

Effect of Iron-Chelator Deferiprone on the In Vitro Growth of Staphylococci

The standard iron-chelator deferoxamine is known to prevent the growth of coagulase-negative *staphylococci* (CoNS) which are major pathogens in iron-overloaded patients. However, we found that deferoxamine rather promotes the growth of coagulase-positive *Staphylococcus aureus*. Accordingly, we tested whether deferiprone, a new clinically-available iron-chelator, can prevent the growth of *S. aureus* strains as well as CoNS. Deferiprone did not at least promote the growth of all *S. aureus* strains (n=26) and CoNS (n=27) at relatively low doses; moreover, it could significantly inhibit the growth of all *staphylococci* on non-transferrin-bound-iron and the growth of all CoNS on transferrin-bound iron at relatively high doses. At the same doses, it did not at least promote the growth of all *S. aureus* strains on transferrin-bound-iron. These findings indicate that deferiprone can be useful to prevent staphylococcal infections, as well as to improve iron overload, in iron-overloaded patients.

Key Words : *Staphylococcus*; Iron; Deferoxamine; Deferiprone; Transferrin

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INTRODUCTION

The human body is potentially so highly nutritious that it can well nourish some bacteria. However, it maintains an iron-restricted condition, as most iron is bound to iron-withholding glycoproteins such as transferrin and lactoferrin, or is sequestered within cells. This low iron-availability provides a non-specific defense mechanism that limits bacterial growth within the human body. Conversely, in order for bacteria to grow within the human body, they must be able to acquire iron effectively. For this purpose, most bacteria have developed their specific high-affinity iron-uptake systems. Among these systems, the siderophore-mediated iron-uptake system is basic and essential in most bacteria (1, 2). The recent increase in the incidence of infections caused by multidrug-resistant bacteria including *staphylococci*, underlines the importance of identifying novel preventive or therapeutic agents. Bacterial iron-uptake systems are attractive targets for the development of preventive or therapeutic vaccines, especially for multidrug-resistant bacteria. Similarly, iron-chelation therapies capable of preventing bacterial iron-uptake have also received attention as attractive novel preventive or therapeutic modalities (3-7).

In vivo iron-availability increases under some pathological conditions. In vivo bacterial growth can be stimulated by increased iron-availability, and conversely, bacterial growth can be suppressed by reducing iron-availability. Coagulase-

negative *staphylococci* (CoNS), especially *S. epidermidis*, are known to be major causative agents of septicemia in patients who have received anticancer chemotherapy or stem cell transplantation, because serum iron levels are elevated in these patients (8-10). Recently, it was reported that *S. epidermidis* infections in such patients can be prevented by administering human apotransferrin, which is a major iron-withholding protein (11). Moreover, it was reported that the growth of coagulase-positive *Staphylococcus aureus* in human serum is suppressed by administering lactoferrin, another iron-withholding protein (12). These findings suggest a possibility that chemical iron-chelating agents can also be used to prevent staphylococcal infections, as well as to improve iron overload in such patients.

Deferoxamine (C₂₅H₄₈N₆O₈ · CH₄O₅S) is the best known hydroxamate siderophore derived from *Streptomyces pilosus*, and is currently being used as the standard parenteral iron-chelator for the treatment of iron-overload (13). In addition, deferoxamine is known to have an antimicrobial effect, mainly because it competes with bacteria for available iron (14). In vitro studies have demonstrated that deferoxamine has bacteriostatic activity against some pathogenic bacteria including *S. epidermidis*, especially in the presence of ascorbic acid (14-18). However, one of the drawbacks of deferoxamine is that some pathogenic bacteria, including *Yersinia enterocolitica*, *Vibrio vulnificus* and *S. aureus*, are able to utilize the drug for iron-uptake, in the same manner as they use their own

siderophores via specific receptors (19-23). This cautions that deferoxamine therapy in iron-overloaded patients can facilitate fatal infections caused by *Y. enterocolitica*, *V. vulnificus* and *S. aureus*.

Recently, a new synthetic oral iron-chelator deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one) became clinically available (24, 25). Deferiprone forms strong complexes with iron at physiological pH. Deferiprone captures iron in vitro from transferrin, lactoferrin, ferritin, and hemosiderin, and in vivo after its parenteral or intragastric administration, and the effect of deferiprone is similar to or greater than that of parenteral deferoxamine. Moreover, deferiprone has been found to inhibit the growths of *Y. enterocolitica* and *V. vulnificus* capable of utilizing deferoxamine because structurally it is completely unlike deferoxamine (20, 26). However, whether deferiprone can inhibit or facilitate the growth of *staphylococci*, especially *S. aureus*, has not been determined yet. Accordingly, in this study, we determined the effect of deferiprone on the *in vitro* growth of staphylococci.

MATERIALS AND METHODS

Bacterial strains, media and reagents

S. aureus KCTC1927 (ACTC6538), *S. epidermidis* KCTC-1917 (ACTC12228) and *S. saprophyticus* KCTC3345 (ACTC-15305) strains were purchased from the Korean Collection for Type Cultures (<http://kctc.kribb.re.kr/>). CoNS (n=25) and *S. aureus* strains (n=25) isolated from various clinical samples were used in this study. Production of coagulase was tested using Staphaurex Plus kits (Murex Biotech Limited, Dartford, U.K.). CoPS included methicillin-susceptible strains (n=11) and methicillin-resistant strains (n=14). Brain Heart Infusion (BHI, BD, Sparks, MD, U.S.A.) agar was used to subculture all *staphylococci*. Staphylococcal siderophore detection (SSD) medium, which was recommended as a minimal medium containing low phosphate and citrate for studying staphylococcal iron-uptake systems (27), was used without deferration, because its iron concentration was less than 1 $\mu\text{g}/\text{dL}$ (28). When necessary, 1 μM of ferric chloride (FC) as a non-transferrin-bound-iron source or 0.5 mg/mL of human holotransferrin (HT; 1,200-1,600 μg of iron per 1 g of protein) as a transferrin-bound-iron source was added to SSD agars or broths. Deferoxamine was purchased from Ciba-Geigy (Basel, Switzerland) and deferiprone from Apotex Inc. (Toronto, Canada). Unless otherwise noted, all reagents were purchased from Sigma (Sparks, MO, U.S.A.).

Observations of the effect of deferoxamine on staphylococcal growth

Staphylococci were preconditioned in BHI broth containing 200 μM dipyrldyl, an iron chelator, at 37°C overnight

in order to adapt them to iron-restricted conditions and to reduce intracellular iron stores. In order to observe the effect of deferoxamine on staphylococcal growth, about 1×10^4 cfu of the preconditioned *staphylococci* were spread onto SSD agars supplemented with 1 μM FC (or 0.5 mg/mL HT). Paper discs containing 30 μL of deferoxamine solution (100 μM) or phosphate-buffered saline were then placed on agar surfaces. Because *S. aureus* strains generally grew more rapidly than CoNS on SSD agars, we observed *S. aureus* growths after 24 hr but CoNS growths after 48 hr. Response to deferoxamine was determined by the presence of a growth-stimulatory zone or

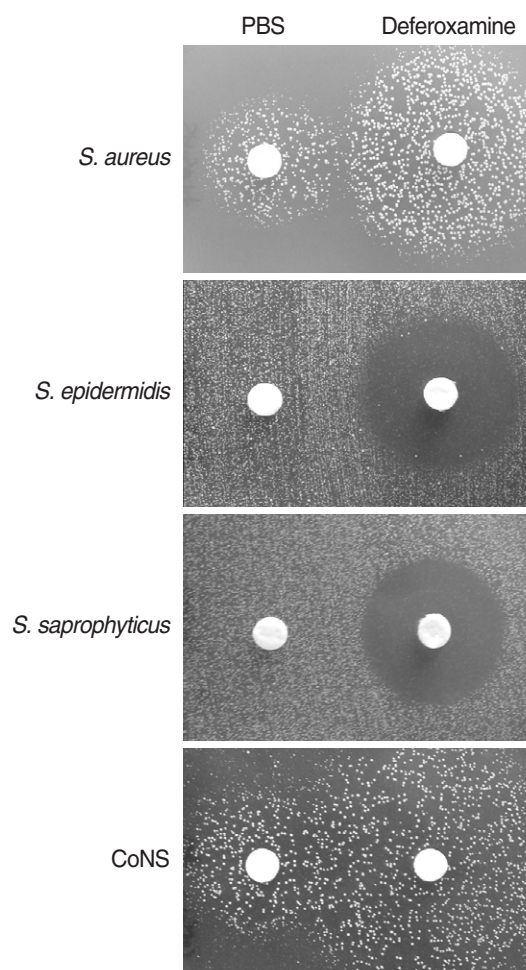


Fig. 1. Examples of responses of staphylococci to deferoxamine. *Staphylococcus aureus* KCTC1927, *Staphylococcus epidermidis* KCTC1917, *Staphylococcus saprophyticus* KCTC3345 and a coagulase-negative staphylococcus (CoNS: *S. epidermidis*) strain were preconditioned by culturing in BHI broth containing 200 μM of dipyrldyl at 37°C overnight to adapt to iron-restricted conditions and to reduce intracellular iron stores. About 10^{3-4} cfu of these three strains were spread onto the surface of SSD agar containing 1 μM FeCl₃, and then discs containing 30 μL of phosphate-buffered saline (PBS) or 100 μM deferoxamine were placed on the agar surface. *S. aureus* KCTC1927 was incubated at 37°C for 24 hr, and *S. epidermidis* KCTC1917, *S. saprophyticus* KCTC3345 and the CoNS strain for 48 hr.

a growth-inhibitory zone around discs containing deferoxamine, as shown in Fig. 1.

Observations of the effect of deferiprone on staphylococcal growths

Staphylococci, preconditioned as above, were inoculated into SSD broths containing 1 μ M FC (or 0.5 mg/mL HT) or into SSD broths containing 1 μ M FC (or 0.5 mg/mL HT) plus 0.5-1.5 mM deferiprone at about 1×10^6 cfu/mL, and then cultured with vigorous shaking (220 rpm) for 6 hr or 24 hr at 37°C. During culture, aliquots were withdrawn at appropriate times, and bacterial growths were measured by optical densities at a wavelength of 600 nm (OD₆₀₀). Response to deferiprone was determined by comparing the OD₆₀₀ ratios of strains after culture for 6 hr in broths with/without deferiprone. Response was arbitrarily expressed using OD ratios,

Table 1. Responses of *staphylococci* to deferoxamine

Bacteria	Response to deferoxamine (number of strains)		
	Growth enhancement	No response	Growth inhibition
<i>S. aureus</i> (n=25)	15	10	0
CoNS (n=25)	2	0	23

Details were described in the section of Materials and Methods. Response to deferoxamine was determined by the presence of a growth-enhancing zone or a growth-inhibitory zone around a disc containing deferoxamine as shown in Fig. 1. The experiment was repeated twice and results were consistent.

CoNS, coagulase-negative *staphylococci*.

as; less than 0.8 (growth inhibition), 0.8-1.2 (no response) and more than 1.2 (growth stimulation). The responses of *S. aureus* KCTC1927, *S. epidermidis* KCTC1917, and *S. saprophyticus* KCTC3345 strains are presented as growth kinetics over 24 hr.

6 M urea-gel electrophoresis

During culture in SSD broths containing HT, culture supernatants were obtained by centrifuging culture aliquots obtained at appropriate times at 10,000 rpm for 5 min. Equal volumes (20 μ L) of culture supernatants were reacted with sample buffer containing 8 M urea, but not SDS or mercaptoethanol, at 37°C for 30 min, and then electrophoresed on 5% stacking gel and 6% running gel containing 6 M urea (28). Proteins were visualized by staining with Coomassie brilliant blue R-250. On 6 M urea-gel, transferrin molecules are separated into four forms according to their iron-saturation levels, i.e., apoferric (AP), C-terminal monoferric (MC), N-terminal monoferric (MN), and diferric (DF) forms.

RESULTS

We first tested the responses of *staphylococci* to deferoxamine (Fig. 1, Table 1). The growth of *S. aureus* KCTC1927 was enhanced but those of *S. epidermidis* KCTC1917 and *S. saprophyticus* KCTC3345 were inhibited by deferoxamine. The growths of 15 *S. aureus* strains were enhanced around discs containing deferoxamine, but the other 10 strains did not respond. No *S. aureus* strain showed 'growth inhibition' by

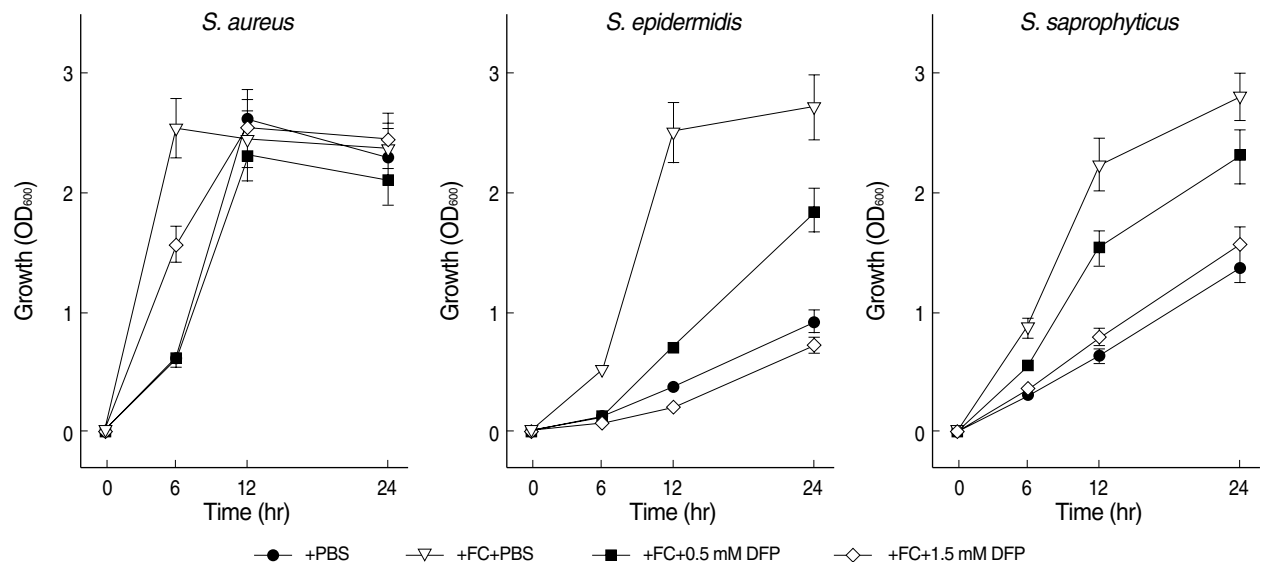


Fig. 2. Effect of deferiprone on the growths of staphylococci on non-transferrin-bound iron. The preconditioned *S. aureus* KCTC1927, *Staphylococcus epidermidis* KCTC1917 and *Staphylococcus saprophyticus* KCTC3345 strains were inoculated into SSD broth containing 1 μ M FC or in SSD broth containing 1 μ M FC plus 0.5-1.5 mM deferiprone (DFP) at ca. 1×10^6 cfu/mL, and then cultured with vigorous shaking (220 rpm) at 37°C for 24 hr. During culture, aliquots were withdrawn at appropriate times, and bacterial growths were monitored by measuring OD₆₀₀ values. Results are expressed as means \pm standard errors.

deferoxamine. In contrast, only two CoNS (*S. epidermidis*) strains showed 'growth enhancement' by deferoxamine, whereas the growths of the remaining 23 strains were inhibited. The response of one of the two CoNS strains to deferoxamine was shown in the Fig. 1. Similar results were observed when SSD broths containing HT plus deferoxamine were used (data not shown). Moreover, the responses of *S. aureus* strains to deferoxamine were not associated with their susceptibilities to methicillin (data not shown). As shown in the Fig. 1, some *S. aureus* and CoNS strains appeared to respond to phosphate-buffered saline alone, indicating that they can use phosphate for iron-uptake.

To determine whether deferiprone can inhibit the growths of *staphylococci* on non-transferrin-bound-iron, we cultured *S. aureus* KCTC1927, *S. epidermidis* KCTC1917 and *S. saprophyticus* KCTC3345 strains in SSD broths only, in SSD broths containing 1 μ M FC, or in SSD broths containing 1 μ M FC

Table 2. Responses of *staphylococci* to deferiprone

Bacteria	Iron sources	Response to deferiprone (number of strains)		
		Growth enhancement	No response	Growth inhibition
<i>S. aureus</i> (n=25)	FeCl ₃	0	0	25
	Holo-transferrin	0	25	0
CoNS (n=25)	FeCl ₃	0	0	25
	Holo-transferrin	0	0	25

Details were described in the section of Materials and Methods. The experiment was repeated twice and results were consistent. CoNS, coagulase-negative *staphylococci*.

plus 0.5-1.5 mM deferiprone. The growths of the three strains were stimulated by the addition of FC (Fig. 2). *S. aureus* KCTC1927 grew more actively than *S. epidermidis* KCTC1917 or *S. saprophyticus* KCTC3345 in iron-limited SSD broths or in SSD broths containing FC. These growth stimulations by FC were inhibited dose-dependently by deferiprone. Deferiprone showed a greater growth-inhibitory effect against *S. epidermidis* KCTC1917 and *S. saprophyticus* KCTC3345 than against *S. aureus* KCTC1927. At less than 0.5 mM, deferiprone did not affect the growths of all the three staphylococci; that is to say, it did not facilitate the growths of all the three staphylococci in contrast with deferoxamine (data not shown).

To determine whether deferiprone inhibits the growths of *S. aureus* (n=25) and CoNS (n=25) strains on non-transferrin-bound-iron, we cultured all 50 *staphylococci* in SSD broths containing 1 μ M FC or in SSD broths containing 1 μ M FC plus 1.5 mM deferiprone at 37°C for 6 hr. Deferiprone inhibited the growths of all *S. aureus* and CoNS strains on non-transferrin-bound-iron regardless of the ability to utilize deferoxamine (Table 2) and methicillin susceptibility (data not shown).

To determine whether deferiprone can also inhibit the growths of *S. aureus* KCTC1927, *S. epidermidis* KCTC1917 and *S. saprophyticus* KCTC3345 strains on transferrin-bound-iron, we cultured these strains in SSD broths only, in SSD broths containing 0.5 mg/mL HT or in SSD broths containing 0.5 mg/mL HT plus 0.5-1.5 mM deferiprone for 24 hr. The growths of all three strains were stimulated by the addition of HT (Fig. 3). *S. aureus* KCTC1927 grew more actively than *S. epidermidis* KCTC1917 or *S. saprophyticus* KCTC-

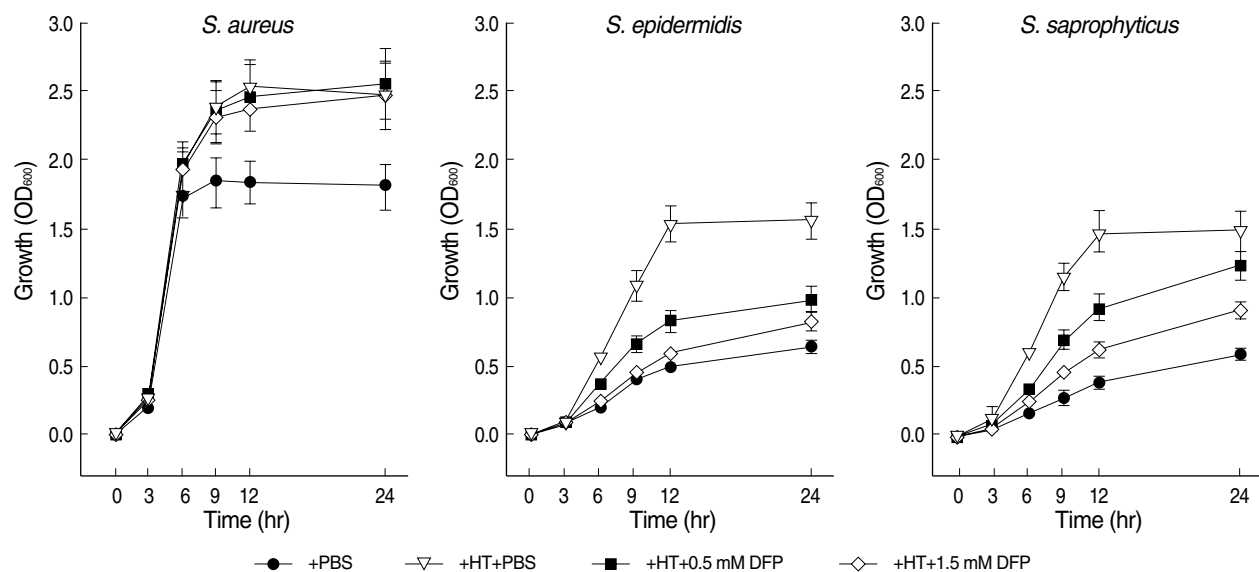


Fig. 3. Effect of deferiprone on the growths of staphylococci on transferrin-bound iron. The preconditioned *Staphylococcus aureus* KCTC1927, *Staphylococcus epidermidis* KCTC1917 and *Staphylococcus saprophyticus* KCTC3345 were inoculated into SSD broth containing 0.5 mg/mL HT or in SSD broth containing 0.5 mg/mL HT plus 0.5-1.5 mM deferiprone (DFP) at ca. 1×10^6 cfu/mL, and then cultured with vigorous shaking (220 rpm) at 37°C for 24 hr. The other abbreviations and symbols are as in Fig. 2.

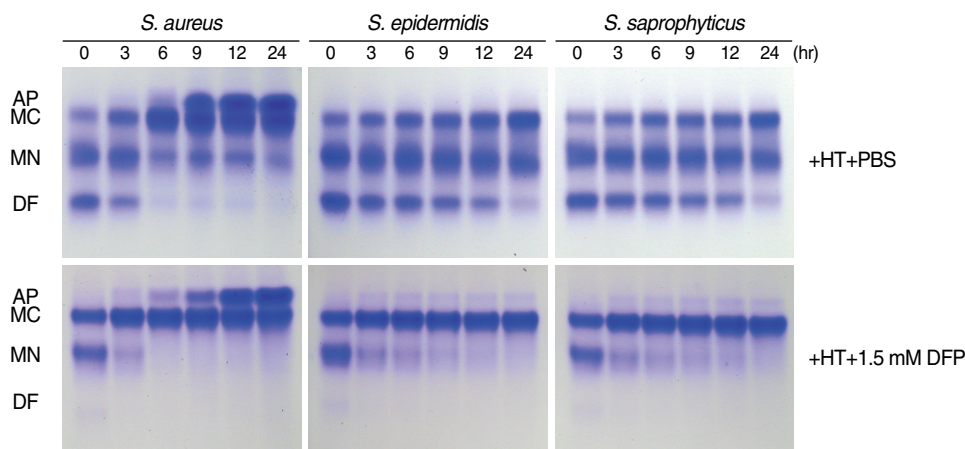


Fig. 4. Utilization of transferrin-bound iron by staphylococci in the absence or presence of deferiprone. During culture in SSD broth containing holotransferrin (HT) or in SSD broth containing HT plus deferiprone (DFP), as shown in Fig. 3, culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min at appropriate times. Equal volumes (20 μ L) of culture supernatants were electrophoresed on 6 M urea-polyacrylamide gels, and stained with Coomassie blue. AP, MC, MN, and DF indicate apoferrous, C-terminal monoferric, N-terminal monoferric and diferric transferrins, respectively.

3345 in SSD broths containing HT (Fig. 3), and it also utilized iron from transferrin more efficiently than the other two *staphylococci* (Fig. 4). On 6 M urea-gel electrophoresis, transferrin bands were changed from DF to MN or MC to AP forms as the result of the removal of iron from transferrin molecules by staphylococci or deferiprone. The growth stimulations of *S. epidermidis* KCTC1917 and *S. saprophyticus* KCTC3345 by HT were inhibited dose-dependently by deferiprone. In contrast, the growth stimulation of *S. aureus* KCTC1927 by HT was not inhibited by deferiprone. As shown at 0 hr in Fig. 4, deferiprone evidently reduced the iron-saturation level of transferrin by removing iron from diferric transferrin. Nevertheless, *S. aureus* KCTC1927 still utilized iron from transferrin more efficiently than the other two staphylococci.

To determine whether deferiprone inhibits the growths of *S. aureus* (n=25) and CoNS (n=25) strains on transferrin-bound-iron, we cultured all 50 *staphylococci* in SSD broths containing 0.5 mg/mL HT or in SSD broths containing 0.5 mg/mL HT plus 1.5 mM deferiprone at 37°C for 6 hr. The growths of all CoNS strains were inhibited by deferiprone. In contrast, the growths of all *S. aureus* strains were not inhibited by deferiprone.

DISCUSSION

Hartzen et al. reported that deferoxamine can inhibit the growths of *staphylococci*, especially in the presence of ascorbic acid (16-18). However, according to our results, the responses of *staphylococci* to deferoxamine appear to vary among staphylococcal species and strains. Deferoxamine was found to stimulate the growths of a few CoNS strains as well as of most *S. aureus* strains (Table 1). Our results are supported by recent

reports, in which *S. aureus* was found to utilize deferoxamine for iron-acquisition and growth via a specific ATP-binding cassette (ABC) transporter system (23, 29). One iron-regulated ABC transporter has also been reported in *S. epidermidis* (30). However, it is not determined whether this transporter system is involved in the uptake of deferoxamine-iron complex by *S. epidermidis*. Overall, it appears that deferoxamine therapy in patients with iron-overload can facilitate staphylococcal infections, especially *S. aureus* infections. Moreover, deferoxamine is known to stimulate the growths of other pathogenic bacteria including *Y. enterocolitica* and *V. vulnificus* (19-22, 26). These problems severely limit the use of deferoxamine for the treatment of iron-overload, although the drug has been used for some time as a standard iron-chelator for the treatment of iron-overload (13).

S. aureus appear to possess more efficient iron-uptake systems than CoNS. In our previous studies (31-34), *S. aureus* was found to acquire iron efficiently from partially iron-saturated transferrin as well as highly iron-saturated transferrin, whereas *S. epidermidis* and *S. saprophyticus* were able to acquire iron only from highly iron-saturated transferrin, but less efficiently than *S. aureus*. Lindsay et al. found that *S. aureus* is able to actively grow and produce siderophores in severely iron-limited SSD medium, whereas CoNS grew poorly and did not produce siderophores (27). Trivier and Courcol reported that *S. aureus* could grow in severely iron-limited media containing 0.04 μ M Fe whereas other bacteria could not (35). Martinaho et al. also found that the growth initiation of *S. epidermidis* is dependent on the presence of readily-available iron, i.e., non-transferrin-bound-iron (10). According to the results of the present study, *S. aureus* grew more actively in SSD broths containing FC or HT and utilized iron from transferrin more efficiently than *S. epidermidis* or *S. saprophyticus* (Fig. 2-4). Similar results were observed for all *S. aureus* and

CoNS strains examined. In addition, deferiprone was found to inhibit the growths of all CoNS strains, but not of *S. aureus* strains, on transferrin-bound-iron. Overall, all these findings indicate that *S. aureus* possess more efficient iron-uptake systems that enable them to grow more actively in severely iron-limited conditions and to utilize transferrin-bound-iron more efficiently than CoNS.

CoNS, but not *S. aureus*, are known to be major causative agents of septicemia in iron-overloaded patients who have received anticancer chemotherapy or stem cell transplantation. CoNS growth proved to be stimulated by elevated non-transferrin-bound-iron or transferrin-bound iron in such iron-overloaded patients (8, 9). According to our results, deferiprone can inhibit significantly the growths of *staphylococci*, especially CoNS, by reducing iron-availability at high doses; moreover, at least it does not promote the growths of *staphylococci*, especially *S. aureus*, at low doses and on transferrin-bound-iron. As shown in Fig. 4, the ability of deferiprone to remove iron from transferrin in vitro and in vivo has been confirmed independently by others under similar conditions (36–38). Accordingly, deferiprone can be useful for the prevention of CoNS infections along with the treatment of iron-overload by reducing both the level of non-transferrin-bound-iron and the iron-saturation level of transferrin.

It has been reported that apotransferrin can prevent *S. epidermidis* growth in patients with iron-overload (11) and that lactoferrin or apotransferrin can prevent *S. aureus* growth in human serum or human peritoneal dialysate (12, 32). However, our results show that *S. aureus* can still efficiently acquire iron from low iron-saturated transferrin (Fig. 3, 4). In addition, apotransferrin and lactoferrin are inevitably destroyed in vivo and must be administered intravenously because of the proteinoic natures. In contrast, deferiprone is a simple chemical oral agent and thus is free of these limitations of apotransferrin and lactoferrin (24, 25).

It was reported that deferiprone can also inhibit the growth of *Y. enterocolitica* by decreasing iron-availability (20). In addition, we recently found that deferiprone can also inhibit the growth of *V. vulnificus*, which causes fatal septicemia with an associated mortality rate of over 50% (26). Moreover, no one has yet found a bacterium capable of utilizing deferiprone for iron uptake. According to our results, deferiprone did not promote the growths of *staphylococci* at less than 0.5 mM in contrast with deferoxamine (data not shown). Relatively higher doses of deferiprone (>0.5 mM) were required to inhibit the growths of *staphylococci* than those were required to inhibit the growth of *Y. enterocolitica* or *V. vulnificus* (<0.2 mM). This is attributed to the more efficient iron-uptake systems of *staphylococci*, which enable them to more actively grow under severely iron-limited conditions than *Y. enterocolitica* or *V. vulnificus*; the latter two are considered archetypal ferrophilic or iron-sensitive bacteria that require higher levels of available iron for growth than other bacteria (26, 39). Ascorbic acid is known to enhance the efficacy of deferiprone as it does for

deferoxamine (14–18, 40). Accordingly, the deferiprone doses required to inhibit the growths of staphylococci may be reduced by administering it in combination with ascorbic acid. Also, more potent iron-chelators capable of efficiently inhibiting the growth of staphylococci, especially *S. aureus*, are required (40).

Overall, the present study indicates that, in contrast with deferoxamine, deferiprone can inhibit the growths of *staphylococci*, especially CoNS, by reducing iron-availability at relatively high doses; moreover, at least it does not promote the growths of staphylococci, especially *S. aureus*, at relatively low doses. Accordingly, it is thought that deferiprone can be a useful iron-chelator to prevent staphylococcal infections, as well as to improve iron overload, in iron-overloaded patients.

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