 4$  K bottom mount camera and a velata  $2 \times 2$  K side-entry CCD camera for image acquisition.

### Transmission electron microscopy

The effect of the LFchimera on the cell morphology of *B. pseudomallei* was investigated by TEM as previous described with some modifications (Shin et al. 1998). *Burkholderia pseudomallei* (approximately  $10^7$  CFU/ml) in 1 mM PPB was incubated with LFchimera at 5, 10, 20  $\mu$ M at 37 °C for 1 h. A bacterial suspension in PPB without peptide served as a control. The bacteria were collected by centrifugation and washed with 0.1 M PPB, pH 7.0. The samples were fixed with 4 % paraformaldehyde and 1 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 and then examined with a FEI Technai G2 Spirit BioTwin microscope operated at 100 kV and equipped with SIS MegaView II camera (Olympus Soft Imaging Solutions GmbH, Germany).

### Effect of LFchimera on biofilm-forming capacity of *B. pseudomallei*

The bacterial suspensions adjusted to an optical density (OD) at 540 nm of 0.8–0.9 in PPB were added to an equal volume of LFchimera to reach final concentrations of 1 and

5  $\mu\text{M}$ . A bacterial suspension in PPB without peptide served as a control. Following incubation at 37 °C for 1 h, LFchimera were removed by two cycles of dilution with PPB and centrifuged, washed and resuspended in PPB. The biofilm-forming capacity was determined according to the method as described previously (Taweechaisupapong et al. 2005). Briefly, 200  $\mu\text{l}$  of each bacterial suspension were added into 96-well polystyrene flat-bottomed plate. Wells containing only PPB served as negative control. Bacteria were incubated aerobically at 37 °C for 3 h to allow adhesion. Next, the supernatant fluid of each well was aspirated gently to remove non-adherent bacteria, and replaced with 200  $\mu\text{l}$  of PPB. After further incubation at 37 °C for an additional 21 h, non-adherent bacteria were removed again and the adherent bacteria were washed with 200  $\mu\text{l}$  of sterilized deionized water and incubation in PPB for an additional 24 h. The supernatant was removed again and the wells were finally washed three times with 200  $\mu\text{l}$  of sterilized deionized water. The attached bacteria, representing a 2-day biofilm culture, were fixed with 200  $\mu\text{l}$  of 99 % methanol for 15 min and the plates were dried at room temperature. The biofilms were stained for 5 min with 200  $\mu\text{l}$  of 2 % Hucker crystal violet. Excess stain was removed with running tap water. The plates were air dried and the dye bound to the adherent biofilm was solubilized with 200  $\mu\text{l}$  of 33 % (v/v) glacial acetic acid per well. The OD in each well was measured at 620 nm using a microplate reader.

#### Antibacterial activities of LFchimera against *B. pseudomallei* grown in biofilm

The antibacterial activities of LFchimera against *B. pseudomallei* grown as a biofilm culture was investigated using a modification of the Calgary Biofilm Device (CBD) as previously described (Ceri et al. 1999; Kanthawong et al. 2012). In brief, the transferable solid-phase (TSP) pin lid (NUNC, Roskilde, Denmark) was placed into the 96-well microtiter plate containing the bacterial inoculum (approximately  $10^7$  CFU/ml) and incubated in an orbital incubator at 37 °C, 150 rpm, for 24 h. Following the period of incubation, the TSP pin lid with grown biofilms was rinsed with saline in another microtiter plate for approx. 1 min to remove loosely adherent bacteria and placed into a new 96-well microtiter plate (challenge plate) containing either 20  $\mu\text{M}$  LFchimera, 20 CAZ or 1873  $\mu\text{M}$  CAZ, then incubated at 37 °C for 24 h. The latter concentration of CAZ, 1873  $\mu\text{M}$  which is about 1 mg/ml, was used as this order of magnitude is often used in antibiotic studies (Sawasdiloln et al. 2010; Kanthawong et al. 2012; Anutrakunchai et al. 2015). Antimicrobial agent-free incubations were included for growth control. After that, the TSP pin lid was removed and rinsed with saline in another

microtiter plate. After rinsing, the TSP pin lid was placed into the new microtiter plate containing Muller–Hinton broth (recovery plate) and biofilms were disrupted from the TSP pin surface using an ultrasonic cleaner (SONOREX; Bandelin, Berlin, Germany) for 5 min. Viability of the biofilm bacteria was determined by plate counts. Colonies were counted after 24 h incubation at 37 °C. The effects of each concentration of LFchimera and CAZ was calculated as percentage killing using the formula  $[1 - (\text{CFU sample} / \text{CFU control})] \times 100 \%$ . Each assay was performed on three separate occasions, with triplicate determinations each time.

#### Statistical analysis

The efficacy of the test agents against each isolate of *B. pseudomallei* grown in biofilm was analyzed using Kruskal–Wallis with Mann–Whitney *U* test to evaluate the differences in antibiofilm efficacy between the CAZ and LFchimera. *P* values <0.05 were considered as statistically significant.

## Results

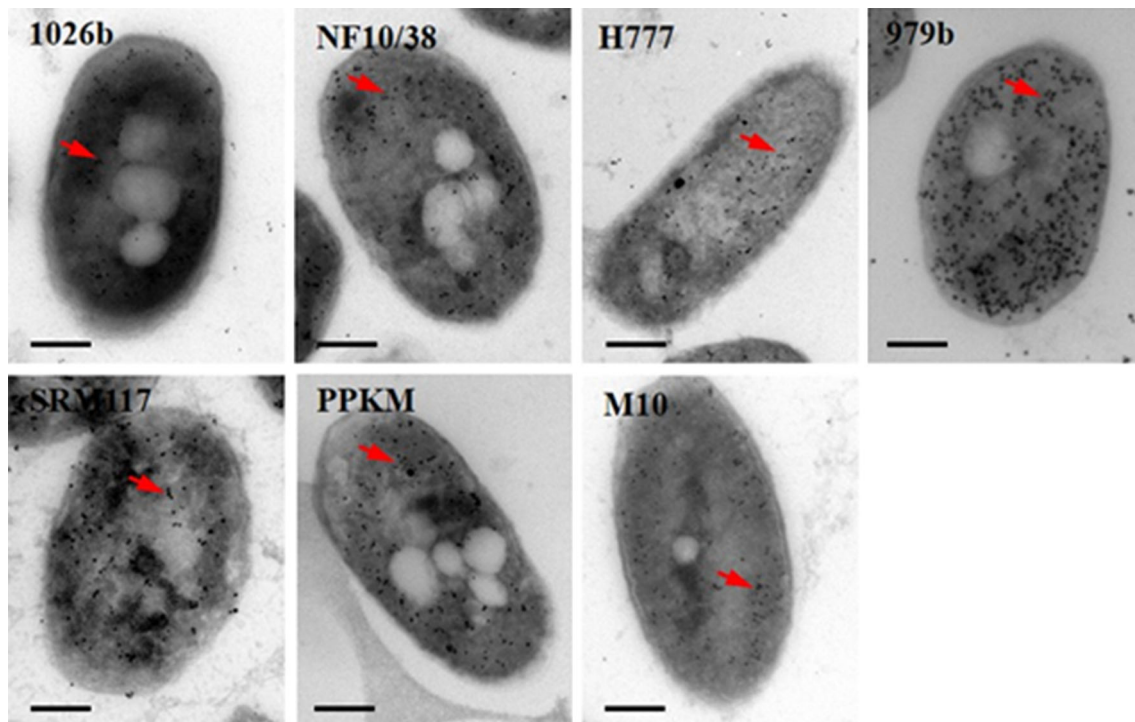
#### Antibacterial activities of LFchimera against planktonic *B. pseudomallei*

Killing activities of LFchimera against planktonic *B. pseudomallei* varied among the bacterial isolates (Table 2). At LFchimera concentration of 20  $\mu\text{M}$ , 74–100 % killing was observed for all isolates tested. Among the wild-type *B. pseudomallei* (1026b, NF10/38 and H777), isolate 1026b was the most sensitive to LFchimera while isolate PPKM was the most sensitive among the mutants.

#### Localization of LFchimera in *B. pseudomallei*

Cellular localization of LFchimera in *B. pseudomallei* was achieved by fluorescence microscopy and immunoelectron microscopy. Both techniques used LFchimera in synthesis labeled with FITC, resulting in a labeling stoichiometry of 1:1, without any free FITC remaining. After incubation with 10  $\mu\text{M}$  FITC-LFchimera for 15 min, all *B. pseudomallei* strains showed a more or less granular labeling intracellularly (data not shown). As shown previously for *B. pseudomallei* 1026b (Kanthawong et al. 2014) no accumulation of LFchimera in the membrane was observed in all strains tested.

To further specify the localization, bacteria treated with 10  $\mu\text{M}$  FITC-LFchimera were incubated with gold-labeled anti-FITC antibodies and analyzed by TEM. The TEM-micrographs clearly show that all *B. pseudomallei* strains



**Fig. 1** Localization of LFchimera in *B. pseudomallei* using immunogold labeling. In LFchimera-treated cells, most gold particles (arrows) are found intracellularly without accumulation on the membranes. Scale bar 500 nm

accumulated gold particles in intracellular structures (Fig. 1). Also here no accumulation in the bacterial membrane was found.

#### Ultrastructural effects of LFchimera on *B. pseudomallei*

TEM micrographs of untreated *B. pseudomallei* in PPB showed a normal rod shape with an intact inner membrane and slightly waved outer membrane (Fig. 2). *Burkholderia pseudomallei* treated with 5–20  $\mu\text{M}$  LFchimera showed membrane extrusion, membrane damage, swelling of periplasmic space and non-membrane-enclosed bodies. The bacteria seemed to lose their integrity and showed disordered structures. Cytoplasmic and DNA region appear to be inhomogeneous when compared to the control (Fig. 2). No major differences were found between the wild-type and mutant strains.

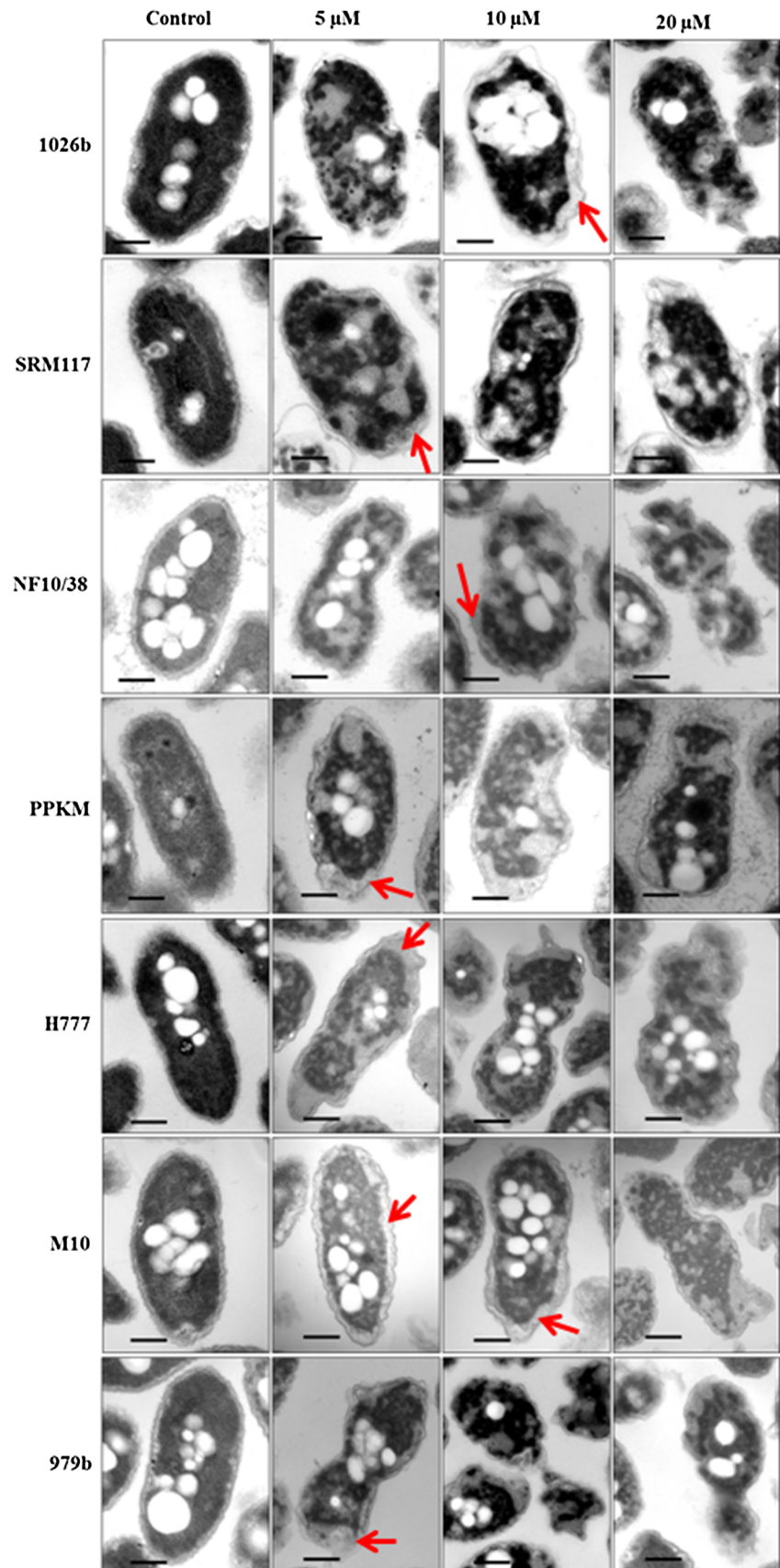
#### Effects of LFchimera on *B. pseudomallei* biofilm formation and established biofilm

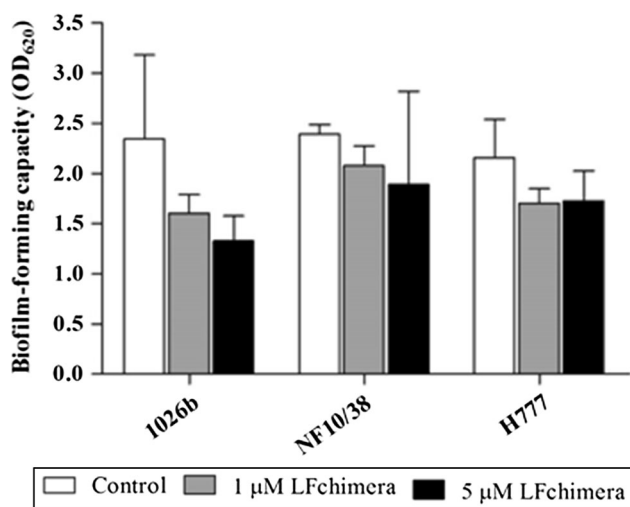
LFchimera at concentration of 1  $\mu\text{M}$  exhibited 8 and 16 % killing activities against *B. pseudomallei* H777 and NF10/38, respectively, while 40 % killing was observed for isolate 1026b (Table 2). In addition, LFchimera at concentration of 5  $\mu\text{M}$  exhibited <80 % killing activities against all isolates tested. Therefore, those subcidal

concentrations of LFchimera (1 and 5  $\mu\text{M}$ ) were selected to determine their inhibitory effects on biofilm formation of *B. pseudomallei* H777, NF10/38 and 1026b after pretreatment of the bacteria with LFchimera for 1 h. The inhibitory effect of LFchimera on biofilm formation of *B. pseudomallei* appeared to be strain-dependent (Fig. 3). LFchimera at concentrations 1 and 5  $\mu\text{M}$  exhibited 31 and 43 % inhibition on biofilm formation of *B. pseudomallei* 1026b, respectively, while the same concentrations of LFchimera showed less active against biofilm formation of *B. pseudomallei* H777 and NF10/38.

The effect of LFchimera against an established biofilm of *B. pseudomallei* was tested and compared with CAZ. The results showed that both agents affected *B. pseudomallei* in a strain-dependent killing activity (Fig. 4). LFchimera exhibited a higher killing effect in all isolates when compared to the CAZ-treated group. The average percentage killing activities of 20  $\mu\text{M}$  LFchimera was about 80–90 % for all *B. pseudomallei* except the isolate 979b. In contrast, all bacterial isolates needed a high concentration of CAZ, 1873  $\mu\text{M}$ , to reach 30–60 % killing activities. Interestingly, low concentration of CAZ (20  $\mu\text{M}$ ) increase CFU/ml of *B. pseudomallei* 1026b, SRM117 and M10 instead of inhibition when compared to untreated control. The difference of % killing between 20  $\mu\text{M}$  LFchimera and 20  $\mu\text{M}$  CAZ was statistically significant ( $P < 0.05$ ) in all isolates. Even when compared to

**Fig. 2** Transmission electron micrographs of *B. pseudomallei* treated with various concentration of LFchimera. *B. pseudomallei* incubated with PPB (control) showed a normal rod shaped morphology. *B. pseudomallei* treated with LFchimera showed swelling of periplasmic space of *B. pseudomallei* cells (arrows) and a highly inhomogeneous electron density in the intracellular DNA region, irregular membranes and a disturbance of the rod shape. Scale bar 500 nm





**Fig. 3** Biofilm-forming capacity of *B. pseudomallei* isolates after pretreatment with LFchimera. Data are presented as the mean and standard deviation of three independent experiments performed in quadruplicate

1873 μM CAZ, significantly higher killing activities of 20 μM LFchimera were observed in 4 isolates (1026b, SRM117, NF10/38 and PPKM). These results indicated that LFchimera is an effective peptide against both planktonic and biofilm forms of *B. pseudomallei* and possessed stronger antibiofilm activity than CAZ.

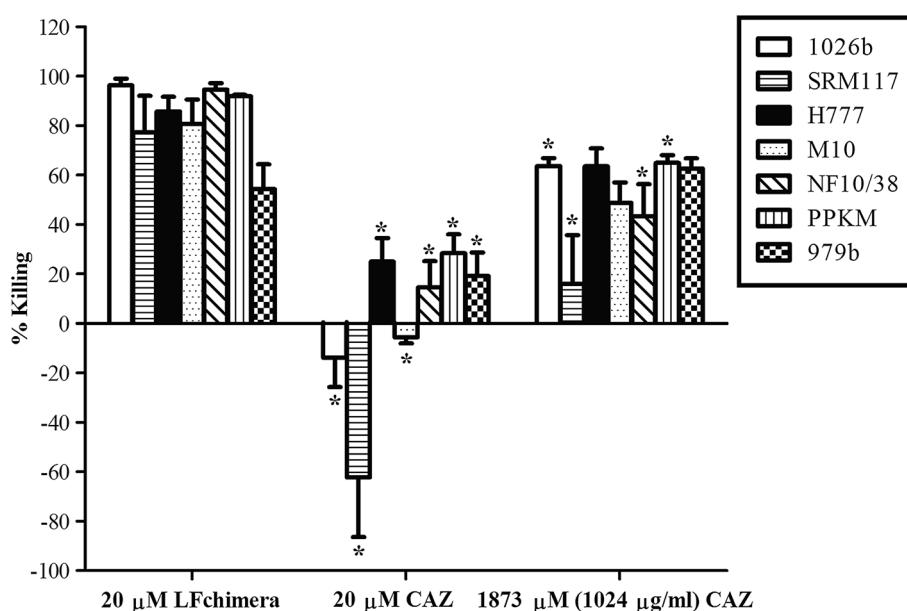
**Discussion**

From our previous study, we found that LFchimera, a chimerical structure containing LFCin17-30 and LFamp- in265-284, is very potent against *B. pseudomallei* (Puknun

et al. 2013). In this study, immunoelectron and transmission electron microscopy were used as complementary techniques to gain insight into peptide action, revealing not only cell surface effects but also intracellular alterations. The cellular localization of LFchimera in all strains tested in this study using fluorescence microscopy and immunogold labeling confirmed our previous reports for *B. pseudomallei* 1026b that LFchimera was found mainly inside bacterial cells with no accumulation in the membranes (Kanthawong et al. 2014). Similar to our study, it has been reported that lactoferricin B (LFcin B), a 25 residue peptide derived from the N-terminal part of bovine lactoferrin, can be traced in the cytoplasm (Haukland et al. 2001) and is capable of inhibiting bacterial macromolecular biosynthesis in both Gram-positive and Gram-negative bacteria (Ulvatne et al. 2004). In this study, TEM images of the bacteria treated with LFchimera showed swelling of periplasmic space and the outer membrane extrusion (Fig. 2). The possible explanation may be that the peptide interacts with the inner membrane leading to a local disruption of the inner membrane and leakage of cytoplasm into the periplasmic space. Moreover, cytoplasmic and DNA region of bacterial cells treated with LFchimera appear to be inhomogeneous when compared to the control. In our previous study, a drastic reduction of intracellular level of nucleotides (AMP, ADP and ATP) was observed after treatment with 20 μM LFchimera for 1 h (Kanthawong et al. 2014). Direct leakage of intracellular nucleotides results in cell-death; whether binding to DNA or other intracellular materials is secondary or the cause of killing needs to be elucidated.

Biofilm formation is a survival strategy of bacteria. Within the biofilm, the bacterial cells are embedded in

**Fig. 4** The killing activity of LFchimera against *B. pseudomallei* in biofilm compared with ceftazidime (CAZ). The biofilm was incubated with tested agents for 24 h and the viability of bacterial cells was determined by plate count technique. Data are presented as the mean and standard deviation of three independent experiments performed in triplicate. \**P* < 0.05 compared to 20 μM LFchimera



extracellular polymeric substances (EPS). EPS provides them with increased resistance to antimicrobial agents, and make them recalcitrant to host immune clearance mechanisms and highly difficult to eradicate with the currently available antimicrobial agents (Flemming and Wingender 2010; de la Fuente-Núñez et al. 2013; Kostakioti et al. 2013). *Burkholderia pseudomallei* is able to form biofilm both in vitro and in vivo (Vorachit et al. 1995). This has been proposed as a possible/main cause of relapse cases in melioidosis patients (Limmathurotsakul et al. 2014). We have demonstrated that *B. pseudomallei* growing in biofilm form was markedly resistant to several antimicrobial agents such as doxycycline, CAZ, imipenem and trimethoprim/sulfamethoxazole when compared to the corresponding planktonic cells of the same isolates (Sawasdidoln et al. 2010). One proposed mechanism of antibiotic resistance in bacterial biofilm is the slow growth rate and low metabolic activity of bacteria in biofilm because most antimicrobial agents, particularly the  $\beta$ -lactams, primarily target metabolically active cells (Lewis 2001; Walters et al. 2003). In the present study, LFchimera showed promising killing activities against biofilms of all *B. pseudomallei* isolates as compared to CAZ. A possible explanation may be that the effect of LFchimera is independent of growth, either slow- or even non-growing bacteria, due to its physico-chemical interaction with bio-membranes (Bolscher et al. 2009; Silva et al. 2013). Its ability to permeabilize and/or to form (transient) pores within the cytoplasmic membrane resulting in the fast penetration of the bacteria leading to cell death.

It has been shown that subinhibitory concentrations of antibiotics alter bacterial physiology, including the generation of genetic and phenotypic variability, synthesis and excretion of bacterial metabolites and their rate of growth (Lorian 1993; Andersson and Hughes 2014). From our previously published works, the minimum biofilm elimination concentration of CAZ for *B. pseudomallei* isolates range from 512 to >2048  $\mu\text{g/ml}$  (Sawasdidoln et al. 2010; Anutrakunchai et al. 2015). In this study, we found that exposing bacteria to subinhibitory concentration of CAZ (20  $\mu\text{M}$  or 10.93  $\mu\text{g/ml}$ ) enhance growth of *B. pseudomallei* 1026b, SRM117 and M10 instead of inhibition. Obviously low concentration of CAZ affect certain biological properties of *B. pseudomallei* which are able to interfere with some important bacterial cell functions. It is interesting to explore this further, especially because continuous growth in the presence of subinhibitory antibiotic levels is a crucial aspect of the current antibiotic resistance crisis.

In the presents study, *B. pseudomallei* H777, NF10/38 and 1026b wild type were selected to test the effects of LFchimera on biofilm formation because *B. pseudomallei* M10 and PPKM mutant were defect in biofilm production (Taweechaisupapong et al. 2005; Tunpiboonsak et al.

2010). It was found that exposure of bacterial cells to subcidal concentration of LFchimera (1 and 5  $\mu\text{M}$ ) reduced biofilm forming capacity of the cells. Therefore, LFchimera might be used to prevent *B. pseudomallei* biofilm associated infection.

Another promising feature of LFchimera is that concentrations up to 20  $\mu\text{M}$  did not show any significant lysis or permeabilization of human red blood cells (Kanthawong et al. 2014) or cytotoxicity to rat hepatocytes (Leon-Sicairos et al. 2009). Recently, LFchimera was proposed to inhibit damage caused by enteropathogenic *E. coli* (EPEC) in HEp-2 cells by suppressing EPEC attachment to HEp-2 cells and blocking EPEC-mediated actin polymerization. Moreover, LFchimera blocked the hemolysis of human red blood cells produced by EPEC (Flores-Villasenor et al. 2012a).

In conclusion, the present work indicates that LFchimera possessed antibacterial and antibiofilm activities and could modulate *B. pseudomallei* colonization. Therefore, it is a promising peptide for the prevention and treatment of melioidosis.

**Acknowledgments** This work was supported by the Commission on Higher Education granting under the CHE-Ph.D.-SW and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health Cluster (SHeP-GMS), Khon Kaen University. JGMB, KN and ECIV are supported by a grant from the University of Amsterdam for research into the focal point Oral Infections and Inflammation.

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