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Bioavailability

Selenite-stress selected mutant strains of probiotic bacteria for Se source production

Tünde Pusztahelyia,*, Szilvia Kovácsa, Jstván Pócsi, József Prokischa

Abstract

Selenium deficiency is a major health problem worldwide for about 1 billion people. Bacterial cells usually possess low tolerance to selenite stress and also low ability to reduce high concentrations of toxic selenite. Here, high tolerance to selenite and selenium bioaccumulation capability were developed in mutated clones of probiotic and starter bacteria including Enterococcus faecium, Bifidobacterium animalis ss. lactis, Lactobacillus casei and Lactococcus lactis ss. lactis by food-level strain development process and clone selection. All mutant clones possessed increased glutathione concentration and glutathione reductase activity. The selenite treatment increased further these values in L. casei mutant strain pointing at a different selenite reduction pathway and/or stress response in this organism. Considerable conversion of selenite to cell bound selenium forms with a concomitant high biomass production was detected in E. faecium and B. animalis ss. lactis cultures. Possible application of these strains as food and feed supplements is under investigation.

Q3 Introduction

Selenium is a micronutrient for all organisms and its deficiency is a major health problem for about 1 billion people worldwide [1]. Selenium status of people varies by country: intakes are usually high in North America and Japan, varies in China, while lower in Europe (especially in Eastern Europe) and Australia. Low selenium status is associated with poor immune function, oxidative stress and increased risk of mortality. Selenium is needed by human brain and cardiovascular diseases [2]. Selenium deficiency is a major health problem worldwide for about 1 billion people. Bacterial cells usually possess low tolerance to selenite stress and also low ability to reduce high concentrations of toxic selenite. Here, high tolerance to selenite and selenium bioaccumulation capability were developed in mutated clones of probiotic and starter bacteria including Enterococcus faecium, Bifidobacterium animalis ss. lactis, Lactobacillus casei and Lactococcus lactis ss. lactis by food-level strain development process and clone selection. All mutant clones possessed increased glutathione concentration and glutathione reductase activity. The selenite treatment increased further these values in L. casei mutant strain pointing at a different selenite reduction pathway and/or stress response in this organism. Considerable conversion of selenite to cell bound selenium forms with a concomitant high biomass production was detected in E. faecium and B. animalis ss. lactis cultures. Possible application of these strains as food and feed supplements is under investigation.

Keywords:
- Selenium
- Selenite
- Selenium supplements
- Bioavailability

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http://dx.doi.org/10.1016/j.jtemb.2014.11.003
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which is reduced by oxidoreductases, and this mechanism maintains cellular forms and levels of GSH [11].

In the present work mutants with high selenite tolerance and selenium accumulation were selected from well-known probiotic bacterium cultures, including Enterococcus faecium W54, Lactococcus lactis ssp. lactis R703, Bifidobacterium animalis ssp. lactis BB12 and Lactobacillus casei 431. These organisms are always preferred for biotechnological application (reviewed in [13]); however, have not been developed for selenite reduction. Characterization of growth and optimization of the cultivation were done. The possible correlation of the GSH level and the GR activity to selenium accumulation were also demonstrated and discussed.

Materials and methods

Medium and strains

E. faecium W54 (Ef; from ProGastro, Winchle Bio Industries, Amsterdam, The Netherlands), L. lactis ssp. lactis R703 (R703; Chr. Hansen A/S, Denmark), B. animalis ssp. lactis BB12 (BB12; Chr. Hansen A/S, Denmark), L. casei 431 (L. casei; Chr. Hansen A/S, Denmark) were used in the experiments. The bacterial strains were cultivated in de Man, Rogosa and Sharpe (MRS) [14] medium {containing 10 g L\(^{-1}\) proteose peptone, 8 g L\(^{-1}\) meat extract, 4 g L\(^{-1}\) yeast extract, 20 g L\(^{-1}\) glucose, 5 g L\(^{-1}\) sodium acetate, 2 g L\(^{-1}\) triammonium citrate, 0.2 g L\(^{-1}\) magnesium sulphate, 0.05 g L\(^{-1}\) manganese sulphate, 2 g L\(^{-1}\) dipotassium hydrogen phosphate, 1 g L\(^{-1}\) Polysorbate 80 (OXOID, UK) pH 6.8.}

Selection of selenite tolerant mutant strains

Strains were inoculated into MRS medium (pH 6.8) supplemented first with 0.331 mmol L\(^{-1}\) selenium (50 mg L\(^{-1}\) NaHSeO\(_3\)). After 24 h cultivation time, the cultures were inoculated into MRS without NaHSeO\(_3\). Afterward, the concentration of selenite was increased up to 10,000 mg L\(^{-1}\) NaHSeO\(_3\) (66.24 mmol L\(^{-1}\) final Se concentration) step-wise in each round of the transfers (inoculation into MRS with and without NaHSeO\(_3\)) (Fig. 1). The NaHSeO\(_3\) stock solution (45 g L\(^{-1}\)) was sterilized by filtration and applied immediately.

Cultures with the highest selenite tolerance were pour-plated onto solid MRS medium, and single mutant colonies of each strain were chosen. Following the differential characterization protocol of lactic acid bacteria the mutant clones were tested, and also were identified with API\(^{®}\)/rapid ID32 Strept test (bioMérieux, France) according to the instructions of the manufacturer using fresh bacterial cultures cultivated on MRS plates. Reading of the test strips was evaluated with miniAPI (bioMérieux, France) equipment.

Differential characterization of the parental and mutant strains

The growth was tested in MRS medium after 48 h cultivation time at different pHs (pH 6.8, pH 4.4 and 9.6) and temperatures (10°C, 45°C), and in the presence of high (6.5%) NaCl concentration. The morphology and catalase activity of the cells were also recorded. The catalase activity was tested in the H\(_2\)O\(_2\) drop test, where the appearance of bubbles indicated catalase positive cells.

Characterization of growth of the selenium tolerant mutant strains

The mutant strains were inoculated into 10 mL liquid MRS aliquots, and after 16 h incubation at 32°C, 10 µL inoculums (cell suspensions with 8 McFarland units cell density) were loaded to 200 µL aliquots of MRS media in microtiter plates. The growths of the strains were measured at different pHs (pH 5.97, pH 6.79, pH 8.01, pH 8.96) and temperatures (28°C, 32°C, 37°C). The cell density was checked spectrophotometrically at \(\lambda = 630\) nm. The growth was tested also with 100 mg L\(^{-1}\) and 200 mg L\(^{-1}\) sodium hydrogen selenite supplementation. All experiments were repeated three times.

Selenium production of the mutant strains

Bacteria were cultivated in 10 mL MRS medium for 16 h at 32°C. From the culture media, 100 µL aliquots of the cultures (cell suspensions with 8 McFarland units cell density) were inoculated into 50 mL MRS medium supplemented with 600 mg L\(^{-1}\) NaHSeO\(_3\). After 24 h cultivation, bacterial cells were centrifuged (5000 rpm, 10 min), the pellets were washed twice with phosphate buffered saline (PBS) and were frozen at -20°C. After lyophilization, dry cell mass and selenium content of the biomass were determined.

The lyophilized samples were refilled to 1 mL with cc. HNO\(_3\) and the samples were digested first with 3 mL cm\(^{-3}\)H\(_2\)NO\(_3\) at 120°C for 50 min then with 2 mL 30% H\(_2\)O\(_2\) at 120°C for 50 min again. After cooling back to room temperature, the digested samples were filtered and adjusted to 10 mL with deionized water and stored in closed tubes. Each sample was analyzed in triplicates for selenium content on a flame emission atomic absorption spectrometer (Thermo Fischer Scientific, iCE 3000 Series AA Spectrometer, SOLAAR House, Cambridge, United Kingdom) and mean values of the selenium contents were calculated.

Total GSH and glutathione reductase assay

Bacteria were cultured in 10 mL MRS medium for 16 h at 32°C. Following that, 50 µL aliquots of the MRS medium, which was supplemented with 600 mg L\(^{-1}\) NaHSeO\(_3\), were inoculated with 100 µL cell suspensions of 8 McFarland units cell density. The cultures were grown for 24 h and the biomass was harvested by centrifugation (5000 rpm, 10 min). The cells were washed twice with 1 mL phosphate buffered saline (PBS) and were re-suspended in 500 µL 20 mmol L\(^{-1}\) TEA-HCl, pH 7.6, also containing 0.1 mmol L\(^{-1}\) EDTA. The cells were broken by sonication (five 30 s pulses with 30 s cooling periods) performed on ice. Crude homogenates were clarified by centrifugation and 20 µL of 5% phosphosalicylic acid was added to 200 µL aliquots of the supernatants to precipitate protein, which was removed by centrifugation (10,000 rpm, 10 min).

Before total GSH determination, 10 µL TEOA was added to 200 µL sample, and the mixture was kept at 4°C for 1 h. Reaction mixtures contained 125 mmol L\(^{-1}\) sodium phosphate buffer pH 7.5, 6.3 mmol L\(^{-1}\) EDTA, 0.21 mmol L\(^{-1}\) NADPH, 0.66 mmol L\(^{-1}\) DTNB and 0.9 U baker’s yeast glutathione reductase (205 units mg\(^{-1}\) protein;
The stress tolerance of the mutant strains was different from that of the parental cultures. Especially the changes in the thermal and pH tolerances were obvious as the optimal growth of the mutant strains was pushed to higher temperatures comparing to the parental strains. In *L. casei* 431 mutant strain, the salt and pH resistance were also important (Table 1).

Investigating at different pHs (5.97, 6.79, 8.01 and 8.96) and temperatures (28, 32 or 37 °C) R703 and BB12 mutants possessed wider thermo- and pH tolerances than *L. casei* 431 or the *E. faecium* mutant strains as they showed similar growths at all tested temperatures and pH values (Fig. 3A). *L. casei* 431 showed decreased growth at alkaline pHs at 28 °C and 32 °C while the differences in the pH tolerance diminished when it was incubated at 37 °C. Under selenium stress (100 mg L⁻¹ and 200 mg L⁻¹ NaHSeO₃) the pH changes of the medium became more important for the bacterial growth especially for *L. casei* mutant strain (Fig. 3B and C).

The stress tolerance of the *E. faecium* mutant strain was rather versatile. Increasing the SeO₄²⁻ concentration, the optimal growth temperature range became wider; meanwhile, without the stress agent the mutant strain was sensitive to the pH changes and showed narrow temperature tolerance, e.g., did not grow properly at 28 °C and 37 °C (Fig. 3B and C). The 32 °C incubation temperature was applicable for all strains on the basis of our results; therefore, all strains were cultivated on this temperature at pH 8.01 for *E. faecium* or at pH 6.79 for further experiments.

The bioaccumulation of selenium was measured in the selenium-tolerant mutant strains applying 600 mg kg⁻¹ NaHSeO₃. Dark red pigmentation of the cells was observed in all cultures, especially when the mutant R703 or the mutant *L. casei* strains were cultivated with selenium. The biomass production was high (above 400 mg L⁻¹) for all mutant strains except *L. casei* (below 100 mg L⁻¹) after 24 h cultivation (Fig. 4). Remarkable high selenium contents were measured in the lyophilized biomasses (above 40,000 mg Se kg⁻¹ dry biomass) and significantly (*p < 0.05) less selenium were accumulated in the mutant *L. casei* strain (Fig. 4).

Intracellular GSH contents and GR activities also were measured in parental and mutant strains of the bacteria. In all mutant strains increased GSH contents and GR activities were calculated comparing to that of the parental strains (Table 2). Interestingly, the parental and the mutant *E. faecium* strain possessed exception-ally increased total GSH content and GR specific activity (Table 2) in contrast to the other strains. Under selenite stress, both the GSH content and the GR activities decreased in all strains with the exception of *L. casei*, where both values increased. In the MRS culture medium extracellular GSH content (3.437 ± 0.01 μmol mL⁻¹) was detected at the inoculation time and increased concentrations were detected from the culture fluid after 24 h cultivation time, except in *E. faecium* (data not shown).

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**Table 1** Differential characterization of the parental and the selenite tolerant mutant bacteria. The bacterial growth was tested at different temperatures, with 6.5% NaCl or at different pH values in MRS medium after 48 h cultivation time.

<table>
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<tr>
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1: *L. casei*, *Bifidobacterium animalis* sp. lactis BB12, *Lacticoccus lactis* sp. R703, *Lactobacillus casei* 431; R703, *Lactococcus lactis* subsp. lactis; BB12, *Bifidobacterium animalis* sp. lactis; *E. faecium*, *Enterococcus faecium*.  
2: +, Growth in 24 h; -, no sign of growth up to 48 h cultivation; ±, weak growth.
Fig. 3. Growth of the selenite-tolerant mutant strains. Biomass productions were determined at different starting pH values (pH 5.97, 6.79, 8.01 and 8.96) and temperatures (28, 32 or 37°C). Optical densities (OD630 values after 24 h cultivation) were determined spectrophotometrically. The *Lactobacillus casei* 431 (*L. casei*), *Bifidobacterium animalis* ssp. *lactis* BB12, *Lactococcus lactis* ssp. *lactis* R703 and *Enterococcus faecium* (Ef) mutant strains were incubated in MRS medium without aeration. Bacterial growth was recorded without selenite stress (A), in the presence of 100 mg L\(^{-1}\) (B) or 200 mg L\(^{-1}\) NaHSeO\(_3\) (C), respectively. All experiments were carried out in triplicates, and the standard deviations of the mean values were below 10%.
Fig. 4. Biomass production (■) and selenium accumulation (gray bars) of the mutant strains. Cultures of Lactobacillus casei 431 (Lcasei), Bifidobacterium animalis ssp. lactis BB12, Lactococcus lactis ssp. lactis R703 and Enterococcus faecium (Ef) mutant strains were supplemented with 600 mg L⁻¹ NaHSeO₃. All experiments were carried out in triplicates; the statistical significance was calculated in Student t-test (p < 0.05). All selenium concentration was significantly different from each other, while the dry biomass of BB12 and Ef were not.

Discussion

Bacterial cells usually possess low tolerance against selenite stress and show limited capability to reduce high concentrations of selenite to elemental selenium. Selenite stress elicits adaptive response in lactic acid bacteria [16–18], therefore, repetitive selection of selenite tolerant L. casei 431, B. animalis ssp. lactis BB12, L. lactis ssp. lactis R703 and Enterococcus faecium W54 strains under increasing selenite stress resulted in promising candidate microbes for future industrial applications.

Optimization of the growth of the selenite tolerant strains revealed significant differences in their thermo tolerance and pH tolerance, which may influence any further utilization of the microbes. Lactococci and lactobacilli are known to have low tolerance against alkaline conditions; however, the R703 and BB12 mutant strains showed remarkable growths in the pH range of 4.4–9.6. Interestingly, Shah [19] reported an optimum growth for Bifidobacterium at pH 6–7; meanwhile, no growth was observed above pH 8–8.5, which contrasted strikingly to our results. Pieniz et al. [8] observed growth and selenium bioaccumulation in E. faecium cultures between 25 and 45 °C, while, under selenite stress the growth of the E. faecium mutant strain decreased considerably below 32 °C. It was also found that the addition of 15 mg L⁻¹ Na₂SeO₃ (0.086 mmol L⁻¹ Se) to E. faecium cultures increased the elemental selenium content of the biomass up to 475.9 ± 19.8 mg kg⁻¹ dry biomass [8]. Importantly, significantly higher quantities of selenium (54,362 ± 594 mg kg⁻¹ dry biomass) were measured in our mutated E. faecium strain when exposed to 3.97 mmol L⁻¹ Se (600 mg L⁻¹ NaHSeO₃) under alkaline pH.

L. casei ssp. casei was reported to accumulate 400 mg Se kg⁻¹ dry biomass, and at least 80% of it was associated with organic molecules like selenocysteine [20]. However, the L. casei 431 strain was also described as a potential nanoselenium producer probiotic organism, which produced 1500–3500 mg Se kg⁻¹ dry biomass in the presence of 200 mg L⁻¹ NaHSeO₃ (1.32 mmol L⁻¹ Se) at 37 °C [21]. In our experiments, the mutant of L. casei 431 strain accumulated about 10 times more total selenium (37,428 ± 978 mg kg⁻¹ dry biomass). While, R703 mutant strain was characterized with wider thermo- and pH tolerances and with higher biomass yields accumulated selenium at a higher level (64,726 ± 545 mg kg⁻¹ dry biomass).

GSH has long been suspected to be involved in selenium metabolism, and while there is no detailed investigation in relation of selenite reduction in Gram positive bacteria, there is a detailed work that outlined the processes in Gram negative organisms [10]. There, biotic and abiotic nanoselenium production takes place simultaneously. The main proposed process is the abiotic reduction of selenite that needs high GSH concentration and selenodiglutathione (GS-Se-GS) is the first product of a quick reaction beside superoxide anion (O₂⁻). Kessi and Hanselmann [10] concluded that the higher the GSH: selenite ratio (>2), the higher the abiotic nanoselenium production. The next reactions are much slower steps leading to elemental selenium formation. In contrast to these processes biotic reactions need enzymes like glutathione reductase (GR), oxidoreductases and ROS eliminating enzymes like NADH peroxidases. GR reduce GSSG to GSH while oxidizing NADPH to NADP⁺, which is reduced by oxidoreductases [11].

Only a few lactic acid bacteria and other Gram positive prokaryotes were proven to possess glutathione biosynthetic pathways and most of the lactic acid bacteria were shown to import GSH from their environment [12]. For lactococci, the available data on GSH synthesis were controversial because numerous lactococci were described not to have any capability to perform de novo GSH synthesis on minimal medium; while, for some of the lactococci, GSH synthesis was proposed when their genomes were annotated and functionally analyzed [12]. A GSH biosynthetic fusion protein is coded in the genome of L. casei (GI:301066250); however, the activity of the gene product has not been demonstrated yet [12]. Moreover, GSH is synthesized by a fusion protein (GSHF or GSHB; [22]) in E. faecium when grown in MRS or in other complex media, and this could explain the high GSH content of the parental and mutant E. faecium strains. Meanwhile, GSH was under the detection level in the other parental strains, GSH was detected in all studied mutant strains meaning a possible elevated GSH uptake. The selenite treatment increased further GR and GSH values in L. casei mutant strain pointing at a different selenite reduction pathway and/or stress response in this organism. Glutathione uptake could activate a GSHₐ glutathione peroxidase (Gpxₐ GR system, which catalyze the reduction of H₂O₂ (e.g. [23]) and GSH protected the cells against acid stress [24] or heat stress and prevented peroxidation of membrane fatty acids [25]. Similarly, a complete glutathione system against oxidative stress was demonstrated in Lactobacillus fermentum [26]. Selenite resistance induced stress response systems that lead up-regulation of GR production In the Gram-positive bacteria E. faecalis, GR has been purified to homogeneity [27]; meanwhile, no GR activity has been described in E. faecium until now. Here, GR activities were detected in E. faecium W54 strain but

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<tr>
<td>R703</td>
<td>0.369 ± 0.012</td>
<td>0.152 ± 0.017</td>
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</tr>
<tr>
<td>BB12</td>
<td>1.227 ± 0.010</td>
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</tr>
<tr>
<td>E. faecium</td>
<td>0.43 ± 0.007</td>
<td>5.022 ± 0.014</td>
<td>1.136 ± 0.021</td>
</tr>
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this enzyme activity was minute compared to the activity of the mutant strain.

The selenite supplementation significantly decreased intracellular GSH concentrations and GR activities. On the basis of the proposed reactions assumed by Kessi and Hanselmann on Gram negative bacteria [10], the low starting GSH concentration, the culmination of GSSG and excess in superoxide anion concentration in case of shortage of ROS eliminating enzymes could disorder abiotic reaction and the cellular homeostasis.

Safe bacterial cultures as functional foods or micronutrient supplements always attract considerable interest from both academic and industrial levels. Significant uptake and transformation of selenite to both organic and inorganic forms of selenium by E. faecium and the BB12 mutant strains with concomitantly high biomass production indicated that these strains could be good sources for direct dietary Se delivery [20], e.g. in dried form or in dairy products. These bacterial cells, as an addition to the decreased toxicity of selenite to elementary selenium [28,29] possess positive physiological and/or synthetic effects on the consumer’s health (e.g. antimutagenic activity [30]). The another downstream products of selenium supplementation, selenoproteins (e.g. glutathione peroxidase) containing oxidative selenocysteine. Moreover, high GSH accumulation and/or synthesis means greater stability of the strains under storage conditions and quicker growth upon inoculation. Probiotic cells with high antioxidant capacity without selenium fortification also can be used in medicine to modulate antioxidant status, pro-/anti-apoptotic proteins, caspases, and DNA damage [31].

We concluded that our strains are suitable for selenium enriched biomass production can be recommended as food or feed supplement with beneficial effects after further investigation.

Conflict of interest

The authors state that there is no conflict of interest.

Acknowledgement

The authors thank the technical assistance in bacterial identification they received from the Laboratory of Bacteriological Diagnostics at the Institute of Medical Microbiology, Medical and Health Center, University of Debrecen.

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