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Evaluation of Selected Features of *Staphylococcus cohnii* Enabling Colonization of Humans

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ABSTRACT. Based on iron utilization, sensitivity to skin fatty acids, lipolytic and proteolytic activity the potential abilities of *Staphylococcus cohnii* strains to colonize humans were evaluated. The investigation included 60 strains that belong to both subspecies, *viz. S. cohnii* ssp. *cohnii* and *S. cohnii* ssp. *urealyticus*. Strains were isolated from different sources of the *Intensive Care Unit* and from non-hospital environment. Most of the strains were multiple antibiotic-resistant. Strains of both subspecies revealed a relatively low iron requirement. These strains were capable of utilizing iron bound in oxo acids and from host iron-binding proteins. *S. cohnii* ssp. *urealyticus* were more effective in iron uptake than *S. cohnii* ssp. *cohnii*. All investigated strains revealed sensitivity to skin fatty acids, but *S. cohnii* ssp. *urealyticus* strains were more resistant. Special features of strains of this subspecies promote colonization of humans.

The number of nosocomial infections caused by coagulase-negative staphylococci has increased in recent years. Although for some time the involvement of these microorganisms was questioned, nowadays some species are well documented to cause these infections. *Staphylococcus cohnii*, which is not commonly known and linked with human diseases, has become the object of our studies especially because of the latest reports of serious consequences of infections caused by these bacteria (Jarlov *et al.* 1996; Mastroianni *et al.* 1995; Taylor-Robinson 1999) and of the fact that many of the multiple antibiotic-resistant strains of *S. cohnii* were isolated from the *Intensive Care Unit* of pædiatric hospital environment (Szewczyk *et al.* 2000; Szewczyk and Różalska 2000). *S. cohnii* is considered to be part of human skin flora but because of irregularity of its isolation, is thought to be only a transient resident.

The analysis of *S. cohnii* strain characteristics may help in evaluating the possible danger to the patients due to the common presence of this microorganism in hospital environment and their multiresistance. The estimation of the colonization ability may include many features. One of them is the evaluation of the iron requirements and abilities to utilize bound iron from different compounds. High iron requirements may suggest a high level of parasitism, which is characteristic of many strains of *S. aureus*. On the other hand, strains of low iron requirements under conditions of iron restrictions in the human body may be more effective in successful colonization. Another investigated feature was sensitivity of *S. cohnii* to fatty acids, which are an important limiting skin colonization factor especially for bacteria, such as staphylococci that use skin to penetrate the macroorganism. The estimation of these features in a large and representative group of *S. cohnii* strains isolated from different environments was the aim of this work.

MATERIALS AND METHODS

Bacterial strains. Sixty strains of *Staphylococcus cohnii*, *viz.* 37 strains of ssp. *cohnii* and 23 strains of ssp. *urealyticus* from the collection of the *Department of Pharmaceutical Microbiology, Medical University in Łódź* (Poland) were investigated. Strains were isolated from hospital environment, patients and personnel of the *Intensive Care Unit of Teaching Pediatric Hospital in Łódź*, and from non-hospital environments. Strains were stored at −70 °C in glycerol and cultivated on agar plates supplemented with 5 % sheep blood at 37 °C in ambient air for 1 d.

Iron-limited media. One series of solid Mueller–Hinton 2 medium (*bioMérieux*) was used to prepare two types of media with iron chelators added. The first medium consisted of 1 g/L conalbumin (*Sigma*) and 200 μmol/L 2,2'-bipyridyl (*Sigma*) (Heuck *et al.* 1995). The second medium was supple-

mented with experimentally determined concentration of *N,N'*-bis-(2-hydroxyphenylacetyl)-1,2-ethanediamine (AED; *Sigma*) according to Marcelis *et al.* (1978).

Growth on conalbumin and 2,2'-bipyridyl plates. Standardized bacterial suspensions of A_{580} = 0.05 were starved in 50 mmol/L Tris-buffer (pH 7.4). After ½ h at 37 °C the suspensions were spot inoculated (5 µL) onto conalbumin and 2,2'-bipyridyl plates. Growth intensity was determined after incubation for 1 d at 37 °C.

Determination of the ability to utilize the iron bound in compounds. Two types of compounds were used. The first group consisted of 2-oxo- and hydroxy acids: 5-aminolevulinic, DL-2-oxo-3-methylvaleric, 2-oxoisocaproic, 2-oxoadipic, pyruvic, 2-oxoglutaric, 2-oxobutyric, mesoxalic, 2-oxoisovaleric (all *Sigma*), oxaloacetic (*Koch-Light*), 2-hydroxyisovaleric (*Aldrich*), and phenylpyruvic (*Fluka*). Iron solutions ($FeCl_3$, 0.2 mmol/L) mixed with oxo-acid solutions were used to load filter paper disks. Ratio of iron to ligand was 1:30 (50 µg of iron chelator per disk) (Drechsel *et al.* 1993).

The second group of tested substances were host iron-binding proteins. Single disks were loaded with iron-saturated (in µg): human transferrin 1000, ovotransferrin 1000, bovine hemoglobin 400, bovine hemin 200 (all from *Sigma*), horse myoglobin 400, horse cytochrome *c* 400 (*Serva*).

Mueller–Hinton medium (20 mL) with appropriate concentration of AED, and 50 µL of standardized bacterial suspension (A_{580} = 0.05) was poured on ion-free plastic plates (Ø 140 mm; *Medlab*). After 1 d preincubation at 4 °C, to let the iron present in the medium to be bound by AED, filter paper disks were placed on plates and loaded with water solutions of different iron sources. The negative control was the disk with 500 µg apotransferrin (*Sigma*) and the control of bacterial growth (positive control) was the disk with 25 µg $FeSO_4 \cdot 7H_2O$ (*BDH*). The plates were incubated at 37 °C for 2 d. Growth around the disks demonstrated the ability of the tested strain to utilize iron sources.

Media with fatty acid supplements. Linolenic ($C_{18:2}$), oleic ($C_{18:1}$) and palmitic acids (C_{16}) were predissolved in ethanol (50 g/L). Sets of Mueller–Hinton medium 2 plates with increasing concentration of acids (15.6 mg/L to 10 g/L) were prepared (Lacey and Lord 1981; Koneman *et al.* 1997).

Determination of MIC. Strains freshly cultivated on agar were suspended in 0.85 % NaCl, standardized according to McFarland's scale 0.5 and then diluted 50 times. Bacterial suspensions were spot inoculated (5 µL) onto the appropriate section of every type of medium. The sensitivity to tested compounds was evaluated according to the *National Committee for Clinical Laboratory Standards* (NCCLS) (*Performance Standards for Antimicrobial Disk Susceptibility Tests* 1997).

Determination of lipolytic and proteolytic activity. Lipolytic activity was tested on agar plates supplemented with 1 % Tween and 0.01 % $CaCl_2 \cdot H_2O$, and on P agar (*Difco*) with egg-yolk emulsion (Freney *et al.* 1999). Proteolytic activity was determined with gelatin and casein. Gelatin hydrolysis was evaluated on an SM-110 medium (*Difco*) with 10 % gelatin. Casein utilization was analyzed on P agar with 10 % skimmed milk. The ability to break down one or both substrates was considered to be a positive result.

RESULTS AND DISCUSSION

Colonization of macroorganisms by *S. cohnii* ssp. *cohnii* and *S. cohnii* ssp. *urealyticus* is limited by the deficit of free iron ions necessary for their metabolism. These ions are mainly components of enzymes that take part in the oxidoreduction processes. Iron requirement in staphylococci is usually high and depends on the type of metabolism and the ability of storing it in cells. The efficiency of iron uptake depends on the ability to use iron from complexes present in the host organism as ferroproteins: heme, transferrin and intracellular enzymes, and also from oxo acids (Wooldridge and Williams 1993; Heuck *et al.* 1995; Drechsel *et al.* 1993).

Analysis of the iron requirement of *S. cohnii* strains was carried out indirectly by determining their ability to grow on iron-poor media supplemented with the chelator — 2,2'-bipyridyl. Depending on iron requirement or on the endogenous iron level, bacterial growth was more or less limited. Of all the 65 investigated strains, 39 (65 %) grew well, which suggests their low iron requirements, while the other 21 (35 %) were characterized by very limited growth, which suggests their relatively high iron requirement (Table I). There was a visible difference between the two analyzed subspecies. *S. cohnii* ssp. *cohnii* strains were placed in both high and low iron requirement groups in similar percentage (59 and 41 %, respectively). Most the investigated *S. cohnii* ssp. *urealyticus* strains (74 %) belonged to the low iron requirement group.

Table I. Correlation between iron requirement of *Staphylococcus cohnii* strains and number of exogenous iron sources (*n*)

Strains of low-iron requirement (65 %)			Strains of high-iron requirement (35 %)		
ssp. *cohnii* (59 %)	*n*	ssp. *urealyticus* (74 %)	ssp. *cohnii* (41 %)	*n*	ssp. *urealyticus* (26 %)
	9		* *	9	
	10		* *	10	
* * *	11		*	11	
* * *	12			12	
* * *	13	•	* * *	13	•
* * * *	14	•	* * * * *	14	• •
* * * * * *	15	• •		15	
	16	• • • • •	*	16	• • •
* * *	17	• • • • • • •	*	17	
	18	•		18	

In order to assess the ability of *S. cohnii* to utilize iron bound in different compounds, it was necessary to prepare a medium that would completely suppress bacterial growth due to binding iron to a strong chelator — AED. Although its concentration of 1.5 mmol/L was enough to stop the growth of *S. cohnii* ssp. *cohnii*, for some strains of *S. cohnii* ssp. *urealyticus* 4.4 mmol/L was needed.

Almost all of the studied compounds (iron-saturated oxo acids and human and animal iron-carrier proteins) were iron sources for strains that were able to use the average 15 iron compounds. There was no difference between the strains isolated from various sources. The group of strains that use a relatively limited number of iron carriers (9–10 compounds) as well as those utilizing almost all of them (17–18) were represented by strains isolated from patients, hospital personnel, hospital and non-hospital environments. Only minor variations were observed between strains at different levels of iron requirement. Strains of low iron requirement were capable of utilizing 11–18 different compounds while those of high iron requirement were able to use 9–17. However, there are distinct differences between the two subspecies (Table I). *S. cohnii* ssp. *urealyticus* strains were capable of utilizing more iron saturated compounds than strains of ssp. *cohnii*.

Table II. Utilization of different iron sources by *S. cohnii* ssp. *cohnii* (*n* = 37) and *S. cohnii* ssp. *urealyticus* (*n* = 23) (percentage of active strains)

Iron source	Ssp. *cohnii*	Ssp. *urealyticus*
Iron saturated 2-oxo- and hydroxyacids		
Phenylpyruvic acid	22	56
2-Oxoadipic acid	40	43
2-Oxoglutaric acid	57	91
2-Oxobutyric acid	65	91
2-Oxoisocaproic acid	78	91
Oxaloacetic acid	78	100
DL-2-Oxo-3-methylvaleric acid	81	96
5-Aminolevulinic acid	81	100
2-Hydroxyisovaleric acid	92	100
Pyruvic acid	100	100
2-Oxoisovaleric acid	100	100
Mesoxalic acid	100	100
Host iron-binding proteins		
Horse cytochrome *c*	0	4
Ovotransferrin	67	87
Bovine hemin	89	96
Horse myoglobin	95	96
Bovine hemoglobin	100	100
Human transferrin	100	100

Comparison of the percentage of strains able to use iron from different compounds is presented in Table II. Under conditions of amino acid abundance bacteria carry out the deamination processes, changing amino acids into oxo acids, which successfully bind iron from the environment and therefore may act as siderophores (Drechsel *et al.* 1993). It is obvious that this process is enhanced by proteolytic activity. This was a feature of many investigated *S. cohnii* strains. The majority of tested oxo acids appeared to be a good source of iron to both subspecies (Table II). This applied to oxo acids with short polar lateral chains like pyruvate, as well as to oxo acids with long nonpolar chains (*e.g.* 2-oxoisocaproate) and to the only tested 2-hydroxy acid. The smallest number of strains were able to utilize iron bound to an aromatic oxo acid — phenylpyruvate. 2-Oxoadipate and 2-oxoglutarate were

also poorly used. The differences between the two subspecies were most significant in the utilization of 2-oxo acids. *S. cohnii* ssp. *urealyticus* more often used iron bound to 2-oxoglutarate, 2-oxobutyrate, oxaloacetate and 5-aminolevulinate than ssp. *cohnii* did.

Table III. Sensitivity of *S. cohnii* ssp. *cohnii* strains and other features permitting colonization[a]

Strain ZMF[d]	Source[c]	Sensitivity to fatty acids[b]			Activity		Effectiveness of iron uptake
		linoleic	oleic	palmitic	lipases	proteinases	
16	H	0.12	2.5	3.1	+	−	low
22	H	0.12	2.5	3.7	++	−	low
24	H	0.12	2.5	2.5	−	+	low
42	I	0.12	0.12	2.5	−	++	low
51	H	0.12	3.1	3.7	++	−	low
59	H	0.12	3.1	1.0	+	−	low
72	I	0.19	3.7	3.1	−	+	medium
77	I	0.25	0.7	1.0	++	+	low
78	I	0.19	2.4	0.7	++	+	low
82	I	**0.19**	**5.0**	**3.1**	−	+	**high**
84	I	**0.12**	**5.0**	**3.1**	−	+	**high**
85	I	**0.25**	**5.0**	**3.7**	−	+	**high**
89	I	**0.4**	**5.0**	**3.7**	++	+	**medium**
93	H	0.12	2.1	3.1	−	+	medium
100	H	0.25	5.0	1.7	+	+	low
101	I	0.4	3.1	0.01	−	+	low
102	I	**0.4**	**3.1**	**0.6**	+	+	**medium**
115	I	0.25	1.7	3.1	−	+	medium
129	H	0.12	3.7	1.0	++	−	low
132	H	0.12	2.5	3.7	++	−	high
134	I	0.4	3.1	2.5	−	+	low
158	I	0.19	1.7	3.1	−	+	low
722	E	0.19	4.4	3.7	+	−	low
726	E	0.19	3.7	3.7	−	+	low
728	E	0.09	3.1	0.06	−	+	medium
729	E	0.12	2.1	2.5	−	+	low
730	E	0.12	2.1	2.5	−	+	high
731	E	0.12	4.4	3.7	+	+	low
732	E	0.12	5.0	4.4	+	−	medium
734	E	0.12	2.1	2.5	−	+	medium
805	P	0.19	3.1	1.0	+	+	low
807	P	0.09	3.7	3.1	+	+	high
814	P	**0.19**	**3.7**	**2.1**	−	+	**high**
816	P	**0.25**	**4.4**	**2.5**	−	++	**high**
828	P	0.25	2.1	2.1	−	+	medium
833	P	**0.25**	**5.0**	**4.4**	−	+	**high**
835	P	0.19	5.0	3.1	++	+	low

[a]Strains of supposed better colonization ability are in **boldface**. [b]Mean MIC value, mg/mL.
[c]E — environment (non hospital), H — hospital environment, I — infants, P — personnel.
[d]Zakład Mikrobiologii Farmaceutycznej (*Department of Pharmaceutical Microbiology*).

S. cohnii was capable to use the majority of iron carrier proteins (Tables I, II). Human transferrin and ovotransferrin are well known host iron compounds used by many bacteria (Mickelsen *et al.* 1981; Morton and Williams 1989; Pidock *et al.* 1988; Verweij-Van *et al.* 1988). Many species of staphylococci can utilize iron from hemin and hemoglobin (*Lisiecki et al.* 1997). All of the tested *S. cohnii* strains were able to use iron from bovine hemoglobin and human transferrin, and almost all of them from bovine hemin and horse myoglobin. Although 87 % of ssp. *urealyticus* strains showed the ability to use iron from ovotransferrin, only 67 % of ssp. *cohnii* possessed this feature. Only one strain belonging to ssp. *urealyticus* was able to use iron from horse cytochrome *c*.

Most strains showed proteolytic activity (Tables III, IV) — 78 % of ssp. *cohnii* strains utilized gelatin but only a few also casein. All ssp. *urealyticus* strains secreted proteinases active to casein and

many also to gelatin. The ssp. *cohnii* strains, that did not show proteolytic activity were able to take-up iron bound to proteins at the same level as proteolyticaly active strains, which might suggest the involvement of their own bacterial siderophores.

The fatty acids and the low pH of the skin caused by them play an important role as a barrier to bacterial infections. Sensitivity to these acids may indicate the level of bacterial colonization ability. Three fatty acids (palmitic, oleic, and linoleic) which are considered to possess the most active antibacterial properties (Pestchow *et al.* 1996; Wang and Johnson 1992) were chosen (Tables III, IV).

Table IV. Sensitivity of *S. cohnii* ssp. *urealyticus* strains and other features permitting colonization[a]

Strain ZMF	Source	Sensitivity to fatty acids			Activity		Effectiveness of iron uptake
		linoleic	oleic	palmitic	lipases	proteinases	
5	H	0.12	2.5	0.5	++	+	medium
65	H	0.19	3.7	3.7	–	+	high
92	H	0.12	2.5	3.7	+	+	medium
94	H	0.19	2.5	5.0	–	+	high
137	I	0.25	5.0	3.1	+	++	high
139	I	0.19	4.4	3.1	–	++	high
701	E	0.19	5.0	3.7	–	+	high
703	E	0.19	5.0	5.0	–	++	high
704	E	0.25	5.0	4.4	–	++	high
705	E	0.25	5.0	5.0	–	++	medium
713	E	0.19	5.0	3.7	–	+	medium
719	E	0.25	4.4	3.7	–	+	medium
723	E	0.19	2.5	0.03	–	+	low
725	E	0.12	3.7	3.7	+	++	medium
740	E	0.12	4.4	3.7	+	++	medium
741	E	0.12	5.0	3.7	+	++	medium
802	P	0.19	5.0	4.4	–	++	high
838	P	0.19	2.1	4.4	–	++	high
843	P	0.25	3.1	2.1	–	++	high
844	P	0.25	3.1	2.1	–	++	high
847	P	0.19	5.0	4.4	–	+	high
852	P	0.19	4.4	3.7	–	++	medium
860	P	0.19	4.4	4.4	+	++	medium

[a]*See* footnotes to Table III.

Sensitivity of *S. cohnii* to fatty acids differed, depending on their structure. The highest sensitivity was observed to linoleic acid. Lacey and Lord (1981) found that coagulase-negative staphylococci were resistant to linoleic acid (MIC > 10 mg/mL), while *S. aureus* strains remained sensitive with MIC = 0.25 mg/mL. Our *S. cohnii* strains were even more sensitive with the average MIC = 0.19 mg/mL. There were only subtle differences between analyzed strains, usually not greater than twice the MIC value. For both ssp. *cohnii* and ssp. *urealyticus* strains a similar level of sensitivity was observed. High sensitivity of *S. cohnii* to linoleic acid may explain the transient colonization of human skin. It is thought that this acid plays a major role in protecting skin from pathogen colonization and infections (Donald *et al.* 1986). Among 17 strains of MIC value above average, 13 were isolated from humans. Three strains (most resistant to linoleic acid; MIC = 0.4 mg/mL) belonged to ssp. *cohnii* and were isolated from infants — patients of the *Intensive Care Unit.*

Inhibitory concentration of oleic and palmitic acids was much higher. The mean values for ssp. *cohnii* strains were 3.3 mg/mL (oleic acid) and 2.6 mg/mL (palmitic acid), while for ssp. *urealyticus* they were 4.0 and 3.5 mg/mL, respectively. As oleic acid dominates over other fatty acids in abscesses, sensitivity to it might be crucial when evaluating the potential danger of studied strains. It is believed that antibacterial activity of fatty acids increases with their unsaturation (Lacey and Lord 1981; Butcher *et al.* 1976). In spite of the difference between oleic and palmitic acid in their structure (length and the presence or absence of double bond), both subspecies of *S. cohnii* were more resistant to oleate than to palmitate. In comparison of two subspecies to both fatty acids, ssp. *urealyticus* was more resistant, especially to palmitate.

Lipolytic activity of bacteria plays an important role in the processes of bacterial survival on skin. Staphylococci produce lipases which release fatty acids from triacylglycerols in sebum secretions, increasing antimicrobial skin activity. Staphylococci often also secrete a fatty-acid modifying enzyme which is responsible for esterification of fatty acids (Long *et al.* 1992). Less than half of the investigated *S. cohnii* strains showed lipolytic activity. Only 46 % of ssp. *cohnii* and 30 % ssp. *urealyticus* produced lipases, while almost 100 % of *S. aureus* strains were lipase-positive. The lack of lipolytic enzymes limits the utilization of different energy sources. On the other hand, there is no release of fatty acids deadly to bacteria.

Among all of studied strains those with effective iron uptake from the environment and higher than average resistance to studied fatty acids for the particular analyzed subspecies, show some regularity (Tables III, IV). From ssp. *cohnii* 22 % were of that type, all of them isolated from the skin, of infant patients and *Intensive Care Unit* personnel. Almost half of ssp. *urealyticus* strains showed such ability. These strains were mainly isolated from the personnel and non-hospital environment. *S. cohnii* ssp. *cohnii* dominated in hospital environment (patients and personnel), while ssp. *urealyticus* was mainly isolated from non-hospital sources.

Both subspecies of *S. cohnii* show a great independence from the host-protective mechanisms, such as restriction of free iron or antimicrobial activity of fatty acids present in skin secretion. Moreover, *S. cohnii* ssp. *urealyticus* seems to have features that enable effective human skin colonization.

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