Nguyen et al | Article 8631
J Pure Appl Microbiol. 2023;17(3):1605-1611. doi: 10.22207/JPAM.17.3.21
Received: 17 April 2023 | Accepted: 08 June 2023
Published Online: 01 September 2023

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Environmentally Responsible Bioengineering for Spore Surface Expression of *Helicobacter pylori* Antigen

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Abstract

The development of genetic technologies and bioengineering are creating an increasing number of genetically engineered microorganisms with new traits for diverse industrial applications such as vaccines, drugs and pollutant degraders. However, the destiny of genetically engineered bacterial spores released into the environment as long-life organisms has remained a big environmental challenge. In this study, an environmentally responsible and sustainable gene technology solution based on the concept of thymine starvation is successfully applied for cloning and expression of a *Helicobacter pylori* antigen on *Bacillus subtilis* spore surface. As an example, a recombinant *Bacillus subtilis* strain A1.13 has been created from a gene fusion of the corresponding N-terminal fragment of spore coat protein CotB in *B. subtilis* and the entire urease subunit A (UreA) in *H. pylori* and the fusion showed a high stability of spore surface expression. The outcomes can open the door for developing highly safe spore vectored vaccines against this kind of pathogen and contributing to reduced potential risks of genetically engineered microorganisms released in the environment.

Keywords: *Bacillus subtilis,* Genetically Engineered Microorganism, Spore Vectored Vaccine, Thymine Starvation, Urease

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Citation: Nguyen TKC, Pham TL, Nguyen TC, Pham TT, Nguyen VD. Environmentally Responsible Bioengineering for Spore Surface Expression of *Helicobacter pylori* Antigen. *J Pure Appl Microbiol.* 2023;17(3):1605-1611. doi: 10.22207/JPAM.17.3.21

INTRODUCTION

The creation of genetically engineered microbes (GEMs), which are made by bioengineering a more robust protein into microorganisms for improved features of interest, is thought to be a more cost-effective and safe way to improve the environment and finally human health. Bioengineered microorganisms are more powerful than naturally occurring ones and may be either faster degraders to contaminants in the environment, or next-generation vaccines, probiotics, enzyme producers, which have been produced in the industry and health sectors.[1] However, the most challenging environmental issue is the ultimate fate of GEMs, especially bioengineered long-life spores, once released in the environment because these spores can live indefinitely in soil and harsh environmental conditions.[2,3]

It has been suggested by an innovative approach that spores under thymine starvation could be germination-deficient and die.[2] The concept of death without thymine has been introduced for *B. subtilis*, the most popular industrial microbe.[4] Based on the existence of two genes *thyA* and *thyB* discovered to encode for thymidylate synthase enzymes,[5] recombinant spores of this bacterium have been designed by using the insertion of new gene sequences into these thymine genes, causing a strict thymine-dependent growth.[2]

This concept is used for the first time in the present study to introduce an antigen or virulence factor from the human pathogenic bacterium *Helicobacter pylori*, a major risk factor for stomach cancer,[6] on the surface of *B. subtilis* spores. This may lead to the creation and advancement of recombinant vaccines against this pathogen that are environmentally sound, ultimately enhancing both human and environmental health.

MATERIALS AND METHODS

Bacterial strains and medium

B. subtilis strain PY79 is a prototrophic laboratory strain derived from the type strain 168.[7] The two-step transformation approach and other standard methods for working with *B. subtilis* were carried out as previously mentioned.[8] DSM is the recommended medium for the growth and sporulation of *B. subtilis*,[9] supplemented with thymine (50 µg/ml) and trimethoprim (3 µg/ml). *Escherichia coli* DH5α (Invitrogen) was cultured in Luria-Bertani (LB). Competent cells of *E. coli* DH5α were prepared as described by Sambrook and Russell[10] and those of *B. subtilis* PY79 were performed according to Zhang et al.[11]

H. pylori strains (HP24, HP28, HP30, HP34, and HP37) isolated from patients with chronic gastritis in 2020 and provided by the Hospital of University of Medicine and Pharmacy, Hue University, Vietnam, were used for the amplification of gene sequence encoding for urease A antigen. *H. pylori* strains were cultured using either selective Horse Blood Agar (HBA) or Brain Heart Infusion (BHI) medium (Oxoid) containing 5% (v/v) fetal bovine serum (FBS, Thermofisher Scientific). Incubation was made in a microaerophilic chamber using an Oxoid CampyGen 2.5 L Sachet (5-7% O2, 5-10% CO2, and 85% N2) at 37°C, with passaging every 48 h. The strains were preserved in BHI supplemented with 15% (v/v) glycerol at -80°C.

Oligonucleotides, plasmids, and enzymes

Table provides a list of the DNA primers used for PCR amplification. Plasmid pThyA (4,274 bp) (Life Technologies) composes of the *thyA* gene that is located around a multiple cloning site (MCS). Enzymes such as Vent DNA polymerase (New England Biolabs, USA), My Taq DNA polymerase (Bioline, UK), T4 DNA ligase (New England Biolabs, USA) and restriction enzymes *Bam*HI, *Nde*I, *Sca*I and EcoRI (New England Biolabs, USA) were used.

Construction of *cotB-ureA* gene fusion in *B. subtilis*

The *cotB* gene was amplified by PCR from *B. subtilis* PY79. The *cotB* gene was then cloned into plasmid pThyA in *E. coli* DH5α and confirmed by colony PCR and digestion of restriction enzymes. For cloning in pThyA-*cot*B, the *ureA* gene was isolated from clinical *H. pylori* strains by PCR. Two steps made up the cloning process. *E. coli* DH5 cells were employed in the first stage for heat shock transformation at 42°C. After being linearized using *Sca*I digestion and electroporation

Table. PCR primer list

Primer name	Primer sequence (5′ – 3′)	Restriction enzyme	Expected PCR product (bp)
CotB-F	AAA CAT ATG GGC GAT GTA TGA ACG GAT T	*Nde*I	1130
CotB-R	AAA GGA TCC ACG ACC AGA TTT GGA TGA TTG	*Bam*HI	
UreAV-F1	AAA GGA TCC AAA AGT GCG GCT GAA TTG AT	*Bam*HI	732
UreAV-R	GGC GAA TTC TTA CTC CTT AAT TGT TTT TAC ATA GTT GTC	*Eco*RI	

into *B. subtilis* PY79, recombinant pThyA plasmids containing the *cotB-ureA* fused gene were used. Cells were then plated on Supplemented Minimal Medium agar (SMM) added with thymine (50 µg/ml) and trimethoprim (3 µg/ml). Single colonies were purified after 48 hours of culture and tested for growth on SMM added with trimethoprim (3 µg/ml) and with or without thymine (50 µg/ml) at 37°C and 46°C. A *cotB-ureA* chimeric gene at the *thyA* locus allowed cells to grow at 37°C with or without thymine but not at 46°C without additional thymine. The resulting *thyA*:: insertion strain is a trimethoprim-resistant transformant that is thymine-dependent. To confirm the existence of the chimeric gene from transformants, the insert DNA fragment was amplified using colony PCR and then sequenced. Fused *cotB-ureA* strains were also confirmed by spore preparation, spore coat protein extraction, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and dot blot analysis.

Spore preparation

The exhaustion method was used to cause sporulation as described previously in *B. subtilis* strains.[9] After being incubated at 37°C for 48 hours, spores were harvested, thoroughly washed, and purified using lysozyme to break down any remaining sporangial cells.[9] To guarantee that there were no viable vegetative cells, each batch of spores was heated at 65°C for one hour. They were then floated in cool, sterile water and kept in aliquots (1×10^{10} spores/mL) at -20°C until they were needed. Serial dilution and plate counting were used to calculate spore counts.

Spore coat protein extraction and SDS-PAGE analysis

Aliquots of 1×10^{10} spores suspended in distilled water were used to extract spore coat proteins in *B. subtilis* by addition of 100 µl extraction buffer (0.05 M Tris-HCl pH 6.8, 1% SDS (sodium dodecyl sulphate), 0.05 M DTT (dithiothreitol)) to spore pellet and then incubation at 68°C for 1 h, as previously reported.9 These supernatant protein samples were mixed 1:1 (v/v) with the 2X treatment sample buffer (1% DTT used instead of 2-ME) for SDS-PAGE and then heated for 15 min at 98°C. Using a Mini Protein II device (Bio-Rad Laboratories, Inc., Richmond, CA, USA), protein samples are loaded onto polyacrylamide gels with a 12% running gel and a 4% stacking gel before being electrophoresed at 15 mA/gel of steady-state current. The gels were stained with 0.05% Coomassie Blue in a solution of 25% v/v ethanol and 10% v/v acetic acid for 4 hours after electrophoresis. The gels were finally destained with destaining solution (25% (v/v) methanol and 10% (v/v) acetic acid).

Dot blotting assay

Spore coat protein extracts were 2-fold serially diluted with phosphate-buffered saline (PBS) and blotted onto nitrocellulose membrane. The experiments were replicated three times. For negative controls, either PBS or supernatant of PY79 cells was used. For positive controls, either *Ure*A protein or supernatant of *H. pylori* HP37 cells was used. The membrane was blocked overnight at 4°C by immersion in 5% Skim milk and PBST (PBS with 0.05% Tween-20), and then three PBST washes on the membrane were performed. The membrane was probed with rabbit anti-*Ure*A primary antibody (PA14347A0Rb, Cusabio, USA) at 1:4000 ratio for 1 h at room temperature, and then three washed with PBST. Goat anti-rabbit IgG-HRP (Sigma-Aldrich, Germany) at 1:40,000 ratio was used as the secondary antibody, and the membrane was incubated for 1 hour at room temperature with this antibody in PBST containing

0.5% BSA. The membrane was then washed again before incubated with SuperSignalTM West Substrate (Thermo Scientific, USA). Image of the blot was captured using the Fusion Solo S (Vilber, France) and signals were analysed by Evolution Capt Edge Software (Vilber, France).

The stability of spore surface expression

The stability of spore surface expression of *Ure*A on A1.13 by using dot blots were tested at different preservation/delivery temperatures (-20°C, 4°C, 25°C and 37°C) for 28 days, as well as in the conditions treated with simulated gastric fluids (SGF) (Merck, Germany) or simulated intestinal fluids (SIF) (Merck, Germany) at 1:1 (v/v) ratio for 1 h and then stored at 4°C for the same days.

RESULTS AND DISCUSSION

Synthesis and integration of the *cotB*-ure*A* gene fusion in the *B. subtilis* chromosome

The first spore coat protein employed in *B. subtilis* spore surface display, *CotB*, is still widely used today.[12-14] In this study, *CotB* was designed as a carrier protein in order to create recombinant *B. subtilis* spores with *Ure*A expressed on their surface. In order to do this, the coding portion of the *cotB* gene and the *ure*A gene of *H. pylori* were fused in frame. In particular, the *cotB* gene fragment (1130 bp) was cloned from *B. subtilis* PY79 with primer pair *CotB*-F and *CotB*-R (Figure 1A), in which three 27 amino acid repeats at the C-terminus were removed because these fragments cause genetic instability of CotB,[12] and an upstream sequence of the *cotB* gene and a restriction enzyme recognition sequence at the two ends were added. The *ure*A gene (732 bp), which consists of the entire nucleotide sequence coding for the urease A subunit and restriction enzyme recognition sequences added at both ends, was PCR amplified successfully from different clinical *H. pylori* strains by using primer pair *Ure*AV-F1 and *Ure*AV-R and the results of single PCR product band were shown (Figure 1B). The *cotB* gene was then purified from PCR, cleaved by *Nde*I and *Bam*HI enzymes before incorporated into

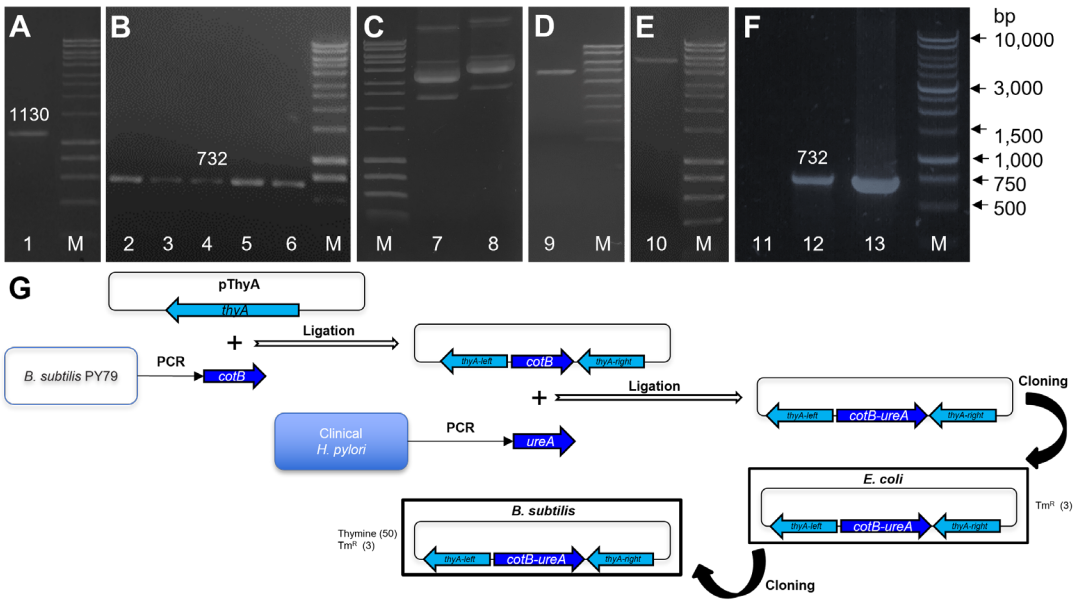

Figure 1. Agarose gel electrophoresis (1% agarose gel) of PCR products and plasmids obtained for construction of *cotB-ureA* gene fusion in *B. subtilis* (A-F); and schematics of the design of *cotB-ureA* gene fusion and cloning steps (G). M: 1 kb DNA marker; 1: PCR product of *cotB* gene from *B. subtilis* PY79; 2 to 6: PCR products of *ureA* gene from clinical *H. pylori* strains; 7: plasmid pThyA-cotB extracted from *E. coli* DH5α; 8: plasmid pThyA-*cotB-ureA* extracted from *E. coli* DH5-α; 9: purified plasmid pThyA-*cotB*; 10: purified plasmid pThyA-*cotB-ureA*; 11: negative control (without template DNA); 12: positive control (ureA); 13: Colony PCR product of *ureA* gene from recombinant *B. subtilis* A1.13

the pThyA vector. The *ure*A gene, after also being purified from PCR, was cut with *Bam*HI and *Eco*RI enzymes before being inserted into the pThyA-*cotB* vector.

The pThyA vector and the *cotB* gene were joined with the T4 DNA ligase enzyme to generate the recombinant pThyA-*cotB* vector, which is followed by the transformation into competent cells *E. coli* DH5α cloned on LB agar including ampicillin (100 µg/ml) (Figure 1C,D). Colonies obtained after 24 h of culture at 37 °C were further screened by colony PCR with the same primer pair *CotB*-F and *CotB*-R to identify recombinant strains.

The *ure*A gene and the pThyA-*cotB* vector were linked by T4 DNA ligase to generate a plasmid called pThyA-*cotB-ureA* which is then also transformed into *E. coli* on ampicillin-supplemented LB agar (Figure 1C,E). The colonies obtained after 24 h of culture at 37°C were further screened by colony PCR method with the same primer pairs *Ure*AV-F1 and *Ure*AV-R to identify *E. coli* strains carrying the recombinant gene.

After transformation in *B. subtilis*, cells that successfully performed double-crossover of homologous segments of the *thyA* gene cannot survive in the medium without thymine because of the inactivation of the *thyA* gene.[2] Individual clones with the presence of *ure*A gene were detected by PCR (Figure 1F). Also, to verify the sequence of *ure*A gene, these clones were sequenced by using the same primers. The results indicated that a selected clone carrying the *ure*A gene from *H. pylori* strain HP37 shared 100% identity with that of *H. pylori* strain dRdM2addM2 (Genbank Accession No. CP026515) (not shown). This clone is named A1.13 and used for further analysis.

The isogenic parental strain PY79 and the

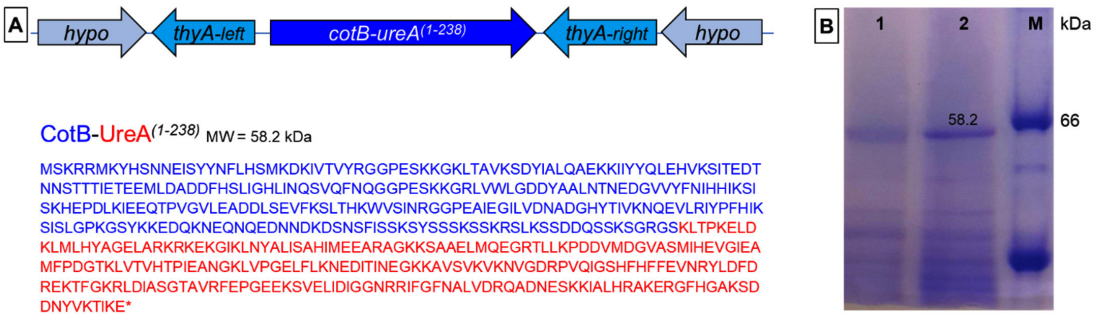

Figure 2. Gene structure and deduced amino acid sequences of the *cotB-ureA* fusion region (A) and SDS – PAGE (12% gel) of *CotB-UreA* (1-238) recombinant protein from spores *B. subtilis* A1.13 (B). Chimeric genes inserted at the *B. subtilis* thyA loci are shown. Urease A from *H. pylori* HP37 was fused in frame to the C-terminus of the spore coat protein *CotB.* The estimated MW of the chimera are shown. Lane 1: extracted protein of *B. subtilis* PY79 spores; lane 2: extracted protein of *B. subtilis* A1.13 spores; M: Protein marker

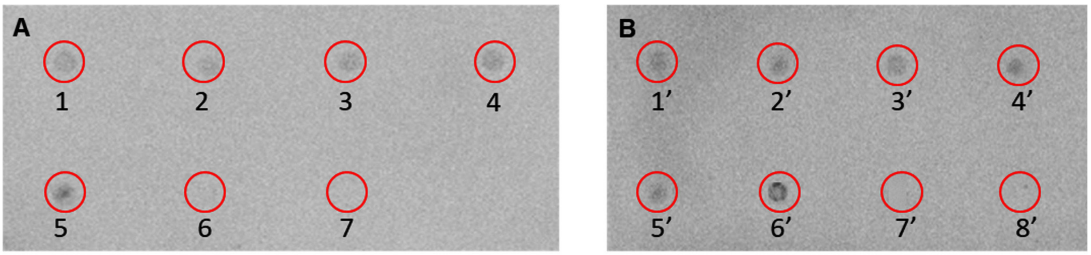

Figure 3. Dot blots present the stability of surface expression of UreA on spores *B. subtilis* A1.13 at different vaccine preservation and delivery conditions. At temperatures of -20°C (1), 4°C (2), 25°C (3) and 37°C (4) for 28 days. Treated with simulated intestinal fluids (1' & 2') or simulated gastric fluids (3' & 4') for 1 h and then stored at 4°C for 28 days. Positive controls: UreA (5 & 5'), *H. pylori* HP37 (6'). Negative controls: spores coats protein extract of PY79 stored at 4°C for 28 days (6 & 7'), PBS (7 & 8'). The number of spores was 2×10^7 spores per sample

recombinant strain A1.13 both displayed similar levels of sporulation, germination efficiency, and spore resistance to trimethoprim and heat (not shown), which is relevant to a previous study.[13] Authors in that research developed a fusion of CotB with an N-terminal length of 275 amino acid residues (825 bp) and a shortened UreA sequence that lacked 49 amino acids at the N-terminal end, but failed to generate any fusion products of CotB with the entire protein UreA. Although still removed DNA region encoding the three repeats in the C terminus of CotB, this study used a longer fragment of *cotB* (1130 bp) with additional upstream sequences, thus likely contributing to improved genetic stability to successfully clone the entire protein UreA in *B. subtilis*.

In addition, compared to *E. coli* expression system, *B. subtilis* host is more relatively difficult in plasmid transformation and less stability of plasmids.[15] In the present study, the plasmid pThyA-*cotB* which was firstly transformed in *E. coli* before fused with *ure*A and then transformed in *B. subtilis*, thus, possibly improved transformation efficiency in the latter host.

To verify that the gene fusion was surface expressed, spore coat protein extraction, SDS-PAGE and dot blot analysis were performed with the use of anti-UreA antibodies. An approximately 58.2-kDa band was found after SDS-PAGE examination of spore coat protein extracts from wild type and recombinant strains bearing the *cotB-ureA* fusion (Figure 2B), which is relevant to the gene structure designed and amino acid sequences deduced from DNA sequences (Figure 2A). Dot blot analysis showed the surface expression of *Ure*A on spores A1.13 and its stability maintained in the different conditions of temperatures and simulated harsh environments of gastrointestinal (GI) tract for a minimum of 28 days (Figure 3).

In order to further test a robust immune response, an oral vaccine candidate must overcome preservation conditions (e.g. -20°C, 4°C) and multiple physicochemical and biological barriers in the GI tract such as the human body temperature at 37°C, a high acidity in the stomach, an alkaline pH in the intestine tract, and proteolytic enzymes available for the degradation of antigenic proteins or peptides.[16] In this study, combined with genetic structure stability, the surface expression stability of the recombinant spores at different oral vaccine preservation and delivery conditions has proven spore as a cutting-edge and effective mucosal vaccination delivery method,[13,14] and suggested A1.13 as a spore vectored vaccine candidate against *H. pylori*. Findings from this study indicate that CotB is an appropriate carrier for displaying the full protein UreA on the surface of spores. However, further immunological experiments, preclinical and clinical trials will now be required to assess protective immune responses and its application potential.

Finally, following genetic manipulation, an environmentally sustainable and innovative gene-based solution for a critical problem of GEMs in the environment has been introduced here to be applied for the development of next-generation vaccines against *H. pylori* and other pathogens. If discharged, the recombinant spores A1.13 would spawn bacteria that could not survive in the environment habitats in the absence of thymine since they were made to be dependent on thymine, thus likely stopping potential risks and controversies surrounding the use of GEMs. However, it now still lacks evidence for a "permanent" thymine-less death of microbes in nature.

CONCLUSION

We used here the N-terminal fragment of spore coat protein *CotB* in *B. subtilis* and the entire antigen *Ure*A in *H. pylori* as examples showing that chimeric proteins comprised of these two heterologous proteins/peptides can be displayed on the spore surface with high stability of genetic structure and expression. The *ure*A gene was inserted into the *thyA* gene in the wild type strain, thus the recombinant spores A1.13 grows dependently on thymine and become a promising spore vectored vaccine candidate for oral administration against *H. pylori* with higher safety when released in the environment.

ACKNOWLEDGMENTS

The authors would like to acknowledge Prof. Simon Cutting at Royal Holloway University of London (UK) for providing *B. subtilis* strain PY79 and plasmid *pThyA*, and Yen Linh Nguyen at

Institute of Vaccines and Medical Biologicals (IVAC, Vietnam) for technical support to dot blot assays.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

VDN conceptualized the study. TLP, TKCN and TCN carried out the study and collected the data. TPP and VDN applied methodology. TTP collected resources and performed validation. VDN supervised, project administration, and funding acquisition. VDN wrote the manuscript. TKCN, VDN and TTP reviewed the manuscript. VDN edited the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

This study was supported by the Vietnam Ministry of Science & Technology (MOST) under grant number NDT.79.GB/20 to VDN.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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