Polish Journal of Microbiology
2006, Vol. 55, No 3, 195–202

Iron Supply of Enterococci by 2-oxoacids and Hydroxyacids

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Received 28 December 2005, revised 9 May 2006, accepted 19 May 2006

Abstract

Only 9 (11.2%) out of 80 studied bacterial strains were able to utilize iron saturated 2-oxo acids and hydroxyacids and grow on o-phenantroline containing media. These strains belonged to *Enterococcus faecalis* and *Enterococcus faecium* species and were isolated from clinical material. Iron sources utilized by all of these strains were Fe(III) complexes with pyruvic, 2-oxobutyric, 4-methylthio-2-oxobutyric, 2-oxo-3-methylvaleric, 2-oxoisocaproic and 2-oxoadipic acids. None of the nine strains released 2-oxoacids to environment during growth in iron excess Fe^{+} medium and iron deficient – Fe^{-} (Chelex) medium. In Fe^{-} (phenantroline) medium, when the growth was strongly inhibited, only pyruvic acid was released. Iron uptake from ^{59}Fe(III)-pyruvate was depended on iron deficiency during growth: cells harvested from Fe^{-} (phenantroline) medium have acquired the most amount of iron. 2,4-Dinitrofenol was a strong inhibitor of ^{59}Fe(III) iron uptake. Release of pyruvic acid is not subject to negative derepression and does not require the presense of iron as its inductor. It appears in the environment as a response to growth inhibiting stress caused by the iron deficiency but contrary to siderophores are not specially synthesized for iron assimilation. Therefore, it is only primary metabolism products released by damaged, but metabolic active cells.

Key words: *Enterococcus* spp., 2-oxoacids, siderophores, iron acquisition

Introduction

The free iron concentration – 10^{-18} M in human body is not sufficient to support bacterial growth (Ratledge and Dover, 2000). Therefore they need specific mechanisms for the acquisition of iron from their host. At least four different mechanisms by which bacteria acquire iron from its carriers have been proposed: the proteolytic cleavage of carrier resulting in the disruption of the iron-binding sites, reduction of bound Fe (III) to Fe(II) and consequent release of Fe(II), through a direct surface interaction between carrier and bacterial receptor and secretion of low molecular mass iron chelating agents – siderophores (Williams and Griffiths, 1992).

Growth promotion and iron transport studies revealed that certain primary metabolites – 2-oxoacids and hydroxyacids may serve as bacterial siderophores. The first evidence came from studies of *Proteus mirabilis* (Evanylo *et al*.,1984) and later the other *Proteus* species, related genera as *Providencia* and *Morganella* (Drechsel *et al*., 1993) and from *E. coli* (Reissbrodt *et al*., 1993), *Pasteurella hemolytica* and *P. multocida* (Reissbrodt *et al*., 1994), staphylococci and micrococci (Heuck *et al*., 1995). Moreoever, strains of *P. hemolytica* and *P. multocida* that were unable to synthesize classical siderophores produced large amounts of pyruvic acid (Reissbrodt *et al*., 1994) while some producing siderophore coagulase-negative staphylococci excreted 2-oxo acids in high concentration under iron-restricted conditions (Lisiecki *et al.*, 1994; Heuck *et al*., 1995).

Enterococci have produced siderophore belonging to hydroxamate chelator class (Lisiecki and Mikucki, 1999). Growth promotion test has indicated that Fe(III)-dicitrate complex stimulated growth of enterococci (Lisiecki and Mikucki, 2004). This paper reports investigations on 2-oxoacids and hydroxyacids functioning as siderophores in *Enterococcus* genus.

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Experimental

Materials and Methods

Bacterial strains. Eighty strains of *Enterococcus* genus were used in the study. Forty nine of them originated from the departmental collection, and others from the National Institute of Public Health in Warsaw, Czech National Collection of Type Culture (CNCTC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and American Type Culture Collection (ATTC). Enterococci from departmental collection, were identified with API-STREP system (bioMerieux).

Growth media. Strains were grown in the medium composed of (per litre): 3 g Casamino Acids Vitamin Free (Difco); 3 g Yeast Extract (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 (Sigma). pH of medium was adjusted to 7.2. Concentration of iron was reduced either by using polyaminocarboxylate resin Chelex 100 (200–400 mesh, BioRad) to approximately 3.5×10^{-7} M or by adding o-phenantroline at concentration corresponding to MIC or 50% MIC of the investigated strain. Solid medium contained 2% Agar Nº 1 (Oxoid).

Suspensions density and viable count determination. The optical density of suspension and cultures was measured in UV/VIS Cary 1 (Varian) spectrophotometer at the 580 nm and viable count was estimated by using serial dilutions in buffered 0.155 M NaCl, pH 7.2 and standard plate methods on 4% Trypticase Soy Agar (Difco).

Growth conditions. Strains were initially iron-starved in Fe^- (Chelex) medium for 18 hours at 37°C with constant shaking. The starved suspensions were used to inoculate (5% v/v) media with different iron content: Fe^+ with 100 μM iron in the form of $FeSO_4 \times 7H_2O$, Fe^- (Chelex) subject to Chelex resin and Fe^- (phenantroline) containing o-phenantroline at concentration corresponding to 50% MIC of tested strains. Media were incubated at 37°C for 24 hours under constant shaking. Cultures were centrifuged (9500 × g, 15 min, 4°C) then the supernatant was filtered through the membrane filter (0.22 μm, Millipore).

2-oxo acids and hydroxyacids used. The following chemicals were used: pyruvic acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, 3-indolylopyruvic acid, oxaloacetic acid, mesoxalic acid, 2-oxobutyric acid, 4-methylthio-2-oxobutyric acid, 2-oxoglutaric acid, 2-oxovaleric acid, 2-oxoisovaleric acid, 5-aminolevulinic acid, 2-oxo-3-methylvaleric acid, 2-oxoisocaproic acid, 2-hydroxyisocaproic acid, 2-oxoadipic acid (Sigma). Complexes of chelators with iron Fe(III) were prepared according to Dreschel *et al.* method (1993) to achieve the ratio 1:30 of iron to ligand.

Control iron solutions and siderophores. The following control iron solutions were used: ferric sulfate, ferric ammonium sulfate and ferric ammonium citrate (Sigma). Complexes of siderophores: rhodotorulic acid (Sigma), 2,3-dihydroxybenzoic acid (Sigma) and desferrioxoamine B (Ciba-Geigy) with iron were prepared according to Dreschel *et al.* method (1993) to achieve the ratio 1:1of iron to ligand.

Determination of MIC for o-phenantroline. MIC for o-phenantroline was determined by agar dilution method on Mueller-Hinton 2 medium (bioMerieux) using twofold serial dilution of o-phenantroline at a concentrations ranging from 10 μg mL^{-1} to 130 μg mL^{-1} in accordance with CLSI standards (Clinical Laboratory Standards Institute, 2005).

Growth promotion tests. 0.1 mL of standardized suspension of the iron-starved strain ($A_{580nm} = 0.1$) was added to 20 mL of melted agar medium with o-phenantroline at concentration corresponding to MIC for tested strains and then poured on plastic Petri dishes (Ø14 cm, Nunc). After agar setting and 24 h storage of plates at 4°C, tissue paper discs (Whatman Nº 3, Ø 6 mm) were placed on their surface and saturated either with 5 μl of iron complex with 2-oxo acid and hydroxyacids (25 μg iron chelator per disc) or iron complexes of siderophores (25 μg iron chelator per disc), control iron solution (25 μg iron per disc) was also used. Growth around the discs demonstrating the ability of the tested strain to utilize iron sources was estimated after 24 and 48 h of incubation at 37°C.

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

Determination of 2-oxoacids by high-performance liquid chromatography (HPLC). The assay of 2-oxoacids in supernatants of cultures was performed with HPLC of their 2-quinoxalinole derivatives according to the Hayashi *et al.* methods (1982). Chromatographic separation was carried out using liquid chromatograph (Varian) with UV/VIS Varian 9050 detector and Li-Chrosorb RP-8 column (250 × 4 mm, 5 μm, Supelco). Pyruvic acid, oxaloacetic acid, 2-oxobutyric acid, 4-methylthio-2-oxobutyric acid, 2-oxoglutaric acid, 2-oxovaleric acid, 2-oxo-3-methylvaleric acid, 2-oxoisocaproic acid and 2-oxoadipic acid at 160 nmol mL^{-1} concentration were used as standards. 2-Oxocaprylic acid (120 nmol mL^{-1}, Sigma) was used as an internal standard. Detection of investigated substances was done at the wavelength of 340 nm.

Time-depended uptake of ^{59}Fe (III) iron. The tested strains were grown overnight at 37°C in a Fe^+, Fe^- (Chelex) and Fe^- (phenantroline) medium. The culture was centrifuged (9500 × g, 15 min, 4°C), cells were washed three times with cold buffered 0.155 M NaCl, pH 7.2 and then suspended in Fe^- (Chelex) medium up to the optical density of $OD_{580} = 0.5$. The assay sample contained 5 mL of bacterial suspension, 2 μM of iron ^{59}Fe (specific activity 0.5 Bq μmol^{-1})(NEN) and 40 μM of 2-oxoacid ligand (the ratio of iron to ligand was 1:20) (Dreschel *et al*., 1993). The complex ^{59}Fe-ligand was added at t_o time, and consecutive 0.5 mL of samples were taken after 5, 10, 15 and 20 minutes and filtered (0.22 μm, Millipore). Cells harvested on filter were washed with 2 ml of 0.155 M NaCl solution. Their radioactivity was measured in Wallac 1470 Wizard gamma counter. The results were presented as nanomoles of iron uptake per mg of bacteria dry weight.

Preparation of cells lysate. Protoplast of enterococci was prepared according to the method of Zorzi *et al.* (1996) and were lysed as described by Lindberg (1981). Total protein concentration in lysates was determined by the method of Lowry (1951).

Enzyme assay. Deamination of L-alanine by cell lysates was assayed spectrophotometrically at 340 nm with L-alanine (Sigma) and NAD (Sigma) as substrates according to Bergmeyer (1985) method.

Statistical analysis. Statistical analysis was performed with the Statistica PL computer programme (StatSoft). Statistical significance was defined as $p < 0.05$.

Results

All strains utilized iron from its control sources in form of Fe (II) and Fe (III) salts and were able to grow in media containing o-phenantroline. The minimal inhibitory concentration of o-phenantrolin (MIC) values for investigated strains were varying within a range of 30–120 μg mL^{-1} (Table I). Therefore, these strains

Table I
Growth promoting activities of ferric complexes of 2-oxo and hydroxyacids

Bacterial strains	MIC o-phenantroline (μg mL^{-1})	Ferric complexes of 2-oxo and hydroxyacids															
		Pyruvic acid	Phenylpyruvic acid	4-hydroxyphenyl-pyruvic acid	4-indolylpyruvic acid	Oxaloacetic acid	Mesoxalic acid	2-oxobutyric acid	4-methylthio-2-oxobutyric acid	2-oxoglutaric acid	2-oxovaleric acid	2-oxoisovaleric acid	DL-2-oxo-3-methyl-valeric acid	5-aminolevulenic acid	2-oxoisocaproic acid	2-hydroxyisocaproic acid	2-oxoadipic acid
E. faecalis BD 122	100	++	(+)	–	–	+++	(+)	++	(+)	(+)	(+)	(+)	++	++	++	–	++
E. faecalis BD 123	70	+	–	–	++	++	(+)	++	++	(+)	++	–	++	+	+	+	+
E. faecalis BD 160	30	+	–	–	–	++	–	+	+	–	(+)	–	+	+	+	(+)	++
E. faecalis 449	70	++	–	–	+	++	(+)	++	+	+	+	–	+++	+	++	+	++
E. faecalis 605	110	++	–	–	–	–	–	(+)	++	–	++	–	++	–	(+)	–	(+)
E. faecium BY 1	120	++	–	–	–	+	(+)	++	++	(+)	–	–	++	+	+	–	++
E. faecium BY 6	70	++	+	(+)	++	+	+	+	++	+	++	(+)	++	+	+	++	+
E. faecium BY 13	115	+	(+)	(+)	–	+	(+)	+	+	+	+	(+)	+	+	+	+	+
E. faecium BY 49	115	+	(+)	–	(+)	+	–	+	(+)	–	(+)	–	+	+	+	–	+

–, no growth; (+), poor growth; +, growth zone 10–15 mm; ++, growth zone 16–20 mm; +++, growth zone 21–30 mm.

absolutely required iron in form of ferric and ferrous salts for the growth. The best growth was obtained for the organic Fe(III) salt – ferric ammonium citrate. In 63.3% of studied strains the growth zone diameter was larger than 20 mm. The smallest growth stimulating effect was observed for inorganic Fe(II) – ferric ammonium sulfate because in more than 98% of strains the growth zone diameter was at the range from 10 to 15 mm.

Ferric complexes with 2-oxo acids and hydroxyacids were utilized by only 9 (11.2%) out of 80 investigated enterococcal strains which growth was stimulated in o-phenantroline medium (Table I). These strains belong to *E. faecalis* and *E. faecium* species and were isolated from clinical material. Only *E. faecium* BY 6 strain utilized Fe(III) of all iron complexes. *E. faecalis* 605 used the smallest number of investigated iron sources and its growth was stimulated by pyruvic, 2-oxobutyric, 2-oxo-3-methylvaleric, 2-oxo-isocaproic, 2-oxoadipic, 2-oxovaleric and 4-methylthio-2-oxobutyric acids only (Table I). Iron Fe(III) sources used by all strains were complexes with pyruvic, 2-oxobutyric, 4-methylthio-2-oxobutyric, 2-oxo-3-methylvaleric, 2-oxoisocaproic and 2-oxoadipic acids (Table I). Strong growth stimulating effect (indicated as +++ or ++) of all or almost all strains was observed for complexes with pyruvic, oxaloacetic, 2-oxobutyric, 4-methylthio-2-oxobutyric, 2-oxo-3-methylvaleric and 2-oxoadipic acids. Phenylpyruvic, 4-hydroxyphenyl-pyruvic, 2-oxomalonic, 2-oxoglutaric and 2-oxoisovaleric acids were only weakly stimulating growth complexes (indicated as + or –) utilized only by few investigated strains (Table I).

Strains that used iron Fe(III) from its complexes with pyruvic acid were not always able to acquire it from its derivatives – phenylpyruvic acid (*E. faecalis* BD 123, BD 160, 449, 605 and *E. faecium* BY 1), 4-hydroxyphenylpyruvic acid (all *E. faecalis* strains and *E. faecium* BY 1, BY 49) and 3-indolylpyruvic acid (*E. faecalis* BD 122, BD 160, 605 strains and *E. faecium* BY 1, BY 49). Oxaloacetic and 2-oxoglutaric acids, intermediate metabolites of tricarboxylic acids cycle, differently stimulated growth of enterococci. Iron Fe(III) from oxaloacetic acid complex was used by all, except one (*E. faecalis* 605) investigated strains and had a stronger stimulating effect on *E. faecalis* growth (indicated as +++ and ++). 2-Oxoglutaric acid a weakly (indicated as + and (+)) promoted the growth of six strains except for *E. faecalis* BD 160, 605 and *E. faecium* BY 49. Iron Fe(III) complexes with branched 2-oxoacid: 2-oxo-3-methylvaleric and 2-oxo-isocaproic acids stimulated the growth of all strains. 2-Oxo-3-methylvaleric acid had the strongest effect (indicated as +++ and ++) while 2-oxoisovaleric acid was a very weak growth stimulator of only three strains. Iron Fe(III) complexes with 2-oxoadipic and 5-aminolevulinic acids were utilized by all examined strains, however 2-oxoadipic acid had a stronger growth stimulating effect (indicated as ++) (Table I) .

Table II
Growth promoting activities of ferric complexes of siderophores

Iron saturated siderophores	Bacterial strains								
	E. faecalis BD 122	*E. faecalis* BD 123	*E. faecalis* BD 160	*E. faecalis* 449	*E. faecalis* 605	*E. faecium* BY 1	*E. faecium* BY 6	*E. faecium* BY 13	*E. faecium* BY 49
	MIC o-phenantroline (μg mL^{-1})								
	100	70	30	70	110	120	70	115	115
Ferrioxamine B	+	+	+	+	+	(+)	+	+	(+)
Rhodotorulic acid	+	+	+	++	++	+	+	+	(+)
2,3-dihydroxybenzoic acid	+	+	+++	++	++	+	+	++	+

–, no growth; (+), poor growth; +, growth zone 10–15 mm; ++, growth zone 16–20 mm; +++, growth zone 21–30 mm.

Investigated strains were also able to utilize iron from its complexes with hydroxamate siderophores – ferrioxamine B and rhodotorulic acid, and catecholate siderophore – 2,3-dihydroxybenzoic acid. The lowest stimulated growth was observed for ferrioxamine B (Table II).

None of the nine strains released 2-oxoacids to the environment during growth on Fe^{+} medium with the iron excess and in Fe^{-} (Chelex) medium with the iron deficiency. Only the culture in Fe^{-} (phenantroline) medium containing chelator at 50% of minimal inhibitory concentration (MIC), with the high iron deficiency released 2-oxoacids (pyruvic acid) into the environment (Table III, Fig. 1.). Strains of *E. faecalis* released its low amounts: from 0.05 (BD 123 and 449 strains) to 0.21 nmol 10^6cfu^{-1} (strain 605), an average 0.1 nmol 10^6cfu^{-1}. *E. faecium* strains released almost 60-times more of this metabolite: from 0.66 (strain BY 49) to 14.61 (strain BY 49) nmol 10^6 cfu^{-1}, an average 5.9 nmol 10^6 cfu^{-1}. On the other hand data calculated per mL of culture supernatants showed that *E. faecalis* strains released more of pyruvic acid: from 4.36 (strain 449) to 27.78 (strain 605) nmol mL^{-1} (an average 11.58 nmol mL^{-1}). *E. faecium* strains released less of this metabolite: from 2.67 (strain BY 1) to 5.26 (strain BY 6) nmol mL^{-1}, (an average 3.79 nmol mL^{-1}). The differences in the amount of released pyruvic acid to the Fe^{-} (phenantroline) medium by *E. faecalis* and *E. faecium* strains were statistically significant ($p<0.05$). The ability of L-alanine deamination to pyruvic acid could not be detected in none of the enterococcal strains. The growth of all strains in Fe^{-} (phenantroline) medium was inhibited, less in *E. faecalis* and much more in *E. faecium* strains. In comparison with cultures in Fe^{+} medium, for *E. faecalis* strains it ranged from 39.1% (strain BD 122) to 80.9% (strain 449) of the number of the cfu mL^{-1} and for *E. faecium* strains from 7.7% (BY 1) to 33.7% (BY 49) of the cfu mL^{-1}.

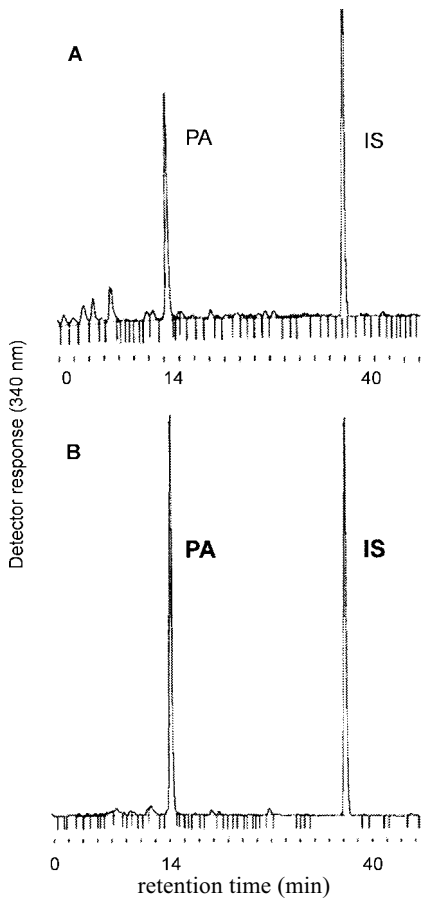

Fig. 1. HPLC profiles of 2-quinoxalinol derivatives of 2-oxoacids in culture supernatant of *E. faecalis* BD 123 strains (A) and standard pyruvic acid (B). PA, pyruvic acid; IS, internal standard (2-oxocaprylic acid)

In the studies on 2-oxoacids participation in iron Fe(III) assimilation by enterococci and elucidation of the process kinetic ^{59}Fe(III) – pyruvic complex and resting cells of three strains: *E. faecalis* 449, *E. faecium* BY 1 and BY 49 were used. The cells were harvested from cultures with different iron concentration: Fe^{+}, iron excess and iron deficiency of various degrees Fe^{-} (Chelex) and Fe^{-} (phenantroline). The crucial differences between these strains were the amounts of pyruvic acid released per 10^6cfu^{-1} during the growth in Fe^{-} (phenantroline) medium: *E. faecalis* 449 has released 4.63 nmol mL^{-1} and 0.05 nmol 10^6cfu^{-1} of pyruvic acid, *E. faecium* BY 1 – 2.67 nmol mL^{-1} and 4.23 nmol 10^6cfu^{-1} and *E. feacium* BY 49 – 2.69 nmol mL^{-1} and 0.66 nmol 10^6cfu^{-1}.

All strains harvested from Fe^{+} medium assimilated isotope ^{59}Fe(III). *E. faecalis* 449 strain took more quickly greater amounts

Table III
Growth and pyruvic acids release into medium

Bacterial strains	Fe^+ medium		Fe^- medium				
			Fe^- (Chelex 100) medium		Fe^- (phenantroline) medium**		
	Growth* cfu mL^{-1} 24 h	Pyruvic acid nmol 10^6 cfu^{-1}	Growth cfu mL^{-1} 24 h	Pyruvic acid nmol 10^6 cfu^{-1}	Growth cfu mL^{-1} 24 h	Pyruvic acid nmol mL^{-1}	Pyruvic acid nmol 10^6 cfu $^{-1}$
E. faecalis BD 122	156×10^6	0	87×10^6	0	61×10^6	6.81	0.12
E. faecalis BD 123	545×10^6	0	540×10^6	0	266×10^6	13.43	0.05
E. faecalis BD 160	78×10^6	0	51×10^6	0	71×10^6	5.22	0.07
E. faecalis 449	126×10^6	0	126×10^6	0	102×10^6	4.36	0.05
E. faecalis 605	233×10^6	0	173×10^6	0	134×10^6	27.78	0.21
E. faecium BY 1	8.2×10^6	0	7.40×10^6	0	0.63×10^6	2.67	4.23
E. faecium BY 6	4.1×10^6	0	4.43×10^6	0	0.36×10^6	5.26	14.61
E. faecium BY 13	3.68×10^6	0	1.96×10^6	0	0.77×10^6	2.88	3.74
E. faecium BY 49	19.6×10^6	0	18.40×10^6	0	6.60×10^6	2.69	0.66

* cfu – colony forming units; ** contained 50% of MIC o-phenantroline

of iron than *E. faecium* BY 49 and BY 1: after 30 minutes cells contained 13.29, 3.2 and 2.18 nmol ^{59}Fe(III) per mg of dry weight of bacteria, respectively (28.3%, 7.3% and 4.6% of initial isotope dose) (Fig. 2, 3, 4). Bacteria harvested from Fe^- (Chelex) medium with lower level of iron deficiency, did not uptake ^{59}Fe(III) more actively. *E. faecalis* 449 strain harvested from Fe^+ medium utilized 13.29 nmol ^{59}Fe(III) (28.3 % of initial isotope dose), and from Fe^- (Chelex) medium – 8.82 nmol ^{59}Fe(III) (18.8 % of initial isotope dose). Small and not increasing with time amounts of isotope taken by *E. faecium* BY 1 and BY 49 growing in Fe^- (Chelex) were 2–3 times lower than those assimilated by cells derived from the Fe^+ medium (Fig. 3, 4).

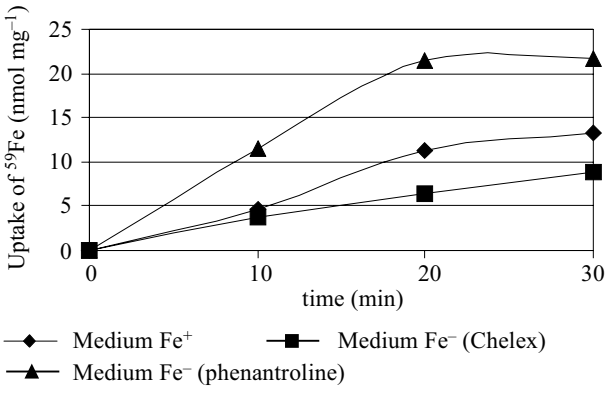

Fig. 2. Iron uptake by *E. faecalis* 449 from ^{59}Fe(III)-pyruvate

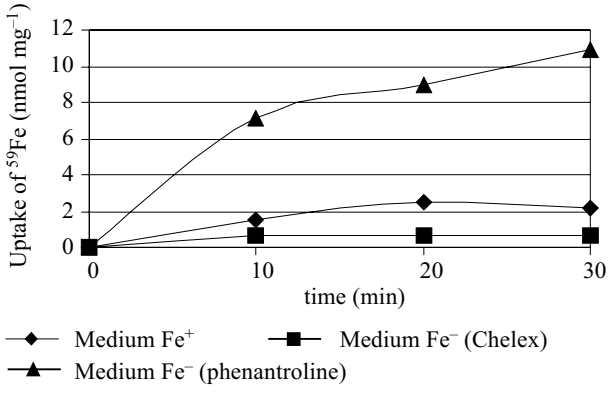

Fig. 3. Iron uptake by *E. faecium* BY 1 from ^{59}Fe(III)-pyruvate

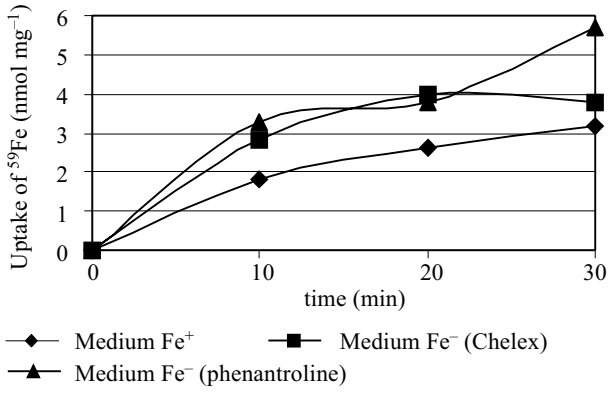

Fig. 4. Iron uptake by *E. faecium* BY 49 from ^{59}Fe(III)-pyruvate

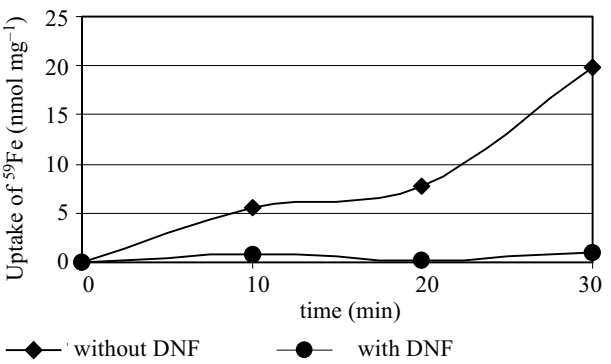

Fig. 5. Iron uptake by *E. faecalis* 449 from ^{59}Fe(III)-pyruvate in the presence of 2,4-dinitrophenol (DNF). Cells were harvested from Fe^- (phenantroline) medium.

High iron deficiency in cultures in Fe^{-} (phenantroline) medium caused more active uptake of iron $^{59}Fe(III)$ by all strains. After 30 minutes *E. faecalis* 449 strain harvested from Fe^{+} medium, used 13.29 nmol $^{59}Fe(III)$ (28.3 % of initial isotope dose) per mg of bacteria dry weight, while from Fe^{-} (phenantroline) medium – 21.62 nmol $^{59}Fe(III)$ (46.1% of initial isotope dose) (Fig. 2). *E. faecium* BY 1 utilized 2.18 nmol (4.6 % of initial isotope dose) and 10.87 nmol (5.1 % of initial isotope dose) $^{59}Fe(III)$ per mg of dry weight of bacteria (Fig. 3). *E. faecium* BY 49 strain harvested from Fe^{+} medium utilized 3.2 nmol (7.3 % of initial isotope dose) and from Fe^{-} (phenantroline) medium 5.7 nmol (13.1 % of initial isotope dose) (Fig. 4).

The influence of 2,4-dinitrophenol on the process of iron assimilation was tested using *E. faecalis* 449 strain cultured in Fe^{-} (phenantroline) medium and actively uptaking iron from its $^{59}Fe(III)$-pyruvic acid complex. This compound was a strong inhibitor of iron $^{59}Fe(III)$ uptake. After 30 minute, in the presence of 2,4-dinitrophenol the cells were taking up 1.08 nmol $^{59}Fe(III)$ (2.36 % of initial isotope dose) while those in absence of this compound – 19.9 nmol $^{59}Fe(III)$ (42.4 % of initial isotope dose) per mg of dry weight of bacteria (Fig. 5).

Discussion

Only 9 (11.2%) out of 80 studied bacterial strains utilized iron Fe(III) complexes with 2-oxo and hydroxyacids. These strains belong to two species – *E. faecalis* and *E. faecium* isolated from clinical material.

The comparison of the iron sources utilization in the form of salts and complexes by enterococci, revealed that the iron salts are the best assimilated sources, subsequently followed by siderophores and 2-oxo and hydroxyacid complexes. Among the last two iron 2-oxoacid complexes were more often used. Fe(III) complexes with pyruvic acid and 2-oxo-3-methylvaleric acid stimulated the growth most effectively.

The structure of 2-oxo or hydroxyacid, particularly its side chain, can influence the transport of iron Fe(III) complex into the cell and growth stimulation. So far only bacteria from the genera *Staphylococcus, Micrococcus* (Heuck *et al.*, 1995), *Proteus, Providencia* and *Morganella* (Drechsel *et al*., 1993) have been tested for this ability.

Aromatic side chain of phenylpyruvic and 2-hydroxyphenylpyruvic acids and heteroaromatic chain of indolylpyruvic acid decreased the growth stimulation of enterococci, as compared to pyruvic acid. For *S. epidermidis* phenylpyruvic acid was, similarly to pyruvic acid, the effective iron Fe(III) carrier (Heuck *et al.*, 1995). Genera *Proteus*, *Providencia and Morganella* utilized iron Fe(III) from phenylpyruvic and indolylpyruvic acids complexes only which stimulated growth the most effectively. Lipophility of side chains of these chelators did not influence the assimilation of iron Fe(III) by enterococci.

Most iron Fe(III) complexes with 2-oxoacids with an elongated non-polar side chain actively stimulated growth of enterococci. Only 2-oxoisovaleric and 2-hydroxyisocaproic acids were very weak iron carriers but not for all strains. 2-Oxoisovaleric acid is valine precursor, 2-oxoisocaproic acid – leucine and 2-oxo-3-methylvaleric acid – isoleucine. The source of these 2-oxoacids in enterococci is the reversible transamination of mentioned aminoacids with 2-oxoglutaric acid and the own cytoplasmic membrane. They may also be taken up from the environment (Chesbro and Evans, 1962). *S. epidermidis* uses only some 2-oxoacids from this group as iron carriers: 2-oxoisocaproic, 2-oxoisovaleric, 2-oxo-3-methylvaleric and 2-hydroxyisovaleric acid (Heuck *et al.*, 1995). The ability of valine and leucine deamination and corresponding 2-oxoacids release in the genera of *Proteus, Providencia* and *Morganella*, results in active utilization of these chelators as iron carriers (Drechsel *et al*., 1993).

The length of the carrier carbon chain in enterococci had no effect on the growth stimulation. Iron Fe(III) complexes with short 2-oxoacids with polar side chains – pyruvic (C_3) or oxaloacetic (C_4) acids equally actively stimulated growth as complexes with C_5 or C_6 oxoacids – 2-oxovaleric, 2-oxo-3-methylvaleric and 2-oxoisocaproic acids. Similarly, such complexes influenced the growth of *S. epidermidis* (Heuck *et al.*, 1995). Genera *Proteus*, *Providencia and Morganella* either did not use these complexes as iron carriers or their growth stimulation was insignificant (Drechsel *et al*., 1993).

However, lipophility does not always facilitate assimilation of iron Fe(III) complexes. Only hydrophobic 2-oxoacids with an elongated non-polar side chain were excellent (with some exceptions) iron Fe(III) carriers for enterococci, *S. epidermidis* (Heuck *et al.*, 1995) and bacteria from the genera *Proteus*, *Providencia* and *Morganella* (Drechsel *et al*., 1993). Although pyruvic acid, the most active Fe(III) carrier in enterococci and *S. epidermidis*, did not stimulate the growth of bacteria from the genus *Proteus*, *Providencia* and *Morganella*, its aromatic and heteroaromatic derivatives having practically no influence on enterococci, well stimulated growth of mentioned bacteria (Heuck *et al*., 1995; Drechsel *et al*., 1993).

The attempts to detect the ability of alanine deamination in enterococci, which leads to the formation of pyruvic acid, the most effective iron Fe(III) carrier, failed. Enterococci do not deaminate aminoacids, only arginine can be deaminated as an energetic substrate of these bacteria (Holt *et al.*, 1986). Genera *Escherichia* and *Salmonella* are also unable to deaminate aminoacids. In consequence, 2-oxoacids are not utilized as iron Fe(III) carriers in its transport to the cell (Reissbrodt *et al.*, 1997). The analogous phenomenon is thus likely to occur in enterococci as the uses 2-oxo and hydroxyacids in iron transport by only 9 of 80 studied strains cannot be a common mechanism of iron uptake. Instead of it hydroxamate siderophores synthesis was found in all eighty examined strains (Lisiecki and Mikucki, 1999).

The decrease of iron concentration in a Fe^- (Chelex) medium to approximately 10^{-6}–10^{-7} M, commonly known as the inducing derepression of siderophores synthesis, did not caused the release of pyruvic acid or any 2-oxo acids into the medium by the enterococci. In the similar conditions *Salmonella* Typhimurium secreted not only pyruvic acid at concentration 5 μmol mL^{-1} but also 2-oxoglutaric, 2-oxoisovaleric and 2-oxoisocaproic acids (Reissbrodt *et al.*, 1997). The increasing iron stress in a Fe^- (phenantroline) medium significantly reduced the growth of *E. faecalis* and inhibited it strongly in *E. faecium*, made the pyruvic acid appear in the supernatant of cultures. Similarly, in *S.* Typhimurium the release of 2-oxoacids was increasing, for pyruvic acid to 5 μmol mL^{-1} when limited iron availability inhibited the growth of bacteria (Reissbrodt *et al.*, 1997). Coagulase-negative staphylococci released a lot of 2-oxoacids (*S. cohnii* 80 nmol mL^{-1} of pyruvic acids), when their growth was inhibited by the presence of iron chelator (Heuck *et al.*, 1995). In comparison to these data the enterococci released more less amounts of pyruvic acid from 2.67 nmol mL^{-1} to 27.78 nmol mL^{-1}. The synthesis and secretion of siderohpores occur only during the bacterial growth as a result of an inductive signal while 2-oxoacids are products of primary metabolism and are similar to citric acid which promotes growth of *E. coli* (Ratledge and Dover, 2000; Silver and Walderhaug, 1992; Waggeg and Braun, 1981) and enterococci (Lisiecki and Mikucki, 2004). The increasing iron unavailability strongly reduced the growth of enterococci and the amount of released pyruvic acid was rising. *E. faecalis* cultures released greater amounts of pyruvic per mL of culture because it contained more cells releasing this metabolite. On the other hand, per number of cfu, *E. faecium* strains, strongly inhibited during growth, released more amount of pyruvic acid proving the main role of growth inhibition in this phenomenon. These relationships do not support the idea that pyruvic acid release is a process induced by the low iron availability which triggers chelator synthesis derepression or more active primary metabolite release. Pyruvic acid appears after 24 h in Fe^- (phenantroline) medium during growth inhibition and cells cover could be less tight (*E. faecalis*) or/and metabolic activity was continued even with growth inhibition (*E. faecium*). Therefore, pyruvic acid, in enterococci iron uptake system could be a primary metabolite secreted by metabolically active cells whose growth was strongly inhibited.

The ability of iron uptake from ^{59}Fe(III)-pyruvic acid complex by resting cells of three strains of *E. faecalis* and *E. faecium* has supported the results of biotests showing that this complex actively stimulated growth of enterococci. Growth promotion then depended on the iron uptake. The activity of assimilation depended, as in *S.* Typhimurium (Reissbrodt *et al.*, 1997), on the level of iron reserves in the cell. Enterococci cultivated in medium containing o-phenantroline, a strong cell penetrating chelator and probably with the lowest iron reserves, were the most active. The cells harvested from a Fe^- (Chelex) medium were not more active in iron uptaking than cells from a Fe^+ medium, which can be linked with intact endogenous iron reserves. It is necessary to discuss the contradiction between pyruvic acid releasing and iron acquisition from ^{59}Fe(III)-pyruvate complex by *E. faecalis* 449 and *E. faecium* BY 1 strains. In this two experiments the different bacterial model was used. The resting cells harvested from medium containing 50% MIC of o-phenantroline were used in iron uptake experiment and pyruvic acid was released by the cells growing in presence of 50% MIC of o-phenantroline. It seems that at such experimental differences simple relationship between pyruvic acid releasing and iron acquisition could not be expected. The comparison strains *E. faecium* 49 and BY 1 has supported this supposition. Both of them have released the same amounts of pyruvic acid – 2.67 and 2.69 nmol mL^{-1} and acquired different amount of iron from ^{59}Fe(III)-pyruvate complex, respectively – 10.87 nmol mL^{-1} and 5.7 nmol mL^{-1} dry weight of cells.

In conditions of limited iron availability, 2-oxo and hydroxyacids take part in iron delivery only in few enterococci. The pyruvic acid release is not subject to negative derepression and does not require the presense of iron as its inductor. It appears in the environment as a response to growth inhibiting stress caused by the iron deficiency but contrary to siderophores are not specially synthesized for iron assimilation. Therefore, it is only primary metabolism products released by damaged, but metabolic active cells.

Acknowledgments. This research was supported by the grant from Medical University at Łódź (No 502-13-224)

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