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Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* spp

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The production, detection and the effects of iron concentration on the siderophore production of *Staphylococcus* strains used as meat starter cultures were studied. Non-pathogenic *Staphylococcus* strains produce extracellular low molecular weight compounds which exhibited positive reactivity when measured by a universal detection method for siderophores. The production of siderophores was very closely associated with the iron concentration in the medium, and very low additions considerably reduced siderophore production. Although the production of siderophores was highly iron-dependent, the antimicrobial activity of spent medium from *Staphylococcus* cultures against selected yeasts and moulds remained considerable under high iron concentrations.

Keywords: *Staphylococcus*; siderophore; iron; starter cultures; fermented meat products; antagonism

Introduction

Iron is one of the essential elements for all microorganisms except lactobacilli [3] and plays an important role in membrane-bound electron transport chains and in cytoplasmic redox enzymes. Despite its natural abundance, iron (Fe^{3+}) under aerobic conditions is present as highly insoluble oxyhydroxides in neutral and alkaline environments. Most aerobic and facultatively anaerobic bacteria, moulds and fungi produce iron chelates (siderophores) to secure their iron availability. Siderophores are iron-specific, relatively low molecular weight compounds which are secreted under low iron stress and which capture iron from the environment. The siderophore–iron complex is transported into the microorganism and iron is released for microbial metabolism [10, 19, 23].

The biosynthesis and secretion of siderophores are strictly regulated by environmental factors, eg iron concentration, temperature and pH, of which iron concentration is the most important. Siderophores exhibit considerable structural variability and affinity for iron, which determines the growth of a microorganism under competitive conditions when iron availability is a limiting factor [23, 32].

Siderophores produced by soil *Pseudomonas* species have been widely studied as biocontrol agents [32]. Much less is known of the function and properties of siderophores produced by *Staphylococcus* spp. Pathogenic staphylococci produce siderophores and in some cases a direct correlation between virulence and siderophore production has been established [4, 10, 14]. The association between virulence of pathogens and their siderophore production has furthered the view that siderophores as iron catchers play an important role in plant and animal pathology. Two staphylococcal siderophores, staphyloferrin A and B, have been isolated and chemically characterized [8, 20]. Staphyloferrin A, a siderophore from *S. hyicus*, has been characterized as a trihydroxamate-type, highly hydrophilic, acid-labile compound composed of two citric acid molecules bound by D-ornithine [14, 20]. The structural components of staphyloferrin B are 2,3-diaminopropionic acid, citrate, ethylenediamine and 2-ketoglutaric acid [11]. In addition to staphyloferrin A and B, *Staphylococcus* spp probably also produce other uncharacterized siderophores [15]. Much less is known of the production and role of siderophores formed by the non-pathogenic *Staphylococcus* strains used as starters in fermented meat products together with lactic acid bacteria.

Iron level is critical for siderophore production and secretion in pathogenic *Staphylococcus* strains as well in other microbes. Iron also plays an important role in the antimicrobial activity of siderophore-producing microorganisms [22]. The aim of the present work was to determine the effects of iron on the production and secretion of siderophores and on the antagonistic properties of non-pathogenic *Staphylococcus* strains against moulds and yeasts.

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Materials and methods

Bacterial strains

Staphylococcus carnosus I (VTTE-94524), *Staphylococcus carnosus* II (VTTE-94525) and *Staphylococcus saprophyticus* (VTTE-82146) from the culture collection of VTT Biotechnology and Food Research were used in the experiments and were maintained as lyophilized cells. The *S. carnosus* strains originated from meat and *S. saprophyticus* from wort (cooked barley malt).

Culture medium and growth conditions for siderophore production

The *Staphylococcus* strains were grown in a semi-synthetic minimal medium [1, 4]. The buffer solution was prepared by dissolving 75.64 g of PIPES (Sigma, St Louis, MO, USA) in 939 ml of a salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl and 1.0 g NH_4Cl (Merck, Darmstadt, Germany).

The pH was adjusted to pH 6.8 and the solution was autoclaved. The mineral solution (in 70 ml) contained 493 mg $MgSO_4 \cdot 7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4 \cdot H_2O$, 1.4 mg H_3BO_4, 0.04 mg $CuSO_4 \cdot H_2O$, 1.2 mg $ZnSO_4 \cdot 7H_2O$ and 1.0 mg $Na_2MoO_4 \cdot H_2O$ (Merck). The mineral solution was autoclaved separately and from the 100× concentrate 10 ml L^{-1} was added to the buffer solution in addition to 30 ml filter-sterilized 10% (w:v) casamino acids (Difco, Detroit, MI, USA) and 20 ml 50% (w:v) glucose solution (BDH, Poole, Dorset, UK). The vitamin solution (in 1 L) contained 30 mg L-tryptophan (Sigma), 0.5 mg nicotinic acid (Merck), 0.5 mg Ca-pantothenate (Sigma), 0.5 mg thiamine (Merck) and 0.003 mg biotin (Sigma). The vitamin solution was prepared as a 1000× concentrate, filter-sterilized and 1 ml of this concentrate was added to 1 L of buffer solution along with 1 mM D-ornithine (Sigma). All glassware was acid-washed in 6 N HCl for 24 h.

The *Staphylococcus* strains were grown in flask cultures (25 ml/100 ml) with shaking (150–170 rpm) for three days at 37° C. The bacterial inocula were grown in deferrated minimal medium for 24 h at 37° C with shaking (150–170 rpm). This actively growing culture was used as an inoculum for experiments (250 μl/25 ml). The effects of temperature on growth and siderophore production were studied by growing *Staphylococcus* strains at 25, 30, 37 and 45° C for 16 h.

Deferration of the medium

The casamino acids and buffer solution contained 65.2 μM and 4.3 μM of iron respectively which could interfere with the production of *Staphylococcus* siderophores. The deferration of siderophore production medium was performed by Chelex-100 resin [7]. Casamino acids together with vitamin solution and D-ornithine were deferrated by 40 g Chelex-100 resin in a 2.2 × 8.0-cm column. The column was washed with 400 ml of 2 N HCl and rinsed until neutral. A solution of 20 g casamino acids in 100 ml distilled water was deferrated in the column and eluted with water until 186.6 ml of solution was collected.

The PIPES buffer solution was deferrated by 80 g Chelex-100 resin in a 2.2 × 16-cm column. The column was washed with 1 L of 2 N HCl and neutralized with 60 ml 1 N NaOH and 1 L distilled water. Nine hundred millilitres of buffer solution brought up to 1 L after deferration was slowly filtered through the column and a final volume of 940 ml collected. The pH of the buffer solution was adjusted to pH 6.8 before autoclaving it.

By this method about 58% of the iron in the buffer solution and about 81% in the casamino acids solution was removed. The iron content of the medium after deferration was 2.4–2.9 μM.

Total iron measurements

The deferrated growth medium was supplemented with various amounts (0, 0.1, 1, 5, 10 and 50 μM) of iron ($FeSO_4$). The total iron concentration in the medium was determined before inoculation by atomic absorption spectrometry.

Turbidity measurements

The cell density in cultures grown at different temperatures were determined 16 h after the beginning of the growth period by a Multiskan MCC microplate reader (Labsystems, Finland) at 630 nm. Simultaneously bacterial counts were performed on Plate Count agar (Difco) at 37° C for 24 h to normalize the siderophore production with the cell density. Changes in medium pH were monitored simultaneously.

Siderophore detection in culture supernatants

Siderophores were detected in supernatant medium from cultures according to the method of Alexander and Zuberer [1] by the CAS reagent (Fluka Chemicals, Buchs, Switzerland) from appropriate dilutions (MES buffer(2-[*N*-morpholino]ethanesulfonic acid, Sigma) after a 24-h incubation at room temperature using a Multiskan MCC microplate reader at 690 nm [25].

The production of siderophores was determined in the minimal medium supplemented with different concentrations of iron 0, 16, 24, 48 and 72 h after the beginning of the growth period. The cells were removed by centrifugation and the spent medium was filtered through an Ultrafree-MC filter (PLGC filter, NMWL (nominal molecular weight limit) 10 000, Millipore, Bedford, MA, USA) to remove the CAS assay-interfering components from the samples. The samples were collected, neutralized and stored at –25° C for subsequent CAS assay.

Testing of antimicrobial activity

An automated turbidometer, Bioscreen (Labsystems, Finland), was used for testing the antimicrobial activity of spent medium [30, 31] against selected moulds and yeasts. Moulds were induced to sporulate on yeast–malt extract medium (20 g L^{-1} malt extract, 1 g L^{-1} yeast extract, 15 g L^{-1} agar, Difco) at 25° C for 5–7 days. The inoculum used was calibrated to 10^6 spores ml^{-1} by counting the spores in a Thoma chamber (Baltimore, MD, USA) using a light microscope. The yeasts were grown in yeast–malt extract medium at 25° C for 1 day and the turbidity of the inoculum was calibrated to 0.1 at 630 nm. Sixty μl of the substance to be studied and 30 μl of the test organism in saline were dispensed to microtitre plate wells with 210 μl of the growth medium supplemented with varying amounts of iron (0–2 mM $FeSO_4$). In the control sample wells the antimicrobial agent was replaced by an equal volume of simultaneously treated minimal medium. The yeasts were incubated in King's Medium B broth (20 g L^{-1} protease peptone, 10 g L^{-1} glycerol, 0.3 g L^{-1} K_2HPO_4, 1.5 g L^{-1} $MgSO_4$, 1 g L^{-1} PIPES free acid, PIPES Na_2-salt, pH 6.8, Sigma) at 25° C for 48 h. The moulds were grown with gentle shaking in sucrose-L-asparagine (SA) broth [28] at 25° C for 72 h. All determinations were carried out with two or three replicates and results are expressed as average values. The area under the growth curve was used as a measure of microbial growth, and the per cent reduction in area was used to describe the inhibitory effects of the siderophores studied. The standard deviation between replicate samples ranged from 1 to 2%.

Results

Effects of temperature on growth and siderophore production

The optimum growth temperature for these *Staphylococcus* strains was 37° C although they also grew well at 30° C, while at 45° C growth was totally inhibited. The production of siderophores was associated with growth of the strains and was less at 25° C than at 37° C and 30° C although differences in the production level between strains were detected (Figure 1). *S. carnosus* II was the most efficient siderophore producer at 37° C. Interestingly *S. carnosus* I produced only low levels of siderophores both at 30° C and 37° C. *S. saprophyticus*, although it grew better at 37° C, produced significant levels of siderophores at 30° C.

Effect of iron concentration on siderophore production

The effects of iron concentration on the siderophore production of *S. carnosus* I, *S. carnosus* II and *S. saprophyticus* were studied in minimal medium supplemented with varying amounts of iron. The highest siderophore production was detected in the culture medium of *S. carnosus* I (Figure 2a) which also seemed to be less sensitive to the increase of iron concentration in the medium compared with the other *Staphylococcus* strains studied (Figure 2). The production of siderophores attained a high level 16 h after the beginning of the growth period and did not significantly increase during further incubation in *S. carnosus* strains (Figure 2a, b). The production of siderophores in *S. saprophyticus* increased during the incubation time (Figure 2c) although all the strains studied attained a stationary growth phase after 16 h despite the presence of various iron levels in the medium (data not shown). The cell density in cultures was 2.5–5.8 $\times 10^9$ CFU ml^{-1}. The pH of the minimal medium supplemented with varying amounts of iron dropped during the first 16 h of growth from pH 6.8 to pH 5.5–5.8, after which it gradually began to rise again (data not shown).

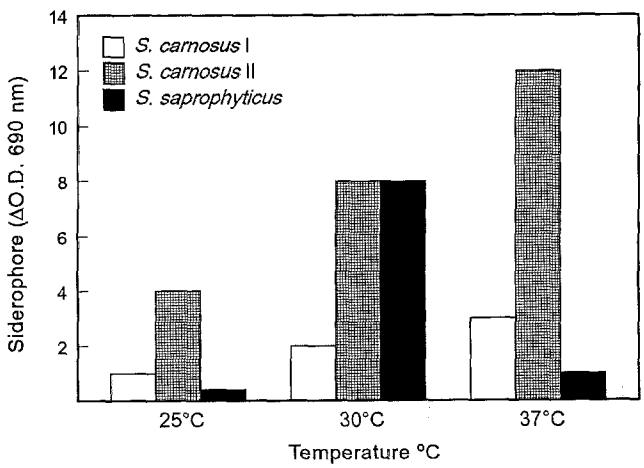

Figure 1 Effects of temperature on the siderophore production of *Staphylococcus* strains. Values normalized to 10^9 CFU ml^{-1}

Effects of iron on antimicrobial activity of Staphylococcus *siderophores*

The antimicrobial activity of the supernatant medium from cultures of *S. carnosus* I, *S. carnosus* II and *S. saprophyticus* was tested against selected yeasts and moulds (Table 1). The supernatant medium was ultrafiltered (PLGC filter, NMWL 10 000) for removal of CAS assay-interfering compounds and other antimicrobial proteins possibly produced by *Staphylococcus* strains. The siderophore-containing extracts inhibited the growth of *Fusarium* strains 12–34%, especially the *F. avenaceum* strain, which was inhibited 34% by *S. carnosus* II and *S. saprophyticus*. *Aspergillus* strains and yeasts were less sensitive to spent medium from *Staphylococcus* cultures (Table 1). However, the growth of *Rhodotorula glutinis* was inhibited 30% by these *Staphylococcus* strains (Table 2). The inhibition range of *S. carnosus* strains and *S. saprophyticus* were quite similar, although some differences existed in the extent of inhibition. The iron concentration of the medium played an important role in the antimicrobial activity of *Staphylococcus* siderophores. The growth inhibition of indicator moulds and yeasts was reversed by much higher concentrations of iron than was the production of *Staphylococcus* siderophores. In some cases a total iron concentration of 2 mM in the medium did not totally reverse the growth inhibition caused by *Staphylococcus* siderophore-containing extract.

Discussion

Research in the production of antimicrobial agents and microbial antagonisms is important because these agents can play a significant role in reducing the use of so-called 'hard' agents in various industrial fields: food processing and preservation, the paper and pulp and medical industries and plant pathology. Considerable research efforts have been devoted towards the study of lactic acid bacteria and their metabolites, but much less to the effects and production of other antibacterial factors.

The present paper focused on the production of siderophores in non-pathogenic *Staphylococcus* strains which have been studied to a much lesser degree than other siderophore producers. Siderophores produced by *Staphylococcus* strains used as starter cultures in fermented meat products have a GRAS (generally regarded as safe) status which opens new potential application possibilities for using these strains and their antimicrobial agents in biocontrol of microbes in a wide spectrum of industrial fields. The siderophore production of *Staphylococcus* strains was detected by the CAS assay [1, 29]. Phosphates interfere with the CAS assay, and in consequence simple growth media are recommended for use in studying siderophore production [22, 29]. Compounds other than phosphates also interfere with the CAS reaction and consequently give inaccurate results. This introduces new problems because many siderophore producers do not grow sufficiently in minimal growth media. In the present study the *Staphylococcus* strains were grown in a minimal medium from which the removal of antimicrobial proteins was necessary for studying the antagonistic activities of siderophores. The detection of siderophores by the CAS assay was possible after protein removal from the samples. Protein removal before

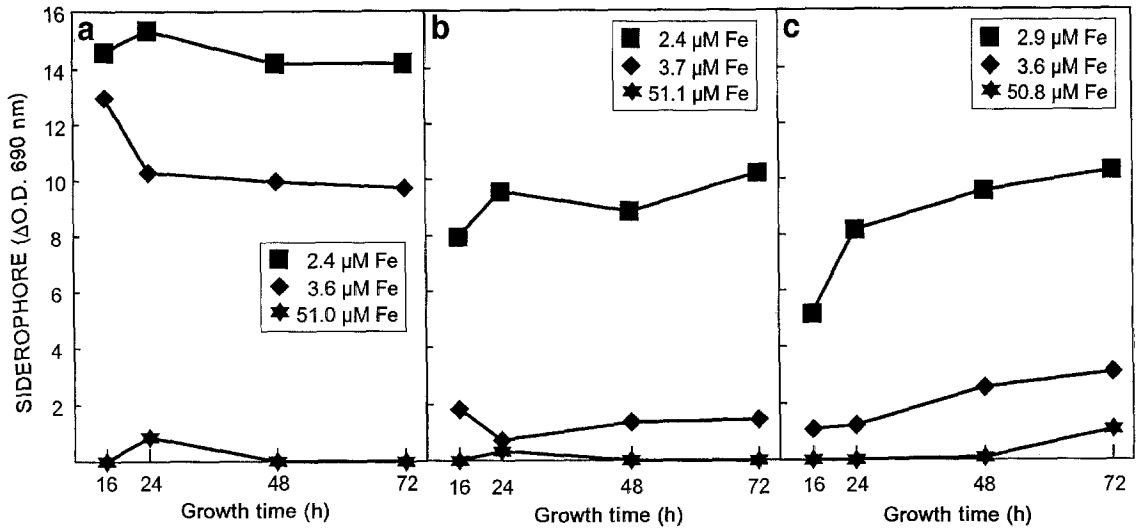

Figure 2 Effects of iron concentration in the growth medium on siderophore production of (a) *S. carnosus* I, (b) *S. carnosus* II and (c) *S. saprophyticus* during three-day growth time

Table 1 Indicator yeasts and moulds selected for antagonistic assays

Strain	Culture code	Origin/Cause	Effect of cell-free extracts
Saccharomyces cerevisiae	C-94**203**	ATCC 9763	No effect
Rhodotorula glutinis	C-920**11**	Malting	Growth inhibition
Cryptococcus albidus	C-920**12**	Malting	Growth stimulation
Pichia anomala	C-931**89**	Feed	No effect
Candida zeylanoides	C-931**90**	Feed	Growth stimulation No effect (*S. carnosus* II)
Fusarium avenaceum	D-801**47**	Barley/gushing	Growth inhibition
Fusarium culmorum	D-801**48**	Barley/gushing	Growth inhibition
Fusarium tricinctum	D-801**39**	Cereal	No effect
Aspergillus clavatus	D-760**36**	Gushing	No effect Growth stimulation (*S. saprophyticus*)
Aspergillus clavatus	D-832**16**	Malting/gushing	No effect Growth stimulation (*S. saprophyticus*)

the CAS assay was not necessary for *Pseudomonas* strains (results not shown) grown in a simple medium (SA medium, [28]).

The siderophore production of non-pathogenic *Staphylococcus* strains was greatly influenced by the iron level in the medium. Differences between the strains in their responses towards iron level were detected but only minor amounts of siderophores were produced at an added iron concentration of 50 μM. Interestingly, the two *S. carnosus* strains studied responded quite differently towards iron in the medium. *Staphylococcus* strains studied reached the stationary growth phase after 16 h growth. *S. carnosus* strains produced notable amounts of siderophores after they had reached the stationary phase, while *S. saprophyticus* produced low amounts of siderophores during the exponential growth phase (results not shown, in preparation). Iron regulates siderophore production in several microorganisms, eg *Pseudomonas*, *Rhizobium* and pathogenic *Staphylococcus* strains [5, 14, 20, 26, 27]. In *Staphylococcus hyicus* a concentration of iron less than 2 μM in the growth medium was needed for sufficient siderophore production. Higher concentrations of iron considerably decreased the production of siderophores [14, 20]. Iron is found in nature mostly as a constituent of insoluble oxyhydroxide polymers and in animals it is tightly bound to transferrin, lactoferrin and haeme. Thus although very low concentrations are needed for siderophore production, it should be kept in mind that iron is always tightly bound and must be actively acquired. Siderophore production is also affected by growth rate and the degree to which iron is a growth-limiting nutrient for the siderophore producer [9]. In addition to ferric iron, siderophores can bind manganese, copper and cobalt and high concentrations of these elements can interfere with siderophore production and acquisition of iron [10].

The role of iron on the antimicrobial activity of *Staphylococcus* siderophores was quite variable. At rather high levels (2 mM) antimicrobial activity could still be detected,

Table 2 Growth inhibition of *Fusarium avenaceum*, *F. culmorum* and *Rhodotorula glutinis* by *Staphylococcus* cell-free extracts supplemented with varying concentrations of iron

Siderophore cell-free extract	Fe (μM)	*Fusarium avenaceum*	*Fusarium culmorum*	*Rhodotorula glutinis*
S. carnosus I	0	21.3	9.6	29.2
VTTE-94524	100	21.5	16.9	35.5
	500	18.3	17.1	31.0
	1000	2.0	11.7	37.7
	2000	−13.6	−2.5	−4.3
S. carnosus II	0	32.2	17.4	9.5
VTTE-94525	100	34.7	23.7	29.8
	500	32.5	22.8	13.2
	1000	25.3	20.6	38.9
	2000	11.9	16.1	0.5
S. saprophyticus	0	31.5	12.4	10.7
VTTE-82146	100	34.2	15.4	30.7
	500	12.2	−0.3	13.7
	1000	−8.5	−8.1	17.5
	2000	−19.5	−9.9	−11.0

although the degree of growth inhibition was clearly diminished. In these cases the possibility of other antimicrobial agents than siderophores should be kept in mind. The growth inhibitions achieved by siderophore-containing culture medium was significant since the growth of moulds and yeasts is considered very difficult to control in food and feed products where they are important and common contaminants [12, 18]. The detection method for the antagonistic action of siderophores was quite different here from that generally used in plant pathology studies where cells are allowed to grow and simultaneously prevent the growth of indicator organisms in agar media. Liquid medium was chosen because in previous studies with lactic acid bacteria it was shown to be more accurate than agar tests [30].

In fermented sausages the combined use of starter cultures of lactic acid bacteria, micrococci and staphylococci has a long tradition. The role of staphylococci has mostly been to enhance the formation of aroma and flavor and to stabilize the color of the final product [24]. *Staphylococcus* starter cultures have interesting possibilities as siderophore producers. The iron levels in fermented sausages are about 5–50 mg kg^{-1}, depending on the species of meat in question and the muscle structure. However the iron is bound to haem compounds, especially myoglobin, haemoglobin, haematin and storage complexes of ferritin and haemosiderin [13]. Many microorganisms can utilize siderophores produced by other microorganisms, as shown between enterobacterial strains and *S. aureus* and *S. epidermidis* [17] or between several *Pseudomonas* species [16]. *Staphylococcus* starters could, however, provide interesting results if combined with lactic acid bacteria which produce inhibitors but are not sensitive to iron restriction [3]. In fact this is already the case in fermented sausages. Siderophores may also play a role in affecting cell permeability. Chelating agents increase the permeability of Gram-negative microbes, allowing antimicrobials such as nisin to penetrate the cells [2]. The chelating agents, however, nonspecifically also bind calcium and magnesium and play a role in cell integrity whereas siderophores bind iron specifically and their activity is receptor-bound [6]. However, the conceptual use of siderophores conjugated to drugs to promote antibacterial and antifungal drug delivery into pathogenic organisms has been successfully studied [21].

It is known that the proper combination of starter cultures and their antibacterial factors can result in an optimal process and products. The mechanisms of these antagonistic activities, however, need further research before safe and reliable applications can be demonstrated in meat fermentation.

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