Polish Journal of Microbiology
2017, Vol. 66, No 4, 419–425

ORIGINAL PAPER

Transferrin and Lactoferrin – Human Iron Sources for Enterococci

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Submitted 9 February 2017, revised and accepted 17 May 2017

Abstract

To overcome limitations in iron acquisition, enterococci have evolved a number of mechanisms to scavenge iron from the host iron-binding proteins – transferrin (TR) and lactoferrin (LF). The aim of this study was to demonstrate the mechanisms by which enterococci utilize human TR and LF bound iron. The study included two strains of *Enterococcus faecalis* grown in iron-deficient and iron-excess media respectively. The binding activity of both proteins was monitored using proteins labelled with ^{125}I. The uptake of iron by enterococci was determined using ^{59}Fe labelled proteins. Reduction of iron bound to TR and LF was assayed with ferrozine. The proteolytic cleavage of TR and LF was visualized by SDS-polyacrylamide gel electrophoresis. The siderophore activity was measured with chrome azurol S. The study revealed that enterococci use several ways to acquire iron from TR and LF, such as iron chelating siderophores, iron reduction – facilitated iron release, protein degradation – promoted iron release, and receptor mediated capture of the iron-host protein complexes. The broad spectrum of iron acquisition mechanisms used by enterococci may play a significant role in the colonization of the human body and the resulting pathogenicity.

Key words: *Enterococcus* spp., iron acquisition, lactoferrin, siderophores, transferrin

Introduction

Members of the genus *Enterococcus* are widely distributed throughout nature as components of human and animal intestinal microbiota, and are also found in vegetables, plant materials and foods (Fisher and Phillips, 2009). Enterococci are typical opportunistic pathogens, and can cause urinary tract, wound, and soft tissue infections. They are associated with bacteremia which can lead to endocarditis in previously damaged cardiac valves (Gilmore *et al.*, 2013; van Tyne *et al.*, 2013). During the past several decades, enterococci, and particularly *Enterococcus faecalis* and *Enterococcus faecium*, have been identified as an important cause of nosocomial infections (Yuen and Ausubel, 2014). The major reason why these organisms survive in the hospital environment may be traced to their intrinsic natural resistance to several commonly used antibiotics. In the hospital setting, they can easily acquire other genes conferring resistance to many other classes of antimicrobial compounds (Gilmore *et al.*, 2013).

Iron is an essential element for bacteria, but is not easily available in host organisms. The concentration of free iron in humans is 10^{-18} M, an amount insufficient for maintaining the production of haem and non-haem iron-containing proteins. *In vivo*, bacteria have to contend with the natural ability of the host to withhold free iron by binding it to iron-protein complexes such as transferrin (TR) and lactoferrin (LF). TR is the iron carrier in the blood, while LF carries iron in secretory fluids. Competition for iron between the host and bacteria is an important factor determining the course of bacterial infections (Weinberg, 2009). At least four different mechanisms by which bacteria are said to acquire iron from host proteins have been suggested: Fe^{3+} chelating siderophore activity; reduction of carrier Fe^{3+} to the lower affinity iron Fe^{2+}resulting in its release; interaction of the Fe^{3+} carrier with a receptor on the cell surface; and proteolytic degradation of the carrier resulting in the splitting of the iron binding site (Krewulak and Vogel, 2008; Sheldon *et al.*, 2016).

Enterococci as lactic acid bacteria (LAB) for a long time were regarded as not requiring iron (Marcelis *et al.*, 1978). This concept, however, is changing in the light of the latest findings (Sobiś-Glinkowska *et al.*, 2001). To survive and multiply in the host, enterococci must possess efficient iron-acquisition mechanisms. It is believed that these mechanisms can be an

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important virulence factor for the enterococci. Still, little is known about enterococcal mechanism of iron assimilation. The goal of the present study was to demonstrate that enterococci may utilize human TR and LF as iron sources, and to detect which mechanism they use to acquire the iron.

Experimental

Materials and Methods

Bacterial strains. Two strains of *E. faecalis*, BD 122 and BD 123, were isolated from the blood of patients with endocarditis after cardiosurgery admitted to the Clinical Hospital No 1 in Lodz, then identified with API 20 Strep (bioMerieaux, France) and stored at –70°C in glycerol (Difco, USA).

Bacteriological media and growth conditions. Strains were grown in complex liquid medium (Lisiecki *et al.*, 1999). Iron deficient-medium was obtained using polyaminocarboxyl resin Chelex 100 (200–400 mesh, BioRad, USA). Strains were initially iron starved in liquid iron-deficient medium for 18 h at 37°C, and continuously shaken. The obtained culture was used to inoculate (5% v/v) a new portion of iron-deficient or iron-excess medium. After 24 h at 37°C with constant shaking, the culture was centrifuged (9500 g, 15 min, 4°C). The supernatant was filtered through a membrane filter (0.22 μm, Millipore, USA).

Determination of the MIC of EDDHA. The minimal inhibitory concentration (MIC) of ironchelator – ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA, Sigma, USA) was determined using the agar dilution method on Mueller-Hinton 2 medium (bioMerieux, France) (EUCAST, 2017).

Determination of ability to utilize iron bound in transferrin and lactoferrin. A standardized bacterial suspension (0.1 ml, $A_{580nm} = 0.1$) was added into 20 ml of melted Mueller-Hinton 2 agar medium with EDDHA, at concentration corresponding to MIC for the tested strains. The mixture was poured into plastic Petri dishes (Ø14 cm). After storing the plates for 24 h at 4°C, filter paper discs (No 3, Whatman, England) were placed on the surface of the plates and saturated with 5 μl of the iron binding host proteins, either TR (1000 μg) or LF (1000 μg, both from Sigma, USA). The negative control used was a disc with 1000 μg of human apo-transferrin (ATR) and apo-lactoferrin (ALF) (both from Sigma, USA). The control for bacterial growth consisted of a disc containing 25 μg of $FeSO_4 \times 7H_2O$ (Sigma, USA). The experiment was also performed with TR and LF filter paper discs separated from the bacteria by a 25,000 Da cutoff dialysis membrane (Visking Dialysis Tubing, Serva, Germany). Growth around the discs demonstrated the ability of the tested strains to utilize iron bound to host protein, and was assessed after 24 h and 48 h of incubation at 37°C.

Bacterial growth in human serum. Serum was obtained from the blood of healthy volunteers and inactivated for 30 min at 56°C. Approximately 10^6 viable iron-starved cells of each strain were added to 0.5 ml of, respectively, unsupplemented serum and serum containing 0.2 mM of ferric nitrilotriacetate (Fluka, Switzerland) to be later saturated with iron serum TR (Brock and Ng, 1983). After 18 h of incubation at 37°C, viable counts were calculated for each serum sample.

Transferrin and lactoferrin binding assay. Iron saturated TR and LF were labelled using the chloramine T method with $Na^{125}I$ (specific activity of 17.4 mCi/mg, NEN Life Science Products, USA) and Iodobeads (Pierce Chemicals, USA) (Markwell, 1982). Bacterial cells (10^9 CFU) were added to 1 ml of the liquid medium (without $MgCl_2$, $CaCl_2$, and glucose, but supplemented with 1 mg/ml of human albumin) and 10 μl of protein labelled with ^{125}I (3 μg protein, radioactivity approximately 10^3 cpm). After 2 h of incubation at 37°C, the suspension was centrifuged at 4500 g for 15 min at 4°C, and the bacterial pellet was washed three times with cold phosphate-buffered saline (PBS). The radioactivity associated with the bacteria was quantified using a gamma counter (Wallac, Sweden). Samples were tested in triplicate along with the control test tubes containing all ingredients except bacteria. The results were presented in units of nanograms of iodine-labelled protein bound per 10^9 bacterial cells (ng protein/10^9 CFU).

Uptake of iron from transferrin and lactoferrin. Human ATR and ALF labelling was performed with $^{59}FeCl_3$ (specific activity of 15.49 mCi/mg, NEN Life Science Products, USA) (Lindsey *et al.*, 1995). Bacterial cells (10^9) were mixed with 1 ml of iron-deficient medium (without $MgCl_2$, $CaCl_2$, and glucose) and 10 μl of protein labelled with ^{59}Fe (15 ng of iron, radioactivity of approximately 10^4 cpm). After 2 h of incubation at 37°C, the suspension was centrifuged at 4500 g for 15 min at 4°C, and the bacterial pellet was washed three times with cold PBS. The radioactivity retained in the bacterial pellet was measured using a gamma counter. Samples were tested in triplicate, along with the control test tubes containing all ingredients except bacteria. The results were presented in units of nanograms of ^{59}Fe bound per 10^9 bacterial cells (ng iron/10^9 CFU).

Iron reductase assay. The reduction of Fe^{3+} was assayed using ferrozine [3-(2-pirydyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] (Sigma, USA) (Deneer *et al.*, 1995). Whole bacterial cells were suspended in 8 ml of medium (without $MgCl_2$, $CaCl_2$, and glucose), and 1 ml of the suspension was removed to measure its optical density at 580 nm. In the final volume of 7 ml, the reaction mixture contained: 50 μM NADH,

3 μM FMN, and 2 mM ferrozine (all reagents by Sigma, USA). After adding 100 μl of either TR or LF (100 μg/ml final concentration), the polystyrene tubes were shaken and incubated at 37°C. At 10, 20, and 30 min, 1 ml of reaction mixture was withdrawn, centrifuged (9500 g, 15 min, 4°C), and the absorbance at 562 nm was measured with a spectrophotometer (UV/VIS Cary 1, Varian, USA). The control tube contained all of the reagents except the enzyme source. The amount of Fe^{2+}-ferrozine complex was calculated as the difference between the absorbance values for the whole reaction mixture and the control mixture. Samples were tested in triplicate. The specific activity of ferric reductase was expressed as micromole Fe^{2+}-ferrozine complex formed per optical density unit (ODU) per minute (μmol Fe^{2+}-ferrozine/min/ODU). The results represent the mean values of three separate experiments.

Proteolytic activity of culture supernatants. Proteolytic activity was determined with gelatin and casein. Gelatin hydrolysis was evaluated on Todd-Hewitt agar (Difco) containing 3% gelatin. Casein utilization was analyzed on Trypticase Soya Agar (Difco) with 1.5% skim milk. Wells (Ø2 mm) were cut into the agar medium and 10 μl of the culture supernatants were pooled into these wells. Agar plates were incubated at 37°C for 24 h. The appearance of a turbid halo around the wells was considered positive for gelatin cleavage. The presence of a clear zone around the wells indicated caseinase activity (Kanemitsu *et al.*, 2001).

Proteolytic degradation of transferrin and lactoferrin. The culture supernatants and bacterial cells of the tested strains harvested from both the iron-deficient and iron-excess medium were tested. The culture supernatant (900 μl) was mixed with 100 μl of either TR or LF, to obtain a final concentration 1 mg/ml. After 2 h of incubation at 37°C, 20 μl was electrophoresed. Roughly 1×10^6 CFU/ml of whole cells was resuspended in 900 μl of iron-deficient medium (without $MgCl_2$, $CaCl_2$, and glucose). Either TR or LF (100 μl) was added to this suspension to a final concentration of 1 mg/ml. The mixture was incubated at 37°C for 2 h. After removing the bacteria with centrifugation (9500 g, 15 min, 4°C), 20 μl of the supernatant was subjected to electrophoresis. To visualize the destruction of LF or TR, SDS-polyacrylamide gel electrophoresis was performed with 12.5% resolving gels (Amersham Biosciences, Sweden). The gels were stained with Coomassie blue.

Determination of siderophores in the culture supernatants. Total siderophore activity in the culture supernatants was measured using chrome azurol S (CAS) (Schwyn and Neilands, 1987). Hydroxamate siderophores were assayed using a chemical-specific assay (Lisiecki *et al.*, 1999). The results were expressed as micrograms of desferrioxamine mesylate (Sigma, USA) per millilitre of culture supernatant.

Determination of iron concentration. Concentrations of iron in the medium were determined by spectrophotometric assaying, using iron test kits containing 1,10-phenanthroline (Merck, USA). Fe^{3+} was first reduced to Fe^{2+} by ascorbic acid, and the total amount of iron was measured as above.

Suspension density and viable count. The optical densities of suspensions and cultures were measured using a UV/VIS Cary 1 spectrophotometer at 580 nm, and standardized according to McFarland's scale. The viable count was estimated by using serial dilutions in PBS and standard plate methods on 4% Trypticase Soy Agar (Difco, USA).

Statistical analysis. Bacterial counts were compared with the non-parametric Mann-Whitney U test. Calculations were performed using the Statistica 7 (StatSoft®, Poland) software, and statistical significance was defined as $p \leq 0.05$.

Results

Multiplication of enterococci in human serum. Both the tested strains multiplied in Fe^{3+} non-enriched human serum (Table I). The serum enriched with Fe^{3+} to a concentration of 0.2 mM did not significantly ($p > 0.05$) stimulate the growth of the strains (Table I). The main iron binding-protein in human serum is TR.

Table I
Growth of enterococci in normal human serum

Strain	Inoculum (CFU/ml)	Unsupplemented serum iron (CFU/ml)	Supplemented serum iron (CFU/ml)
E. faecalis BD 122	1.9×10^6	3.8×10^6	3.0×10^6
E. faecalis BD 123	1.0×10^6	1.7×10^6	2.4×10^6

CFU, colony forming unit.

Ability of transferrin and lactoferrin to support the growth of enterococci. It was demonstrated, using the disc-diffusion technique, that human iron saturated TR and LF can support the growth of the tested enterococcal strains even under iron-deficient condition (Table II). Using apo-TR and apo-LF, the iron-free form of these proteins, instead of TR and LF did not promote growth of the tested enterococcal strains (Table II). The above results indicated that TR and LF can be used as iron sources for the tested enterococcal strains. Human TR and LF stimulated the acquisition of iron even when separated from the bacteria by a 25,000 Da cutoff dialysis membrane (data not shown). This experimental data suggests the involvement of siderophores – iron chelators – in the uptake of TR and LF bound iron. Only

Table II
Promotion the growth of enterococci by transferrin and lactoferrin

Iron sources	*E. faecalis* BD 122	*E. faecalis* BD 123
	Growth (mm)*	
None	0	0
Apo-transferrin	0	0
Transferrin	6	8
Apo-lactoferrin	0	0
Lactoferrin	7	9
Ferrous sulfate	10	11

* Details were described in the section of Materials and Methods. Response to iron-binding proteins was determined by the presence of a growth zone (mm) around a disc containing transferrin and lactoferrin. The negative control was disc containing apo-transferrin and apo-lactoferrin – iron-free form of these proteins. The control of bacterial growth consisted of a disc containing ferrous sulfate. The experiment was repeated twice and the results were consistent.

siderophores, as low molecular weight compounds with high affinity constants for iron, are able to overcome the barrier of a dialysis membrane, extract iron bound to TR and LF and provide them into bacterial cell.

Iron-chelators produced by enterococci. Under iron-deficient growth conditions, siderophore activity was detected in the culture supernatants of both strains, using a universal chemical assay with CAS. The growth medium contained the iron concentration of approximately 3.5×10^{-7} M. The siderophore activity of both strains was similar – 21.38 μg/ml and 19.71 μg/ml, respectively (Table III). The source of iron chelators, was a siderophore containing N-hydroxyamide groups belonging to the hydroxamate siderophore class (Table III). The enterococcal strains did not produce siderophores in the iron-excess medium (10^{-4} M).

Reduction of iron bound to transferrin and lactoferrin by enterococci. The iron bound to TR and LF was reduced by ferric reductases of whole cells in both strains (Table III). The culture supernatants did not exhibit this activity. Separation of the substrate from the cells by a dialysis membrane during incubation inhibited its reduction (data not shown). This finding indicates that the enterococcal ferric reductases are cell surface enzymes, and can be involved in enterococcal iron uptake.

Ability of enterococci to take up iron from transferrin and lactoferrin. To correlate the ability of TR and LF to support the growth of enterococcal strains with uptake of bound iron, the assimilation of iron from TR and LF was also tested. Whole cells of both strains actively acquired iron from the ^{59}Fe-labelled TR (Table III and IV). *E. faecalis* BD 122 acquired 57.5% and *E. faecalis* BD 123 acquired 50.7% of the initial isotope content, respectively. Iron from the ^{59}Fe-labelled LF was also absorbed by both strains, though to a considerably lesser extent: 3.1% of the initial isotope dose for strain BD 122 and 1.8% for strain BD 123 (Table IV).

Transferrin and lactoferrin binding activity of enterococci. Binding of TR and LF to the tested strains was quantified with iodine-labelled proteins. Human ^{125}I-LF was bound by the whole cells of both strains. *E. faecalis* BD 122 and BD 123 bound 2.5% and 2.4% of the initial isotope dose, respectively (Table III and V). The control value in these experiments was 33.00 ± 10.11 ng per 10^9 CFU, which corresponded to 1% of the initial isotope dose (Table V). Human ^{125}I-TR was not bound by the cells (Table III and V). The binding value for *E. faecalis* BD 122 was 30.00 ± 25.10 ng per 10^9 CFU, while for *E. faecalis* BD 123 it was 30.10 ± 13.20 ng per 10^9 CFU, amounting to 0.6% and 0.6% of the initial isotope doses, respectively. These values were identical with the control value (30.00 ± 15.10 ng per 10^9 CFU), corresponding to 0.6% of the initial isotope dose (Table V).

Table III
Suggested mechanisms of lactoferrin (LF) and transferrin (TR) bound iron acquisition by enterococci

Mechanism of iron acquisition		Strain	
		E. faecalis BD 122	*E. faecalis* BD 123
Hydroxamate siderophore (μg/ml)		21.38	19.73
Fe^{3+} reduction of carrier (μM Fe(II)-ferrozine/min/ODU*)	LF	0.10 ± 0.04	0.08 ± 0.05
	TR	0.01 ± 0.01	0.02 ± 0.01
^{125}I Carrier binding (ng protein per 109 CFU†)	LF	92.40 ± 19.9	79.20 ± 5.04
	TR	0	0
^{59}Fe iron-uptake (ng ^{59} Fe per 10^9 CFU)	LF	0.47 ± 0.23	0.28 ± 0.05
	TR	8.62 ± 1.17	7.6 ± 0.62
Proteolytic cleavage of iron bound to protein	LF	+	+
	TR	–	–

* ODU, optical density unit; † CFU, colony forming unit.

Table IV
Lactoferrin and transferrin binding by whole cells of the tested enterococci*

Protein	*E. faecalis* BD 122	*E. faecalis* BD 123	Control value
	ng iodine labelled protein per 10^9 CFU†		
Lactoferrin	82.50 ± 20.05	79.20 ± 15.08	33.00 ± 10.11
Transferrin	30.00 ± 25.08	30.07 ± 13.23	30.00 ± 15.12

* Cells were harvested from the iron-deficient medium. ^{125}I-labelled proteins were used. All experiments were completed using 1 ml of culture at 10^9 CFU/ml. Radioactivity is represented as ng of iodine labelled protein binding by the cells. The control mixture contained all of the reagents except the bacteria. Each column displays the mean values of three determinations from one experimental run.
† CFU, colony forming unit.

Table V
Iron uptake from lactoferrin and transferrin by whole cells of the tested enterococci*

Protein	*E. faecalis* BD 122	*E. faecalis* BD 123	Control value
	ng ^{59}Fe per 10^9 CFU†		
Lactoferrin	0.47 ± 0.23	0.28 ± 0.05	0.05 ± 0.03
Transferrin	8.62 ± 1.17	7.60 ± 0.62	0.29 ± 0.03

* Cells were harvested from the iron-deficient medium. All experiments were completed using 1 ml of culture at 10^9 CFU/ml. Radioactivity is represented as ng of ^{59}Fe transported into the cell. The control mixture contained all of the reagents except the bacteria. Each column displays the mean values of three determinations from one experimental run.
† CFU, colony forming unit

Proteolytic degradation of transferrin and lactoferrin by enterococci. Finally, the susceptibility of TR and LF to proteolytic degradation by the tested strains was investigated using SDS-polyacrylamide gel electrophoresis. The strains were proteolytically active towards the standard proteolysis substrates: casein and gelatin. Only the culture supernatants of both strains were active with LF. The products of degradation of LF into lower molecular weight fragments were detected after 2 h of incubation (Fig. 1 and 2). The excessive amount of Fe^{3+} (10^{-4} M) in the culture did not affect the proteolytic activity. Human TR was not proteolytically degraded by either strain in both the presence of the culture supernatants or whole cells (Fig. 1 and 2). The obtained data suggests that proteases active on TR are not produced by the tested enterococcal strains.

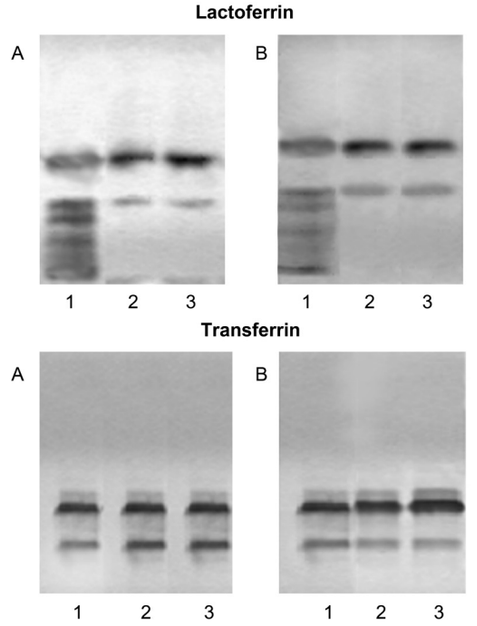

Fig. 1. SDS-polyacrylamide gel electrophoresis of the proteolytic degradation of iron-binding proteins – lactoferrin and transferrin by *E. faecalis* BD 122 grown in iron-deficient (A) or iron excess medium (B).

Lane 1, culture supernatant and iron-binding protein; lane 2, whole cell and iron-binding protein; lane 3, liquid growth medium and iron-binding protein. Approximately 20 µg of protein was separated on 12.5% polyacrylamide gel and stained with Coomassie blue.

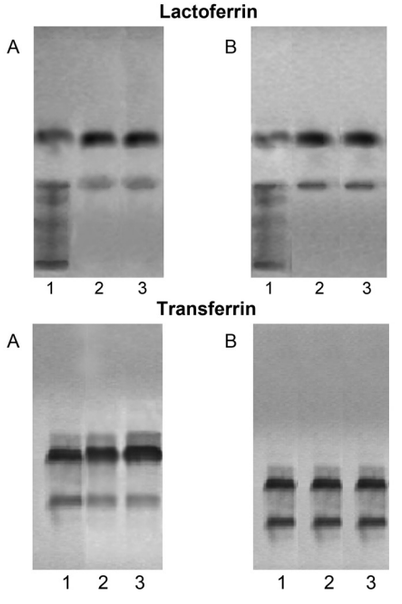

Fig. 2. SDS-polyacrylamide gel electrophoresis of the proteolytic degradation of iron-binding proteins – lactoferrin and transferrin by *E. faecalis* BD 123 grown in iron-deficient (A) or iron excess medium (B).

Lane 1, culture supernatant and iron-binding protein; lane 2, whole cell and iron-binding protein; lane 3, liquid growth medium and iron-binding protein. Approximately 20 µg of protein was separated on 12.5% polyacrylamide gel and stained with Coomassie blue.

Discussion

Numerous pathogenic and opportunistic bacteria utilize the human iron binding proteins – transferrin (TR) and lactoferrin (LF) as a source of iron (Mietzner and Morse, 1994; Morgenthau *et al.*, 2013; Parker Siburt

et al., 2012). Prior to the present work, there was no data in the available literature on *Enterococcus* spp. In this study, we evaluated the ability of two strains of *E. faecalis* to utilize human TR and LF as a sources of iron, and investigated the mechanism by which iron can be obtained from this protein. The tested enterococci strains acquired ^{59}Fe from human TR and LF. Iron saturated TR and LF were found to support growth of the tested strains. Bacteria growth or survival in human serum was, thus, associated with their ability to acquire iron from TR, the main source of iron in the blood (Mietzner and Morse, 1994). Lactoferrin has been identified in various secretory fluids, such as mammalian breast milk, saliva, tears, and mucosal secretions. Lactoferrin is also present in specific granules of neutrophils, and can also be find in stool, after being released from fecal leukocytes (García-Montoya *et al.*, 2012). Enterococci are inhabitants of the gastrointestinal tract of human microbiota (Fisher K. and C. Phillips, 2009). The bacteria can acquire iron not only from human TR and LF, but also from the iron carriers of other animals. Previous studies show that enterococci have the ability to acquire iron from ovotransferrin and bovine TR and LF (Sobiś-Glinkowska *et al.*, 2001).

Siderophores, extracellular iron chelators, are produced in response to an environmental iron deficiency by numerous, but not all, bacteria (Krewulak and Vogel, 2008; Kurth *et al.*, 2016.). The binding of Fe^{3+} by these compounds, with a stability constant (K) of 10^{22} to 10^{50}, is considered sufficient for iron uptake from body carriers such as TR, LF, and ferritin, but not from haem proteins (Harris *et al.*, 1979; Drechsel and Winkelman, 1997). The TR and LF stability constants at a pH of 7.4 (*i.e.* physiological conditions) equal $10^{25.6}$. Until now, enterococci have been regarded as not having the ability to produce siderophores. However, it has been stated that the bacteria produce iron chelators belonging to the hydroxamate class of siderophores (Lisiecki *et al.*, 1999). We demonstrated that production of the hydroxamate siderophores in the growth medium by both of the tested strains was induced by iron deficiency at concentration of 3.5×10^{-7} M. Even lower free iron concentration of the human body (10^{-18} M) may induce the production of hydroxamate siderophores, which may bind the iron of TR and LF and transport it into the cells.

Iron bound to TR and LF may be released by bacteria through reduction by assimilating the ferric reductases, which are not a part of the respiratory chain. The stability constant of Fe^{2+}-carrier complexes is then reduced to $K = 10^8$, and the complexes undergo dissociation, with the Fe^{2+} ion being released in a form easily assimilated by bacteria (Clarke *et al.*, 2001). Assimilation of iron by ferric reductases is widespread amongst bacteria living in aerobic environments with neutral pH (Fontecave *et al.*, 1994; Schröder *et al.*, 2003). The bacterial ferric reductases possess wide substrate specificities, and also reduce the Fe^{3+} of siderophores, TR, LF, or ferritin (Deneer *et al.*, 1995; Schröder *et al.*, 2003; Vartivarian and Cowart, 1999). Our results show the presence of ferric reductase activity toward TR and LF in the tested strains. The higher activity of reductases towards LF compared to TR is likely to be associated with the adaptation of enterococci for colonization of the digestive tract and vaginal vault mucosa, where LF is the most available iron source. Thus, the reduction of iron bound to TR and LF requires enterococcal cells to have direct access to these substrates. This access limits the involvement of reductases in the mobilization of iron sources from these iron carriers in the body. However, the majority of bacteria have only cell-related assimilating ferric reductases at their disposal (Schröder *et al.*, 2003).

Bacteria can acquire TR and LF bound iron to bind the protein on its surface *via* the specific receptor (Schröder *et al.*, 2003). Some reports have suggested that enterococci can bind to TR and LF (Zareba *et al.*, 1997; Styriak *et al.*, 2004). However, the investigated enterococci strains bound only iodine-labelled ^{125}I-LF, and acquired ^{59}Fe from both LF and TR. Therefore, it can be assumed that iron bound to LF might be assimilated into the cell after direct binding to the carrier through the receptor, whereas iron bound to TR, after reduction with ferric reductases, might be acquired in the form of Fe^{2+} through simple diffusion. Experimental conditions used in our experiments which utilized whole cells did not promote siderophore synthesis, which requires growth and multiplication of cells (Ratledge and Dover, 2000).

Bacterial extracellular proteases can similarly be involved in the iron uptake. These enzymes can cleave TR and LF into smaller fragments, and may result in the loosening of the Fe^{3+} bond to the protein, releasing the iron and allowing it to be more easily sequestered by siderophores. Iron (Fe^{3+}) can be also reduced to Fe^{2+} by ferric reductases, resulting in their release. The extracellular proteases in enterococci culture supernatants have cleaved only LF. The source of proteolytic activity of the enterococcal supernatants was probably gelatinase (Gel E). Both tested strains show proteolytic activity towards gelatine. Presence in enterococci of two additional proeteases, SprE and Deg P, was reported (Strzelecki *et al.*, 2011). It was also demonstrated that enterococci are proteolytically active towards haemoglobin – haem iron carrier (Sobiś-Glinkowska *et al.*, 2001).

To summarize, these experimental results are the first ever to indicate that human TR and LF are the source of iron for enterococci. The present study revealed that enterococci can use several strategies to acquire iron from TR and LF, such as iron chelating siderophores, iron reduction – facilitated iron release, receptor medi-

ated capture of the iron-host protein complexes and protein degradation – promoted iron release. It is possible that interactions exist between these mechanisms in enterococci. Enterococci used more mechanisms of iron aquisition from LF compared with those used to acquire TF bound iron. It probably results from the bacterial adaptation to niches settled in the organism. Bacteria of the genus *Enterococcus* are characterized by a natural and acquired resistance to many antimicrobial agents (Fisher and Phillips, 2009; Gilmore *et al*., 2013). For these reasons, treatment of enterococcal infections poses significant difficulties. It is crucial for us to intensify research into the mechanisms responsible for facilitating iron uptake by these pathogens. Inhibition of iron uptake in enterococci can be helpful in reducing and combating enterococcal infections. It is worth pointing out that it can be the next, new target for medication against infection caused by enterococci.

Acknowledgements

This research was supported by grant from Medical University of Lodz (No 503/3-012-03/503-01).

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