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# Acquisition of Iron by Enterococci: Some Properties and Role of Assimilating Ferric Iron Reductases

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Abstract

Ferric iron reductases activities have been occurred in 91% of investigated enterococci strains. Maximum activity occurred with coenzyme NADH as the reductant and the presence of cofactor FMN was necessary. Mg(II) ions has not stimulated reductases activity. Treatment of cells with proteolytic enzymes had not effect on iron reduction. The whole cells and cell fraction – cytoplasmic membrane and cytoplasm showed Fe(III) – reducing activity. The highest specific activity was associated with cytoplasm. The activity in cytoplasmic membrane was not related to iron concentration in the growth medium. In cytoplasm the activity was stimulated after growth in low-iron medium. Ferric iron reductases of enterococci characterized the broad substrate specificity. The iron in form of ferric ammonium citrate, lactoferrin and ferrioxamine B were the best iron sources for enterococcal ferric iron reductases.

Key words: *Enterococcus* spp., ferric iron reductases, iron acquisition

## Introduction

Reduction of complexed Fe (III) to Fe (II) decreases its affinity to the carrier: stability constant $K = 10^{30}$ of Fe(III)-siderophore after reduction amounts to $K = 10^{8}$ (Silver and Walderhaug, 1992; Clarke *et al.*, 2001). The complex dissociates and iron is released in the Fe(II) form easily assimilated by bacteria. In the cell in the presence of ferrochelatase it is used for the synthesis of haeme and non haeme iron-containing proteins. It is also a corepressor of Fur protein – a negative repressor controlling expression of iron uptake and transport systems (Clarke *et al.*, 2001; Schröder *et al.*, 2003). The ferric iron reductases are used for assimilating Fe(III) for the purpose of intracellular incorporation into protein. They have occurred in all bacteria excluding a small group of lactic acid bacteria (Schröder *et al.*, 2003).

Assimilating ferric iron reductases can be localized in bacterial cytoplasm, cytoplasmic membrane and periplasmic space (Schröder *et al.*, 2003). Some pathogenic bacteria release ferric iron reductases to the environment or expose them on the cell surface (Barchini and Cowart, 1996; Deneer *et al.*, 1995; Homuth *et al.*, 1998). These reductases permit bacteria to reduce in the environment iron Fe(III) free and bound to various carriers. The cytoplasmic reductases release Fe(II) by reduction from Fe(III)-siderophore complex introduced to the cell (Ratledge and Dover, 2000). Assimilating iron ferric reductases were detected in a number of pathogenic and facultative pathogenic bacteria (Barchini and Cowart, 1996; Coves and Fontecave, 1993; Johnson *et al.*, 1991; Le Faou and Morse, 1991). However, no data are available on ferric iron reductases in enterococci. The part of own research we have published earlier (Lisiecki and Mikucki, 2005). The results of these studies reveal more information on the occurrence, some properties and the role of these enzymes.

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## Experimental

### Materials and Methods

**Bacterial strains.** One hundred twenty strains of *Enterococcus* genus were used. Forty-nine of them originated from our own collection (Department of Pharmaceutical Microbiology, Medical University, Łódź) , and rest from the National Institute of Public Health in Warsaw, Microbiology Department Collegium Medical University of Toruń in Bydgoszcz, Czech National Collection of Type Cultures (CNCTC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and American Type Culture Collection (ATTC). Enterococci from our collection, were identified with API-STREP system (bioMerieux).

**Suspensions density and viable count.** The optical density of suspension and cultures was measured in UV/VIS Cary 1 (Varian) spectrophotometer at 580 nm. It was expressed in form of ODU (optical density unit) corresponding to absorbance value $A_{580}$ = 1.0. Mc Farland scale (Difco) and calibrated loops were used also. Viable count was estimated by using serial dilutions in buffered 0.85% NaCl, pH 7.2 and standard plate methods on 4% Trypticase Soy Agar (Difco).

**Growth media.** Strains were grown on medium containing (per litre): 3 g Casamino Acids, Vitamin Free (Difco); 3 g Yeast Extraxt (Difco); 3 g $KH_2PO_4$; 5 g NaCl; 1 g $NH_4Cl$; 0.09 g $MgCl_2$; 0.01 g $CaCl_2$; 12.1 g Tris (BDH) and 20 g glucose. pH of medium was adjusted to 7.2. Concentration of iron was reduced using polyaminocarboxylate resin Chelex 100 (200–400 mesh, BioRad). In some experiments the medium was supplemented with 5 μM haeme (Sigma) and 0.1 μM haemoglobin (Sigma). Solid medium contained 1.5% Noble Agar N° 1 (Oxoid). Mueller-Hinton 2 agar medium contained 50 μM o-phenantroline (Fluka) was used also.

**Growth conditions.** Strains were initially iron-starved for 18 hours at 37°C on Mueller-Hinton 2 agar medium (Difco) containing 50 μM o-phenantroline (Fluka). The optical density of prepared suspension corresponded to standard N° 2 of Mc Farland scale and contained $6\times10^8$ cells per ml. This starved suspensions were used to inoculate (5% v/v) media of different iron content: $Fe^+$ with $10^{-4}$ M of iron in the form of $FeSO_4\times7H_2O$ and $Fe^-$ (Chelex) subject to Chelex 100 (BioRad) resin and containing approximately $1.2\times10^{-6}$ M – $3.5\times10^{-6}$ of iron. Cultures were incubated at 37°C for 24 hours under constant shaking and centrifuged (9500×g, 15 min, 4°C). The cells were washed with buffered 0.155 M NaCl, pH 7.2 and supernatant was filtered through the membrane filter (0.22 μm, Millipore).

**Protoplast formation and fractionation.** Protoplast of enterococci was prepared with lysozyme (Serva) according to the method of Zorzi *et al.* (1996) and Lindberg (1981). The protoplast were lysed in 0.01 M TRIS-HCl (pH 7.2) buffer, DNase (1 μg/ml; Sigma) and RNase (2.5 μg/ml; Sigma) were added and the suspension was incubated in an ice water bath for 30 min. The protoplast lysate was pre-centrifuged (3000×g, 15 min, 4°C) the pellet discarded and the supernatant was dialysed against deionized water and centrifuged at 100 000×g for 60 min at 9°C (Beckman L8-70M) to obtain membrane (pellet) and cytoplasmic (supernatant) fraction. The protein content was estimated by the method of Lowry *et al.* (1951) and both fraction were lyophilized.

**Ferric iron reductase assay**. The reduction of Fe(III) was assayed with ferrozine [3-(2-pirydyl)-5,6-bis(4-phenylsulfonic acid)-1,2,3-triazine] (Sigma) as Fe(II) trapping reagent (Deneer *et al*., 1995). Ferrozine is water soluble, does not react with Fe(III) iron. The ferrous complex of ferrozine has an absorbance maximum at 562 nm (Stookey, 1970; Cowart *et al.*, 1993).

**Ferric iron reductases activity detection.** Standardized suspension of starved enterococci was spotted with calibrated loop (0.01 ml) on the surface of solid medium. After 24 h of incubation in 37°C the plate was flooded with 15 ml of 0.8% agarose solution (Sigma) containing: 2 mM ferrozine (Sigma), 50 μM NADH (Sigma), 50 μM FMN (Sigma) and 100 μg/ml of substrate – ferric ammonium citrate (Sigma). The plates were incubated in 37°C for 3 h in darkness. The violet zone around the bacteria proved the presence of Fe(II)-ferrozine complex and, indirectly, iron reductase activity (Mazoy and Lemos, 1999).

**Determination of ferric iron reductases activity of whole cells.** The cells were suspended in 8 ml of medium without $MgCl_2$, $CaCl_2$ and glucose and 1 ml of suspension was withdrawn to measure optical density of at 580 nm. The reaction mixture in final volume of 7 ml contained: bacteria, 50 μM NADH (Sigma), 3 μM FMN (Sigma) and 2 mM ferrozine (Sigma). In some experiments the reaction mixture contained 10 mM $MgCl_2$ (Sigma). After adding 100 μl substrate of reduction (100 μg/ml final concentration) polystyrene tubes were shaked and incubated in 37°C. After 10, 20 and 30 minutes 1 ml of reaction mixture was withdrawn, centrifuged (9500×g, 15 min, 4°C) and absorbance at 562 nm was measured in spectrophotometer UV/VIS Cary 1 (Varian). The control of assay contained all of the reagents except source of enzyme. The amount of Fe(II)-ferrozine complex was calculated as difference between the absorbance value of whole reaction mixture and control. Specific activity of ferric iron reductases was expressed as amount of μmol Fe(II)-ferrozine complex formed per ODU (optical density units) per minute (μmol Fe(II)-ferrozine/min/ODU) (Deneer *et al*., 1995). The results represent the means of three separate experiments.

**Determination of ferric iron reductases activity in cell fractions.** The sources of enzyme was cell fractions – membrane or cytoplasm. The reaction mixture contained in final of 2 ml volume: 0.5 ml of source of enzyme, 250 μM NADH (Sigma), 50 μM FMN (Sigma) and 250 μM ferrozine (Sigma) and of 0.01 M TRIS-HCl (pH 7.4) buffer. After adding 10 μl substrate of reduction (100 μg/ml final concentration) to polystyrene cuvettes the change in absorbance at 562 nm after 10, 20 and 30 minutes was measured in spectrophotometer UV/VIS Cary 1 (Varian). The assay was performed at 37°C. The control of assay contained all of the reagents except source of enzyme. The amount of Fe(II)-ferrozine complex was calculated as difference between the absorbance value of whole reaction mixture and control. Specific activity of ferric iron reductases was expressed as amount of μmol Fe(II)-ferrozine complex formed per g of protein per minute [μmol Fe(II)-ferrozine/min/g of protein] (Mazoy and Lemos, 1999). The results represent the means of three separate experiments.

**Determination of iron concentration.** Iron concentration in media was determined as described by Gadia and Mehra (1977) with ferrozine (Sigma) using UV/VIS Cary 1 (Varian) spectrophotometer at the 526 nm.

**Substrate of reduction reactions.** The following substrates were used: ferric ammonium sulphate (Sigma), ferric nitrate (POCH), ferric ammonium citrate (Sigma), ferric versenate (POCH), ferric pyruvate (Sigma), transferrin (Sigma), lactoferrin (Sigma), haemoglobin (Sigma), ferritin (Sigma) and ferrioxamin B (Sigma).

**Treatment of cells with proteolytic enzymes.** This experiment was carried out according to Deneer *et al.* (1995). The cells were suspended in 5 ml of 0.1 M Tris/HCl (pH 7.4) buffer supplemented with 1 mM $MgCl_2$ (Sigma) and proteinase K (0.5 mg/ml)

(Sigma) or trypsine (0.5 mg/ml) (Sigma) and the suspension was incubated at 37°C for 45 min. The cells were washed twice with buffered 0.155 M NaCl (pH 7.2) and resuspended in medium without salt and glucose and assayed for iron reductase activity.

**Statistical analysis**. Statistical analysis was performed with the Statistica PL computer programme (StatSoft) and nonparametric test of Kruskal-Wallis and U Mann-Whitney (Dixon and Massey, 1951). Statistical significance was defined as $p<0.05$.

## Results

Screening of strains of enterococci on the solid medium with ferrozine as Fe(II) iron trapping agent showed that 111 strains (91%) possessed reductase activity towards ferric ammonium citrate as a substrate. These data were confirmed by ferrozine test with resting cells. The studied strains differed in the activity in a wide range between 0.06 µmol of Fe(II)-ferrozine/min/ODU and 1.03 µmol of Fe(II)-ferrozine/min/ODU. There was no correlation between origin of strain, its species affiliation and reductase activity ($p>0.05$). For further investigation 6 strains with high reductase activity were selected: 4 out of them were derived from clinical material – *E. faecalis* BY 56 and BD 123 and *E. faecium* 97-0 and BY 9, and two from the environment – *E. saccharolyticus* DSM 20726 and *E. sulfureus* DSM 6905. Only *E. saccharolyticus* DSM 20726 reduced Fe(III) in absences of exogenous coenzymes such as NADH or NADPH and FMN cofactor. In the remaining strains NADH or NADPH alone did not activate ferric reductases. The presence of FMN as cofactor was necessary and activated ferric iron reductase itself but the most effectively when NADH was a donor of electrons (Table I). Magnesium ions did not affect a degree of reduction ($p>0.05$).

Fe (III) deficiency at the concentration of $3.5\times10^{-7}$ M in the medium did not influence the activity of ferric reductase in whole cells ($p>0.05$) (Table II). The presence of ferroporphyrin-haemin in the concentration of 5 µM or haemoglobin in the concentration of 0.1 µM did not change the activity also ($p>0.05$). Reduction of Fe (III) connected with a carrier required its contact with the cell. Separation of lactoferrin from the growing cells by a dialyzing membrane hindered its reduction. Modification of proteins on the

Table I
Effect of NADH, NADPH and FMN as reductans on ferric reductase activity in whole cells of enterococci

| Strains | Ferric iron reductese activity* | | | | | |
|---|---|---|---|---|---|---|
| | – | NADH (50 µM) | NADPH (50 µM) | FMN (3 µM) | NADH + FMN | NADPH + FMN |
| *E. faecalis* BD 123 | 0 | 0 | 0 | 0.046 | 0.18 | 0.07 |
| *E. feacalis* BY 56 | 0 | 0 | 0 | 0.043 | 0.09 | 0.075 |
| *E. faecium* BY 9 | 0 | 0 | 0 | 0.14 | 0.21 | 0.09 |
| *E. faecium* 97–0 | 0 | 0 | 0 | 0.08 | 0.11 | 0.12 |
| *E. sulfureus* DSM 6905 | 1.55 | 1.50 | 1.10 | 2.50 | 2.90 | 3.20 |
| *E. saccharolyticus* DSM 20726 | 0 | 0.28 | 0.032 | 0.32 | 0.36 | 0.14 |

* expressed as µM Fe(II)-ferrozine/min/ODU

Table II
Effect of iron concentration in medium on ferric iron reductase activity in whole cells of enterococci

| Strains | Ferric iron reductese activity* | |
|---|---|---|
| | Fe + medium | Fe- (Chelex) medium |
| *E. faecalis* BD 123 | 0.35 | 0.37 |
| *E. feacalis* BY 56 | 0.14 | 0.12 |
| *E. faecium* BY 9 | 0.25 | 0.11 |
| *E. faecium* 97–0 | 0.15 | 0.12 |
| *E. sulfureus* DSM 6905 | 1.78 | 1.70 |
| *E. saccharolyticus* DSM 20726 | 0.28 | 0.18 |

* expressed as µM Fe(II)-ferrozine/min/ODU

Table III
Effect of trypsin or proteinase K treatment of cells on ferric iron reductase activity in enterococci

| Strains | Ferric iron reductese activity* | | |
|---|---|---|---|
| | Intact cells | Modificated cell | |
| | | Trypsin | Proteinase K |
| *E. feacalis* BD 123 | 0.18 | 0.22 | 0.15 |
| *E. faecalis* BY 56 | 0.03 | 0.01 | 0.04 |
| *E. faecium* BY 9 | 0.01 | 0.02 | 0.01 |
| *E. faecium* 97–0 | 0.02 | 0.02 | 0.04 |
| *E. sulfureus* DSM 6905 | 0.06 | 0.12 | 0.05 |
| *E. saccharolyticus* DSM 20726 | 0.14 | 0.25 | 0.03 |

* expressed as μM Fe(II)-ferrozine/min/ODU

surface of the cells decomposed by trypsin or proteinase K did not influence the activity of ferric reductase activity ($p > 0.05$) (Table III).

The concentrated and non-concentrated culture supernatants did not show ferric iron reductase activity. It was present in whole cells and two subcellular fractions: cytoplasm and cytoplasmic membrane obtained after cells protoplastization and lysis of protoplasts. Ferric reductase activity in the cytoplasm was higher either in cells grown in the medium with iron deficiency [Fe- (Chelex)] and its abundance (Fe+) ($p < 0.05$). It amounted in mean value to 63 and 41 μmol of Fe(II)-ferrozine/min/g of protein) when corresponding values of activity in cytoplasmic membranes amounted to12.0 and 7.2 μmol of Fe(II)-ferrozine min/g of protein. Iron deficiency during the growth caused a significant increase in the activity of ferric iron reductases only in the cytoplasmic fraction ($p < 0.1$) (Table IV).

Ferric iron reductases were characterized by wide substrate specificity. They reduced inorganic compounds of iron – ferric ammonium sulfate and ferric nitrate, organic compounds – ferric ammonium citrate, ferric versenate and ferric pyruvate, body sources of iron – transferrin, lactoferrin, hemoglobin and ferritin, and bacterial siderophore-ferrioxamine B (data not shown). No differences were found in reductase activity of strains towards all substrates ($p > 0.05$). Significant differences were detected only when groups of substrates were assessed ($p < 0.05$). Substrates reduced with the highest activity such as ferrioxamine B, lactoferrin and ferric ammonium citrate belonged to the first group (0.26, 0.18, 0.17 μmol of Fe(II)-ferrozine/min/ODU, respectively). The second group involved ferric ammonium sulphate, ferric versenate and ferric nitrate (0.12, 0.09 and 0.042 μmol of Fe(II)-ferrozine min/ODU). The third group involved substrates reduced with the lowest activity: transferrin, ferric pyruvate, haemoglobin and ferritin (0.02, 0.019, 0.016, 0.016 μmol of Fe(II)-ferrozine/min/ODU).

Table IV
Effect of iron concentration in medium on specific activity of ferric iron reductase in cell fraction of enterococci

| Strains | Ferric iron reductese activity* | | | |
|---|---|---|---|---|
| | Cytoplasm | | Cytoplasmic membrane | |
| | Fe + | Fe- (Chelex) | Fe + | Fe- (Chelex) |
| *E. faecalis* BD 123 | 49.0 | 140.0 | 10.0 | 8.0 |
| *E. feacalis* BY 56 | 17.0 | 27.0 | 13.0 | 13.0 |
| *E. faecium* BY 9 | 35.0 | 25.0 | 6.0 | 10.0 |
| *E. faecium* 97–0 | 26.0 | 38.0 | 5.0 | 6.0 |
| *E. sulfureus* DSM 6905 | 66.0 | 56.0 | 1.0 | 15.0 |
| *E. saccharolyticus* DSM 20726 | 53.0 | 91.0 | 8.0 | 20.0 |

* expressed as μM Fe(II)-ferrozine/min/g of protein

## Discussion

Assimilating ferric iron reductases are widely spread in Gram-positive and Gram-negative bacteria (Johnson *et al.*, 1991; Le Faou and Morse, 1991; Deneer *et al.*, 1995; Coves and Fontecave, 1993; Barchini and Cowart, 1996; Homuth *et al.*, 1998). In more than 90% of enterococci of various species and origin reductase activity of ferric ammonium citrate was found. Bacterial ferric reductases were characterized by wide substrate specificity. They reduced Fe(III) in inorganic and organic compounds, complexes with natural and synthetic chelators, bound to the host body carriers and even free Fe(III) ions (Schröder *et al.*, 2003; Ratledge and Dover, 2000). Enterococci reduced Fe(III) of inorganic and organic compounds, siderophores and body iron carriers also. The ability to reduce Fe(III) of lactoferrin was crucial for colonization of the macroorganism but a minor participation of the rest of body carriers-transferrin, hemoglobin and ferritin in the reduction was enigmatic.

Assimilating ferric iron reductases use NADH and NADP coenzymes and sometimes glutathione as electron donors. The presence of free cofactors in the form of flavins – FMN, FAD or riboflavin is necessary for the reduction (Schröder *et al.*, 2003). NADH as a coenzyme and FMN as a cofactor occur more often in bacteria (Schröder *et al.*, 2003; Ratledge and Dover, 2000). Resting cells of enterococci did not reduce ferric ammonium citrate even in the presence of NADH or NADPH. Only free flavin (FMN) stimulated ferric reduction, and endogenous coenzymes associated with cell surface were electron donors. Exogenous coenzymes stimulated ferric reduction only in the presence of flavin (FMN), and NADH did it stronger as compared with NADPH. Same as in *E. coli* (Fontecave *et al.*, 1987) and *P. aeruginosa* (Halle and Meyer, 1992) free flavin FMN could be reduced by enterococci and in this form served as direct, chemical reductant for numerous substrates. This reductase would be in fact a flavin reductase, not a ferric reductase.

Assimilating ferric iron reductases are constitutive enzymes. In any studies a regulating role of iron was not noted in their production (Arceneaux and Byers,1980; Johnson *et al.*, 1991; Deneer *et al.*, 1995; Cowart, 2002). Activity of ferric iron reductases of whole enterococcal cells was not regulated by iron availability. Some bacterial ferric reductases are released to the environment (Homuth *et al.*, 1998). Most often ferric reduction occurs when iron ions or their carriers contact with reductases exposed on the cell surface. In test with ferrozine only iron Fe(II) bound to the surface of resting cells and released to the environment after reduction was detected because this compound did not penetrate through cytoplasmic membrane (Stookey, 1970; Cowart *et al.*, 1993; Deneer *et al.*, 1995). Enterococci like *Listeria monocytogenes* (Deneer et *al.*, 1995) did not reduce Fe (III) in lactoferrin when were separated from the substrate by a dialysis membrane so, the contact between substrate and cell was necessary. Modification of the cell surface of enterococci by means of the breakdown of proteins neither eliminated nor inhibited cooperation between cells and substrates same as in *Legionella pnemophila* (Johnson *et al.*, 1991) and *Listeria monocytogenes* (Deneer *et al.*, 1995). Enterococcal reductases can be localized deeper in the cell cover and less available for proteolytic enzymes. Bacterial assimilating ferric iron reductases can be detected in the cytoplasmic membrane, periplasmic space and cytoplasm (Schröder *et al*., 2003). They can be found in all subcellular fractions (Schröder *et al.* 2003; Cowart, 2002) and differ in the use of electron donors (Schröder *et al.*, 2003). Location of reductases in the cytoplasmic membrane and periplasmic space has a strategic role: permits a contact with the substrate. Reductases occurring in the growth environment can be enzymes actively secreted or because of loose binding to the membrane and periplasmic spaces released in to the growth medium (Barchini and Cowart, 1996; Deneer *et al.*, 1995). Detection of more and more exogenous ferric iron reductases allowed us to draw a conclusion that iron acquisition by means of their parcipitation could be a common bacterial strategy. However enterococci neither released from the cell surface nor secreted ferric reductases to the environment. It diminished their strategic role in the efficacy of iron acquisition.

Ferric iron reductases occur in enterococci in two subcellular fractions: cytoplasm and cytoplasmic membrane. It is a localization of ferric reductases in the majority of bacteria. In enterococci as in other bacteria these reductases create two groups of enzymes: constitutive, connected with cytoplasmic membrane and inductive occurred in cytoplasm. Specific activity of iron ferric reductase in cytoplasm was higher as compared to the cytoplasmic membrane enzymes.

Recently published data on cooperation between siderophores and ferric iron reductases can change our attitude towards their significance for iron acquisition by bacteria (Vartivarian and Cowart, 1999; Cowart, 2002). Siderophores are induced chelators while assimilating reductases are constitutive enzymes which synthesis in contradistinction to siderophores takes place independently of iron availability. Siderophores have been classified as secondary metabolites recently, because their production occurs in late phases of growth such as stationary and death phase. Ferric iron reductases are produced at all phases of growth.

These features cause that they can play more spectacular role as compared with siderophores in mobilization of Fe(III) sources in the environment of bacteria. Therefore, the presence of at least two separate pathways of iron acquisition by bacteria, which produce siderophores and ferric iron reductases, have been postulated. Enterococci also belong to this group. In conditions of iron availability in the environment ferric iron reductases transform Fe(III) into Fe(II) which can be transported to the cell through Nramp transporter (natural resistance-associated macrophages proteins) or/and a pathway of simple diffusion (Halle and Meyer, 1992). With limited iron Fe(III) availability and therefore decreasing Fe(II) cell concentration derepression of siderophore system occurs and with participation of these chelators a second way of iron acquisition is opened. Siderophores bind free and complexed Fe(III) and also free Fe(II) after reduction. Binding of the latter to siderophores leads to their immediate oxidation to Fe (III) and Fe (III)-siderophores complex can be transferred to cytoplasm. The action of these two ways requires the presence of constitutive ferric iron reductases (Cowart, 2002).

In enterococci a tight binding of reductases to cell can be a limitation in their ascribed action. Therefore they cannot mobilize Fe(III) sources through reduction outside the cell. They reduce substrates only adjoining to the cell surface and Fe(II) transportation into cytoplasm could take place with simple diffusion. Gram-positive bacteria such as *L. monocytogenes* (Barchini and Cowart, 1996) and Gram-negative as *E. coli* and *P. aeruginosa* (Vartivarian and Cowart, 1999) make use of such individual way of Fe(II) ions transport, separated from the way of transport of Fe (III)-siderophore complexes.

Cytoplasmic ferric iron reductases in enterococci are probably an induced enzymes. They can be a final part of induced siderophore system. After introduction with a receptor of Fe(III)-siderophore complex to cytoplasm they release iron through its reduction to Fe(II) which in the presence of ferrochelatase can be built in haeme and non-haeme proteins. Cytoplasmic ferric reductases dependent on NADH or NADPH for Fe(III)-schizokinen, ferrioxamine B and Fe(III)-aerobactin were detected in *Mycobacterium, Bacillus* and *Acinetobacter* genera (Clarke *et al*., 2001; Ratledge and Dover, 2000)

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