Mucosal Adjuvant Activity of IL-2 Presenting Spores of Bacillus subtilis in a Murine Model of Helicobacter pylori Vaccination

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Abstract
The endospores of Bacillus subtilis are now widely used as a platform for presentation of heterologous proteins and due to their safety record and high resistance to harsh environmental conditions can be considered as potential vehicles for oral vaccination. In this research we show that recombinant B. subtilis spores presenting a fragment of the Helicobacter acinonychis UreB protein and expressing the ureB gene under vegetative promoter elicit a strong cellular immune response in orally immunized mice when co-administered with spores presenting IL-2. We show for the first time the successful application of two types of recombinant spores, one carrying an antigen and the other an adjuvant, in a single oral immunization.

Introduction
The display of active molecules on the surface of microorganisms is a promising technology to be used in the biotechnology and medicine [1,2]. A special attention is paid to bacterial endospores as carriers of heterologous proteins [3], which are advantageous to whole-cell display systems because of their unique properties.

Endospores are dormant forms of bacteria belonging to different genera, but most extensively studied surface display systems are based on Bacillus subtilis endospores [4]. B. subtilis spores are highly resistant to non-physiological and harsh environmental conditions. Such properties mainly result from the presence of protective structure surrounding spore called the coat. Multilayered coat is formed by at least seventy different proteins (Cot proteins) and composes of an inner and outer coat [5] as well as the outermost layer called the crust [6,7]. Three coat proteins, CotB, CotC and CotG have been used for display of heterologous enzymes and antigens on the spore surface [8–11].

So far B. subtilis spores have been successfully used to develop protection in animal models against various pathogens such as Clostridium perfringens [12], Clostridium difficile [13], Clostridium tetani [14] or Rotavirus [15]. In all these examples spore-based vaccines have been delivered by a mucosal route and have been shown to stimulate both systemic and localized immune responses. B. subtilis spores have also been shown to induce balanced Th1/Th2 response [16] and could be used as a mucosal adjuvant in some applications [17]. Moreover, taking into account probiotic properties of B. subtilis and its spores [18], these features make them very attractive candidates as vaccine carriers, especially in oral immunizations.

Helicobacter pylori is a major factor causing chronic gastritis and significantly increases the risk of developing peptic ulcer disease and gastric cancer [19]. Current treatments of H. pylori infections are encountering problems caused by antibiotic resistance (especially to metronidazole and clarithromycin) leading to growing difficulties in eradication of this bacterium [20]. Infection with H. pylori is related to Th1-biased T-cell response and generally elicits robust cellular and humoral immune responses. In spite of these facts, spontaneous eradication of these bacteria form human body is very rare. Moreover, the research conducted on animal models suggests, that establishing humoral immunity does not protect against infection [19].

Several approaches to the construction of a vaccine against H. pylori infections have been undertaken. One of the strategies used subunit A of urease (UreA) as an antigen the use of which has been patented (OraVax Inc., Cambridge, MA, US) and the vaccine based on this protein has been used in clinical studies (phase I) [21–23]. Another successful approach to immunization against H. pylori infection has been based on multi-epitope DNA vaccine with CpG oligonucleotides and LTB as adjuvants [24]. The results of other trials to immunize mice with H. pylori oipA gene-encoded...
construct co-delivered by IL-2 gene-encoded construct and LTB [25], as well as Salmonella vector construct that expressed fusion proteins complexed with H. pylori CagA, VacA and UreB in different arrangements suggested an important role of use of multiple antigen in formulation along with an adjuvant leading to Th1 shift of cellular response [26].

Here we report that recombinant Bacillus subtilis spores presenting UreB protein elicit cellular immune response in orally immunized mice when administered along with spores presenting human IL-2. Such formulation seems to be a promising vaccine candidate against Helicobacter pylori infections.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the institutional and national guidelines for animal care and use. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Medical University of Gdańsk (Permit Number: 4/2010). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Bacterial strains and transformation

Bacillus subtilis strains used in this study are listed in Table 1. Plasmid amplifications for nucleotide sequencing and subcloning experiments were performed with Escherichia coli strain DH5α [27]. Bacterial strains were transformed by previously described procedures: CaCl2-mediated transformation of E. coli competent cells [27] and transformation of B. subtilis [28].

Construction of gene fusions

DNA coding for CotC coat protein was PCR amplified using the B. subtilis chromosome as a template and oligonucleotides pair cotC-F/cotC-R (Table 2) as primers. Amplification product of 383 bp was cloned into the pDL vector [29] obtained from Bacillus Genetic Stock Center yielding plasmid pKH29.

A 655 bp DNA fragment coding for a fragment of UreB was PCR amplified using Helicobacter acinonychis chromosome as a template and oligonucleotides ureB-F and ureB-R (Table 2) as primers. The PCR product was sequentially digested with BamHI and SacI and cloned in frame to the 3’ end of the cotC gene carried by plasmid pKH29 yielding plasmid pKH108.

A plasmid enabling integration of pnov2 fusion with ureB gene into thrC locus was constructed as follows. A 303 bp fragment of B. subtilis chromosome containing promoter of rntO operon was PCR amplified using oligonucleotides rop2-F and rop2-R (Table 2) as primers. The PCR product was sequentially digested with HindIII and EcoRI and cloned into the pDG1663 vector [30] obtained from Bacillus Genetic Stock Center yielding plasmid pKH100. Next, a 1730 bp fragment encoding entire UreB protein was PCR amplified using H. acinonychis chromosome as a template and oligonucleotides ure-Bw-F and ure-Bw-R (Table 2) as primers. Obtained PCR product was sequentially digested with HindIII and PstI and cloned into the pKH100 plasmid yielding pKH101 plasmid.

DNA coding for CotB coat protein was PCR amplified using the B. subtilis chromosome as a template and oligonucleotides pair cotB-F/cotB-R (Table 2) as primers. Amplification product of 1094 bp was cloned into the pDG1663 vector obtained from Bacillus Genetic Stock Center yielding plasmid pKH117.

A gene encoding human IL-2 with B. subtilis optimized codon usage flanked by restriction enzyme sites with the sequence coding for GGGGAAAKGGG peptide linker at the N-terminus was synthesized at Eurogentec (Belgium) and delivered in pUC19 vector. A 457 bp fragment coding for IL-2 with peptide linker at N-terminus was PCR amplified using oligonucleotides IL-2-F and IL-2-R (Table 2) as primers and the plasmid containing synthetic gene as template. Obtained PCR product was sequentially digested with BamHI and PstI and cloned into the pKH117 vector yielding pKH122 plasmid.

Chromosomal integration

Appropriate plasmids were linearized by digestion with a single cutting restriction enzyme. Linearized DNA was used to transform competent cells of the B. subtilis strain 168. In case of pKH108 plasmid chloramphenicol-resistant (CmR) clones were the result of a double-crossover recombination event, resulting in the interruption of the non-essential amyE gene on the B. subtilis chromosome. Several CmR clones were tested by PCR. Selected clones were called BKH48 and used for subsequent transformation with linearized pKH122 plasmid.

Obtained erythromycin-resistant (ErmR) clones were the result of double-crossover recombination in the non-essential thrC gene. Several ErmR clones were tested by PCR. Selected clones were called BKH108 and stored for further experiments.

In case of transformation with linearized pKH122 plasmid the verification of obtained clones followed the same procedure as for the construction of BKH108 strain with selection for erythromycin-resistant colonies. Selected clones were called BKH121 and stored for further research.

Preparation of spores

Sporulation was induced by the exhaustion method in DS (Difco-Sporulation) medium as described elsewhere [32]. PMSF

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**Table 1. Strain list.**

| Strain | Relevant genotype | Reference |
|--------|-------------------|-----------|
| DH5α   | thrA2 lacZΔ(U169) pheA glu44 O8O' lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | [27] |
| **Bacillus subtilis** | | |
| 168    | trpC2             | [49]     |
| BKH48  | amyE::cotC-ureB3  | This work |
| BKH108 | thrC::rrnOP2-ureB, amyE::cotC-ureB3 | This work |
| BKH121 | thrC::cotB-linker-IL-2 | This work |

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**Mucosal Adjuvant Activity of IL-2 Presenting Spores**
Table 2. Oligonucleotide list.

| Name     | Sequence (5’-3’) | Restriction site |
|----------|-----------------|-----------------|
| cotB-F   | GCGGATCCGATGATTGAT | BamH1 |
| cotB-R   | GATGAAATTCCAGGATTTAGG | EcoRI |
| cotC-F   | GGGGATCCGATGTTTGTATGC | BamH1 |
| cotC-R   | GAGAATTCCAGGATTTAGG | EcoRI |
| ureB-F   | GCTACGGATCCAAATAACACCTAAACCG | BamH1 |
| ureB-R   | GCACCTGAGCTCCTACTTTTGTGGTAC | SacI |
| IL-2linker-F | GCCATACATTTCATGCTAGCTAGATAGATAGATAGTGC | Peo |
| IL-2linker-R | CATATGGCAGTCGGTGGGAAGAGCAGCGG | BamH1 |
| rOP2-F   | GATGGCATAAGCCTTCATGGGTCTCACCCTCGTGTTC | HindIII |
| rOP2-R   | GGGGATGAGGAATTCGAGCTGATGAGCCTACATGAC | EcoRI |
| ureBw-F  | CCATTAAAGCTAAAAAGATACAGGAAAAG | HindIII |
| ureBw-R  | CTCCACATGATCTTTAGAATAGCTAAG | Peo |
| hisureB-F | CTGGAAATTCGGTGGCTTCTATTTTCA | EcoRI |
| hisureB-R | CATATGGCAGTCGATCACCACCTACATCAGGAGATTACAGGAAAAGA | NheI |

The recognition sites for the restriction enzymes are in bold.
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(0.05 M) was included to inhibit proteolysis. After the final suspension in water spores were treated at 65°C for 1 h to kill any residual cells. The spore suspension was titrated immediately for CFU/ml before freezing at −20°C. By this method we could reliably produce 6×10^10 spores per litre of DSM culture.

**Spore germination**

Spore germination measurements in the presence of l-alanine or AGFK solution were performed as follows. Spores were heat activated at 80°C for 10 min and diluted to an OD_{600} of 1 in 10 mM l-alanine and 10 mM Tris-HCl at pH 7.5 (for l-alanine-induced spore germination) or in 10 mM Tris-HCl at pH 7.5 with 3.3 mM l-asparagine, 5.6 mM d-glucose, 5.6 mM d-fructose, and 10 mM KCl (for AGFK-induced spore germination). Germination was then monitored by following the loss of absorbance of spore suspensions at 600 nm.

**Extraction of spore coat proteins**

Spore coat proteins were extracted from 50 μl of a suspensions of spores at high density (1×10^10 spores per ml) using a decoating extraction buffer as described elsewhere [33]. Extracted proteins were assessed for integrity by SDS-polyacrylamide gel electrophoresis (PAGE) and for concentration by two independent methods: the Pierce BCA Protein Assay (Pierce, USA) and the BioRad DC Protein Assay kit (Bio-Rad, USA).

**Western and dot blotting analyses**

Western blotting analyses were performed as described elsewhere [10]. Dot blotting analyses were performed as previously described [11] and followed by densitometric analysis with Chemidoc XRS (Bio-Rad, USA) and the Multi Analyst software.

**Immunofluorescence microscopy**

Samples were prepared as previously described [11]. The coverslip was mounted onto a microscope slide and viewed using a Zeiss Axioplan fluorescence microscope with the same exposure time for all samples. Images were captured using a camera connected to the microscope, processed with Corel Photo-Paint software and saved in TIFF format.

**Purification of UreB and antibody production**

The ureB gene of *H. acinonichis* was PCR amplified using chromosomal DNA as a template and oligonucleotides hisureB-up and hisureB-dn (Table 2) as primes. DNA encoding six histidines (His6-tag) was carried by oligonucleotide hisureA-dn. The obtained PCR product of 1730 bp was digested with enzymes EcoRI and NheI and cloned into the commercial vector pBAD (Stratagene). The resulting plasmid, pJK01, was verified by restriction analysis and nucleotide sequencing. The protein was purified and used for antibody production following method described previously [10].

**Immunizations**

Five groups of eight mice (female, BALB/c, 8 weeks) were immunized by oral route with suspensions of either spores expressing CotC-UreB3 (BKH108), CotB-linker-IL-2 (BKH121), both CotC-UreB3 and CotB-linker-IL-2 (1:1) or control, non-expressing spores (strain 168). A naive, non-immunized control group was included. Oral immunizations contained 1×10^10 spores in a volume of 0.2 ml and were administered by intragastric lavage on days 1, 5, 22, 24, 26, 43, 45, 47. Serum samples and spleen were collected on days 1, 22, 43 and 61 from two animals per group.

**Indirect ELISA for detection of antigen-specific serum**

Plates were coated with 100 μl per well of the specific antigen (2 μg/ml in carbonate/bicarbonate buffer) and left at room temperature overnight. Antigen was UreB purified protein. After blocking with 0.5% BSA in PBS for 1 h at 37°C serum samples were applied using a two-fold dilution series starting with a 1/20 dilution in ELISA diluent buffer (0.1 M Tris-HCl, pH 7.4; 3% (w/v) NaCl; 0.5% (w/v) BSA; 10% (v/v) sheep serum (Sigma); 0.1% (v/v) Triton-X-100; 0.05% (v/v) Tween-20). Every plate carried replicate wells of a negative control (a 1/20 diluted pre-immune serum), a positive control (serum from mice immunized intraperitoneally with
UreB purified protein. Plates were incubated for 2 h at 37°C before addition of anti-mouse AP conjugates (Sigma). Plates were incubated for a further 1 h at 37°C then developed using the substrate pNPP (para-Nitrophenylphosphate; Sigma).

Reactions were stopped using 2 M H2SO4.

Isolation of splenocytes

Mice were sacrificed and spleen was aseptically removed. The spleens were then perfused with RPMI-1640 (supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin) using 5 ml syringe fitted with 26 G needle to obtain single cell suspension of splenocytes. The splenocytes suspension was then centrifuged at 300×g for 15 min. The RBCs were lysed by hypotonic shock using 3 ml of 0.84% of sterile NH4Cl or ACK lysis buffer for 5 min. The cells were then washed thrice with RPMI 1640 to remove lysed RBCs and NH4Cl.

IFN-γ and IL-4 ELISpot assay

The numbers of IFN-γ and IL-4 secreting cells were determined by using mouse IFN-γ or IL-4 ELISpot respectively kit according to manufacturer’s instructions (BD ELISpot). Splenocytes (2×10⁴/mL) were cultured in presence or absence of UreB antigen for 48 h. The spots were counted using automated ELISpot plate reader (CTL-ImmunoSpot S6 Micro Analyzer, USA). ELISpot tests have been performed for each animal in three technical repeats. Results were statistically evaluated using Student’s t-test.

Results

Construction and chromosomal integration of gene fusions

To obtain recombinant B. subtilis spores expressing UreB the coding part of the ureB gene of H. acinonychis was fused in frame with the coding part of cotC. The gene fusion retained the promoter of the cot gene to ensure proper timing of expression during the sporulation process. Gene fusions were integrated into the B. subtilis chromosome at the non-essential locus amyE. As heterologous part we used a fragment of UreB that encompassed 166 amino acids (residues 418 to 584). This fragment of ureB gene coding for putative most immunogenic regions was designated with Antigen program (a part of EMBOSS package; http://emboss.sourceforge.net/).

In addition, to obtain the expression of full-length UreB in vegetative cells the ureB gene was fused to the constitutive promoter of rRNA operon (rRNAOP) [34] and integrated into B. subtilis chromosome at the non-essential locus thrC (Fig. 1A).

To achieve recombinant B. subtilis spores expressing IL-2 the coding sequence of the IL-2 gene of Homo sapiens was fused in frame with the coding part of cotB. The C-terminus of CotB is formed of three 27 amino acid repeats that confer genetic instability to chimeric proteins containing them [31]. For this reason, in case of CotB fusions a fragment of DNA coding for these three repeats was omitted leaving only part of this gene encoding the N-terminal 275 amino acid residues (Fig. 1A). We also added the strong alpha-helix motif (-GGGEEAAKGGG-) [10,35] between the C-terminus of CotB and N-terminus of IL-2 (Fig. 1B).

Figure 1. Schematic representation of the three gene fusions constructed. Panel A – gene fusions present in the chromosome of BKH108 strain, Panel B – gene fusion present in the chromosome of BKH121 strain.

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The constructed strains were named BKH108 (CotC-UreB, rrnOP2-UreB) and BKH121 (CotB-GGGEAAKGGG-IL-2) and used for further analysis.

The two recombinant strains and their isogenic parental strain 168 showed comparable sporulation and germination (Figure 2) efficiencies and their spores were equally resistant to chloroform and lysozyme treatment (not shown). Therefore, limited to the spore properties that we have analysed, the presence of CotC-UreB and CotB-linker-IL-2 fusions did not affect spore structure or functionality.

**Spore coat expression**

The localization of fusion proteins on the spore coat was tested by western blotting with anti-CotC, anti-UreB, anti-CotB and anti-IL-2 antibodies. The analysis of strain BKH108 showed the presence of an about 28-kDa protein which reacted with both UreB- and CotC-specific antibodies (Fig. 2AB). A standard pattern of CotC and CotU proteins [31,36] was observed in wild type spores with and without fusion CotC-UreB (Fig. 3A, lanes 1–2). In agreement with a previous report [37], the fusion of a heterologous protein at the C-terminus of CotC impaired the formation of CotC homodimer and CotC-CotU heterodimer. As a consequence, when fused to UreB CotC was only found as a monomer. In addition, the analysis of strain BKH108 showed the presence of an about 62-kDa protein detected by anti-UreB antibodies and corresponding in size to the entire UreB (Fig. 3B, lane 4). As expected this protein was present in extracts from vegetative cells. Western blot analysis of spore coat proteins purified from wild type

![Figure 2](image1.png)

**Figure 2. Germination of spore suspensions in l-alanine and AGFK solutions.** Spores prepared from cells of 168 (diamonds) and BKH108 (squares) grown in DS medium were heat activated and subsequently incubated in 10 mM Tris-HCl (pH 7.5) with 10 mM l-alanine or with 3.3 mM l-asparaginate, 5.6 mM d-glucose, 5.6 mM d-fructose, and 10 mM KCl (AGFK). Germination was followed by measuring the A600 of the spore suspension.

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![Figure 3](image2.png)

**Figure 3. Western blotting analysis of expression of the cot-ureB fusion gene and the vegetative expression of the ureB gene.** Panel A - Spore coat proteins were extracted analysed by western blotting with anti-CotC antibody. Spore coat proteins from spores of the 168 (lane 1) or the BKH108 (fusion CotC-UreB) (lane 2) strain. Panel B - Western blotting with anti-UreB antibody of spore coat proteins from spores of the 168 (lane 1) or BKH108 (fusion CotC-UreB) (lane 2) and of total cell protein extracts of 168 (lane 3) or BKH108 (lane 4) strain. Each lane of panel A and B was loaded with 20 μg of total proteins. Arrows points to fusion proteins.

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and recombinant strains BKH121 revealed the presence of an about 55-kDa band which reacted with both IL-2 and CotB-specific antibodies (Fig. 4AB). A 66-kDa band, only reacting with CotB-specific antibody, was present in extracts from wild type and recombinant spores (Fig. 2A), indicating the presence of intact CotB molecules in the spore coat together with CotB-IL-2 fusion protein. A 45-kDa protein, reacting with anti-UreB antibody was observed in strain carrying fusion CotB-linker-IL-2 (Fig. 4B, lane 2). This protein was also recognized by anti-CotB antibody (Fig. 4A, lane 2), we therefore hypothesize that it is a degradation product of CotB-linker-IL-2 fusion.

In both cases the recombinant proteins observed showed apparent molecular weights that correlated well with the deduced molecular weights: Fusion CotC-UreB, 26.9/28; CotB-linker-IL-2, 52.7/55; (deduced/apparent kDa)

Surface display

The surface localization of CotC-UreB (BKH108) and CotB- GGGEAAAKGGG-IL-2 (BKH121) fusion proteins was analyzed...
by immunofluorescence microscopy of dormant spores of wild type and recombinant strains using anti-UreB and anti-IL-2 (Abcam, UK) primary antibodies and anti-mouse IgG-Cy3 (Jackson ImmunoResearch Laboratories, Inc). We observed a fluorescent signal around purified dormant spores of both BKH108 and BKH121 strains (Fig. 5). These results indicate that both fusion proteins are present on the spore coat surface and are available for antibody binding.

Efficiency of spore surface display

The amounts of CotC-UreB and CotB-GGGEAAKGGG-IL-2 fusion proteins present on the spore coat surface was assessed using a dot-blotting method. The fusion protein CotC-UreB

Table 3. Densitometric analysis.

| Protein source | Amount of protein used (ng) | Density in OD/mm² (standard deviation) | Protein concentration (ng) in extracts (% of total) | n° of recombinant molecules extracted from each spore |
|---------------|--------------------------|----------------------------------------|-------------------------------------------------|----------------------------------|
| Purified UreB | 25.0 ng                  | 172.1 (±0.02)                          | NA                                              | NA                               |
|               | 12.5 ng                  | 88.2 (±0.03)                           | NA                                              | NA                               |
|               | 6.25 ng                  | 45.7 (±0.12)                           | NA                                              | NA                               |
| BKH108 (CotC-UreB) | 2.50 µg          | 132.5 (±0.03)                          | 19.25 (0.77)                                     | 1.5 x 10⁴                         |
|               | 1.25 µg                  | 67.3 (±0.02)                           | 9.54 (0.76)                                      |                                   |
|               | 0.625 µg                 | 34.9 (±0.06)                           | 4.77 (0.76)                                      |                                   |
| Purified IL-2 | 25.0 ng                  | 202.6 (±0.01)                          | NA                                              |                                   |
|               | 12.5 ng                  | 102.1 (±0.02)                          | NA                                              |                                   |
|               | 6.25 ng                  | 51.3 (±0.01)                           | NA                                              |                                   |
| BKH121 (CotB-linker-IL-2) | 2.50 µg          | 169.6 (±0.01)                          | 20.93 (0.84)                                     | 9.5 x 10³                         |
|               | 1.25 µg                  | 85.4 (±0.02)                           | 10.45 (0.84)                                     |                                   |
|               | 0.625 µg                 | 43.6 (±0.02)                           | 5.31 (0.85)                                      |                                   |

Figure 6. IFN-γ response of sensitized mouse splenocytes to UreB as assessed by the ELISpot. The splenocytes were isolated from naïve mice (open bars), mice orally immunized with 168 spores (dotted open bars), BKH108 (CotC-UreB, vegetative expression of UreB) (bars with horizontal hatching), BKH122 (CotB-linker-IL-2) (bars with vertical hatching) or 1:1 mixture of BKH108 and BKH122 (dotted closed bars). Cells were treated with purified UreB protein for 72 h and then the IFN-γ cells were enumerated by ELISpot procedure. Error bars represent standard deviation. * p-value <0.05, ** p-value <0.005. I – day 22, II – day 43, III – day 61.

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constituted 0.7% of total spore coat proteins from strain BKH108 (Table 3). From these results, we calculated that the number of fusion protein molecules extracted from a single spore was $1.5 \times 10^9$. In the case of CotB-GGGEAAAKGGG-IL-2 we calculated that the fusion constituted 0.8% of total spore coat proteins, which translates into $9.3 \times 10^8$ molecules of fusion protein per single dormant spore (Table 3).

**Immune response to recombinant spores**

To verify whether such recombinant spores were able to elicit an immune response and whether spores presenting IL-2 acted as adjuvant we orally immunize groups of mice with spores presenting UreB or IL-2 or with a combination of the two recombinant spores. Production of UreB-specific serum antibody and of IFN-γ and IL-4 by sensitized splenocytes isolated from immunized mice was followed. We were not able to detect UreB-specific antibodies in any of the tested groups of mice (data not shown) suggesting that the immunizations did not induce a humoral response. However, the results of IFN-γ ELISpot experiments revealed that BKH108 spores induced a strong cellular immune response when co-administered with BKH121 spores, presenting human IL-2 (Fig. 6). Moreover, there was clearly visible increase in the immune response along with subsequent immunizations. Interestingly, we were not able to detect IL-4 produced by UreB-sensitized splenocytes isolated from immunized animals (data not shown).

**Discussion**

The use of *Bacillus subtilis* spores as mucosal vaccine vehicles has already been tested with various antigens (for review see [4]). The successful applications of spores as vaccines so far reported were based on the use of strong antigens such as tetanus toxin or heat-labile toxin of *Escherichia coli*, that in addition to the strong antigenicity, also serve as efficient adjuvants [38]. Recombinant spores of *B. subtilis* seem to be a perfect choice for *Helicobacter pylori* oral vaccine candidate. Due to unique properties of spores such as heat-stability and ability to safely pass through harsh stomach environment the delivery of *H. pylori* antigens should be much more efficient than in case of other mucosal vaccine systems. Not to be omitted is also the fact that administration of oral vaccines eliminates the need of needles usage and the assistance of trained medical personnel.

We combined three approaches in our design of *H. pylori* spore-based vaccine. First, we used the coat protein CotC as a carrier for a fragment of subunit B of *Helicobacter acinonychis* urease. UreB protein has already been used for immunizations against *H. pylori* infections and is well-characterized antigen of this bacterium [39,40]. UreB of *H. acinonychis* shares 72% identity with UreB of *H. pylori* and was used to avoid any possible intellectual property issues caused by patents restricting the use of this protein in oral vaccine formulations. BKH108 recombinant spores proved to efficiently display a fragment of UreB protein on the spore surface (Fig. 3A).

*B. subtilis* spores were shown to germinate and most probably undergo re-sporulation inside the gastrointestinal tract (GIT) of laboratory animals [6]. BKH108 spores, apart from presentation of UreB fragment, harbor ureB gene of *H. acinonychis* under control of vegetative promoter of rmOP operon. This enables for production of full-length UreB protein in the vegetative cells, which can appear inside the GIT upon germination of spores and increase the amount of antigen in the site of immunization. Indeed, we observed efficient production of UreB protein in BKH108 cells during vegetative growth (Fig. 3B). It is worth notifying, that use of pแนะนำ for vegetative expression of an antigen has already been applied for spore-based orally administered vaccines and proved to lead to induction of immune response [41,42].

An efficient immunization usually requires usage of an appropriate adjuvant. In case of *H. pylori* vaccine such adjuvant should shift the immune response towards Th1 type [43]. Recently IL-2 has been used for that purpose [23]. This cytokine is mainly produced by Th1-polarized helper T-cells and is imposes strong shift towards the cellular immune response [44,45]. On the other hand, the cellular response has been proposed as a leading one in protecting against *H. pylori* infection [46]. Having in mind these facts we decided to use IL-2-presenting spores, which should serve as an adjuvant helping in the development of immune response. IL-2 has been linked with CotB spore coat protein via previously described linker [47] to additionally improve the display. As a result we obtained spores, which efficiently presented IL-2 (Fig. 4).

The results of oral immunizations of mice suggest that such combination of recombinant spores presenting a fragment of UreB protein and enabling for the expression of this protein in vegetative cells along with IL-2-presenting spores is able to elicit immune response to UreB. The magnitude of response was increasing with each subsequent immunization showing the development of immune memory (Fig. 6). Interestingly, we did not observe production of UreB specific antibodies. However, this observation combined with the lack of IL-4 produced by sensitized mouse splenocytes is not entirely surprising. IL-2, as mentioned above, imposes strong shift towards cellular response. This fact can explain the lack of humoral response to administered antigen. Moreover, when administered alone, BKH108 spores led to the induction of distinct, but statistically significant cellular immune response. In case of IL-2-presenting spores (BKH121) we observed similar phenomenon, which in that case may suggest unspecific induction of immune response by IL-2. This last observation should be verified in further experiments. Nevertheless, when administered together, BKH108 and BKH121 spores led to much stronger induction of cellular immune response suggesting such formulation as a very promising vaccine.

The key question regards a potential risk of IL-2 usage in vaccine formulations. Because of its biological activity an uncontrolled administration of this cytokine may lead to vascular leak syndrome [for review see [46]]. Such adverse effect of immunization is undesired therefore it is important to carefully assess safe amount of IL-2 administered along with a vaccine. The formulation of vaccine based on recombinant spores enables for convenient optimization of its composition. In our research we used spores carrying an antigen (UreB) along with spores serving as an adjuvant (IL-2). By changing of proportion of administered spores we can modify the amount of both, an antigen and an adjuvant in final formulation.

In conclusion, *B. subtilis* spores seem to be a promising platform for vaccine candidates against *H. pylori*. Although the application of IL-2 as an adjuvant increases the efficiency of immunization, further research is required to assess protective and therapeutic potentials of such formulation.

**Author Contributions**

Conceived and designed the experiments: MO KH AI ER. Performed the experiments: KH AI MS IP GP-S. Analyzed the data: KH AI MO ER RI. Contributed reagents/materials/analysis tools: MO KH ER. Wrote the paper: AI ER RI.
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