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A STUDY OF THE REPAIR ACTIVITY OF THE XTHA AP *ENDONUCLEASE FROM HELICOBACTER PYLORI* IN MUTANT *ESCHERICHIA COLI*

Turgimbayeva A.M.[1,2], Abeldenov S.K.[1], Saparbayev M.K.[3], Ramankulov Ye.M.[1], Khassenov B.B.[1]

[1] *National Center for Biotechnology*
Korgalzhyn road, 13/5, Astana, 010000, Kazakhstan
[2] *L.N. Gumilyov Eurasian National University*
Satpayev str., 2, Astana, 010000, Kazakhstan
[3] *Institute of Gustav Roussy*
CNRS UMR 8200, 114 Rue Edouard Vaillant, Villejuif, 94805, France
turgimbayeva@gmail.com

ABSTRACT

Oxidized and alkylated DNA damage occurs in the *Helicobacter pylori* genome during its life, just as it does in the genomes of other pathogens. Reactive oxygen species cause single-stranded DNA breaks, and the alkylation of purine bases leads to the formation of an apurinic/apyrimidinic site (AP site). Base excision repair enzymes, in particular AP endonucleases, play an important role in the removal of cytotoxic oxidized DNA damage and AP sites. *H. pylori* has a single XthA AP-endonuclease (HpXthA), unlike *E. coli*, which has two.

We aimed to obtain rabbit polyclonal antibodies against HpXthA, and optimize western blot conditions with the obtained antibodies. We carried out a comparative analysis of *H. pylori xthA* gene expression in the pET-system vectors relative to pBlueScript SK(+). We confirmed that the replacement of an aspartic acid residue with an asparagine residue at position 144 is essential for the enzymatic activity of the HpXthA AP endonuclease. We also showed the importance of the HpXthA AP endonuclease in the survival of cells treated with methyl methanesulfonate and hydrogen peroxide. For this, *E. coli xthA*$^-$, *nfo*$^-$ double mutants were complemented with the *xthA* gene from *H. pylori*. As a result of *H. pylori xthA* gene expression, *E. coli* survival increased in the presence of mutagens. In mutant *E. coli*, the repair of oxidized and methylated DNA bases occurred owing to HpXthA expression, which was confirmed by western blotting with anti-HpXthA antibodies.

Keywords: base excision repair, *Helicobacter pylori*, AP endonuclease XthA, AP-site, oxidized DNA damage, alkylated DNA damage

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INTRODUCTION

Reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH), are known to damage DNA, proteins and lipids [1]. Bacterial cells have two strategies against ROS exposure. The first strategy is based on the activity of

superoxide dismutase and catalase, which directly neutralize ROS. The second strategy is based on the activity of enzymes that restore or destroy oxidized components. DNA repair enzymes and stress-responsive proteases inhere to the second strategy of bacterial cell protection against ROS exposure [2,3].

Base excision repair (BER) plays an important role in elimination of DNA damages in *Escherichia coli* [4]. DNA glycosylases recognize and remove the damaged nitrogenous bases, leaving an apurinic/apyrimidinic site (AP site). AP endonucleases create a nick at the 5'-end of the damaged nucleotide, producing a free 3'-hydroxyl group on the adjacent nucleotide. It allows to DNA polymerase and ligase to restore DNA. *xthA* gene encodes XthA AP endonuclease, which provides approximately 90% of AP endonuclease activity in *E. coli* [5]. The rest of AP endonuclease activity is performed by Nfo AP endonuclease in *E. coli* [6]. The importance of the BER pathway in the recovery of oxidized DNA damage is confirmed by *E. coli xthA*$^-$ and *xthA*$^-$, *nfo*$^-$-mutants, which are hypersensitive to H_2O_2 [6,7]. As a result of H_2O_2 exposure, *xthA, nfo*$^-$ mutants die 20 times faster than wild-type bacteria [7].

ROS, generated from H_2O_2, cause single-stranded DNA breaks. The 3'-ends of these breaks are framed by phosphate groups. Such ends are not amenable to further recovery until the phosphate groups are removed by XthA, which also has a phosphatase function. It requires more than 99% of all 3'-phosphatase activity in *E. coli* [7]. In addition to H_2O_2 hypersensitivity, *xthA*$^-$, *nfo*$^-$ *double* mutants are sensitive to methyl methanesulfonate (MMS). MMS alkylates the purine bases, as a result AP sites are generated. AP sites can be fatal to *xthA*$^-$, *nfo*$^-$ double mutants [6], while *E. coli xthA*$^-$ mutants are less susceptible to MMS [8]. It is related with the ability of other AP endonucleases and AP lyases to replace the function of XthA AP endonuclease to restore N-alkylated DNA [8,9].

It is known that more than half of the world's population is infected with *H. pylori*. However, in most people, infection does not lead to the appearance of clinical symptoms of gastric and duodenal ulcers, gastritis, duodenitis, gastric cancer, etc. Chronic gastritis associated with infection of the pathogen is one of the initial stages of inflammation. In the further, inflammation can lead to epithelial cells' damage and the development of carcinogenesis. *H. pylori* infection is a significant risk factor for gastric carcinogenesis. Being in a very aggressive stomach environment, *H. pylori* should avoid the acidity of the lumen and penetrate the gastric mucosa. In this extracellular space, the pathogen encounters oxidative damage of the immune response. BER is the main mechanism for repairing oxidative DNA base lesions. The key enzymes of this mechanism are AP endonucleases.

It is known that *H. pylori* has a limited number of genes encoding enzymes of the BER pathway [10]. An analysis of the pathogen genome revealed only one AP endonuclease - XthA (HpXthA), which is homologous to *E. coli* XthA and encoded by the HP1526 gene [11,12]. Cell-free extracts of mutant *H. pylori* strain with impaired open reading frame of the HP1526 gene did not possess Mg^{2+}-dependent AP endonuclease activity [13]. *H. pylori xthA*$^-$ mutants and *xthA*$^-$, *mutY* double mutants showed 4- and 37-fold increase of spontaneous mutation rate, respectively, in comparison with the wild type [14]. These data indicate that HpXthA is actively involved in the repair of AP sites and other mutagenic DNA damages. Earlier, we have already studied the biochemical activity of *H. pylori* XthA AP endonuclease *in vitro* [15]. The goal of this research is to determine the role of *H. pylori* XthA AP endonuclease in resistance of the bacterium to the effects of DNA-methylating agents and oxygen radicals. To achieve this goal, the following tasks are solved: obtaining HpXthA-specific antibodies, complementation of *E. coli xthA*$^-$, *nfo*$^-$ double mutant strain with recombinant HpXthA, determining HpXthA influence on the survival of *E. coli* treated with MMS and H_2O_2 *in vivo*.

Materials and methods

Strains, vectors and reagents. *Escherichia coli* DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–), Rosetta2(DE3) (F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pRARE2 (Cam^R) Kan^R.), BH110 (nfo::kan^R [Δ(XthA-pncA)90 X::Tn10]) strains and pBlueScript II SK+ (Addgene), pET11a/HpXthA и pET28c(+)/HpXthA vectors were used in the research.

Media and reagents. Low-salt Luria Bertani broth (1% tryptone, 0,5% yeast extract, 0,5% NaCl) was used for cultivation *E. coli* strains. SOC (2% tryptone, 0,5% yeast extract, 0,05% NaCl, 2,5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose, pH 7.5) medium was used for incubation of the transformed cells. In the media the concentration of kanamycin and ampicillin was 50 μg/ml and 150 μg/ml, respectively. The preparation of media was carried out in accordance with standard protocol [16]. Restriction endonucleases, T4 DNA ligase, Phusion High-fidelity DNA polymerase (Thermo Scientific) were used to clone and amplify the target gene. *Taq* DNA polymerase of own preparation was used for PCR screening of transformed colonies [17]. BH110 strain was lysogenized by helper phage (λDE3), carrying a copy of the T7 RNA polymerase gene, using the DE3 lysogenization kit (Novagen, Merck4Biosciences, France). Methyl methanesulfonate (MMS) and H_2O_2 (Sigma-Aldrich) were used as agents causing DNA damage *in vivo*. Freund's complete and incomplete adjuvants (Sigma-Aldrich) were used for rabbit immunization.

Oligonucleotides. Used oligonucleotides are shown in table 1.

Table 1 - Oligonucleotides for cloning

Oligonucleotide	Sequence
5BamHI-rbsHpXthA	5’-CGGGATCCAAGGAGATATAATGAAACTGATTTCATG GAATGT-3’
3NotI-HpXthA	5’-ATAGTTTAGCGGCCGCTTAAACTAATTCCAACCCT ACC-3’
5HpXthA_D144N	5’-GTCATTGTGTGTGGG**AAC**TTGAATGTGGCCC-3’
3HpXthA_D144N	5’-GGGCCACATTCAA**GTT**CCCACACACAATGAC-3’
M13fw	5’-GTAAAACGACGGCCAG-3’
M13rv	5’-CAGGAAACAGCTATGAC-3’

Immobilized metal ion affinity chromatography of HpXthA recombinant protein. Earlier, during the study of the biochemical activity of XthA AP endonuclease from *H. pylori*, we constructed pET-28c (+)/HpXthA vector carrying *xthA* gene from *H. pylori* [15]. Rosetta2 (DE3) cells were transformed with the obtained vector. 200 ml culture of transformed strain was incubated with 0.5 mM isopropyl-β-D1-thiogalactopyranoside (IPTG) for 16 hours at 22°C. Cells were harvested at + 4°C, 6000 × g for 7 minutes. The pellet was suspended in 20 mM NaCl, 20 mM Tris-HCl pH 8.0 buffer with a protease inhibitor cocktail (Roche Diagnostics). Cells were incubated with 3 mg/ml lysozyme for 20 minutes at room temperature and further treated with ultrasound (50 kHz) in a pulsed mode on ice. The lysate was centrifuged at +4°C, 40 000 × g for 60 minutes, the precipitate was removed. NaCl concentration was adjusted to 500 mM in the clarified lysate. HiTrap Chelating HP column (GE Healthcare) was prepared according to the manufacturer's protocol and activated with 100 mM $NiCl_2$. The lysate was loaded manually, and FPLC AKTA Purifier 10 (GE Healthcare) was used for purification and fractionation of the eluate. Recombinant protein was eluated in a linear imidazole gradient from 20 mM to 500 mM. Eluate fractions were pooled and loaded into a HiTrap Heparin HP column (GE Healthcare) and eluted with a linear NaCl gradient from 50 mM to 1M. The homogeneity of the obtained fractions was checked by separation in 12% of SDS-PAGE.

Immunization of the rabbit and production of anti-HpXthA polyclonal antibodies. Obtaining of polyclonal antibodies was performed as previously described [18].

A six-month-old rabbit was used; 1 ml of blood was taken from the blood ear vein prior to immunization to use serum as a negative control. On the first day of immunization, 300 μg of recombinant HpXthA protein, diluted in phosphate buffer, and Freund's complete adjuvant were subcutaneously injected into the back of a rabbit. After 7 days, immunization with 150 μg of recombinant HpXthA protein diluted in phosphate buffer with Freund's incomplete adjuvant was repeated. The third, fourth and fifth immunizations were carried out every 7 days, 150 μg antigen was injected into the rabbit. Two days later, the sixth immunization was performed and the next day, blood was taken.

Immunoglobulins were salted with 35% ammonium sulfate for 16 hours at +4°C, followed by precipitation at 40,000 × g for 30 minutes. The precipitate was suspended in 10 ml phosphate buffer and dialyzed against 1 liter of the same buffer. The resulting antibodies were stored at -20°C in 50% glycerol solution. The specificity and sensitivity of purified rabbit antibodies were tested by western blot with 1:250, 1:500, 1:2500, 1:5000 antibody dilutions and various concentrations of the recombinant HpXthA protein (2 μg, 1 μg, 500 ng, 100 ng, 50 ng).

Western blot. Western blot was performed according to a known method [16]. Samples were separated in 12% polyacrylamide gel with SDS and transferred to the PVDF membrane by electro transfer. To control the quality of sample transfer to the PVDF membrane, the membrane was stained by non-specific protein Ponceau S dye (Sigma-Aldrich). After blocking the membrane with fat-free milk, HpXthA protein was detected by rabbit anti-HpXthA polyclonal antibodies. Goat anti-rabbit horseradish peroxidase conjugated antibodies (Thermo Scientific) were used as secondary antibodies at a dilution of 1:10 000. The membrane was treated with a chemiluminescent substrate (Applichem) and exposed to X-ray film (Agfa).

Cloning of *xthA* gene from *H. pylori* into pBlueScriptII SK(+) and site-directed mutagenesis. To clone *xthA* gene into pBlueScriptII SK(+) vector, the target gene was amplified from pET28c(+)/HpXthA vector. PCR conditions: 74 ng pET28 (+)/HpXthA, 0.8 mM dNTP mix, 0.2 μM of each primer (5BamHI-rbsHpXthA and 3NotI-HpXthA), 1x HF buffer (containing 1.5 mM $MgCl_2$), 1 unit of Phusion High-Fidelity DNA polymerase. Temperature mode of PCR: + 98°C - 1 min; 30 cycles: +98°C - 15 s, + 55°C - 30 s, + 72°C - 40 s; + 72°C - 5 min. Amplified *xthA* gene from *H. pylori* was purified by phenol-chloroform extraction. The gene and expression vector were digested by BamHI and NotI restriction enzymes in 2x Tango buffer. Ligation was performed using T4 ligase in T4 Ligase buffer for 16 hours, at + 16°C.

Competent DH5α cells were transformed with the resulting ligase mixture by heat shock method. Selection of transformed colonies was performed by white-blue selection method on the agar medium contained X-gal, IPTG and ampicillin. After PCR screening of M13 region, positive colonies were inoculated in LB broth with ampicillin. Plasmid DNA was isolated using MiniPrep kit (Thermo Scientific). The insert was sequenced by Sanger method with M13 primers.

To obtain mutant HpXthA protein with D144N substitution, site-directed mutagenesis of pBlueScriptII SK(+)/HpXthA vector was hold using QuickChange site-directed mutagenesis kit (Stratagene) and 5HpXthA_D144N and 3HpXthA_D144N primers. pBlueScriptII SK(+)/HpXthA and pBlueScriptII SK(+)/HpXthA D144N expression vectors were obtained as a result.

Sensitivity to alkylating and oxidizing DNA agents. Cell treatment with MMS and H_2O_2 was performed as previously described [19]. The diluted overnight culture of *E. coli* BH110 (DE3) strain was incubated at 28°C in LB broth containing 150 μg/ml ampicillin and 0.05 mM IPTG. Upon reaching the exponential growth phase (OD_{600}=0.6), the cells were

collected by centrifugation (6 000 × g, 7 min, + 4°C), washed and suspended in phosphate buffer. At +46°C an alkylating agent MMS was added to 2.5 ml of 0.6% molten LB agar containing 1 mM IPTG. 250 µl of each cell dilution was immediately added to the mixture, poured onto 25 ml 1.5% LB agar. To test the effectiveness of elimination of oxidized DNA damages by AP endonucleases, each cell dilution was incubated with 10 mM H_2O_2 for 5, 10, 15, and 20 minutes. Then the cells were mixed with molten 0.6% LB agar containing 1 mM IPTG, and poured onto 1.5% LB agar. All cells were incubated at +37°C, the counting of colonies was carried out after 2 days.

RESULTS AND DISCUSSION

Purification of HpXthA antigen was hold to obtain antibodies. At the first stage of purification by metal affinity chromatography, HpXthA eluted at 220 mM imidazole. At the second stage HpXthA eluted at 345 mM NaCl on heparin affinity column (fig. 1, fractions 12, 13). The yield of purified HpXthA protein was 1 mg from 200 ml culture.

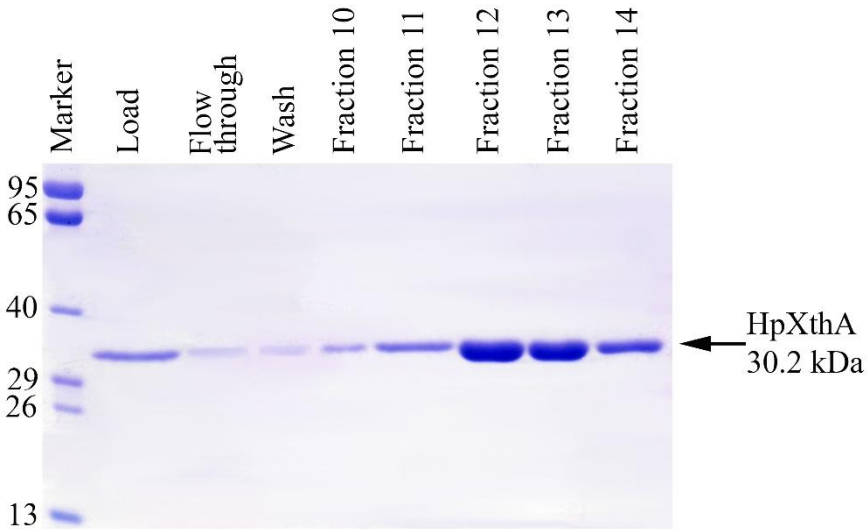


Fig. 1. Electrophoregram of recombinant HpXthA AP endonuclease purification on heparin affinity column

A six-month-old rabbit was immunized with purified recombinant HpXthA protein to obtain an immune response. After precipitation with ammonium sulfate and dialysis, immunoglobulins were tested for sensitivity and specificity by western blot with different concentrations of recombinant HpXthA protein 50 ng-2 µg (fig. 2). Dilution of anti-HpXthA antibodies was selected empirically (1:250, 1:500, 1: 2500, 1: 5000) and it was determined that the optimal dilution of the antibodies and buffer is 1:5000.

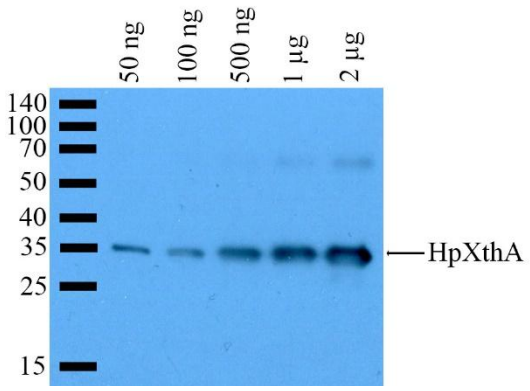
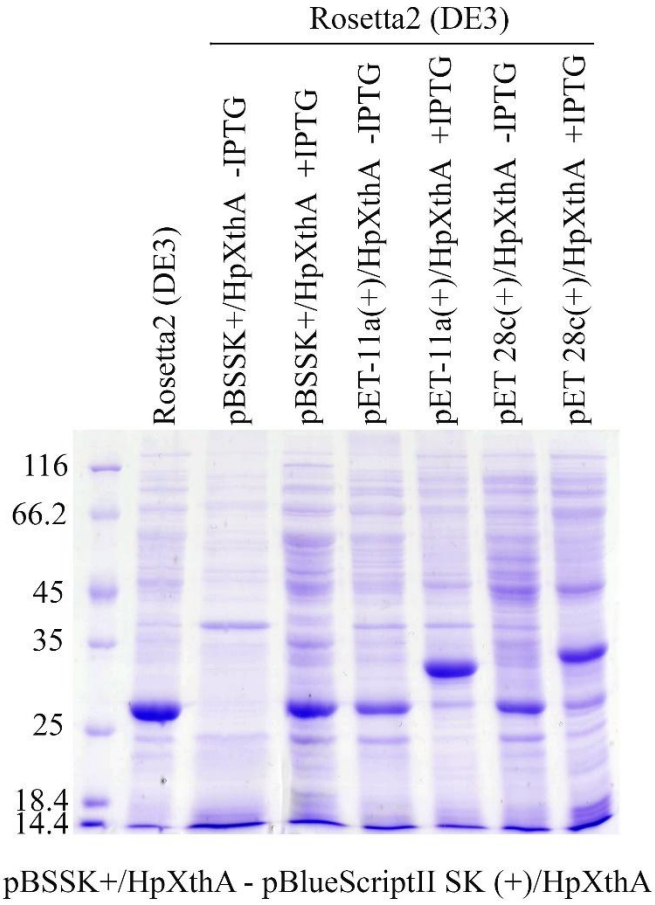


Fig. 2. Results of western blot with different concentrations of HpXthA and 1:5000 anti-HpXthA antibodies

According to the results of western blot, obtained antibodies are sufficiently sensitive to HpXthA protein.

xthA gene from *H. pylori* was cloned into pBlueScriptII SK(+) plasmid vector on the sites BamHI / NotI under the control of the T7 promoter. The choice of this vector is based on relatively low level of gene expression in comparison with the pET-system vectors. The electrophoresis of induced and uninduced cell lysates of Rosetta2 (DE3) clearly demonstrates this fact (fig. 3). Rosetta2 (DE3) was transformed with pBlueScriptII SK(+)/HpXthA, pET-11a (+)/HpXthA, pET-28c (+)/HpXthA vectors.



Molecular weight of HpXthA in pBlueScriptII SK(+)/HpXthA and pET-11a(+)/HpXthA – 28 kDa, in pET-28(+)/HpXthA – 30.2 kDa.

Fig. 3. Electrophoregram of comparative assessment of *xthA* gene expression level in different vectors

As can be seen in figure 3, *xthA* gene expression level in Rosetta2 (DE3) cells transformed with pBlueScriptSK (+)/HpXthA is much lower than in cells transformed with pET-11a (+)/HpXthA and pET-28c (+)/HpXthA.

pBlueScriptII SK (+)/HpXthA D144N vector was obtained by site-directed mutagenesis. In this vector, encoded HpXthA protein has a mutation in the 144th position: an aspartic acid residue was replaced by an asparagine residue. The choice of this amino acid substitution is related with the fact that D210N replacement in APE1 homologous human AP endonuclease has led to 10,000-fold decrease in activity [20]. It is known that Asp210

coordinates the metal-binding site of the enzyme[21], which is important for the implementation of AP endonuclease activity.

To determine the sensitivity of HpXthA to methylating and oxidizing DNA agents *in vivo*, model experiments were conducted using methyl methanesulfonate (MMS) and hydrogen peroxide (H_2O_2), respectively. *E. coli* BH110 (DE3) cells, mutant for Nfo and XthA AP endonucleases, were transformed with pBlueScriptII SK (+)/HpXthA, pBlueScriptII SK (+)/HpXthA D144N plasmid vectors. Low expression of recombinant XthA protein allowed us to obtain the level of AP endonuclease activity comparable with the physiological significative. This will affect the survival of the transformed cells. Cells, transformed with the empty pBlueScriptII SK (+) vector, were used as negative control. Cells transformed with pBW21/nfo vector, containing *nfo* gene from *E. coli*, were used as positive control. The percentage of survived cells was assessed after exposure of transformed cells to MMS and H_2O_2 (fig. 4).

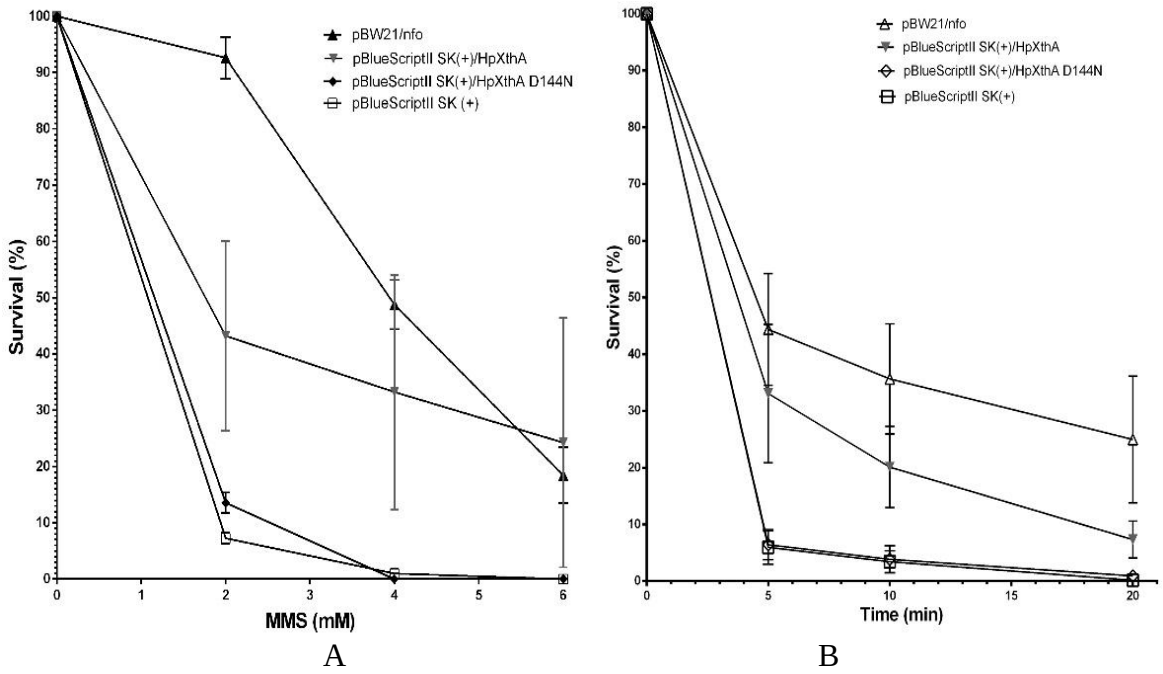


Fig. 4. The survival of *E. coli* BH110 (DE3) strain transformed with different vectors after exposure to MMS (A) and H_2O_2 (B)

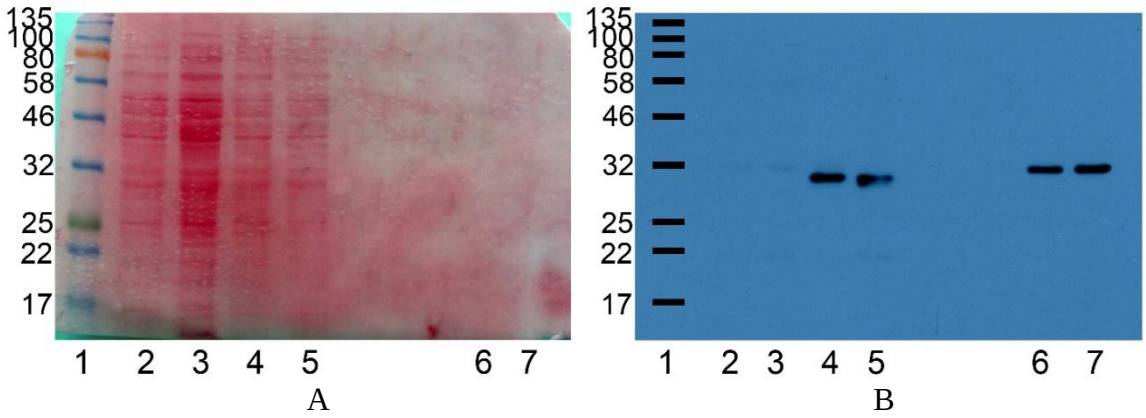
As can be seen from the graph in figure 4A, as the quantity of MMS increased the surviving percentage of *E. coli*, transformed with pBlueScriptII SK (+), dropped sharply. At 2 mM MMS, the survival rate decreased to less than 10%. Induced expression of *xthA* from *H. pylori* increased the MMS resistance of cells to a somewhat lesser extent compared to *nfo* from *E. coli*. The mutant XthA D144N did not contribute to magnification of the cell survival. This indicates the importance of aspartic acid in the 144th position. MMS sensitivity of cells expressing XthA D144N is at the same level as cells that do not contain any AP endonuclease. The number of colonies expressing the mutant HpXthA D144N is at the level of the negative control; only 14% colonies survived after incubation with 2 mM MMS. As the amount of MMS increased (4 mM, 6 mM), the cells completely died.

Similar results were obtained in experiments on the treatment of cells with H_2O_2 (Figure 4B). In the experiments with MMS the concentration of mutagen was changed, in this experiment the incubation time was varied. As the time of cell incubation with 10 mM H_2O_2 increased, the survival of cells decreased. After addition of recombinant HpXthA AP endonuclease the cell survival increased, as in the experiment with MMS. Mutant for XthA

and Nfo AP endonucleases *E. coli* BH110 (DE3) strain is very sensitive to MMS and H_2O_2 [6].

As expected, a plasmid with *nfo* gene from *E. coli* contributed to the survival of BH110 (DE3) strain after MMS and H_2O_2 exposure. pBlueScriptII SK (+)/HpXthA plasmid carrying *xthA* gene from *H. pylori* restored the MMS resistance of *E. coli* close to the level of plasmid with *E. coli nfo* gene. Also, pBlueScriptII SK (+)/HpXthA plasmid partially restored H_2O_2 resistance. It was demonstrated by the increase in cell survival in different periods of H_2O_2 exposure compared with cells with an empty plasmid. These results indicate important biological significance of catalytic activity of XthA enzyme for *H. pylori*.

E. coli BH110 (DE3) were transformed with the vector providing HpXthA expression. Results of western blot with anti-HpXthA antibodies confirmed that, HpXthA expression has contributed to the survival of cells in experiments with MMS and H_2O_2. Complete lysates of *E. coli* BH110 (DE3), *E. coli* BH110 (DE3) / pBlueScriptII SK (+), *E. coli* BH110 (DE3) / pBlueScriptII SK (+)/HpXthA and BH110 (DE3) / pBlueScriptII SK (+)/HpXthA D144N were used. 50 ng and 100 ng of recombinant protein were used as a positive control. All lysates were used in an amount of 4 μg of total protein (Figure 5). Figure 5A shows the results of transferring samples from a 12% polyacrylamide gel to PVDF membrane after staining with Ponceau S; in Figure 5B - western blot results.



A - PVDF membrane with protein samples, stained by Ponceau S, B – XthA detection by antibodies

1 – protein marker, 2 – cell lysate of BH110 (DE3), 3 – cell lysate of BH110 (DE3) / pBlueScriptII SK(+), 4 – cell lysate of BH110 (DE3) / pBlueScriptII SK(+)/HpXthA, 5 – cell lysate of pBlueScriptII SK(+)/HpXthA D144N, 6 - 50 ng HpXthA, 7 – 100 ng HpXthA

Fig. 5. Western blot of experimental *E. coli* cell lysates

Staining with the non-specific Ponceau S dye showed successful samples' transfer from a 12% polyacrylamide gel to PVDF membrane (fig. 5A). Low sensitivity of Ponceau S (<200 ng protein [22]) does not allow detect 50 ng and 100 ng recombinant HpXthA protein. Anti-HpXthA antibodies recognized HpXthA and mutant HpXthA D144N proteins from cell lysates. The absence of XthA expression was confirmed by western blot in *E. coli* BH110 (DE3), *E. coli* BH110 (DE3)/pBlueScriptIISK(+) lysates (Figure 3B). This experiment has confirmed our assumption that HpXthA protein promotes the survival of mutant *E. coli* BH110 (DE3) strain (nfo^-, $xthA^-$) in experiments with alkylating and oxidative stresses.

CONCLUSION

In vivo experiments carried out on *E. coli* BH110 (DE3) strain, which is mutant for AP endonucleases and expresses *xthA* gene from *H. pylori*, showed a decrease in sensitivity to DNA methylating and oxidizing agents. Western blot with specific antibodies showed that HpXthA correctes oxidized and methylated DNA bases in mutant *E. coli* cells. This

improves the survival of cells in the presence of MMS and H_2O_2. The important role of aspartic acid in the 144th position for HpXthA AP endonuclease activity was confirmed. It should be emphasized that XthA is the only AP endonuclease of *H. pylori*. This enzyme protects the bacterium from the genotoxic effects of endogenous and induced AP sites and DNA strand breaks. The obtained survival results contribute to our better understanding of DNA repair mechanism of pathogen microorganism.

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