The Journal of Microbiology, April 2005, p.183-190 Vol. 43, No. 2

Staphylococcus aureus Siderophore-Mediated Iron-Acquisition System Plays a Dominant and Essential Role in the Utilization of Transferrin-Bound Iron

Ra-Young Park[1], Hui-Yu Sun[1], Mi-Hwa Choi[1], Young-Hoon Bai[2] and Sung-Heui Shin[1,*]

[1]Research Center for Resistant Cells and [2]Department of Biology, Chosun University Medical School, Gwangju 501-759, Republic of Korea

(Received December 6, 2004 / Accepted March 18, 2005)

***Staphylococcus aureus* is known to be capable of utilizing transferrin-bound iron, via both siderophore- and transferrin-binding protein (named IsdA)-mediated iron-acquisition systems. This study was designed in order to determine which iron-acquisition system plays the essential or dominant role with respect to the acquisition of iron from human transferrin, in the growth of *S. aureus*. Holotransferrin (HT) and partially iron-saturated transferrin (PT), but not apotransferrin (AT), were found to stimulate the growth of *S. aureus*. *S. aureus* consumed most of the transferrin-bound iron during the exponential growth phase. Extracellular proteases were not, however, involved in the liberation of iron from transferrin. Transferrin-binding to the washed whole cells via IsdA was not observed during the culture. The expression of IsdA was observed only in the deferrated media with AT, but not in the media supplemented with PT or HT. In contrast, siderophores were definitely produced in the deferrated media with PT and HT, as well as in the media supplemented with AT. The siderophores proved to have the ability to remove iron directly from transferrin, but the washed whole cells expressing IsdA did not. In the bioassay, the growth of *S. aureus* on transferrin-bound iron was stimulated by the siderophores alone. These results demonstrate that the siderophore-mediated iron-acquisition system plays a dominant and essential role in the uptake of iron from transferrin, whereas the IsdA-mediated iron-acquisition system may play only an ancillary role in the uptake of iron from transferrin.**

Key words: *Staphylococcus aureus*, Siderophore, Transferrin, Iron, Transferrin-binding protein

Staphylococci are ubiquitous bacteria, and have been implicated in a wide spectrum of diseases. Of the many varieties of staphylococci, *S. aureus* is the leading cause of nosocomial diseases, and is the most predominant pathogen in human infections (Emori and Gaynes, 1993; Sieradski *et al.*, 1999). The recent identification of vancomycin-resistant strains of *S. aureus* (Sieradski *et al.*, 1999; Rybak and Alkins, 2001) underlines the importance of identifying novel therapeutic agents which can directly and specifically target this important pathogen. One area that has received recent attention in this regard is the bacterial iron-acquisition system (Lim *et al.*, 1998; Modun *et al.*, 1998; Braun and Barun, 2002; Skaar and Schneewind, 2004). This is primarily due to the fact that all bacterial pathogens require iron to infect a host, and iron is the only nutrient that limits bacterial growth inside the human host. The limited iron-availability in the host provides one form of non-specific defense which bacterial pathogens must overcome in order to grow and infect the host (Crosa, 1997; Ratledge and Dover, 2000; Bullen *et al.*, 2002; Andrews *et al.*, 2003).

Most pathogenic bacteria utilize high-affinity iron-acquisition systems to counter the limited iron-availability in their hosts. The iron-acquisition systems of Gram-negative bacteria have been extensively studied. These include the direct binding of transferrin, lactoferrin, or hemeproteins to specific receptors, and the secretion of siderophores, which exhibit the ability to bind to specific receptors when complexed with iron (Crosa, 1997; Ratledge and Dover, 2000; Bullen *et al.*, 2002; Andrews *et al.*, 2003). Recently, studies have been undertaken which elucidated the iron-acquisition systems of Gram-positive bacteria (Brown and Holden, 2002).

Siderophore-, transferrin-binding protein (designated IsdA)-, and hemoprotein receptor-mediated iron-acquisition systems have been reported in *S. aureus* (Sebulsky *et al.*, 2000; Taylor and Heinrichs, 2002; Mazmanian *et al.*, 2003). It is of note that *S. aureus* possesses all three of the iron-acquisition systems, because there have, until now, been no reports regarding bacteria possessing all three iron-acquisition systems. Most pathogenic bacteria possess siderophore- and/or hemoprotein receptor-mediated iron-acquisition systems, but only the *Haemophilus* and *Neisseria* species, which do not produce siderophores, have

* To whom correspondence should be addressed.
(Tel) 82-62-233-6052; (Fax) 82-62-230-6352
(E-mail) shsin@chosun.ac.kr

been reported to exploit the transferrin-binding protein-mediated iron-acquisition system (Crosa, 1997; Ratledge and Dover, 2000; Brown and Holden, 2002; Andrews *et al.*, 2003). *S. aureus* produces several types of siderophores, as well as retaining the ability to use heterologous hydroxamate-siderophores, and is also capable of utilizing hemoproteins as a sole iron source, via specific receptors on the cell wall (Konetschny-Rapp *et al.*, 1990; Drechsel *et al.*, 1993; Courcol *et al.*, 1997; Morrissey *et al.*, 2000; Cabrera *et al.*, 2001; Sebulsky and Heinrichs 2001; Dryla *et al.*, 2003). However, the transferrin-binding protein-mediated iron-acquisition system in *S. aureus* remains poorly understood (Brock *et al.*, 1991; Modun *et al.*, 1994, 1998; Lindsay *et al.*, 1995; Modun and Williams, 1999; Martinaho *et al.*, 2001; Taylor and Heinrichs, 2002).

The staphylococcal transferrin-binding protein (named Tpn) was first reported to be the multifunctional cell wall glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Modun *et al.*, 1994, 1998; Modun and Williams, 1999). However, Taylor and Heinrichs (2002) disputed the role of GAPDH in transferrin-binding, reporting that the mutation of the *gap* gene encoding for GAPDH had no effect on transferrin-binding. Instead, they proposed that another cell wall-anchored protein (named StbA) may function as a transferrin-binding protein in *S. aureus*. More recently, via DNA sequence homology, StbA has been determined to be one of the iron-regulated surface determinants (IsdA), which functions in the binding and uptake of heme-iron (Brown and Holden, 2002; Mazmanian *et al.*, 2003; Clarke *et al.*, 2004). It is well-known that *S. epidermidis* can also express the same transferrin-binding protein as in *S. aureus*, but is unable to utilize low iron-saturated transferrin (Modun *et al.*, 1994, 1998; Martinaho *et al.*, 2001). Considering these facts, it remains incumbent upon the research community to determine whether or not IsdA is a transferrin-specific receptor for the acquisition of iron in *S. aureus*.

Accordingly, the present study was designed to ascertain which of the IsdA- and the siderophore-mediated iron-acquisition systems plays the essential or dominant role in the acquisition of iron from human transferrin, and whether extracellular proteases affect the liberation of iron from transferrin, via the destruction of transferrin (Potempa *et al.*, 1988; Okuzo *et al.*, 1996). As a result, we determined that the siderophore-mediated iron-acquisition system plays the dominant and essential role in the utilization of transferrin-bound iron, without assistance from extracellular proteases, and that the siderophores were able to independently stimulate the growth of *S. aureus* on transferrin-bound iron.

Materials and Methods

Bacterial strain, media and growth conditions

The *S. aureus* ATCC 6538 strain was used in our experiments. Brain Heart Infusion (BHI, France) was used as the complex medium, and MM9 medium with low phosphate and citrate (Schwyn and Neilands, 1987; Lindsay and Riley, 1994), was used as the chemically-defined minimal medium. BHI broth was deferrated using 8-hydroxyquinoline (Leong and Neilands, 1982). The residual iron concentration of the deferrated (DF) BHI was below 1 μM (Diagnostic Iron and Total Iron-Binding kit, Sigma, USA). MM9 was used without deferration, as its iron concentration was also below 1 μM. When necessary, 0.5 mg/ml of human-apotransferrin (AT; <30 μg of iron per 1 g of protein), -partially iron-saturated transferrin (PT; 300 ~ 600 μg of iron per 1 g of protein) and -holotransferrin (HT; 1,200 ~ 1,600 μg of iron per 1 g of protein), all of which were purchased from Sigma-Aldrich, were supplemented into the DF-BHI and MM9. Deferoxamine, a well-known heterologous hydroxamate-type siderophore, was purchased from Ciba-Geigy (Switzerland).

The bacterium was subcultured in the BHI with 200 μM dipyridyl (Sigma, USA) overnight, in order to adapt the bacterium to iron-restricted conditions, and to lower intracellular iron storage. About 1×10^6 cfu/ml of this preconditioned bacterium was inoculated into the test media, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. At appropriate intervals, culture fluids were withdrawn and used to monitor bacterial growth, via the measurement of optical densities at a wavelength of 600 nm (OD_{600}), and the culture supernatants, which were obtained by centrifugation (10,000 rpm, 5 min) at the same intervals, were used in several assays performed in this study.

Protease assay

The protease activities of the culture supernatants were measured on Skim milk agar (Difco, USA). Skim milk agar was punched out to create small wells with diameters of 3 mm, and 20 μl of the culture supernatants were loaded into these wells. By performing the same procedure five times, a total of 100 μl of the culture supernatants were loaded, and then the agar plates were incubated at 37°C for 24 h. Protease activity was expressed as the followings: - (no activity), ± (not obvious), + (weak activity; less than 7 mm) and ++ (strong activity; more than 7 mm), on the basis of the size of the clear zones formed around the wells.

Siderophore assay

The total siderophore activities of the culture supernatants were measured on the chrome azurol S (CAS) agar diffusion assay, which was described in our previous study (Shin *et al.*, 2001). In brief, CAS agar was punched out to create small wells with diameters of 3 mm, and 20 μl of the culture supernatants were loaded into these wells. By performing the same procedure five times, a total of 100 μl of culture supernatants were loaded, after which the agar plates were incubated at 37°C for 24 h. The size of

yellow-orange haloes around the wells indicated total siderophore activity.

SDS-PAGE

In order to visualize the destruction of transferrin by staphylococcal proteases and the binding of transferrin to the whole cells, SDS-PAGE was performed, as previously described (Lim *et al.*, 1998). In brief, 20 μl of the culture supernatants were electrophoresed on SDS-polyacrylamide gel (10%), and then the proteins were visualized with Coomassie blue-staining.

Receptor-ligand binding assay

In order to observe the expression of IsdA on the cell wall, a receptor-ligand binding assay was preformed, as described previously (Lim *et al.*, 1998). In brief, 100 μg of the cell wall proteins were electrophoresed on SDS-polyacrylamide gel (10%), and transferred to nitrocellulose membranes. The membranes were allowed to react with human transferrin conjugated with horseradish-peroxidase (Sigma, USA), and visualized with diaminobenzidine and hydrogen peroxide (Sigma, USA).

6 M urea-gel electrophoresis

In order to observe the removal of iron from transferrin, we performed 6 M urea-gel electrophoresis, as described previously (Makey and Seal, 1988; Modun *et al.*, 1994). In brief, equal volumes of the culture supernatants were mixed with sample buffer containing 8 M urea, but not containing SDS or mercaptoethanol, then allowed to react at 37°C for 30 min, and electrophoresed on 5% stacking gel and 6% running gel containing 6 M urea.

Removal of iron from transferrin by washed whole cells and siderophores

In order to obtain the whole cells expressing IsdA and the culture supernatant containing siderophores, the preconditioned bacterium was cultured in DF-BHI for 12 h. The bacterial pellet and culture supernatant were obtained by centrifuging at 10,000 rpm for 10 min. A part of the bacterial pellet was separated in order to isolate cell wall proteins, and the expression of IsdA was confirmed by receptor-ligand binding assay (Fig. 5A). The remainder of the bacterial pellet was then washed three times with Tris (pH 7.0)-glucose (1%) medium. About 1×10^{10} cfu/ml of the whole cells were resuspended with Tris-glucose medium containing 0.5 mg/ml of HT, then incubated at 37°C for 0, 10, 30, and 60 min. After the reactions were finished, the supernatants were obtained by centrifugation (10,000 rpm, 10 min). The supernatants (20 μl) were electrophoresed on 6 M urea-gel. The siderophore activity of the culture supernatant was verified by CAS agar diffusion assay (Fig. 5B). The culture supernatant was then ultrafiltrated (Vivaspin, MWCO 10,000, Sartorius, Germany) in order to obtain the crude fraction of the low-molecular-weight siderophores. HT (0.5 mg/ml) was added to the culture ultrafiltrate, and incubated at 37°C for 0, 10, 30, and 60 min. After completion of the reaction, 20

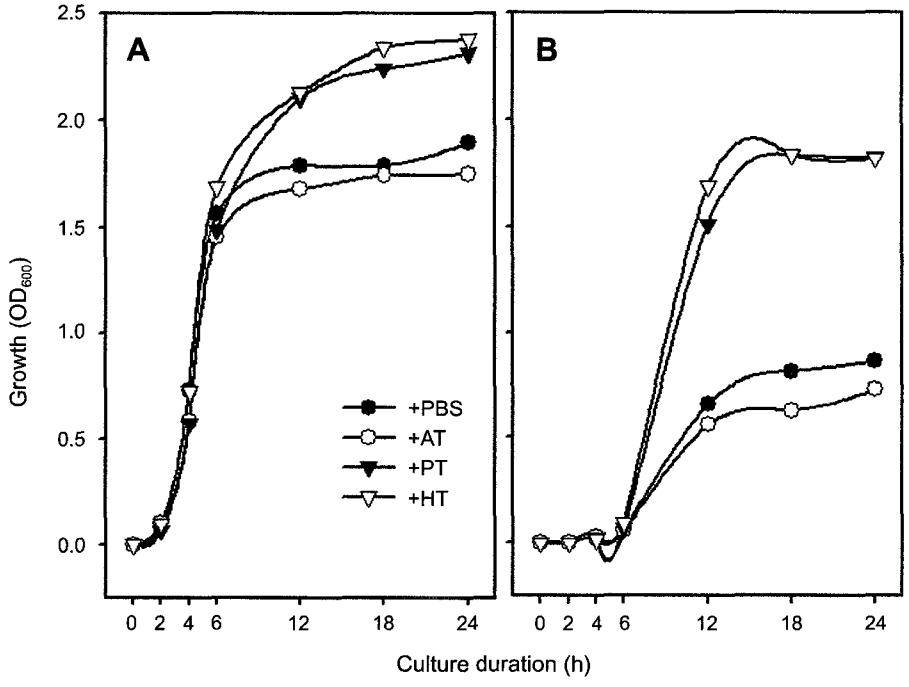

Fig. 1. Utilization of transferrin-bound iron. *Staphylococcus aureus* was cultured in deferrated BHI (A) and MM9 (B) containing phosphate-buffered saline (PBS), human apotransferrin (AT), partially iron-saturated transferrin (PT) and holotransferrin (HT). The culture fluids were withdrawn for monitoring of growth by the measurement of optical density at a wavelength of 600 nm at the indicated times. Of triplicate experiments with similar results, a representative is shown.

μl of the reaction mixture was electrophoresed on 6 M urea-gel.

Bioassay

About 1×10^4 cfu/ml of the preconditioned bacterium was spread onto the surface of MM9 agar containing 0.5 mg/ml of PT, and then the discs containing 30 μl of the culture ultrafiltrate, and the ultrafiltrate of the uninoculated DF-BHI with PBS and deferoxamine (100 μM), designated as our negative and positive controls, were placed onto the agar surface. The agar plates were incubated at 37°C for 24 h.

Results and Discussion

Utilization of transferrin-bound iron for S. aureus growth

When cultured in DF-BHI and MM9 broths containing AT, PT, and HT, *S. aureus* was observed to grow more actively in media containing PT and HT than in media containing AT (Fig. 1). AT, however, did not stimulate the growth of *S. aureus* when supplied to DF-BHI and MM9. No significant differences were noted between PT and HT in the DF-BHI, and this held true for the MM9 as well. Therefore, further experiments in this study were carried out in DF-BHI, as the growth of *S. aureus* in the MM9 containing AT was so severely retarded that the production of siderophores and the expression of transferrin-binding protein were below our experimental threshold of detection. In order to observe the utilization of transferrin-bound iron, equal volumes (20 μl) of the culture supernatants were electrophoresed on 6 M urea gel (Fig. 2). The removal of iron from AT was negligible, whereas the removal of iron from PT and HT increased consistently with bacterial growth. Most transferrin-bound iron was consumed during the exponential growth phase. *S. aureus* was able to utilize iron from HT more easily than from PT. Similar results were shown with the MM9 (data not shown). Despite the fact that the removal of iron from AT was min, *S. aureus* still was able to grow to some extent in the DF-BHI with AT. This was thought to be attributable to the residual iron in the DF-BHI (less than 1 μM).

It has been well established that *S. aureus* is able to utilize transferrin-bound iron (Brock *et al.*, 1991; Modun *et al.*, 1994; Lindsay *et al.*, 1995; Lim *et al.*, 1998), and our present study corroborated this. It has also been reported that the affinity of transferrin for iron can be altered by changes in pH, which is a consequence of metabolic activity (Conrad, 2001). Measurement of pH in the culture supernatants showed that the pH dropped from 7.0 to 6.5 in the early exponential growth phase and dropped further to 6.0 in the late growth phase (Fig. 2). In a separate experiment designed to determine the effect of pH on the liberation of iron from HT, release of iron from HT was observed only at pH 6.0 when HT was added into the DF-BHI, the pH having been adjusted to 7.0, 6.5, and 6.0

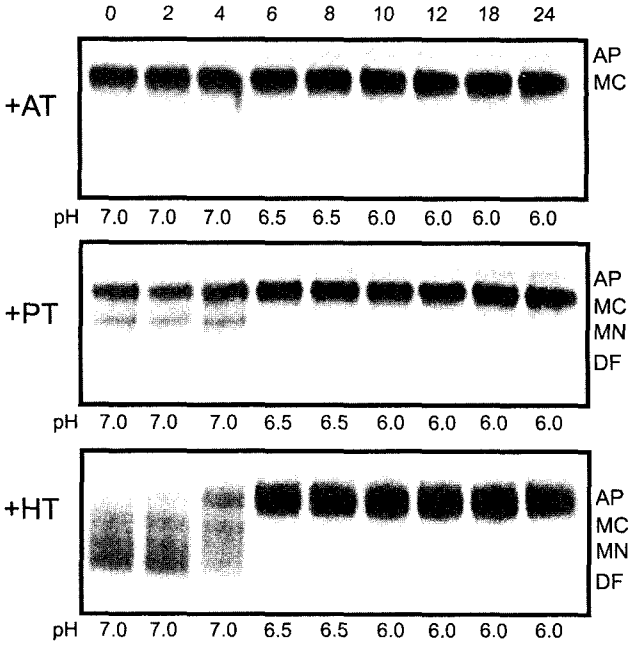

Fig. 2. Iron acquisition from human transferrin. Bacterial growth in the deferrated BHI with AT, PT and HT is shown in Fig. 1. During the cultures, the culture supernatants were obtained at the indicated times (top side). The 20 μl of the culture supernatants obtained were electrophoresed on 6 M urea-gel and visualized by Coomassie-blue staining. Four forms of transferrin were indicated, according to the level of iron-saturation: apo- (AP), C-terminal monoferric- (MC), N-terminal monoferric- (MN) and diferric (DF) forms. The pHs of the culture supernatants were shown. Of triplicate experiments with similar results, a representative is shown.

(data not shown). Therefore, it was concluded that the consumption of most of the transferrin-bound iron in the early exponential growth phase could not have been the result of the slight change in pH from 7.0 to 6.5. The lower pH (around 6.0) in the late growth phase, however, may have affected iron removal rates.

S. aureus extracellular proteases are not involved in the iron-uptake from transferrin

The known major extracellular proteases produced by *S. aureus* include serine protease, cysteine proteases, and aureolysin. *In vitro* studies have shown that the proteases of *S. aureus* induce the cleavage and degradation of several host proteins, implicating bacterial proteases as factors for the bacterial circumvention of the host's defenses (Potempa, 1988). Moreover, it has been suggested that other bacterial extracellular proteases are involved in iron-uptake from transferrin, via the siderophore-mediated iron acquisition system (Okuzo *et al.*, 1996). If transferrin is degraded by these extracellular proteases, the specific interaction between transferrin and IsdA might be inhibited, and transferrin-bound iron could be utilized via siderophores, rather than by IsdA. In the present study, however, the destruction of transferrin was not observed

regardless of the production of extracellular proteases (Fig. 3), implying that the extracellular proteases of *S. aureus* appear not to be involved in the liberation of iron from transferrin. The absence of transferrin degradation does not prove the involvement of IsdA in the uptake of iron from transferrin, as siderophores can capture iron from intact transferrin (Modun *et al.*, 1998). Rather, this indicates that *S. aureus* can uptake iron from transferrin without the assistance of proteases. Results similar to or consistent with these have also been reported by other researchers (Lindsay *et al*, 1995).

Removal of iron from transferrin was correlated with the production of siderophores, but not with the expression of IsdA

If transferrin is able to bind to the cell surface of *S. aureus* via IsdA, the level of transferrin in the culture supernatants should gradually decrease, being consumed more quickly with increased bacterial growth. In the present study, however, when equal volumes of the culture supernatants were electrophoresed, no tapering of transferrin bands was observed, regardless of the degree to which the transferrin was iron-saturated (Fig. 3). It is notable that no tapering of the transferrin bands was observed even though IsdA was expressed on the cell wall in the DF-BHI containing AT (Fig. 4A).

Some controversy surrounds the issue of the iron-uptake of *S. aureus* via the binding of transferrin to the cell surface. Lindsay et al. (Lindsay *et al.*, 1995) reported that *S. aureus* was able to remove iron from ^{55}Fe-labelled transferrin via a process which did not require the binding of transferrin to the cell surface of *S. aureus*, indicating the siderophore-mediated acquisition of iron from transferrin. However, other researchers (Brock *et al.*, 1991) have reported that *S. aureus* was able to utilize transferrin-bound iron when the bacterial cells were separated from transferrin by a dialysis membrane, but only insufficiently. Similar results were also observed in our previous study (Lim *et al.*, 1998). Moreover, Modun *et al.* (1994 and 1998) observed the removal of iron from transferrin by the whole cells of *S. aureus* and *S. epidermidis*. However, it has also been well established that *S. epidermidis*, which has been observed to express a greater quantity of transferrin-binding protein than *S. aureus* (Modun *et al.*, 1994, 1998), is unable to utilize low iron-saturated transferrin (Lindsay *et al.*, 1995; Martinaho *et al.*, 2001). In our other study, *S. epidermidis* was found to be able to utilize low iron-saturated iron, but only when exogenous siderophores were supplemented (data not shown). Therefore, it was thought that the inability of *S. epidermidis* to utilize low iron-saturated transferrin was due to the insufficiency of the siderophore-mediated iron-uptake system, regardless of the expression of transferrin-binding protein. Taken together, these results suggest that the IsdA-mediated iron-acquisition system is not essential for staphylococcal growth on transferrin-bound iron.

Moreover, in the present study, IsdA was expressed only in the DF-BHI containing AT, and not in the DF-BHI

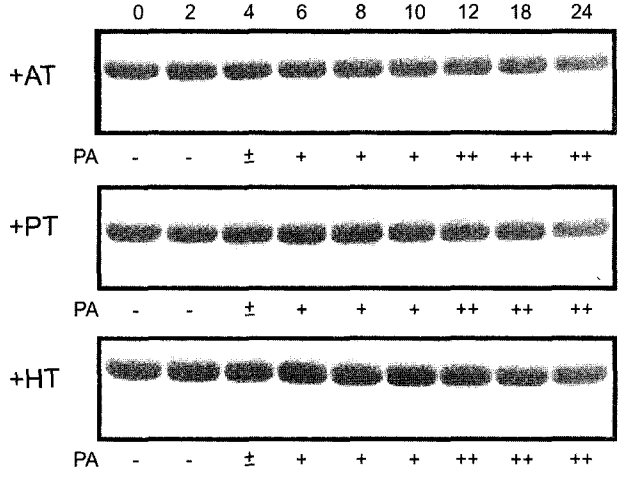

Fig. 3. No binding of transferrin to whole cells and no destruction of transferrin by extracellular proteases. Bacterial growth in the deferrated BHI with AT, PT and HT is shown in Fig. 1. During culturing, the culture supernatants were obtained at the indicated times (top side). The 20 μl of the culture supernatants were electrophoresed on 10% polyacrylamide gel and visualized by Coomassie-blue staining. Protease activities (PA) in the culture supernatants were measured on skim milk agar, as described in Materials and Methods. Of triplicate experiments with similar results, a representative is shown.

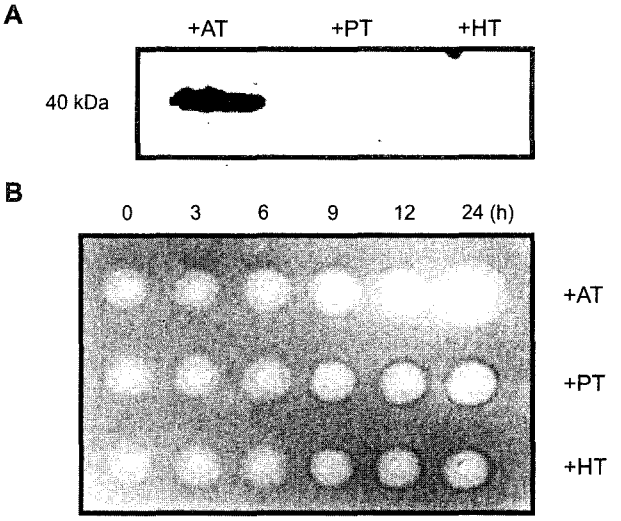

Fig. 4. Expression of cell wall *IsdA* (A) and production of siderophores (B). Bacterial growth in the deferrated BHI with AT, PT and HT is shown in Fig. 1. A: Cell wall proteins were isolated from the bacterial pellets obtained at 12 h during the culture, and 100 μg of cell wall proteins were electrophoresed and transferred to nitrocellulose membrane. Receptor-ligand binding assay was performed using human transferrin conjugated with horseradish-peroxidase. B: Total siderophore activities in the culture supernatants obtained at the indicated times (top side) were measured by CAS agar diffusion assay. Of triplicate experiments with similar results, a representative is shown.

containing either PT or HT (Fig. 4A). By way of contrast, siderophores were produced in the DF-BHI containing HT and PT as well as in the DF-BHI containing AT (Fig. 4B). Due to the iron-chelating activity of DF-BHI itself and the sensitivity of the CAS agar diffusion assay, siderophore production was masked in the early growth phase, but became detectable only in the late growth phase (Courcol *et al.*, 1997). The development of more sensitive and stable methods capable of measuring a very small amount of siderophores in complex media is necessary. More siderophores were produced in the DF-BHI containing AT than in the DF-BHI containing PT or HT. Due to the high affinity of transferrin for iron (Conrad, 2001), the DF-BHI containing PT or HT can also be considered to be relatively iron-restricted. Therefore, it was thought that considerable amounts of siderophores were also produced in the DF-BHI containing PT or HT. In previous studies (Lim *et al.*, 1998; Taylor and Heinrichs, 2002; Lim *et al.*, 2004), it was demonstrated that both the expression of IsdA and the production of siderophores were iron-repressible, indicating Fur regulation (Xiong *et al.*, 2000). Therefore, our results indicated that concentrations of intracellular iron increased as a result of the siderophore-mediated iron acquisition system, which played an essential and dominant role in iron-uptake from transferrin, and the increased intracellular iron concentrations repressed the expression of IsdA via Fur regulation in the DF-BHI containing PT and HT.

Removal of iron from transferrin via siderophores but not with whole cells expressing IsdA

In order to observe directly whether the removal of iron from transferrin could occur via IsdA, the washed whole cells of *S. aureus* expressing IsdA on the cell surface (Fig. 5A), were allowed to react with HT. In this case, the removal of iron from HT was not observed (Fig. 5C). This result was not in accord with the results reported by Modun *et al.* (1994). According to their results, when *S. aureus* was incubated in PBS supplemented with glucose, it converted diferric transferrin into its apotransferrin form within 30 min. In this study, however, PBS was replaced with Tris buffer, as we believed that the phosphates in the PBS might affect the removal of iron from transferrin (Leong and Neilands, 1982). This discrepancy might, then, be due to the differing phosphate concentrations in the buffers. In contrast, when the culture ultrafiltrate containing siderophores (Fig. 5B) was allowed to react with HT, most of the iron was removed from the HT within 10 min (Fig. 5D). Similar results were observed by Modun *et al.* (1994). They reported that the removal of iron from transferrin occurred more rapidly when completed by staphyloferrin A, a siderophore produced by staphylococci, than by the washed whole cells. Overall, these results demonstrated that the siderophore-mediated iron-acquisition system plays a dominant or essential role in the uptake of iron from transferrin, whereas the IsdA-mediated iron-acquisition system, if it is involved at all, plays only an ancillary role in the uptake of iron from transferrin.

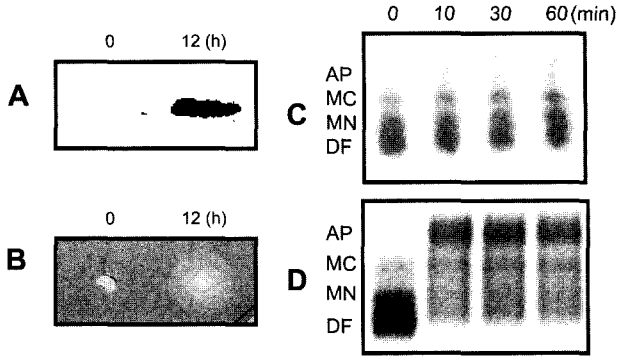

Fig. 5. Removal of iron from human holotransferrin by siderophores but not by washed whole cells expressing IsdA. C: the washed whole cells (1×10^{10} cfu/ml) expressing IsdA (A) were allowed to react with 0.5 mg/ml of holotransferrin at 37°C for 0, 10, 30 and 60 min. D: The culture ultrafiltrate with siderophore activity (B) was allowed to react with holotransferrin under the similar conditions. After the completion of the reaction, 6 M urea-gel electrophoresis was performed. Of triplicate experiments with similar results, a representative is shown.

Growth stimulation of S. aureus on transferrin-bound iron by siderophores only

In order to observe whether the growth of *S. aureus* could be stimulated using only siderophores, bioassays using the ultrafiltrates of the cultures exhibiting siderophore activity were performed. According to our observations, the growth of *S. aureus* was stimulated only in the area surrounding the discs containing deferoxamine, as well as the culture ultrafiltrate with siderophore activity, in the MM9 containing PT (Fig. 6). Similar results were observed, even in the MM9 containing AT as well as HT (data not shown). These results indicated that *S. aureus* possesses the ability to utilize transferrin-bound iron for growth via only the siderophore-mediated iron-acquisition system, and completely independently of the IsdA-mediated iron-acquisition system.

S. aureus has the rare characteristic of a well-developed siderophore-mediated iron-acquisition system. *S. aureus* is able to produce and utilize a variety of siderophore types. Due to the efficient and effective siderophore-mediated iron-acquisition system, *S. aureus* is able to grow, even under severely iron-restricted conditions (Trivier and Courcol, 1996). More recently, it was reported that an isogenic *sbnE*-negative mutant strain exhibiting defective siderophore production, also evidenced a marked growth deficiency in iron-restricted media, and in a murine kidney abscess model (Dale *et al.*, 2004). In addition, in our other study, a streptonigrin-resistant strain of *S. aureus* exhibited delayed siderophore production, the inability to capture iron from PT, and a growth deficiency in the DF-BHI containing PT, as well as in human peritoneal dialy-

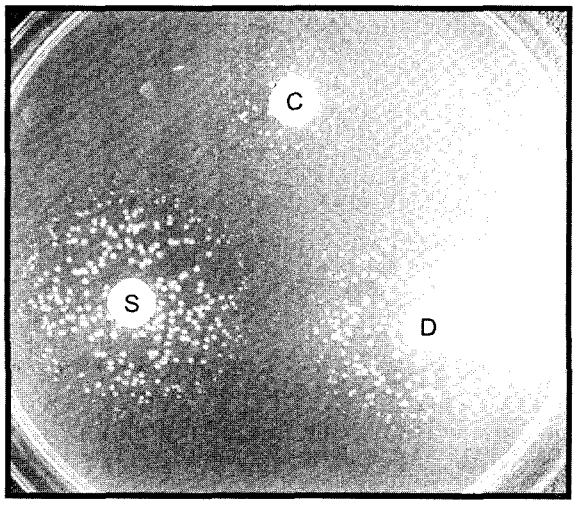

Fig. 6. Stimulation of growth by siderophores. About 1×10^4 cfu/ml of the preconditioned S. aureus was spread onto MM9 agar containing 0.5 mg/ml of partially iron-saturated transferrin, and then a disc containing 30 μl of the culture ultrafiltrate with siderophore activity (S; refer to Fig. 5B), and the ultrafiltrate of the uninoculated BHI with/without 100 μM of deferoxamine (D and C), used as the positive/negative controls, were placed on the surface of the agar, and the agar plate was incubated at 37°C for 24 h. Of triplicate experiments with similar results, a representative is shown.

sate solution, an *ex vivo* experimental model of *S. aureus* infections (Park *et al.*, 2005). These results clearly illustrate that the siderophore-mediated iron-uptake system plays an important role with regard to both the uptake of iron from transferrin, and also the growth of *S. aureus in vivo* and *in vitro*.

Other controversies surrounding the role of IsdA

Other controversies have surrounded IsdA's role as a transferrin-receptor for iron acquisition. The normal iron-saturation level of transferrin is about 30% in the human body, and the binding of transferrin to IsdA is not affected by the iron-saturation level of transferrin (Modun *et al.*, 1994). This fact indicates that the iron-uptake from transferrin via IsdA can be rendered inefficient, as a result of the competition for IsdA between iron-unbound and iron-bound transferrins. In addition, IsdA is expressed *in vivo*, and sera from patients suffering from staphylococcal infections contain antibodies against IsdA, which have proven able to block the binding of transferrin to IsdA (Lim *et al.*, 1998; Modun *et al.*, 1998). This fact also indicates that the uptake of iron from transferrin via IsdA can be inefficient during human infection. Moreover, IsdA shares no significant sequence homology with any other bacterial transferrin-receptors (Taylor and Heinrichs, 2002), and, recently, IsdA has also become known as one of the iron-regulated surface determinants which functions in the uptake of heme iron from the hemoproteins (Mazmanian *et al.*, 2003). More recently, Clarke *et al.* (2004) demonstrated that IsdA was able to bind many different human ligands, including transferrin and fibronectin, providing some evidence that the IsdA binding of transferrin was not specific for the mediation of iron removal from transferrin. All these facts demonstrate that IsdA does not play an important role in the uptake of iron from transferrin inside the human body.

In conclusion, the siderophore-mediated iron-acquisition system of *S. aureus* plays a dominant or essential role in the uptake of iron from transferrin, whereas the IsdA-mediated iron-acquisition system, if it is involved at all, plays only an ancillary role in the uptake of iron from transferrin. Therefore, our results suggest that the siderophore-mediated iron-acquisition system can be a new target for the development of therapeutic agents or preventive vaccines against *S. aureus*.

Acknowledgements

This study was supported by grants (R13-2004-009) from the Ministry of Science and Technology, Korea, and from the Korea Science and Engineering Foundation through the Research Center for Resistant Cells. We also thank Dr. C.S. Ha of the Hanmaum Hospital (Changwon 641-784, Gyeongnam, Republic of Korea) for his helpful discussion.

References

Andrews, S.C., A.K. Robinson, and F. Rodriguez-Quinones. 2003. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27, 215-237.

Braun, V. and M. Barun. 2002. Active transport of iron and siderophore antibiotics. *Curr. Opin. Microbiol.* 5, 194-201.

Brock, J.H., P.H. Williams, J. Liceaga, and K.G. Woolridge. 1991. Relative availability of transferrin-bound iron and cell-driven iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms. *Infect. Immun.* 59, 3185-3190.

Brown, J.S. and D.W. Holden. 2002. Iron acquisition by Gram-positive bacterial pathogens. *Microbes Infect.* 4, 1149-1156.

Bullen, J., E. Griffiths, H. Rogers, and G. Ward. 2002. Sepsis: the critical role of iron. *Microbes Infect.* 2, 409-415.

Cabrera, G., A. Xiong, M. Uebel, V.K. Singh, and R.K. Jayaswal. 2001. Molecular characterization of the iron-hydroxamate uptake system in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 67, 1001-1003.

Clarke, S.R., M.D. Wiltshire, and S.J. Foster. 2004. IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesion. *Mol. Microbiol.* 51, 1509-1519.

Conrad, M.E. 2001. Iron: seminal publications of the twentieth century-transferrin. *J. Tra. Ele. Exp. Med.* 14, 115-117.

Courcol, R.J., D. Trivier, M.C. Bissinger, G.R. Martin, and M.R. Brown. 1997. Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. *Infect. Immun.* 65, 1944-1948.

Crosa, J.H. 1997. Signal transduction and transcriptional and post-transcriptional control of iron-regulated genes in bacteria.

Microbiol. Mol. Biol. Rev. 61, 319-336.

Dale, S.E., A. Doherty-Kirby, G. Lajoie and D.E. Heinrichs. 2004. Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: Identification and characterization of genes involved in production of a siderophore. *Infect. Immun.* 72, 29-37.

Drechsel, H., S. Freund, G. Nicholson, H. Haag, O. Jung, H. Zahner, and G. Jung. 1993. Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci. *Biometals* 6, 185-192.

Dryla, A., D. Gelbmann, A. Von Gabain, and E. Nagy. 2003. Identification of a novel iron regulated staphylococcal surface protein with haptoglobulin-hemoglobin binding activity. *Mol. Microbiol.* 49, 37-53.

Emori, T.G. and R.P. Gaynes. 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin. Microbiol. Rev.* 6, 428-442.

Konetschny-Rapp, S., G. Jung, J. Meiwes, and H. Zahner. 1990. Staphyloferrin A: a structurally new siderophore from staphylococci. *Eur. J. Biochem.* 191, 65-74.

Leong, S.A. and J.B. Neilands. 1982. Siderophore production by phytopathogenic microbial species. *Arch. Biochem. Biophys.* 218, 351-359.

Lim, Y., S.H. Shin, I.Y. Jang, J.H. Rhee, and I.S. Kim. 1998. A human transferrin-binding protein of *Staphylococcus aureus* is immunogenic *in vivo* and has an epitope in common with human transferrin receptor. *FEMS Microbiol. Lett.* 166, 225-230.

Lim, Y., S.H. Shin, S.I. Lee, I.S. Kim, and J.H. Rhee. 1998. Iron-repressibility of siderophore and transferrin-binding protein in *Staphylococccus aureus*. *FEMS Microbiol. Lett.* 163, 19-24.

Lim, Y., S.Y. Cho, N.S. Cho, S.I. Lee, J.Y. Chung, C.H. Chung, and S.H. Shin. 2004. Oxygen stimulates the expression of iron-repressible high-affinity iron-uptake systems of *Staphylococcus aureus*-Application of CAS agar diffusion assay. *Infection and Chemotherapy* 36, 32-39.

Lindsay, J.A. and T.V. Riley. 1994. Staphylococcal iron requirements, siderophore production, and iron-regulated protein expression. *Infect. Immun.* 62, 2309-2314.

Lindsay, J.A., T.V. Riley, and B.J. Mee. 1995. *Staphylococcus aureus* but not *Staphylococcus epidermidis* can acquire iron from transferrin. *Microbiology* 141, 197-203.

Makey, D.G. and U.S. Seal. 1988. The detection of four molecular forms of human transferrin during the iron binding process. *Biochem. Biophys. Acta* 453, 250-256.

Martinaho, S., L. von Bonsdorff, A. Rouhiainen, M. Lonnroth and J. Parkkinen. 2001. Dependence of *Staphylococcus epidermidis* on non transferrin-bound iron for growth. *FEMS Microbiol. Lett.* 196, 177-182.

Mazmanian, S.K., E.P. Skaar, A.H. Gaspar, M. Humayun, P. Gornicki, J. Jelenska, A. Joachmiak, D.M. Missiakas, and O. Schneewind. 2003. Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science* 299, 906-909.

Modun, B.J., A. Cockayne, R. Finch, and P. Williams. 1998. The *Staphylococcus aureus* and *Staphylococcus epidermidis* transferrin-binding proteins are expressed *in vivo* during infection. *Microbiology* 144, 1005-1012.

Modun, B., D. Kendall, and P. Williams. 1994. *Staphylococci* express a receptor for human transferrin: Identification of a 42-kilodalton cell wall transferrin-binding protein. *Infect. Immun.* 62, 3850-3858.

Modun, B. and P. Williams. 1999. The staphylococcal transferrin-binding protein is a cell wall glyceraldehydes-3-phosphate dehydrogenase. *Infect. Immun.* 67, 1086-1092.

Modun, B., R.W. Evans, C.L. Joannou, and P. Williams. 1998. Receptor-mediated recognition and uptake of iron from human transferrin by *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect. Immun.* 66, 3591-3596.

Morrissey, J.A., A. Cockayne, P.J. Hill, and P. Williams. 2000. Molecular cloning and analysis of a putative siderophore ABC transporter from *Staphylococcus aureus*. *Infect. Immun.* 68, 6281-6288.

Okuzo, N., T. Akiyama, S. Miyoshi, S. Shinoda, and S. Yamamoto. 1996. Involvement of vulnibactin and exocellular protease in utilization of transferrin-and lactoferrin-bound iron by *Vibrio vulnificus*. *Microbiol. Immunol.* 40, 595-598.

Park, R.Y., H.Y. Sun, M.H. Choi, Y.H. Bae, and S.H. Shin. 2005. Growth of *Staphylococcus aureus* with defective siderophore production in human peritoneal dialysate solution. *J. Microbiol.* 43, 54-61.

Potempa, J., A. Dubin, G. Korzus, and J. Travis. 1988. Degradation of elastin by a cysteine proteinase from *Staphylococcus aureus*. *J. Biol. Chem.* 263, 2664-2667.

Ratledge, C. and L. Dover. 2000. Iron metabolism in pathogenic bacteria, *Ann. Rev. Microbiol.* 54, 881-941.

Rybak, M.J. and R.L. Akins. 2001. Emergence of methicillin-resistant *Staphylococcus aureus* with intermediate glycopeptide resistance: clinical significance and treatment options. *Drugs* 61, 1-7.

Schwyn, B. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47-56.

Sebulsky, M.T. and D.E. Heinrichs. 2001. Identification and characterization of *fhuD1* and *fhuD2*, two genes involved in iron-hydroxamate uptake in *Staphylococcus aureus*. *J. Bacteriol.* 183, 4994-5000.

Sebulsky, M.T., D. Hohnstein, M.D. Hunter, and D.E. Heinrichs. 2000. Identification and characterization of a membrane permease involved in iron-hydroxamate transport in *Staphylococcus aureus*. *J. Bacteriol.* 182, 4394-4400.

Shin, S.H., Y. Lim, S.E. Lee, N.W. Yang, and J.H. Rhee. 2001. CAS agar diffusion assay for the measurement of siderophores in biological fluids. *J. Microbiol. Methods* 44, 89-95.

Sieradzki, K., R.B. Roberts, S.W. Haber, and A.Tomasz. 1999. The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. *N. Engl. J. Med.* 340, 517-523.

Skaar, E.P. and O. Schneewind. 2004. Iron-regulated surface determinants (Isd) of *Staphylococcus aureus*: stealing iron from heme. *Microbes Infect.* 6, 390-397.

Taylor, J.M. and Heinrichs, D.E. (2002) Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Mol. Microbiol.* 43, 1603-1614.

Trivier, D. and R.J. Courcol. 1996. Iron depletion and virulence in *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 141, 117-127.

Xiong, A., V.K. Singh, G. Cabrera, and R.K. Jayaswal. 2000. Molecular characterization of the ferric-uptake regulator, Fur, from *Staphylococcus aureus*. *Microbiology* 146, 659-668.