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*Research Article*

# The role of *Escherichia coli* bacterioferritin in stress induced conditions

Ebru Çelen[1*], Mehmet Akif KILIÇ[2], Geoffrey R. Moore[3]

[1]*Department of Biology, Faculty of Science and Art, University of Abant İzzet Baysal 14280, Bolu, Turkey*
[2]*Department of Biology, Faculty of Science and Art, University of Akdeniz 07058 Antalya, Turkey*
[3]*Center for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom*

## Abstract

*Escherichia coli* possesses, at least, two iron storage proteins: FtnA (ferritin) and Bfr (Bacterioferritin). FtnA is shown to be a major iron storage protein in *E. coli*. Although Bfr resembles the ferritin in many of its structural and functional features, the role of Bfr in *E. coli* is not clear. The aim of this study was to investigate the role of E. coli Bfr in stress induced conditions. In this study, *E. coli bfr*- (lacking the chromosomal bfr gene) and *E. coli bfr*+ (the same as the former but containing the *E. coli bfr* overexpression vector) mutants were used. In order to examine the role of *E. coli Bfr* in stress-induced conditions, *E. coli* cells were grown in the presence of excess iron. In the case of hydrogen peroxide toxicity, the cells were grown on an agar plate containing hydrogen peroxide discs, and the results of toxicity were expressed as the size of the cell death zone. In order to find out whether Bfr supports cell growth under iron and phosphate starvation, the *E. coli* cells were grown in iron and phosphate depleted media. In *E. coli* the overproduced Bfr did not support the growth of cells under the iron and phosphate deficient conditions. The results showed that overproduced Bfr in *E. coli* does not contribute to the survival of the cell in iron and phosphate starvation. Furthermore, it does not enhance the resistance of *E. coli* against iron and hydrogen peroxide toxicity.

**Keywords:** *Escherichia coli*, Bfr, iron, phosphate, toxicity.
*Corresponding Author: Ebru Çelen (e-mail:celen_e@ibu.edu.tr)
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# İndüklenmiş stres koşullarinda *Escherichia coli* bakterioferritinin rolü

## Özet

*Escherichia coli* en az iki demir depo proteine sahiptir: FtnA (ferritin) ve Bfr (Bakterioferritin). FtnA'nın *E. coli*'de esas demir depo proteini olduğu gösterilmiştir. Bfr'ın yapısal ve fonksiyonel pek çok özelliği ferritine benzemesine rağmen, *E. coli* Bfr'nin rolü belirsizdir. Bu çalışmanın amacı indüklenmiş stres koşullarında *E. coli* Bfr'nin rolünü incelemektir. Bu çalışmada, *E. coli bfr*$^-$ (kromozomal bfr geni bulunmayan) ve *E. coli bfr*$^+$ (bir öncekiyle aynı olmakla birlikte *E. coli bfr* overekspresyon vektörü içeren) mutantlar kullanıldı. İndüklenmiş stres koşullarında *E. coli* Bfr'nin rolünü incelemek için, *E. coli* hücreleri yüksek demir varlığında büyütüldü. Hidrojen peroksit toksitesi için, hücreler hidrojen peroksit

diskleri içeren agar plaklarında büyütüldü ve toksite sonuçları hücre ölüm zon çapı olarak ifade edildi. Bfr'nin demir ve fosfat yetersizliğinde hücre büyümesini destekleyip desteklemediğini belirlemek için, *E. coli* hücreleri demir ve fosfat bulunmayan ortamda büyütüldü. *E. coli*'de aşırı üretilen Bfr demir ve fosfat bulunmayan koşullarda hücre büyümesini desteklemedi. Bu sonuçlar göstermiştir ki *E. coli*'de aşırı üretilen Bfr fosfat ve demir açlığında hücrenin hayatta kalmasına katkıda bulunmuyor. Bununla birlikte Bfr, demir ve hidrojen peroksit toksitesine karşı *E. coli*'nin direncini güçlendirmiyor.

**Anahtar Kelimeler:** *Escherichia coli*, Bfr, demir, fosfat, toksite.

## Introduction

Iron is an essential minor element for most organisms due to its vital role in many important biological processes including photosynthesis, the TCA cycle, respiration, oxygen transportation, nitrogen fixation and DNA biosynthesis. However, it is also potentially toxic as it catalyzes the generation of cell-damaging free radicals. In organisms, iron exists in two oxidation states: ferrous ($Fe^{2+}$) or ferric ($Fe^{3+}$) form and the poor solubility of the ferric form leads to bioavailability problems (Andrews 1998). Organisms overcome iron-related problems by the regulation of highly authentic transport and redox stress resistance systems.

Many bacteria deposit iron within iron storage proteins (Andrews 1998). These iron stores can then be used to enhance growth when external iron supplies are restricted. Three types of iron storage proteins are recognized in bacteria: ferritin (FtnA), which is also found in eukaryotes, haem-containing bacterioferritin (Bfr) found only in bacteria and the smaller Dps proteins present only in prokaryotes. All types can be present in the same bacterium. Both ferritin and Bfr are composed of similar subunits that assemble to form an approximately spherical protein shell surrounding a central cavity that acts as an iron storage reservoir. Each protein can accommodate 2000-3000 iron atoms. In the case of overproduced Bfr, the iron content of overproduced Escherichia coli Bfr was higher than that of FtnA and the overproduced Bfr accumulated more iron per molecule than FtnA in vivo (Andrews et al. 1993; Hudson et al. 1993).

The FtnA of E. coli was shown to be responsible for the post-exponential accumulation and storage of up to 50% of cellular iron during iron sufficient growth. Inactivation of the ferritin gene (ftnA) of E. coli resulted in a 50% reduction in stationary phase cellular iron content following growth under iron sufficient conditions and the rate of cell growth was reduced under iron-restricted conditions (Abdul-Tehrani et al. 1999). FtnA also acts as an iron storage protein in *Porphyromonas gingivalis and Campylobacter jejuni* (Ratnayake et al. 2000; Wai et al. 1996). However, in *Salmonella enterica sv. Typhimurium*, Bfr is responsible for the majority of stored iron (Velayudhan et al. 2007) and in *Neiserria gonorrhoeae*, Bfr serves as an iron source under iron limiting conditions and is required for protection against oxidative stress (Chen and Morse 1999).

Bfr cores are extremely rich in phosphate (Rohrer et al. 1990; Bauminger et al. 1980; Moore et al. 1986) and the phosphate is distributed throughout the core (Rohrer et al. 1990). Therefore, Bfr appears to serve as a phosphate store. In this study, the role of Bfr in high concentrations of iron and hydrogen peroxide was tested using *E. coli bfr*- and *bfr*+ cells. Since the overproduced Bfr contains high amounts of iron and phosphate, whether *E. coli* Bfr supports cell growth in iron or phosphate deficient media was also investigated. The results indicate that overproduced Bfr in *E. coli* does not support cell growth in iron or phosphate deprivation, and it does not enhance the resistance of cells against iron and hydrogen peroxide toxicity.

## Materials and methods

Bacterial strains and plasmids: The genotypes and sources of *E. coli* strains and plasmids are listed in Table 1.

**Table 1:** Properties of E. coli ALO1 strain and plasmids used in this study.

| Strain or plasmid | Properties | References |
|---|---|---|
| *E. coli* ALO1 | *E. coli* BL21, *bfr::kan* | (Kilic et al. 2003; Malone et al. 2004) |
| Plasmids | | |
| pALN1 | bfr amp | (Malone et al. 2004) |
| pALN18 | *bfr* mutant (E128R/E135R) | (Malone et al. 2004) |
| pET21a | Expression vector | Novagen |

The *E. coli* AL01 strain (*bfr*$^-$) was previously created by insertional inactivation of the chromosomal *bfr* gene (Kilic et al. 2003; Malone et al. 2004). The *E. coli* wild type and the double mutant (E128R/E135R) Bfr expression vectors were also previously transferred to the *E. coli* AL01 (13). The protein expression of the cells was controlled by the induction of isopropyl-β-D-thiogalactoside (IPTG), and the addition of 10 μM IPTG resulted in a substantially high amount of protein production in the cells. The *E. coli* pET21 was designed to find out the effect of expression vectors on experimental conditions. The double mutant Bfr was used as the control of the wild-type Bfr iron storage activity. It is known that the mutant E128R/E135R cannot assemble to a 24-meric structure and, therefore, it cannot store iron (Kilic et al. 2003).

Growth media and experimental conditions: For general growth, the *E. coli* cells were inoculated in Luria broth (L broth) or L agar and Minimal medium broth (MM broth) or MM agar supplemented with ampicillin (200 μg/ml). MM contains 20 mM $NH_4Cl$, 2 % $MgSO_4$; 5 ml, 100 mM $CaCl_2$; 1ml, 5 g glucose, 14 g $K_2HPO_4$, 2.7 g $KH_2PO_4$ and 2.5 g NaCl (pH 7.4).

1- Iron-induced radical toxicity; to determine whether high concentrations of iron could be toxic to the *bfr*- cell and also whether the overproduced Bfr could overcome the related problem, the *E. coli* cells were grown in L broth or MM supplemented with various concentrations (1, 2 and 6 mM) of the iron citrate. The iron citrate stock solution was 100 mM $Fe(NH_4)_2(SO_4)_2$ in 1 mM citric acid.

2-Iron or phosphate-limited growth conditions; the iron-depleted medium was prepared by removing iron from the MM using an iron chelator resin overnight at +4°C. To test whether the iron stored in Bfr could support cell growth in iron starvation, the cells were initially grown overnight in iron-rich MM (containing 2 mM iron). The cells were harvested and washed twice with 50 mM phosphate buffer (pH 7.4) by centrifugated at 4000 rpm for 5 min. Before inoculating the cells into the iron deficient medium.

In order to find out whether the Bfr could support cell growth in a medium without phosphate, the *E. coli* cells were initially inoculated in a Trisma base medium (pH 7.4) supplemented with 10 mM of $Na_2HPO_4$. The bacterial cells harvested and washed with the Tris medium and then inoculated into the Tris medium contained no phosphate.

For iron-induced toxicity and iron and phosphate restricted growth, the *E. coli* cells were grown in 250-ml conical flasks containing 50 ml of the relevant medium at 37°C by shaking at 250 rpm. The bacterial growth monitored by measuring the optical density of the cells at 650 nm.

Sensitivity to $H_2O_2$; The sensitivity of *E. coli*

*Bfr*⁻ and *Bfr*⁺ cells to $H_2O_2$ was determined by a disk diffusion assay. All cells were grown to the stationary phase in an aerobic MM medium containing 2 or 6 mM iron citrate, and 100 µl of samples were spread on MM agar plates. Sterile filter paper disks (7 mm in diameter), saturated with 10 µl of 8.8 µM $H_2O_2$, were placed on plates, and the diameters of growth inhibition zones were measured after the aerobic incubation of the plates at 37°C for 24 h.

SDS-PAGE Analysis; Bfr production of the *E. coli* cells was followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 12% polyacrylamide gel was prepared according to a method based on that of Laemmli (1970). The protein samples were boiled for 5 min. and then load into the wells. The gel was run on a Hoeffer minigel system (Pharmacia) according to the manufacturer's and stained with Coomassie Blue stain for at least for 2 hours and then it was destained overnight.

## Results

In all experiments, the main *E. coli* strain used was the *E. coli* AL01 (*bfr*-) and all other plasmids pALN1 (wild type *bfr*), pALN18 (the mutant *bfr*) and expression vectors (pET21) were maintained in the same strain. Wild-type and mutant Bfr production in *E. coli* was followed by SDS-PAGE and as seen in Figure 1, IPTG induced the Bfr expression, and as expected, both the wild type and the mutant Bfr were expressed in high amounts. As the overproduced Bfr protein expression is IPTG-dependent and the protein production rate affects cell growth, various IPTG concentrations (0.01-1 mM) were tested for Bfr expression and it was found that 0.01 mM IPTG succeeds sufficient Bfr production without reducing the cell growth rate. After the optimization of Bfr production and cell growth, the role of Bfr in high iron concentrations was tested by growing cells (AL01, pALN1, pALN18 and pET21) in the presence of 1, 2 and 6 mM iron. Figure 2 shows all cells to have similar growth curves. There were no apparent differences between the growth rates of cells that either had high amounts of wild-type Bfr (pALN1) or completely lacked Bfr (AL01), indicating that the wild-type *E. coli* Bfr is not advantageous in excess iron conditions.

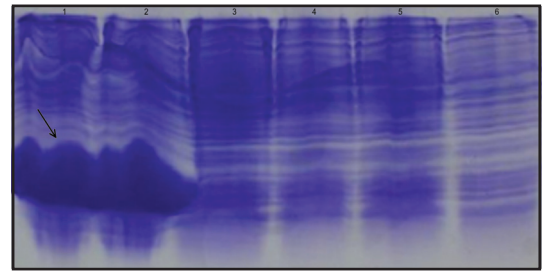


**Figure 1.** shows the overproduced Bfr in *E. coli* mutants on SDS-PAGE. 1; ALO1 pALN1 *bfr*⁺ induced with IPTG, 2; ALO1 pALN18 *bfr*⁺ induced with IPTG, 3; ALO1 pALN1 *bfr*⁺, 4; ALO1 pALN18 *bfr*⁺, 5; ALO1 *bfr*⁻, 6; ALO1 pET21 *bfr*⁻

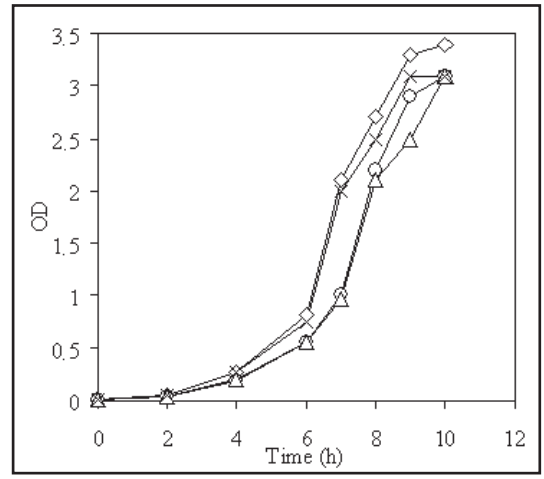


**Figure 2.** Effect of high concentration of iron on the growth of *E. coli* ALO1 Bfr mutants. Cells were grown in MM supplemented with 6 mM iron citrate. □, ALO1 *bfr*⁻; ○, ALO1pALN1 *bfr*⁺; Δ, ALO1pALN18 *bfr*⁺; ×, ALO1pET21 *bfr*⁻.

It is well known that all bacterial ferritins (Ftn and Bfr) contain high amounts of iron, and one expected function of ferritins is to provide iron when required by the cell. In order to investigate whether *E. coli* Bfr can support cell growth under iron starvation, the *bfr*+ and *bfr*- cells were precultured in an iron-rich MM broth media (containing 1, 2, and 6 mM iron citrate) and these cells were subsequently inoculated in an iron-depleted MM broth medium. The growth rates of all cells in the iron-limited medium were almost identical (Figure 3) indicating that wild type Bfr does not support

the growth of cells under iron deficiency.

Similar to ferritins' iron storage functions, bacterial ferritins ought to serve as a phosphate reservoir due to the high phosphate content

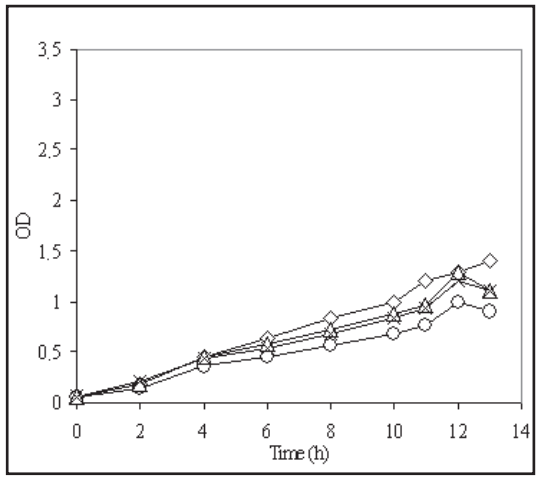


**Figure 3.** Effect of iron limitation on the growth of *E. coli* ALO1 Bfr mutants. Cells were precultured in MM supplemented with 2 mM iron citrate and 10 µM IPTG and subsequently inoculated in MM without iron. □, ALO1 *bfr*⁻; ○, ALO1pALN1 *bfr*⁺; Δ, ALO1pALN18 *bfr*⁺; ×, ALO1pET21 *bfr*⁻.

in their internal cavities. In order to find out whether *E. coli* Bfr could serve as a phosphate reservoir under phosphate-starved growth conditions, Bfr producing and non-producing cells were first grown in MM media containing various concentrations of phosphate (10 µM, 1 mM, 10 mM and 100 mM) and then they were subinoculated in the same medium without phosphate. The growth curves of *E. coli* AL01, pANL1, pALN18 and pET21 under phosphate-starved conditions are shown in Figure 4. As it is seen in the figure, the growth of cells was severely repressed, and the growth rate of wild-type Bfr overproducing cells (pALN1) did not differ from that of the non-producing cells or the double Bfr mutant.

Iron is known to enhance oxidative stress. In this study, we used filter discs saturated with 10 µl of 9 mM $H_2O_2$ on minimal agar containing 1 and 2 mM iron citrate in order to determine the sensitivity of *E. coli* cells (AL01, pALN1, pALN18 and pET21) to $H_2O_2$ toxicity. According to disc diffusion assay, all cells had

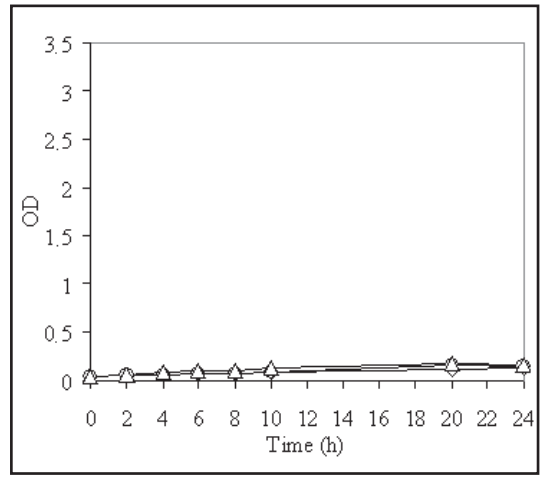


**Figure 4.** Effect of phosphate limitation on the growth of *E. coli* ALO1 *Bfr* mutants. Cells were precultured in Tris medium supplemented with 10 mM $Na_2HPO_4$, 1 mM iron citrate and 10 µM IPTG and subsequently inoculated in Tris medium no phosphate. □, ALO1 *bfr*⁻; ○, ALO1pALN1 *bfr*⁺; Δ, ALO1pALN18 *bfr*⁺; ×, ALO1pET21 *bfr*⁻.

similar inhibition zones (data is not given) indicating that overproduced Bfr does not provide further resistance to the cell in response to the hydrogen peroxide toxicity.

## Discussion

The physiological role of plasmid-coded *E. coli* Bfr was investigated in chromosomally inactivated *bfr*- background *E. coli* strains (AL01). Similar to the study presented here, the function of *E. coli* ferritins (FtnA and Bfr) has also been studied in mutant *E. coli* strains and the study has concluded that in *E. coli*, FtnA acts as a major iron storage protein (4). On the contrary, the function of Bfr in *E. coli* could not be elucidated. Abdul-Tehrani *et al (1999)*. had used chromosomally inactivated *bfr*- strains to investigate the role of Bfr. In the present study, the Bfr content of the cell was enhanced using an overexpression system in order to strengthen the potential role of Bfr in *E. coli*. The SDS-PAGE analysis revealed that the wild-type and mutant Bfrs were expressed in substantially high amounts in the *E. coli* AL01 strain. The previous studies have shown that the iron content of the overproduced *E. coli* Bfr (25-75 iron atom/holomer) is significantly higher

than that of the FtnA (5-20 iron atom/holomer) (Abdul-Tehrani et al. 1999) and furthermore that Bfr overproduction raises the iron content of the cell about 2.6 fold (Andrews et al. 1993), therefore, the overproduction of Bfr in *E. coli* in the present study is also expected to raise the iron content of the cell.

Bacteria with an iron-dependent regulation system, such as *E. coli*, require approximately 0.3-2.8 μM of iron for optimal growth (Braun and Killmann 1999). In *E. coli,* iron regulation is mediated by the ferric-uptake regulator protein (Fur), which controls the iron-dependent expression of more than 90 genes (Hantke 2001; Andrews et al. 2003). It has been reported that Fur may act as a transcriptional activator for iron presenting proteins (such as Bfr, FtnA, fumarase A) (Masse and Gottesman 2002). The inactivation of fur in *E. coli* results in 2.5 fold reduction in the iron content of the cell and similarly it markedly reduces the uptake of iron-loaded sidephores in *Pseudomonas aeruginosa* (Hassett et al. 1996). It is known that Fur plays an important role in the iron homeostasis of cells. In the present study, the absence of growth impairment in the cells lacking Bfr (AL01 *bfr*-) or having high cellular Bfr content (pALN1) could be dependent on two factors: Firstly, the FtnA in *E. coli ALO1 Bfr*- could reduce the stress of intracellular free iron by storing it within its cavity and secondly, the iron content of cells may remain unchanged due to Fur activity. Despite *E. coli* pALN1 having high cellular Bfr content, both *bfr*+ and *bfr*- cells were grown identically in high iron concentration (6 mM). Similar to wild-type *E. coli*, *bfr*- and *ftnA*- mutants were grown in high iron content (1 mM), all cells exhibited similar tolerance to high iron concentration (Abdul-Tehrani et al. 1999). The lack of growth enhancement even in Bfr overproducing *E. coli* suggests that *E. coli* Bfr is not involved in the iron-related oxidative stress response.

Iron storage proteins can provide sufficient iron when required by bacteria and also support growth in iron starvation (Carrondo 2003). Using *FtnA, Bfr* and *FtnA-Bfr* mutants of *Salmonella enterica sv. typhimurium*, it has been shown that Bfr stores the majority of the intracellular iron and the inactivation of *bfr* results in an intracellular iron increase (Velayudhan et al. 2007). For this reason, it has been postulated that *Salmonella enterica sv. typhimurium* Bfr acts as an iron storage protein. However, in this study, it has not been shown whether the stored iron in Bfr could be supplied to the cell in case of starvation. In contrast, in *Neisseria gonorhoea* the mutation of bfrB gene resulted in a decline in cell growth indicating that the BfrB supports bacterial growth in iron-limiting conditions (Chen and Morse 1999). In the present study, the *E. coli* cells (AL01, *bfr*- and pANL1, *bfr*+) contain the chromosomal FtnA gene and therefore in these cells, iron could be also stored in FtnA. As the *E. coli* pALN1 possess high Bfr content, it can store more iron than *E. coli* AL01 and, therefore, the overproduced Bfr is likely to support cell growth in iron starvation. However, similar growth in *E. coli Bfr*- and $Bfr^{+}$ cells in iron limiting conditions indicates that the overproduced Bfr can not sustain cellular iron demand.

The Bfr core is made of phosphate as well as iron. Since *E. coli* Bfr contains substantial amounts of the phosphate, in the present study it has been tested whether the Bfr could supply phosphate to cells in the case of starvation. The *E. coli* $bfr^{-}$ and $bfr^{+}$ cells have shown difficulty when they are growing under phosphate-limiting conditions. Although both cell types have their chromosomal wild-type *FtnA* gene, the result indicated that neither FtnA nor overproduced Bfr can support cell growth in response to demand. Iron is potentially toxic because of its ability to catalyze the generation of the highly toxic hydroxyl radical through the Fenton reaction. Superoxide and hydrogen peroxide are mildly reactive in physiological conditions. When cellular free iron interacts with these molecules, it creates extremely damaging hydroxyl radicals. The main function of the iron storage proteins (such as ferritin) is postulated to sequester the cellular free iron and consequently protect cells from oxidants (Cozzi et al. 1990; Touati 1995). In the previous study (Abdul-Tehrani et al. 1999), the mutants (*bfr, ftnA* and *bfr-, ftnA*) of *E. coli* iron storage proteins showed no sensitivity to hydrogen peroxide. Only the resistance of the *E. coli fur* mutant was found to be lower than the wild type.

When it was assessed whether the overproduced Bfr of *E. coli* protects the cell against hydrogen peroxide, the Bfr overproducing cells did not show any further resistance. Therefore, it has been concluded that neither chromosomal Bfr nor overproduced Bfr reduces the sensitivity of cells to hydrogen peroxide.

Bacterial ferritins are thought to serve in several stress-induced conditions including intracellular free iron related toxicity, oxidative stress and iron starvation. In the present study, the responses of *E. coli bfr*$^-$ and *bfr*$^+$ cells were tested against the high concentration of iron and hydrogen peroxide. It was also investigated whether overproduced Bfr could supply iron and phosphate to cells in an iron or phosphate deficient medium. Obtained results indicate that overproduced Bfr in *E. coli* does not provide support against iron and hydrogen peroxide toxicity, nor does it contribute to cell survival in iron or phosphate starvation. Therefore, the *E. coli* Bfr does not seem to contribute to the welfare of cells in the above-tested conditions.

## Acknowledgements

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