

## Growth and Siderophore Production of Staphylococci in Human Peritoneal Dialysate

Although activity of iron uptake system (IUS) was thought to play an important role in staphylococcal growth in human peritoneal dialysate (HPD) solution, siderophore production, one of the well-known IUS, was not yet detected directly in HPD solution. Therefore, we tried to detect siderophore production directly in HPD solution by using a newly developed chrome azurol S (CAS) agar diffusion assay and to investigate the effect of IUS activity on bacterial growth in HPD solution. According to the susceptibility test for streptonigrin and the productivity of siderophore in the iron-deficient (ID) medium, *Staphylococcus aureus* ATCC 6538 strain and *Staphylococcus epidermidis* clinical isolate had higher IUS activity and grew better than *S. aureus* ATCC 25923 strain in the ID medium. These bacteria did not grow and produce siderophore in the unused chronic ambulatory peritoneal dialysis solution. However, these bacteria grew and produced siderophore in the HPD solution. Moreover, *S. aureus* ATCC 25923 strain with lower activity of IUS grew poorly and produced smaller amount of siderophore in HPD compared to *S. aureus* ATCC 6538 strain and *S. epidermidis* clinical isolate with higher activity of IUS like in the ID medium. To the best of our knowledge, this is the first report that siderophore production is directly detected in the HPD by CAS agar diffusion assay. These results indicated that activity of IUS plays an important role in bacterial growth in the HPD solution and pathogenesis of continuous ambulatory peritoneal dialysis peritonitis.

**Key Words :** *Staphylococcus aureus*; *Staphylococcus epidermidis*; Siderophores; Peritonitis; Peritoneal Dialysis

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## INTRODUCTION

*Staphylococcus epidermidis* and, to lesser extent, *Staphylococcus aureus* are commonly responsible for peritonitis in patients with end-stage renal diseases undergoing continuous ambulatory peritoneal dialysis (CAPD) (1). Although staphylococci cannot grow in commercial peritoneal dialysis solutions, these fluids are modified during dialysis and become enriched by a plasma ultrafiltrate. This modified human peritoneal dialysate (HPD) can support staphylococcal growth (2-4). However, like other extracellular body fluids, HPD represents a severely iron-restricted environment for any infecting pathogen. Most bacteria including staphylococci respond to the environmental cue of restricted iron availability by derepressing high affinity iron uptake systems (IUS), such as siderophore mediated and transferrin-binding protein-mediated IUS (5, 6).

In the context of peritonitis, pathogenesis of staphylococcal infections is likely to be related, at least in part, to the ability of the organism to uptake iron within HPD. Staphylococci were reported to express both IUS, siderophore-mediated and transferrin receptor-mediated IUS, in the iron-deficient (ID) laboratory media (6). However, whether IUS of staphy-

lococci was expressed in HPD is not yet verified directly. Recently, we developed a new method, chrome azurol S (CAS) agar diffusion assay, for measurement of siderophore produced in biological fluids as well as in synthetic minimal media (7). Therefore, we tried to verify directly the production of siderophore in HPD via the new method CAS agar diffusion assay and to investigate the effect of IUS activity on bacterial growth in HPD solution.

## MATERIALS AND METHODS

### Bacteria, Media and HPD solution

Coagulase-positive *S. aureus* ATCC 6538 and ATCC 25923 strains, and coagulase-negative *S. epidermidis* clinical isolate were used. Streptonigrin susceptibility of the two *Staphylococcus aureus* strains was evaluated by conventional broth dilution method for antimicrobial susceptibility test. The three bacteria were enriched in BHI broth. ID medium was prepared from BHI broth [iron-sufficient (IS) medium] according to the method of Lim et al. (6). Residual iron concentra-

tions of ID and IS media were measured by Inductively Coupled Plasma Emission Spectrophotometer (JY 70PLUS, Jobin Yvon Co., France) and 16.85 and 0.2  $\mu\text{M}$ , respectively. HPD solutions (Daniel PD-2 Peritoneal Dialysis Solution with 1.5% Dextrose, FNB9866, Daxter Healthcare PTE LTD, Singapore) were obtained after dialysis from the Department of Nephrology in Chosun University Hospital. Five samples of HPD solutions were pooled immediately before use, and one unused CAPD solution before dialysis was used as the control for comparison with HPD solutions. Iron concentrations of the unused CAPD solution and HPD solution were very low and undetectable levels. Total protein levels (mean  $\pm$  standard error) of the unused CAPD solution and HPD solution were 0 and  $0.0267 \pm 0.0033$  g/dL, respectively, and glucose levels were  $1183.33 \pm 6.01$  (mg/dL) and  $453.00 \pm 3.88$ , respectively.

### Culture condition and growth monitoring

About  $10^5$  cfu/mL of the three bacteria, grown in the BHI containing 0.2 mM dipyriddy, was inoculated into the iron-deficient medium, HPD solution and unused CAPD solution, respectively, and shaking-cultured (150 rpm) at 37°C for 24 hr. At the appropriate intervals, culture fluids were withdrawn and then their optical densities were measured at 600 nm of wavelength. Other culture fluids were centrifuged at 5,000 rpm for 5 min, and then their supernatants were used for siderophore detection.

### CAS agar diffusion assay

CAS agar diffusion assay was performed according to the method of Shin et al. (7) without modification. The modified CAS agar plate was punched with 5-mm-diameter holes by using gel puncher. Each hole was filled with 35  $\mu\text{L}$  of the culture supernatants. After incubation of the plate at 37°C for 4-8 hr, the size of orange halo formed around each hole was measured. Siderophore activity was expressed as the square value of the halo diameter. All experiments were carried out at least three times with similar results, and a representative result was shown.

## RESULTS

Although all staphylococci used in this study grew better in IS medium than in ID medium, siderophore was produced more in the ID medium than in IS medium. And there was shown a more remarkable difference in growth and siderophore production between the two strains in the ID medium than in IS medium. Growth of staphylococci in ID medium was accorded with siderophore production. *S. aureus* ATCC 6538 strain was more susceptible than the 25923 strain against streptonigrin (Table 1), which was known to show stronger bactericidal activity for bacteria with higher IUS activity than

**Table 1.** Streptonigrin susceptibility of the two *Staphylococcus aureus* strains

Strains	MIC (ng/mL)	MBC (ng/mL)
ATCC 25923	7.81	7.81
ATCC 6538	1.95	3.91

MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration.

for bacteria with lower IUS activity (8).

These susceptibilities against streptonigrin indicated that 6538 strain possessed higher activity of IUS compared to 25923 strain. Actually 6538 strain produced larger amount of siderophore than 25923 strain in ID medium as well as in IS medium, moreover, 6538 strain grew more actively than 25923 strain in ID medium (Fig. 1). When both strains were cultured in unused CAPD solution and HPD solution of which iron concentrations were undetectably low, 6538 strain with higher IUS activity grew more actively than 25923 strain with lower IUS activity in the HPD solution. The 6538 strain grew more actively in HPD solution than in unused CAPD solution, while the 25923 strain did not show difference of growth in both solutions (Fig. 2).

Growth of both strains in HPD was accorded with siderophore production. Siderophore production of the 6538 strain in the HPD solution continued to increase after the initiation of growth. However, siderophore production in the unused CAPD solution was not detectable. Like *S. aureus* ATCC 6538, *S. epidermidis* clinical isolate showed similar patterns of growth and siderophore production (Fig. 1, 2). *S. epidermidis* grew more actively and produced more siderophore in the HPD solution than in the unused CAPD solution.

## DISCUSSION

Peritonitis is a common complication of CAPD with two-thirds of patients developing peritonitis during the first year of dialysis. Coagulase-negative staphylococci are the most commonly identified pathogen, accounting for 40 to 60% of all positive cultures, followed by *S. aureus* and streptococci (10 to 20 % each) (1, 9, 10).

Both *S. epidermidis* and *S. aureus* are commonly responsible for peritonitis in renal patients undergoing CAPD. In the context of CAPD peritonitis, the pathogenesis of staphylococcal infection is likely to be related to the ability of the organism to multiply within the dialyzed peritoneum. Although staphylococci cannot grow in commercial peritoneal dialysis solutions, these fluids are modified during dialysis and become enriched by a plasma ultrafiltrate. This modified HPD contains several human serum proteins, including albumin, transferrin, and immunoglobulin, and can support staphylococcal growth (2-4) as shown in our results.

Thus, in common with other extracellular body fluids, HPD

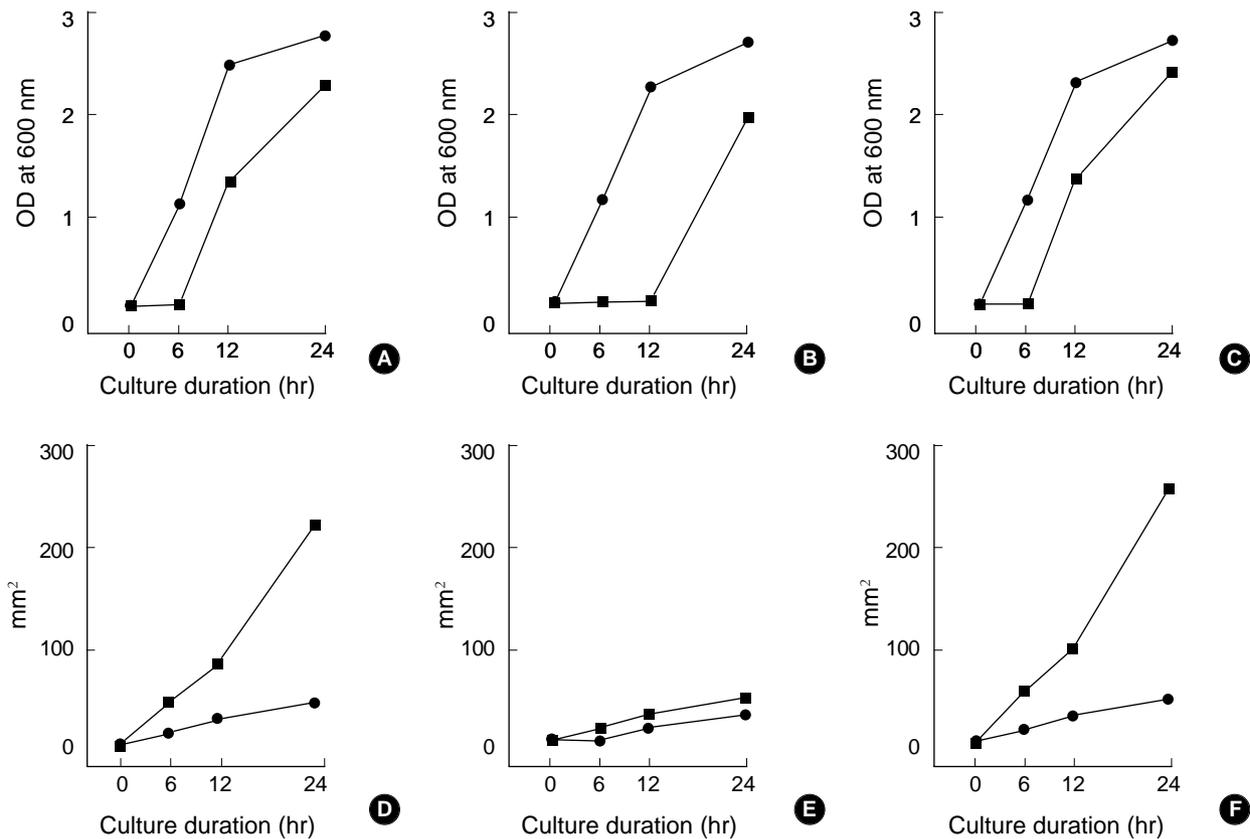


Fig. 1. Growth and siderophore production of staphylococci in conventional laboratory medium. A, B and C indicate growth of *S. aureus* ATCC 6538 (A), *S. aureus* ATCC25923 (B) and *S. epidermidis* clinical isolate (C) in the iron-sufficient medium (●) and iron-deficient medium (■), respectively. D, E and F indicate siderophore production of *S. aureus* ATCC 6538 (D), *S. aureus* ATCC25923 (E) and *S. epidermidis* clinical isolate (F) in the iron-sufficient medium (●) and iron-deficient medium (■), respectively.

represents a severely iron-restricted environment for any infecting pathogen. Iron availability is low in vivo in spite of large amounts being present. Most extracellular iron found in body fluids such as plasma and mucosal secretions is bound to the high-affinity iron-binding glycoproteins such as transferrin and lactoferrin, resulting in little free iron being available. The restricted availability of iron impacts significantly on many biological systems, including survival and proliferation of bacterial pathogens in host environments. Most bacteria respond to the environmental cue of restricted iron availability by derepressing siderophone-mediated iron transport systems (11).

Previously, other researchers (4, 5, 12, 13) have shown that growth of staphylococci in HPD resulted in the induction of two immunodominant, iron-regulated cytoplasmic membrane proteins. These iron-regulated proteins were thought to be putative siderophore receptors and also expressed by *S. epidermidis* grown in vitro in iron-restricted nutrient broth, and in vivo in a chamber implanted intraperitoneally in rats. Some of these proteins are also shared by *S. aureus*. Both *S. aureus* and *S. epidermidis* have been reported to produce siderophores in vitro in the iron-restricted media (6, 14-16).

However, it is not clear until now whether IUS of staphylococci are expressed in vivo including HPD solution. Our previous studies (17) revealed that transferrin receptor of *S. aureus* was expressed in body fluids such as ascitic fluid obtained from liver cirrhosis patients and pleural effusion obtained from pleurisy patients with *Mycobacterium tuberculosis*. Siderophore production of staphylococci, however, could not be detected in these body fluids although the new CAS agar diffusion assay was applied. We thought that complex components of body fluids interfered with siderophore detection. Actually most researchers could detect siderophore production only in chemically defined minimal media (14, 18). In the context of CAPD peritonitis, HPD solution is a transudate that mimics human body fluids and is not as complex as human body fluids, and is relatively good enriched medium for bacterial growth, compared to the unused CAPD solution. Therefore, HPD solution can be used as a valuable 'ex vivo' system for studying IUS of staphylococci. In this study, we could detect siderophore production of staphylococci directly in HPD solution. To the best of our knowledge, this is the first report to detect siderophore production by staphylococci directly in HPD solution, and we also can confirm that

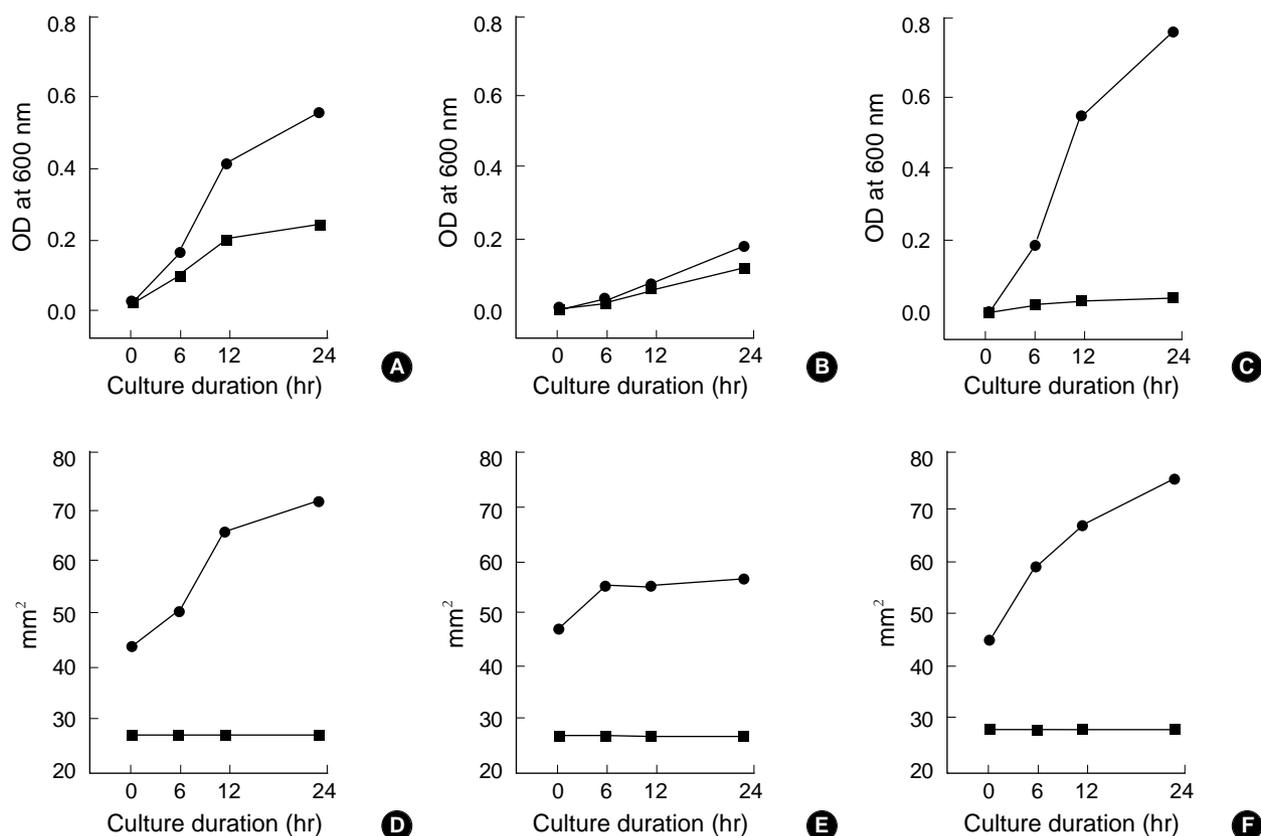


Fig. 2. Growth and siderophore production of staphylococci in human peritoneal dialysate (HPD) solution. A, B and C indicate growth of *S. aureus* ATCC 6538 (A), *S. aureus* ATCC 25923 (B) and *S. epidermidis* clinical isolate (C) in the HPD solution (●) and the unused chronic ambulatory peritoneal dialysis (CAPD) solution (■), respectively. D, E and F indicate siderophore production of *S. aureus* ATCC 6538 (D), *S. aureus* ATCC 25923 (E) and *S. epidermidis* clinical isolate (F) in the HPD solution (●) and the CAPD solution (■), respectively.

CAS agar diffusion assay (7) is simple, stable and highly reproducible method for screening and quantitative siderophore analysis in biological fluids. These results indicated that IUS including siderophore could play an important role in staphylococcal growth in HPD solution and thus in pathogenesis of staphylococcal CAPD peritonitis. Poor growth of *S. aureus* 25923 strain with lower activity IUS supported the importance of IUS for staphylococcal growth in HPD solution.

Moreover, our results suggested the possibility that IUS including siderophore were expressed in other body fluids, and the possibility that several virulence factors were expressed in HPD solution. It has been well-known that restricted iron availability acts as an environmental signal to regulate expression of other genes in vivo, notably virulence factors such as exotoxins, and the coordinate upregulation of virulence factor production and IUS in response to restricted iron availability via the ferric uptake regulatory (Fur) system is a common feature in a number of organisms (11, 19). These possibilities, we thought, should be peeled off through further consecutive studies.

Taken together, staphylococci could not grow and produce siderophore in the unused CAPD solution. This fluid, how-

ever, were modified during dialysis and became enriched by a plasma ultrafiltrate, and thus the enriched HPD solution could eventually support staphylococcal growth. However, HPD solution was still a severely iron-restricted environment. So we thought that staphylococci produced siderophore in the HPD solution like in ID medium, and growth of bacterial strains with higher IUS activity were more stimulated.

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