# GENERATION OF *BACILLUS SUBTILIS* CLONE DISPLAYING METAL-BINDING POLYHISTIDYL PEPTIDE

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## Abstract

A recombinant *Bacillus subtilis* clone displaying hexahistidine oligopeptide (6x-His) was generated to enhance the adsorption of heavy metal ions for environmental treatment application. The clone was generated by genetically fusing a hexa-His-encoding gene with the gene encoding the C-terminal half of *Staplylococcus aureus*-derived fibronectin-binding protein B (FnbB). The latter contains a LPXTG motif, which is necessary for sorting and covalently anchoring of the fused protein on the cell wall of *B. subtilis*. The presence of 6x-His on the cell surface was confirmed by Dot-Blot hybridization of the total protein fraction of the cell wall resulting from lysozyme treatment of the engineered bacterial cells with anti-6x-His mouse IgG In addition, the display of this oligopeptide was also proved by fluorescent immunostaining with the same primary antibody. Metal-binding capability of the engineered *B. subtilis* clone was examined using $Ni^{2+}$ as the model metal ion at some concentrations and pH values. The engineered clone was shown to have a $Ni^{2+}$ relative adsorption from 1.35 to 2.20 as compared to the control strain.

*Keywords: EngineeredBacillus subtilis, Cell surface displaying, Hexa-histidine, Metal-binding bacteria, Fibronectin binding protein B*

## 1. Introduction

Pollution of soil and water by heavy metal ions is a major environmental problem in the world, especially in industrializing countries such as Vietnam. While most physical and chemical remediation processes are either inadequate or very expensive to solve this problem, bioremediation using plants or microorganisms has been attracting the attention of environmental scientists. Particularly, using microbial cells able to display metal-binding peptides on the cell surface as potential novel bioadsorbents for removal of toxic metals from polluted environment has been growingly investigated with Gram-negative bacteria such as *E. coli* (Sousa, 1996, 1998; Kotrba, 1999a, b), *Ralstonia eutropha* (Valls, 2000), Gram-positive *Staphylococcus carnosus* (Stahl, 1997; Samuelson, 2000; Wernerus, 2001), and the yeast *Saccharomyces cerevisiae* (Kuroda, 2001) as cellular carrier.

Gram-positive bacteria as a metal-binding peptide-displaying system to be used for field application such as bioadsorption are more advantageous as compared to Gram-negative bacteria due to their single step for membrane translocation and thick cell wall. On the other hand, bacteria having more rapid growth than yeast are more suitable for field application than the latter.

Recently, we have successfully established an experimental system for covalently displaying proteins on the surface of *Bacillus subtilis* (Nguyen, 2005). We were interested to develop this cell surface protein-displaying system because *B. subtilis* offers several advantages over other Gram-positive bacteria: (i) it is considered as a GRAS organism (generally recognized as safe); (ii) it has a naturally high secretion capacity; (iii) the genetics including transcription, translation, and secretion mechanisms and genetic manipulation are far advanced; (iv) large-scale fermentation techniques have been developed (Westers, 2004; Meima, 2004). The *srtA* sortase-encoding gene of *Listeria monocytogenes*, which recognizes the LPXTG sorting motif of *Staphylococcus aureus* proteins (Ton-That, 1999; Dhar, 2000) and the peptidoglycan crossbridge present in the cell wall of *B. subtilis* (Navarre, 1999), was introduced into *B. subtilis*. The gene encoding exogenous protein to be anchored on the cell wall was fused in frame to the nucleotide sequence encoding the C-terminal half of *S. aureus* fibronectin binding protein B (FnbB) (Jönsson, 1991). Our *B. subtilis* displaying system could anchor up to 240,000 molecules of enzymatically active α-amylase, which is about 20 times more than previously reported for other bacterial species.

Here we report our results on using this *B. subtilis* system to display a hexa-His oligopeptide on the cell wall to generate a recombinant clone with enhanced adsorption of heavy metal ions for environmental treatment application. We could successfully demonstrate the presence of 6x-His on *B. subtilis* cell surface by Dot-Blot hybridization and fluorescent immunostaining. The metal-binding capacity of the engineered *B. subtilis* clone was shown using $Ni^{2+}$ as the model metal ion.

## 2. Materials and methods

*Microbial strains, plasmids and media*

*E. coli* DH5α ($F^-$ *end*A1 *hsd*R17 ($r_{k-}/m_{k-}$) *sup*44 *thi* $\lambda^-$ *rec*A1 *gyr*A96 Δ*lac*U169(ϕ80*lacZ*ΔM15) was used as host cell for cloning of plasmids. *B. subtilis* NDH03 (*leuA8 metB5 trpC2 hsdRM1 amyE::neo*) (Nguyen, 2005) having erythromycin and neomycin resistance was used as the host cell for generating 6x-His oligonucleotide-displaying clone. The latter carries the gene encoding sortase A from *Listeria monocytogenes*, which was induced by IPTG..

Plasmid pNDH11 (Fig. 1A) (Nguyen, 2005) having an expression cassette under the control of a promoter induced by xylose was used for anchoring hexa-His oligopeptide on the cell wall of *B. subtilis* NDH03. This expression cassette includes the secretion signal (*SamyQ*) of α-amylase and a sequence encoding the 94 amino acid C terminal half of *S. aureus*-derived fibronectin-binding protein B (FnbB) (Fig. 1B). This plasmid carries ampicillin-resistance conferring gene for the screening of transformant in *E. coli* and chloramphenicol-resistance conferring gene in *B. subtilis*, respectively.

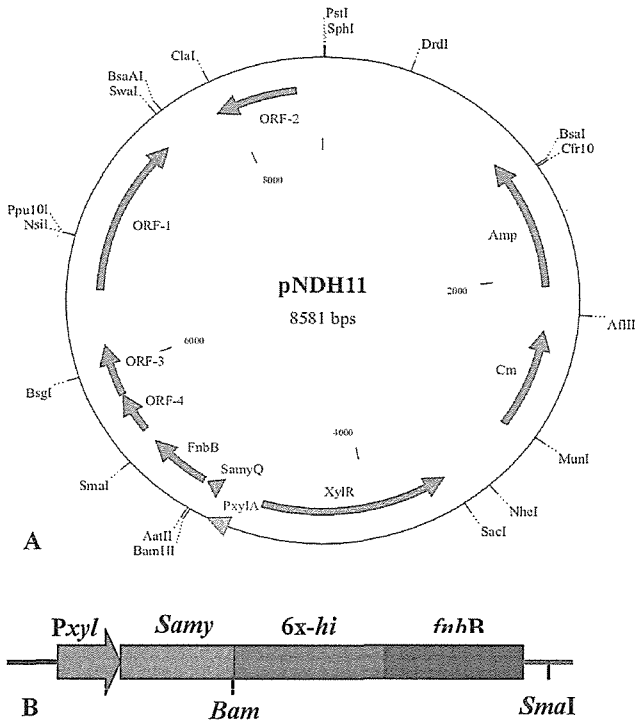


**Fig. 1. Structural map of pNDH11 and expression cassette for displaying 6x-His oligopeptide on B. subtilis cell wall. PxylA: xylose inducible promoter; SamyQ: signal sequence of α-amylase-encoding gene; fnbB: sequence encoding the C-terminal half of fibronectin binding protein B from S. aureus**

Luria-Bertani (LB) medium (5g/l yeast extract, 10g/l trypton, 5g/l NaCl, pH 7.5) was used for cultivation of *E. coli* and *B. subtilis*. HS medium for preparation of *B. subtilis* competent cell was generated by mixing the following determined volume of solutions: 10ml of 10X S-Base, $Mg^{2+}$, 2.5ml of 20% (w/v) glucose, 5ml of 0,1% (w/v) L-tryptophan, 1ml of 2% (w/v) casein, 5ml of 10% (w/v) yeast extract, 10ml of 8% (w/v) arginine and 0.4% (w/v) histidine, 66.5ml of distilled water. LS medium for transformation of *B. subtilis* was prepared by mixing the following solutions: 10ml of 10X S-Base, $Mg^{2+}$, 2.5ml of 20% (w/v) glucose, 0.5ml of 0,1% (w/v) L-tryptophan, 0.5ml of 2% (w/v) casein, 5ml of 2% (w/v) yeast extract, 0.25ml of 1M $MgCl_2$, 0.25ml 1M $CaCl_2$, 80ml of distilled water. In both cases, amino acid solution was sterilized in advance by membrane filtering and was added later to the cooled autoclaved medium.

*Generating B. subtilis strain displaying 6x-His oligopeptide on the cell surface*

The nuceltide sequence encoding 6x-His oligopeptide with *Bam*HI at 5' terminal and *Aat*II at 3' terminal was chemically sunthesized. Both this sequence and the pNDH11 vector were cleavaged with *Bam*HI and *Aat*II and linked by ligase to generate the recombinant pNDH11-6xHis vector, which was then transformed into *E. coli* DH5α. Recombinant vector was isolated from transformant and its structure was analyzed by PCR, restriction enzyme and sequencing. Confirmed recombinant pNDH11-6xHis vector was later transformed into competent *B. subtilis* NDH03.

*B. subtilis* NDH03 with a natural competency was activated overnight in 5ml LB medium containing

100μg/ml erythromycin and 10μg/ml neomycin (LB/Ery+Neo). One ml of the culture was inoculated into 50ml HS medium with the same antibiotics (HS/Ery+Neo). The inoculated culture was shaken at 250rpm, 37°C and growth was monitored by $OD_{578nm}$. Cells were harvested at the beginning of the stationary phase, suspended in 80% glycerol. The cell suspension was kept in ice for 30min, divided into different 1.5ml eppendorf tube and stored at -80°C until being use for transformation.

Suspension of competent *B. subtilis* NDH03 in 1.5ml eppendorf tube was transferred into 10ml LS medium and incubated with low shaking rate at 30°C for 2hr. One ml of the cell suspension was transferred into a sterile eppendorf tube and 10μl of 0,1M EDTA was added. The mixture was kept at room temperature for 5min, then 0.5 - 2μg of pNDH11-6xHis vector DNA was added. The tube was incubated at 37°C for 2 hr and cell suspension was spread on LB plate containing 100μg/ml erythromycin, 10μg/ml neomycin and 10μg/ml chloramphenicol (LB/Ery+Neo+Chl). This LB plates were incubated at 37°C for 24hr for screening of *B. subtilis* NDH03/pNDH11-6xHis transformants.

*Confirmation of the display of hexa-His oligopeptide on NDH03/ pNDH11-6xHis cell wall*

NDH03/ pNDH11-6xHis clone was cultured in LB/Ery+Neo+Chl medium at 37°C under shaking until $OD_{578nm}$ reached 0.7-0.8. The expression and display of 6x-His oligopeptide on the cell surface was induced by adding 1M IPTG (isopropyl β-thiogalactoside) and 50% xylose solution to final concentrations of 0.5mM and 0.5%, respectively, and the culture was continued at the same condition for 1hr. The display of hexa-His oligopeptide on NDH03/ pNDH11-6xHis cell wall was confirmed by both Dot-blot hybridization and fluorescent immunostaining methods.

For Dot-blot hybridization, 6x-His oligopeptide was released from the cell wall by the treatment of 100μl suspension of whole induced cells in distilled water with lysozyme (0.5mg/ml final concentration) at 37°C for 30min. After a centrifugation step, the supernatant was harvested and the precipitant was resuspended in 100μl distilled water. Both the supernatant and resuspended precipitant were boiled at 100°C for 10min, rapidly cooled in ice and 20μl each of the two fractions was directly loaded on the hybridization membrane (Amersham Biosciences Hybond ECL) by Dot blot set. Unloaded part of membrane was blocked with phosphate-buffered saline PBS (80mM $Na_2HPO_4$, 20mM $NaH_2PO_4$, 100mM NaCl, pH 7.5) containing 0.05 Tween 20 and 50% defatted milk for 90min at room temperature. After being washed once with PBS containing 0.1% Tween 20, the membrane was hybridized with anti-6x-His mouse IgG (Amersham Biosciences) solution (1:10,000 in 5% defatted milk) for 90min at room temperature. Unbound IgG on the membrane was washed 3 times with PBS-0.1% Tween 20. The hybridization dot of 6x-His oligopeptide with anti-6x-His mouse antibody was detected by treatment with the secondary anti mouse IgG-goat antibody (Abcam) conjugated with horse radish peroxidase. The enzyme activity was revealed by luminescence with $H_2O_2$ and luminol substances, which could be recorded and observed on sensitive film.

For fluorescent immunostaining, induced cells were washed in PBS and resuspended in PBS 2% bovine serum albumin (BSA ) containing the primary anti-6x-His mouse IgG solution at a dilution of 1:100 for 1hr. Then, cells were washed and incubated with anti-mouse IgG FITC (fluorescein isothiocyanate, Abcam) conjugate at a dilution of 1:100 in PBS 2% BSA. A 3μl aliquot of this cell suspension was spread onto a slide, air dried and observed under a fluorecence microscope (Olympus) with 490-495nm as exciting wavelength and 510nm as fluorescent wavelength, respectively.

*Examining the metal ion-binding capability of NDH03/pNDH11-6xHis B. subtilis clone*

250ml of LB/Ery+Neo+Chl was inoculated with 5ml of activated *B. subtilis* seed, which had been cultured overnight in the same medium at 37°C and 250rpm, and was cultured under the same condition until $OD_{578}$ reached approximately 0.8. Subsequently, IPTG and xylose were added to a final concentration of 0.5mM and 0.5%, respectively, and the culture was continued for 3hr. Cells were harvested by centrifugation at 6,000rpm, 4°C for 10min and washed once with distilled water. Induced cells was added to each Erlenmayer flasks containing 150ml of $Ni(NO_3)_2$ solution at 2ppm or 10ppm and different pH 5.0 and 6.0 to a concentration of 4-5mg cells/ml. Flasks were gently shaken at 30°C for 80min. Samples were taken at 20min interval, centrifuged and supernatants were analyzed for residual $Ni^{2+}$ concentration by atomic adsorption spectrophotometer (Perkin Elmer Analyst 600).

## 3. Results and discussion

*Generating B. subiltis clone displaying 6x-His oligopeptide on cell wall and confirming the display of 6x-His oligopeptide*

NDH03/pNDH11-6xHis transformants could successfully form colonies on LB/Ery+Neo+Chl plate. pNDH11-6xHis in transformants was proved by colony PCR (data not shown). NDH03/pNDH11-6xHis clone was cultured in LB/Ery+Neo+Chl broth and the expression and display of 6x-His oligopeptide on cell wall was induced by IPTG and xylose. The anchorage of 6x-His oligopeptide on cell wall fraction was confirmed by Dot blot hybridization (Fig. 2). After being induced for 1hr and 3hr, cell wall proteins were released by lysozyme treatment and expected to be present in the supernatant after centrifugation. Fig. 2 showed that 6x-His oligopeptide could be successfully detected in the supernatant fraction from NDH03/ pNDH11-6xHis after 3hr (Fig. 2, 3B) induction but not after 1hr (Fig. 2, 1B). On the contrary, positive signal was not detected after 1hr nor 3hr of induction in the case of NDH03/pNDH11 (Fig. 2, 1A, 3A), which does not contain the oligonucleotide sequence encoding 6xHis oligopeptide. The positive signal of precipitant fractions, which composed of intracellular cell components, from NDH03/pNDH11 (Fig. 2, 2A, 4A) as well as from NDH03/pNDH11-6xHis (Fig. 2, 2B, 4B), suggested the presence of endogenous *B. subtilis* proteins containing intramolecular polyhistidyl oligonucleotide.

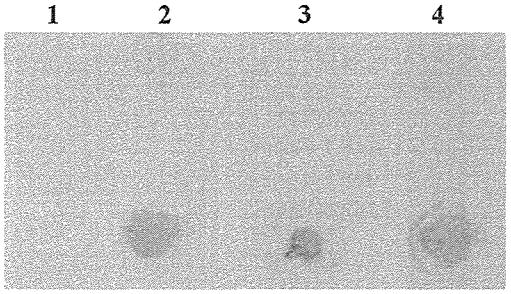


**Fig. 2. Dot blot hybridization to demonstrate the presence of 6x-His oligopeptide on cell wall. 1,3: protein on cell wall (supernatant); 2,4: intracellular protein (precipitant); A: NDH03/ pNDH11; B: NDH03/pNDH11-6xHis**

The presence of 6x-His oligopeptide on cell wall of NDH03/pNDH11-6xHis was also confirmed by fluorescent immunostaining (Fig. 3). Cells with fluorescent signal could be successfully observed with NDH03/pNDH11-6xHis after 3hr of incubation (Fig. 3, 4B) but not after 1hr induction (Fig. 3, 3B). On the contrary, NDH03/pNDH11 could not generate any fluorescence (Fig. 3, 1B, 2B). This result well agreed with that of Dot blot hybridization and also confirmed that 6x-His oligopeptide was successfully displayed on cell wall of NDH03/pNDH11-6xHis.

*Examining the metal ion-binding capability of NDH03/pNDH11-6xHis B. subtilis clone*

Metal ion-binding capability of NDH03/pNDH11-6xHis *B. subtilis* clone was examined basing on the change of $Ni^{2+}$ concentration in solution before and after being incubated with these bacterial cells as compared with control (NDH03/pNDH11) (Fig. 4). In solution containing 2ppm $Ni^{2+}$, NDH03/pNDH11-6xHis cells showed relative adsorptions of 1.35 - 1.40 as compared to that of NDH03/pNDH11 at both pH 5.0 and 6.0. In a solution of 10ppm $Ni^{2+}$, this enhanced adsorption was more apparent with relative adsorptions of 1.70 - 2.20 as compared to control. The relative adsorptions of $Ni^{2+}$ at 10ppm concentration were higher than those at 2ppm concentration due to the non-chelating binding of $Ni^{2+}$ by *B. subtilis* cells. At low concentration of $Ni^{2+}$, this non-chelating binding strongly competed with 6x-His chelating binding, but this competition

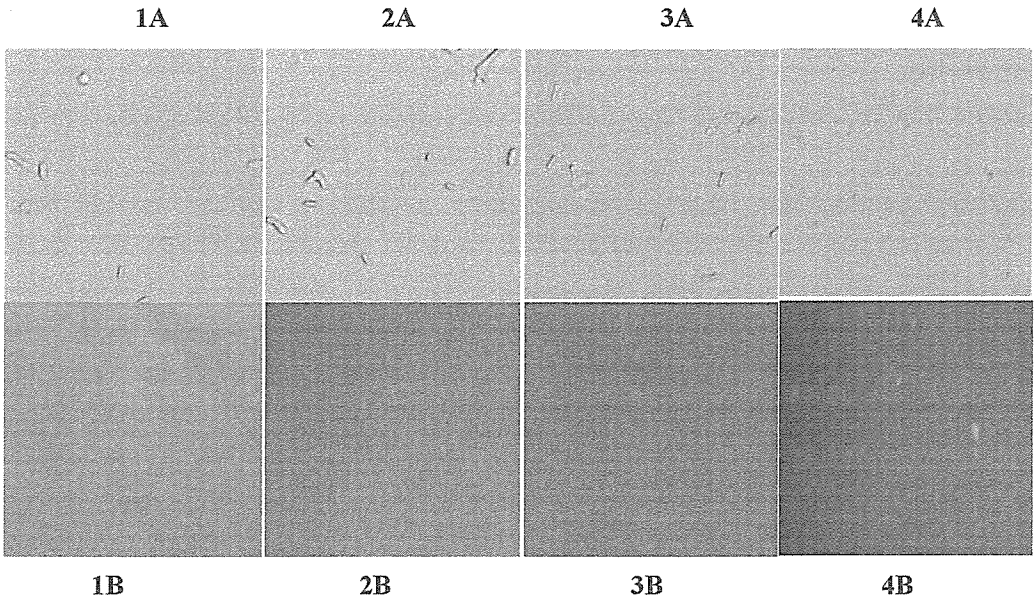


**Fig. 3. Confirming the display of 6x-His oligopeptide on cell wall of NDH03/pNDH11-6xHis by immunostaining under fluorescence microscope (1,500X, Olympus).** ***A: normal light mode; B: fluorescent mode; 1,2: NDH03/pNDH11; 3,4: NDH03/pNDH11-6xHis; 1,3: 1h induction; 2,4: 3hr induction***

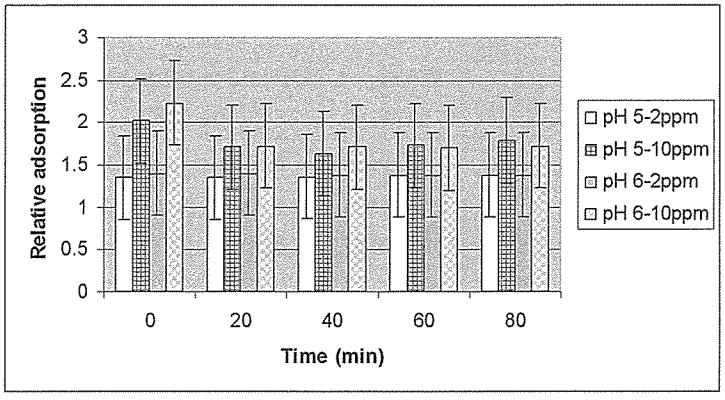


**Fig. 4. Enhanced adsorption of $Ni^{2+}$ by 6x-His oligopeptide-displaying NDH03/pNDH11-6xHis B. subtilis clone. The $Ni^{2+}$-binding capacity of NDH03/pNDH11-6xHis was compared with that of NDH03/pNDH11, whose $Ni^{2+}$-binding capacity was taken as 1.0.**

decreased at higher $Ni^{2+}$ concentration. This enhanced adsorption seemed to be independent on solution pH and time of treatment by *B. subtilis* cells at 2ppm $Ni^{2+}$. However, at 10 ppm $Ni^{2+}$, a slight deadsorption of metal ion was observed after the first 20min and the relative adsorption became stable later up to 2hr (dat not shown).

## 4. Conclusions

We have successfully generating an engineered *B. subtilis* clone displaying 6x-His oligopeptide on the cell wall. The anchorage of this oligopeptide on the cell wall was confirmed both by Dot-Blot hybridization and fluorescent immunostaining using anti-6x-His mouse polyclonal IgG as primary antibody, which could specifically bind to 6x-His oligopeptide on the cell wall. The metal-binding capability of the engineered *B. subtilis* clone was shown using $Ni^{2+}$ as the model metal ion. The 6x-His oligopeptide-displaying strain showed an enhanced adsorption of $Ni^{2+}$ as compared to the control strain, thus proving that 6x-His oligopeptide was functionally anchored on the cell surface and this engineered cell has a potency to be developed into a filter bioadsorbent for treatment of heavy metal-polluted environments. *B. subtilis* as cellular carrier in a displaying system for metal-binding peptides for removal of toxic metals from polluted environments has more advantageous than other Gram-negative, positive bacteria and yeast. In addition, our system was proved to be able to display up to 240,000 protein molecules on one cell wall, about 20 times more than reported bacterial systems, suggesting high-capacity metal bioadsorbent can be developed with this system. However, our preliminary examination on $Ni^{2+}$-binding capability of the 6x-His oliopeptide-displaying *B. subtilis* strain failed to show that excellent metal-binding capacity, which might be due to the unappropriated lenght of 6x-His-FnbB fused protein. More experiments should be carried out to optimize the expression and display of oligohistidyl peptide on the cell wall as well as the chemophysical condition of treatment of metal-containing solution.

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