BACTERIAL CHARACTERISATION

Iron supply of staphylococci and of micrococci by α -ketoacids

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Summary. Most of 232 strains of coagulase-negative staphylococci (CNS) belonging to 16 species, and nine strains of micrococci belonging to four species, could be distinguished by their growth on an iron-poor nutrient medium. α -Keto-/ α -hydroxyacids, as primary metabolites, facilitated iron supply to CNS and micrococci under various iron-limited conditions, as detected by different bioassay methods. Twelve selected CNS strains and two micrococcal strains produced significant amounts of α -ketoacids in iron-poor liquid nutrient media. In particular, strains of *Staphylococcus cohnii*, *S. xylosus* and *S. lentus* unable to grow on iron-poor media, produced high amounts of pyruvic acid and α -ketoglutaric acid. In bioassays, the selected CNS strains being siderophore indicator strains were fed with α -ketoacids at different levels of iron limitation. Strains able to grow under iron deprivation appeared to produce additional siderophores such as staphyloferrins under iron stress. α -Keto-/ α -hydroxyacids may be additionally active in the iron supply of CNS and micrococci.

Introduction

Coagulase-negative staphylococci (CNS) are normal flora of the surface of the human and animal body, and are most commonly encountered as commensal bacteria. The number of reports dealing with nosocomial CNS infection has increased enormously in recent years. If local or general host defence mechanisms are impaired or if foreign bodies such as prosthetic devices, indwelling catheters or shunts are present in the host, CNS may cause severe infection such as septicæmia and endocarditis.¹ Furthermore, in many patients undergoing treatment for renal failure by continuous ambulatory peritoneal dialysis, CNS are the predominant cause of acute, persistent or recurrent peritonitis.²

The emergence of CNS as significant pathogens has aroused much interest in the characterisation of possible virulence determinants. Iron concentration influences the expression of several genes involved in iron uptake and virulence in bacteria.^{3–4} For several micro-organisms, a correlation between siderophore production and virulence has been demonstrated.⁵ For this reason, there is an increasing interest in strategies of iron acquisition in CNS.

Recently, Meiwes *et al.*⁶ and Konetschny-Rapp *et al.*³ described the isolation and structure elucidation of

a novel siderophore from *S. hyicus* DSM 20459, named staphyloferrin A. It belongs to the polycarboxylate-type siderophores, as described by Smith *et al.*⁷ Drechsel *et al.*⁸ and Haag *et al.*⁹ described the purification, characterisation and biological properties of a second siderophore found in staphylococci named staphyloferrin B. It was also isolated from *S. hyicus* DSM 20459 and is produced by a large variety of staphylococci.

Additionally, staphylococci are able to utilise other iron sources including ferric citrate, host iron compounds such as transferrin, lactoferrin and haemoglobin¹⁰ and exogenous siderophores, mostly of the hydroxamate-type, e.g., ferrioxamines, ferrichromes.¹¹ In an agar diffusion test, the staphylococci were shown to utilise dihydroxamate-type siderophores¹⁰ and with the exception of all strains of *S. epidermidis* and some strains of *S. hominis* and *S. capitis*,¹² trihydroxamate-type siderophores. Marcelis *et al.*¹³ concluded from agar-diffusion tests with staphylococcal cell suspensions that siderophores, which could not be utilised by gram-negative bacteria, were excreted.

Little information is available about iron uptake by staphylococci. Like gram-negative bacteria, staphylococci synthesise some additional cell-wall proteins and cytoplasmic proteins (known as iron-repressible cytoplasmic membrane proteins, IRMPs) under iron-deficient conditions.^{2, 14, 15} Their significance as potential iron transport proteins has not been elucidated.

Studies of growth promotion and iron transport

have revealed that certain α -ketoacids and α -hydroxyacids, generated by amino-acid deaminases as primary metabolites, show significant siderophore activity.¹⁶⁻¹⁹ Enterobacteriaceae of the *Proteus-Providencia-Morganella* group, *Salmonella* spp., *Yersinia enterocolitica*, even *Escherichia coli*, as well as *Pasteurella* spp.²⁰ use their iron-binding properties under iron-limited conditions for iron supply.

This paper reports studies with α -ketoacids and α -hydroxyacids acting as siderophores of CNS and of two species of micrococci. They are produced as primary metabolites by CNS and can be utilised, from exogenous sources, by different species of CNS.

Materials and methods

Bacterial strains

The 282 strains studied belonged to the species *S. aureus* (41 isolates), 16 different species of CNS (232) and to four different species of micrococci (9) (table II). They were taken from the collection of the Robert Koch Institute, Wernigerode Branch, Germany. All strains were identified by classical methods according to Kloos and Lambe²¹ and by the micro-method developed by Bräulke *et al.*²² and freshly cultivated on nutrient agar at 37°C overnight before use.

S. epidermidis ATCC 14990 was used as the indicator strain in the bioassays described below.

α -Ketoacids (1-oxoacids), α -hydroxyacids and other siderophores

Ferrioxamines B, E (FoB, E) were kindly provided by Dr H. H. Peter, Ciba Geigy, Basel, Switzerland; ferrioxamine G (FoG) was isolated and purified from *Hafnia alvei* according to Reissbrodt *et al.* (1990);²³ enterobactin (ENT) was produced from *E. coli* according to the method of Neilands and Nakamura;²⁴ rhizoferrin, ferrichrome and staphyloferrin A were kindly provided by G. Winkelmann, University of Tübingen, Tübingen, Germany; pyruvic acid (PA) was purchased from E. Merck GmbH, Darmstadt, Germany; δ -aminolevulinic acid, DL- α -keto- β -methylvaleric acid (KMV), oxalacetic acid (OXO), α -ketoisocaproic acid (KIC), α -ketoadipic acid (KAA), α -hydroxyisovaleric acid (OH-IV), β -phenylpyruvic acid (PPA), β -indolpyruvic acid (β -IP), α -ketoglutaric acid (KGA), α -ketobutyric acid (KBA), α -ketoisovaleric acid (KIV) and α -ketomalonic acid (KMA) were purchased from Sigma. The purity of siderophores was confirmed by high-performance liquid chromatography (HPLC) as described previously.^{25, 26}

Nutrient media

Bioassay medium (medium 1). A modification of the bioassay medium described by Meiwes *et al.*⁶ was used. This medium comprised two components: firstly

Casamino acids (Difco) 2.5 g, Bacto agar (Difco) 7.5 g in 250 ml double-distilled water, and secondly glucose 5.0 g, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 50 mg, $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$ 2.9 mg, N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid 7.5 g, KH_2PO_4 500 mg, $(\text{NH}_4)_2\text{SO}_4$ 1 g and NaCl 2.5 mg in 250 ml of double-distilled water. Both components were combined after sterilisation and the pH was adjusted to 7.5. A sterile stock solution (0.5 ml) of vitamins (mg/l final concentration: folic acid 10; biotin 6; p-aminobenzoic acid 200; thiamin HCl 1000; pantothenic acid 1200; riboflavin 1000; nicotinic acid 2300; pyridoxin HCl 12000; vitamin B12 100) was added. Finally, deferrated ethylenediaminedi-(o-hydroxyphenylacetic acid) (EDDHA; Sigma) 15 mg, purified according to Rogers²⁷, was added for iron stress. Molten agar at 45°C was inoculated with an 18-h culture of the siderophore indicator strain *S. epidermidis* ATCC 14990 in nutrient broth (1 ml/100 ml).

Chemically defined medium (CDM-H; medium 2). This modified, chemically defined medium according to Flossmann *et al.*²⁸ contained (/1 L final concentration): $\text{NaH}_2\text{PO}_4 \cdot 12 \text{H}_2\text{O}$, 32.3 g; KH_2PO_4 , 1.36 g; NaCl, 1.19 g; glucose 6.0 g; L-arginine, 0.2 g; L-cysteine hydrochloride 0.12 g; L-serine, 0.2 g; L-glutamic acid, 0.15 g; L-isoleucine, 0.065 g; L-leucine, 0.065 g; L-phenylalanine, 0.095 g; calcium pantothenate, 0.002 g; nicotinic acid amide, 0.005 g; thiamine hydrochloride, 0.0001 g; orotic acid, 0.0015 g dissolved in 400 ml of double-distilled water, and a second component of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.25 g; L-aspartic acid, 1.6 g; L-tyrosine, 0.09 g dissolved in 600 ml of double-distilled water. These solutions were combined after sterilisation and the pH was adjusted to 7.3.

A sterile stock solution (0.1 ml) of vitamins (mg/10 ml final concentration: Ca-pantothenate, 20; nicotinamide, 50; thiamin.HCl, 1; orotic acid, 15) was added. Finally, yeast extract 0.5 g and purified agar (Oxoid, Agar No. 1) 1 g dissolved in 20 ml of double-distilled water and sterilised were added. This agar medium was seeded (100 μl /10 ml) with an 18-h culture of selected strains in nutrient broth.

Conalbumin/ α, α' -bipyridyl-medium. Conalbumin plates were prepared according to Stevenson and Griffith.²⁹ The agar (13 ml/plate) contained 6.5 ml of double concentrated Trypticase Soy Agar (BBL), 0.65 ml of conalbumin (ovotransferrin solution, Sigma; 4 mg/ml) and 0.45 ml of NaHCO_3 (0.71 M) dissolved in 5.4 ml of double-distilled water (final concentration of conalbumin, 1.0 g/L); 100 μM α, α' -bipyridyl was added for iron limitation. The same components were added to Tryptic Soy Broth (BBL) for liquid medium.

Growth of different CNS and micrococcal strains in iron poor media

Growth on conalbumin/ α, α' -bipyridyl-plates. Growth for the identification of surviving mechanisms under

iron-limited conditions was tested on conalbumin/ α, α' -bipyridyl-plates as described above. Forty-one *S. aureus* strains, 232 CNS and nine micrococcal strains (table II) freshly grown for 48 h at 37°C on Mueller-Hinton Agar (Oxoid) with sheep blood 5% and with a bacterial density of $c. 3 \times 10^8$ cfu/ml were starved in 0.5 ml of Tris-buffer, pH 7.4. After 30 min at 37°C, the starved strains were spot inoculated on to conalbumin/ α, α' -bipyridyl-plates. Growth was read after incubation for 24 h at 37°C (tables II and IV).

Growth in liquid conalbumin/ α, α' -bipyridyl-medium. For testing the individual ability to produce endogenous chelators, selected strains (table IV) were cultivated in 2 ml of conalbumin/ α, α' -bipyridyl liquid medium with shaking overnight at 37°C. After centrifugation (10 min at 3000 rpm) supernates were taken to prepare paper disks (20 μ l/disk of 6 mm diameter) for bioassays and also used for determination of α -ketoacids by HPLC.

Bioassays

Bioassays (also designated as cross-feeding tests) were performed by modifications of the methods described by Miles and Khimji³⁰ and Reissbrodt and Rabsch³¹ with the two different bioassay media (1 and 2).

Bioassay of supernates of CNS and micrococci on *S. epidermidis* ATCC 14990. Bioassay medium 1 was seeded with *S. epidermidis* ATCC 14990 (1 ml/100 ml) to demonstrate siderophore activity of the selected CNS and micrococcal strains. Filter paper disks were loaded with supernates of these strains and placed on the solidified agar which were incubated at 37°C for 18 h. A halo of growth surrounded those disks carrying siderophore activity.

Bioassay of siderophores/ α -ketoacids/ α -hydroxyacids on *S. epidermidis* ATCC 14990. Medium 2 was used to prove the iron chelating effect of diverse siderophores and α -keto-/ α -hydroxyacids in bioassays. Filter paper disks were loaded with siderophore solutions (1 mg/ml), 5 μ l each in the case of natural siderophores and 10 μ l each of α -keto-/ α -hydroxyacids. Growth around the disks demonstrated siderophore activity.

Bioassay of α -ketoacids, ferrioxamine B and enterobactin on several CNS strains under different conditions of iron limitation by EDDHA. Bioassays of selected α -ketoacids and siderophores listed in table III on the CNS strains were performed with medium 2. The individual ability of the CNS strains which had been shown to utilise these siderophores by use of corresponding uptake systems was to be tested. Therefore, the amount of EDDHA in medium 2 was increased from 10 μ M to a final 100 μ M/plate.

Determination of α -ketoacids by HPLC

Six strain-pairs of four CNS species and two micrococcal species (growing and non-growing strains) were selected (table IV). These 14 strains were

analysed by HPLC for their α -ketoacid composition according to Hayashi *et al.*²⁶ The α -ketoacids were determined as Q values. Q value was defined as

$$Q = \frac{\text{peak height* of the } \alpha\text{-ketoacid}}{\text{peak height* of } \alpha\text{-keto-octanoic acid**}}$$

where * showed as 2-chinoxalinoles and ** was the internal standard (120 nmol).

Results

Bioassays and growth of staphylococci and micrococci under different iron-deprived conditions

The polycarboxylate-type siderophores rhizoferrin and staphyloferrin A, δ -aminolevulinic acid and two α -keto-/ α -hydroxyacids cross-fed *S. epidermidis* ATCC 14990 in bioassays (table I). The tri-hydroxamate-type siderophores ferrioxamine B, E, G and ferrichrome, as well as the catecholate-type siderophore enterobactin did not cross-feed; β -indolepyruvic acid inhibited this strain.

Thirty-four of 241 different strains of CNS and micrococci tested did not grow on iron-depleted conalbumin/ α, α' -bipyridyl medium (table II). Of the many strains tested, the common species *S. epidermidis* and *S. haemolyticus* grew on this iron-limited medium, although all the *S. epidermidis* strains grew poorly. All 41 *S. aureus* strains were able to overcome this iron limitation and grew well. Among the other species tested, strains showed varied ability to grow on iron-depleted media. This individual ability is described either as growing (+) or non-growing (–).

Twelve CNS strains and two micrococcal strains were selected and used as pairs of growing or non-growing organisms in further investigations (table IV). These 14 strains were able to grow in shaken liquid conalbumin/ α, α' -bipyridyl medium, and supernates of these cultures cross-fed *S. epidermidis* ATCC 14990 used in the bioassays.

Bioassays were also performed by seeding the CNS-strains listed in table IV as siderophore indicator

Table I. Bioassay with different siderophores on *S. epidermidis* ATCC 14990

Siderophore tested	Growth
Ferrioxamines B, E, G	–
Enterobactin	–
Rhizoferrin	+
Staphyloferrin A	+
Ferrichrome	–
δ -Aminolevulinic acid	+
α -Ketoisocaproic acid	+
α -Ketoisovaleric acid	+
β -Phenylpyruvic acid	+
α -Ketoglutaric acid	–
Pyruvic acid	–
DL- α -Keto- β -methylvaleric acid	+
α -Hydroxyisovaleric acid	+
β -Indolepyruvic acid	Inhibition zone
Oxalacetic acid	–

Table II. Growth of *S. aureus*, CNS strains of 16 different species and of micrococci on conalbumin/ α,α' -bipyridyl-plates

Species	Number of strains tested	Number of strains	
		growing	not growing
<i>S. aureus</i>	41	41	0
<i>S. epidermidis</i>	57	57	0
<i>S. hominis</i>	25	16	9
<i>S. capitis</i>	23	11	12
<i>S. cohnii</i>	15	13	2
<i>S. sciuri</i>	6	6	0
<i>S. lentus</i>	4	3	1
<i>S. haemolyticus</i>	14	14	0
<i>S. intermedius</i>	10	10	0
<i>S. warneri</i>	10	9	1
<i>S. saprophyticus</i>	13	11	2
<i>S. schleiferi</i>	4	4	0
<i>S. xylosus</i>	8	7	1
<i>S. lugdunensis</i>	11	8	3
<i>S. simulans</i>	22	20	2
<i>S. chromogenes</i>	7	7	0
<i>S. hyicus</i>	3	3	0
<i>M. luteus</i>	4	4	0
<i>M. roseus</i>	1	1	0
<i>M. kristinae</i>	1	0	1
<i>M. varians</i>	3	3	0
Total	282	248	34

strains. Here the cross-feeding test was performed under different conditions of iron limitation by the addition of increasing amounts of EDDHA to medium 2. Table III shows a representative example of these experiments. The α -ketoacids KIC, KIV, KMV and PA, as well as ferrioxamine B and enterobactin, cross-fed the *S. lentus* strains on iron-limited medium 2. *S. lentus* 609/91 showed growth enhancement by the α -ketoacids only up to 40 μ M EDDHA, above that level the plate was overgrown, probably as a result of the production of staphyloferrins. *S. lentus* 317/91, while not able to grow on conalbumin/ α,α' -bipyridyl-plates,

showed positive cross-feeding tests under all conditions of iron limitation tested. The same behaviour was detected in the strain-pairs of *S. xylosus* and *S. cohnii*. The *S. saprophyticus* strain-pairs (growing and non-growing) were cross-fed by these α -ketoacids under all the iron-deprived conditions tested (10–100 μ M EDDHA, data not shown). Ferrioxamine B and enterobactin cross-fed all the strains tested under the different iron-limited conditions.

Production of α -ketoacids

Table IV shows the detection of α -ketoacids in the supernates of 14 selected strains after cultivation in liquid conalbumin/ α,α' -bipyridyl nutrient medium. The α -ketoacid composition of investigated strains was found to be different within the species-pairs of *S. xylosus*, *S. cohnii* and *S. lentus*. Non-growing CNS strains exhibited significantly higher amounts of PA when compared with the growing ones. Similar results were seen for KGA and to a lesser extent for KBA. Production of the other α -ketoacids was similar and was independent of growth under conditions of iron limitation. All strains tested produced the α -ketoacids PA, KIC and PPA in varying amounts. Besides significant differences in the composition of α -ketoacids, the total amounts of α -ketoacids produced were significantly higher in these non-growing CNS strains when compared with the growing strains. The *S. saprophyticus* strains, whether capable of growing or not, on conalbumin/ α,α' -bipyridyl-plates, did not show such differences, although higher amounts of KAA were observed in non-growing strains. Also remarkable was the KMA content of *S. saprophyticus* 358/91 (non-growing) and that of KGA in the growing *S. saprophyticus* strain 392/91.

The composition of α -ketoacids produced by both micrococcal species was similar, independent of their

Table III. Bioassay of two selected *S. lentus* strains, growing (+) or non-growing (–), on conalbumin/ α,α' -bipyridyl-plates with different iron limitation

Siderophores	Growth zones (mm) of <i>S. lentus</i> 609/91 (+) with						
	10 μ M EDDHA	15 μ M EDDHA	20 μ M EDDHA	30 μ M EDDHA	40 μ M EDDHA	60 μ M EDDHA	100 μ M EDDHA
KIC	22	16	14	(6)	10	g	g
KIV	20	14	14	(6)	12	g	g
KMV	12	(8)	10	(6)	10	g	g
PA	14	(6)	(8)	(6)	10	g	g
FoB	32	30	28	30	30	30	30
ENT	24	24	24	24	24	24	24
Siderophores	Growth zones (mm) of <i>S. lentus</i> 317/91 (–)						
	10 μ M EDDHA	15 μ M EDDHA	20 μ M EDDHA	30 μ M EDDHA	40 μ M EDDHA	60 μ M EDDHA	100 μ M EDDHA
KIC	14	16	(10)	14	14	20	12
KIV	12	(10)	(14)	18	14	10	12
KMV	18	(8)	20	20	(12)	14	18
PA	12	(6)	(10)	14	18	18	10
FoB	30	30	30	30	30	30	30
ENT	24	24	26	28	24	32	28

Figures in parentheses indicate poor growth around the siderophore disks; g, equal growth on the entire plate, probably the stronger staphylococcal siderophores (staphyloferrin) enable the strain to grow. For abbreviations see *Materials and methods*.

Table IV. Growth of 12 CNS strains and two strains of micrococci on conalbumin/ α,α' -bipyridyl plates and detection of α -ketoacids in supernates of these strains

Strain	Growth on conalbumin/ α,α' -bipyridyl	Q values of α -ketoacids									
		KGA	KMA	KAA	PA	KBA	KIV	KIC	KMV	PPA	Total
<i>S. xylosus</i> 679/90	—	1.89	0	0	32.55	0	0.09	2.09	0.24	0.15	37.01
<i>S. xylosus</i> 527/90	+	0.21	0	0	1.86	0	0.87	5.68	1.51	0.22	10.35
<i>S. cohnii</i> 349/91	+	0	3.38	0	6.60	0	0.26	1.87	0	0.44	12.55
<i>S. cohnii</i> 611/90	+	0	0	1.04	3.24	0	0.12	1.98	0	0.38	6.76
<i>S. cohnii</i> 469/90	—	3.53	0	0	87.54	1.11	0	2.18	0	0.40	94.76
<i>S. cohnii</i> 17/91	—	17.89	0	0	80.58	0.64	0.33	3.84	0.32	1.21	104.81
<i>S. lentus</i> 609/91	+	0	0	0	3.13	0	0.74	4.04	0	1.91	9.82
<i>S. lentus</i> 317/91	—	104.18	6.59	8.10	49.40	0.37	1.13	3.09	1.22	1.11	175.24
<i>S. saprophyticus</i> 392/91	+	6.14	0.68	0	11.79	0	0.36	3.29	0.35	0.87	23.48
<i>S. saprophyticus</i> 179/92	+	0	1.63	0.48	9.06	0	0.31	2.55	0.26	0.72	15.01
<i>S. saprophyticus</i> 357/91	—	0	0	8.04	7.21	0	0.56	3.74	0.27	1.03	20.85
<i>S. saprophyticus</i> 358/91	—	0	9.86	1.05	7.18	0	0.57	3.89	0.41	1.19	24.15
<i>M. varians</i> CCM 2253	+	5.11	0	0	42.81	0	0.24	2.02	0	0.60	50.78
<i>M. kristinae</i> ATCC 27571	—	4.23	0	0	26.50	0	0.59	4.96	0	0.69	36.97

ability to grow on conalbumin/ α,α' -bipyridyl-plates, although *Micrococcus varians* CCM 2253 produced a larger amount of α -ketoacids than did the non-growing *M. kristinae* ATCC 27571.

Discussion

Staphylococci, like other micro-organisms, employ different iron-supplying systems. These systems are adapted to the varying iron concentrations made available by different iron complexes. While it is well known that staphylococci are able to utilise the host iron compounds haemoglobin, lactoferrin and transferrin, the uptake mechanisms have not been precisely determined.³² Use of exogenous trihydroxamate-type siderophores such as ferrioxamine B or E by staphylococci was first described by Zähler *et al.*¹¹ The different utilisation of ferrioxamines B, E and G by staphylococci has been employed as a diagnostic tool by Heuck *et al.*¹²

Hydroxamate- or catecholate-type siderophores have been shown to be absent in staphylococci. Two polyhydroxycarboxylate-type siderophores, staphyloferrin A and B were detected in *S. aureus* as well as in many CNS tested.^{6,8,9} Meiwes *et al.*⁶ described 34 staphylococcal strains producing staphyloferrin A when the medium was supplemented with D-ornithine, but only 11 strains were staphyloferrin A positive without supplementation. In 16 of 29 strains examined,

Haag *et al.*⁹ were able to detect staphyloferrin B. However, in some of the CNS, neither staphyloferrin A nor B was detected. The iron transport systems of the staphyloferrins A and B are commonly found in the staphylococci, and the system may be active also in the uptake of the structurally similar polyhydroxycarboxylate siderophore, rhizoferrin,³³ as shown in table I.

In all, 232 strains of 16 CNS species and nine micrococcal strains of four species, varied in their ability to grow on iron-poor solid nutrient media such as conalbumin/ α,α' -bipyridyl plates (table II). The ability of the six paired strains (growing and non-growing) to grow in shaken liquid conalbumin/ α,α' -bipyridyl medium probably depends on the improved aeration. In particular, the non-growing strains produced significantly higher amounts of α -keto-/ α -hydroxyacids (see below) to overcome iron limitation under those conditions. Recently, Drechsel *et al.*⁸ detected α -keto- and α -hydroxyacids as novel siderophores of the *Proteus-Providencia-Morganella* group. Testing of such compounds on *S. epidermidis* ATCC 14990, with other siderophores as controls, resulted in positive bioassay reactions of the α -keto- and α -hydroxyacids except β -IP acid. Bioassay reactions appeared as expected in the cases of ferrioxamines, rhizoferrin and enterobactin. δ -Aminolevulinic acid as a precursor of haem compounds supported growth of *S. epidermidis* ATCC 14990, exhibiting a possible metabolic pathway for such compounds in this strain.

Relatively high amounts of α -ketoacids were detected in supernates of the 12 selected CNS strains and of *M. varians* CCM 2253 and *M. kristinae* ATCC 27571 (table IV). The amounts of α -ketoacids produced by these strains were significantly higher than those detected in *E. coli*.¹⁷ In particular, the non-growing *S. xylosus*, *S. cohnii* and *S. lentus* exhibited very high total amounts of α -ketoacids. Growing and non-growing *S. cohnii* strains can be distinguished by significant differences in the production of PA, KGA and KBA (table IV) under the same iron-limited conditions. The other α -ketoacids detected behaved quite similarly. Over-production of pyruvic acid by *E. coli* and *S. typhimurium* under iron-limited conditions has also been detected,^{17,18} especially in enterobactin-negative strains.

The α -ketoacids produced by the CNS and micrococcal strains act as weak iron chelators in iron supply. They are not able to remove iron from conalbumin (data not shown). Furthermore, since growth promotion of *S. epidermidis* ATCC 14990 by α -hydroxyacids was detected (table I), there is a need to confirm the presence of such compounds in the same supernates.

Growing and non-growing CNS strains also show differences in their ability to overcome iron stress in various iron-limited nutrient media. The *S. lentus* strain pair selected showed equal growth zones in bioassays of ferrioxamine B and enterobactin under all iron-limited conditions (table III). The α -ketoacids behaved similarly in the case of the non-growing *S. lentus* strain. The decreasing diameters of growth zones due to α -ketoacids and overgrowth in the case of the growing strain at greater iron limitation (60 μ M and 100 μ M EDDHA, respectively) may depend on the production of additional siderophores under these conditions (e.g., staphyloferrins). Furthermore, a possible change in the uptake systems of α -ketoacids at different levels of iron limitation, obtained by addition of increasing amounts of EDDHA, can also be discussed. The other strain pairs behaved similarly (data not shown). Together with the quite similar composition of the detected α -ketoacids, the strain

pairs of *S. saprophyticus* seemed to acquire iron from Fe^{3+} - α -ketoacids over a wide range of iron limitation.

All strains of the common species *S. aureus*, *S. epidermidis* and *S. haemolyticus* tested were able to grow on conalbumin/ α , α' -bipyridyl medium, although *S. epidermidis* grew less well than *S. aureus* (table II). Therefore, they could not be differentiated by their ability to overcome iron stress under these conditions. Further investigations should determine the iron chelating mechanisms, and define the possible role for the common staphylococci as significant pathogens under impaired host defence mechanisms.

Recently, Lindsay and Riley¹⁵ also showed the ability of all *S. aureus* tested, to grow well under iron restricted conditions but poor growth of the CNS strains under these conditions. The *S. aureus* strains produced siderophores detected by the chrome azurol S universal method in large quantities. In contrast, all the *S. epidermidis* strains tested and a few strains of three other CNS species produced low levels of iron chelators. They assume that CNS strains produce low levels of staphyloferrins, citrate or other unknown iron chelators (the last constitutively produced). These authors concluded that the better ability to grow under iron restricted conditions may contribute to the increased pathogenicity of *S. aureus*.

While the utilisation and production of α -ketoacids by CNS are clearly involved in iron supply, further studies are needed to determine the significance of these compounds in the differentiation and characterisation of CNS and their relationships with other iron suppliers, such as staphyloferrins.

α -Keto-/ α -hydroxyacids seem to function in the iron supply of CNS and micrococci under different iron-limited conditions. It is likely that they can substitute as iron supply mechanisms when endogenous or exogenous iron carriers are not available. These compounds occur as primary metabolites in mammalian cells, so they can contribute to the multiplication of CNS and micrococci in eukaryotic hosts.

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