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# *Salmonella* Typhimurium SL1344 Utilizing Human Transferrin-bound Iron as an Iron Source Regardless of Siderophore-mediated Uptake

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Inorganic iron is essential for various metabolic processes, including RNA synthesis, electron transport, and oxygen detoxification in microorganisms. Many bacterial pathogens compete for iron acquisition in diverse environmental condition such as host. *Salmonella* Typhimurium SL1344 also requires inorganic iron as a cofactor for growth. When a M9 minimal liquid medium was supplemented with ethylenediamine di-*o*-hydroxyphenylactic acid (EDDA) which acts as an iron-chelating agent, growth of *Salmonella* Typhimurium SL1344 in the supplemented medium was completely arrested by deficient of useful iron under iron-depleted condition. However, a number of siderophores, which are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria and fungi, were produced for utilization of restricted iron under iron-depleted condition. A M9 minimal liquid medium complemented with human transferrin (hTf)-iron complex turned completely off production of siderophores, but growth of *Salmonella* Typhimurium SL1344 maintained level similar to compare one complemented with iron (III) chloride (FeCl3). This means that human transferrin (hTf)-bound iron can utilize via directly interaction with *Salmonella* Typhimurium SL1344 without productions of siderophores. Through construction and analysis of negative mutant for utilization of human transferrin (hTf)-bound iron, we confirm that the bacterium can directly use human transferrin (hTf)-bound iron without extracellularly intermediated carriers such as siderophores.

**Key words** : Iron, *S.* Typhimurium, siderophore, streptonigrin, Tn *phoA*, transferrin

## Introduction

Inorganic iron was required by microorganisms as a cofactor for various metabolic processes, including RNA synthesis, electron transport, and oxygen detoxification [4]. Although iron is plentiful in nature, it readily oxidizes and precipitates to form biologically unavailable under oxic conditions and at a neutral pH. In the mammalian host, intracellular pools for most iron stores are found in heme compounds, ferritin, and metalloprotein complexes [18], while extracellular sources are sequestered by iron binding proteins, such as transferrin (in serum; Tf) and lactoferrin (at mucosal surfaces and in neutrophils; Lf). In order to overcome these low iron concentrations which may be as low as $10^{-18}$ M [5], pathogenic bacteria have developed a variety of iron acquisition mechanisms [17]. One common way of acquiring iron is via the secretions of siderophores, which are high-affinity iron binding molecules and deliver the bound iron directly to the cell by means of a specific ferric-siderophore receptor [17].

Loss of the ability to produce siderophores is correlated with loss of virulence from *Erwinia chrysanthemi* in plants [10], *Vibrio anguillarum* in fish [8], and *Pseudomonas aeruginosa* [7, 14], *Yersinia enterocolitica* [12], and *Escherichia coli* [19] in mice. The direct interation with host iron-binding glycoproteins such as Tf and Lf at the bacterial cell surface provides an alternative to siderophores as a method to gain access to host iron supplies such as Tbp (transferrin binding protein) system in *Neisseria gonorrhoeae* [3, 20].

In this study, we exhibit that *S.* Typhimurium SL1344 can use transferrin-bound iron under condition lacking biosyntheses of siderophores by treatment of EDDA. This confirmed through construction of Tf-binding negative mutant that iron can't be entered by direct interaction between transferrin and specific sites on cell surface at *S.* Typhimurium SL1344.

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## Materials and Methods

### Media and reagents

*S.* Typhimurium SL1344 was used to investigate uptake of Tf-bound iron. Tn *phoA* [13] was used for construction of insertional mutation to a gene encoding an acceptor, which assumed to play a role as an acceptor for Tf, to interact with Tf-bound iron. *S.* Typhimurium SL1344 was incubated in Luria-Bertani (LB), M9 or Curtiss minimal (CM) media described previously [9, 15]. When required, antibiotics were used by 100 μg/ml ampicillin (Am), 50 μg/ml kanamycin (Km), 50 μg/ml streptomycin (Sm) and streptonigrin (Sn) to indicated concentration, etc. $FeCl_2$ and hTf were purchased from sigma-aldrich and used to 30 μM and 400 μg/ml, respectively.

### Analyses of growth curves for *S.* Typhimurium in MM9 media containing various iron sources

M9 medium was used for analyses of growth curves to *S.* Typhimurium. $FeCl_3$ or hTf (human transferrin) (Sigma-Aldrich, U.S.) as iron sources were mixed with M9 medium. Otherwise, free iron was arrested by chelating agent such as EDDA. *S.* Typhimurium was grown for overnight at 37℃ in LB broth. This broth was washed three times with M9 medium, diluted to 1:100 in prewarmed fresh M9 or M9 media differentially supplemented with each component as shown in Fig. 1, 2 and 4, and incubated until indicated times. Measurements of growth were analyzed by optical density at 600 nm in wavelength.

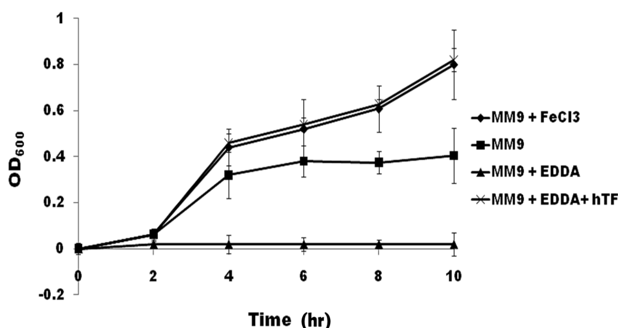


Fig. 1. Growth curves of *S.* Typhimurium in the M9 media containing various sources of iron. Rectangle is growth curve of *S.* Typhimurium incubated in M9 medium, diamond in M9 medium with $FeCl_3$, triangle in M9 medium with EDDA, and remained symbol in M9 medium with EDDA/hTf. $FeCl_3$, EDDA, and hTf were added to 30, 50, and 400 μM, respectively. X-axis indicates incubated times and Y-axis marks optical density at 600 nm in wavelength.

### Detection and identification of siderophores changed by Tf-bound iron

Chrome azurol S (CAS) agar for assessment of siderophores produced from *S.* Typhimurium was prepared as described earlier [1]. The CAS universal siderophore detection assay [16] was used to monitor productions of siderophores by *Salmonella* cells grown in iron-depleted M9 media by measuring the decrease of optical density at 630 nm in wavelength due to the CAS dye reaction as reported previously [1]. In order to investigate productions of siderophores when complemented with Tf, Tf was directly added in M9 media or submerged in M9 media after sealing in dialysis bag.

### Mutant isolation via streptonigrin enrichment culture

*S.* Typhimurium was randomly mutated by conjugation to use Tn *phoA* (pRT733). Constructed mutants were selected on LB supplemented with Km and Sm, collected, and then resuspended to cell mass of $5\times10^9$ CFU (colony forming unit)/ml. Fifty micro liter ($2.5\times10^8$ cells) pool of mutant were inoculated in 10 ml M9 media complemented with 400 μg/ml hTf and 100 μg/ml Am, and then incubated for 4 hr at 37℃. The grown cells were harvested and washed twice with buffered saline-gelatin (BSG). Am-enriched cells were incubated in 10 ml LB medium for 2 hr at 37℃ by shaking to 200 rpm (revolution per min). Each 2 ml cultured broth was subcultured without Sn, with 4 μg/ml, and with 6 μg/ml Sn for 12 hr at 37℃ by shaking. The cultured broths in Sn-treatments were patched onto CM agar medium containing Km with and without EDDA/hTf. Mutated colonies were obtained by selection of isolates to show no growth on medium containing Km with EDDA/hTf.

## Results and Discussion

### *S.* Typhimurium is able to use Tf-bound iron regardless of siderophores

Almost iron in a mammalian host is found intracellularly in the forms of heme, hemosiderin, ferritin or complexed with metalloproteins [18], whereas extracellular iron is stored by Tf, Lf, and hemoglobin. Sequestration of iron by these iron-binding proteins and compounds effectively lowers the concentration of available soluble ferric iron to a level insufficient for bacterial growth. To overcome this limitation, pathogenic microorganisms have evolved to alternative mechanisms to acquire iron from these iron-bound com-

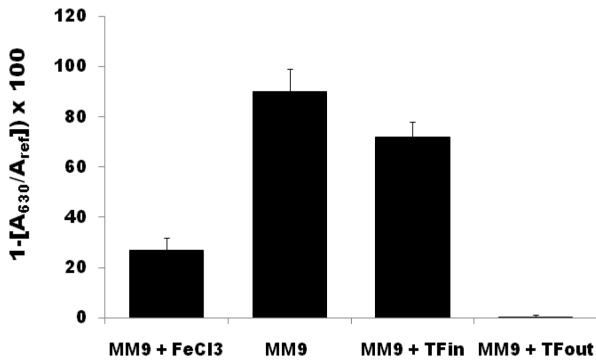


Fig. 2. Productions of siderophores by *S.* Typhimurium grown in M9 media containing differentially supplemented hTf. Cell free culture supernatants of bacteria were tested by using the CAS assay. In or out indicates the location of supplemented Tf; In, inside of dialysis bag; Out, outside of dialysis tubing bag.

pounds [5].

*S.* Typhimurium was investigated whether alternative iron uptake pathway is or not, except for system utilizing siderophores. As shown in Fig. 1, growth of *S.* Typhimurium in M9 media supplemented with EDDA was completely arrested for indicated times. When compared to M9 medium, *Salmonella* growth in M9 supplemented with $FeCl_3$ and EDDA/hTf were not nearly different to that of M9 media until 3 hr from inoculums, but, after its time, showed gradually growth better than that of M9 medium. A medium added with only EDDA was to completely inhibit growth of *S.* Typhimurium, whereas an hTf supplementation to M9 medium with EDDA showed recovery of growth as much as $FeCl_3$. These results indicated that *S.* Typhimurium could directly use iron from Tf-bound iron without siderophores.

## *S.* Typhimurium is a mechanism to directly use hTf-bound iron

Many bacterial species grown in iron-deficient media causes the expression of so-called iron-regulated genes scattered throughout the genome. Among the enteric bacteria, this process is controlled at the transcriptional level by the regulatory protein Fur, a product of the chromosomal *fur* (ferric uptake regulation) gene [2, 11]. Some of the genes controlled by Fur encode high-affinity iron transport systems mediated by siderophores, low-molecular-mass iron (III) chelators which scavenge iron in the external medium and make it available to bacteria.

It has been known that *S.* Typhimurium raises productions of siderphores under iron-depleted condition, but lowers under iron rich medium. Our results also showed that siderophores were highly expressed by M9 medium restricted with iron, but very low when complemented with iron (Fig. 2). These results indicate that productions of siderophores are regulated by iron. Interestingly, when hTf was added by sealing in dialysis bag, productions of siderophores were highly increased, whereas when added directly into the M9 medium, its production shut off completely (Fig. 2). Therefore, it was suggested that hTf-bound iron shuts off production of siderophores and is directly in-

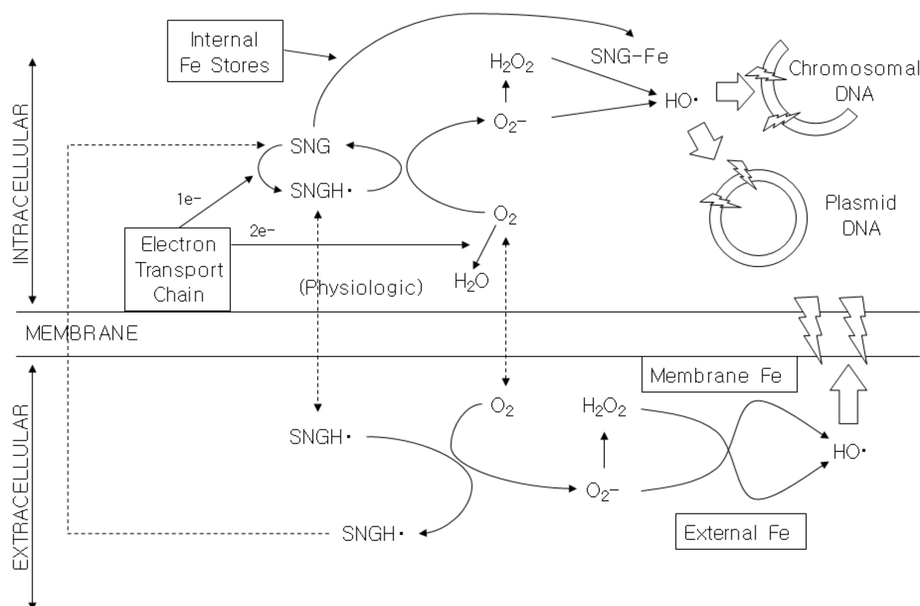


Fig. 3. Model for the effects of streptonigrin on bacteria. SNG, Streptonigrin; SNGH, reduced streptonigrin.

Table 1. Results of streptonigrin enrichment cultures

| Streptonigrin (μg/ml) | Initial cells (CFU) | After 12 hr culture (CFU) |
|---|---|---|
| 0 | $3.2×10^8$ | $9.4×10^9$ |
| 4 | $3.2×10^8$ | $2.8×10^3$ |
| 6 | $3.2×10^8$ | $1.6×10^1$ |

teraction with any materials on cell surface of *S.* Typhimurium for iron utilization.

## A mutant impossible of interacting directly with hTf-bound iron is unable to grow at existence of hTF

Numerous mutations in a single chromosome are generated by Sn treatment, and it has also the great potential to identify iron transport mutants. This chemical has been used in *Neisseria* and *Bordetella* species to identify iron uptake mutant. The quinine antibiotic Sn is believed to kill bacteria by promoting formation of oxygen radical in the presence of iron [6] (Fig. 3).

For construction of mutants to iron transport system(s), the first random mutagenesis was performed by conjugation between *S.* Typhimurium and *E. coli* host containing Tn *phoA* (pRT733). The mutated colonies were selected on LB added with Km and Sm, and then pooled by total volume 5 ml ($5×10^9$ CFU/ml). Am-enrichment culture performed to increase yield of mutant selection as follows. When mutant pools were incubated by M9 media supplemented with hTf and Am, hTf negative mutants could not grow due to deficiency of available iron. However, wild type or other mutants, which unrelated with hTf utilization, would grow rapidly and then be killed due to Am. Mutant pools were incubated in M9 media with hTf and Am for 4 hr at 37℃. The cultured broths were washed twice with BSG, resuspended in 10 ml LB and incubated for 2 hr at 37℃.

Table 2. Biochemical and genetic characterization of Tf mutant

| Cultural conditions | Wild type | Tf mutant | References |
|---|---|---|---|
| Streptonigrin | Resistant | Resistant | *rpsL* |
| Kanamycin | Sensitive | Resistant | Tn *PhoA* mutagenesis |
| CM agar plate | No growth | No growth | *hisG* |
| CM + his plate | Growth | Growth | |
| Indole test | Negative | Negative | Most of *E. coli* are positive |
| P22 sensitivity | susceptible | susceptible | Only susceptible to *S.* Typhimurium |
| CM + lactose plate | Growth (white) | Growth (white) | |
| Virulence plasmid by KEDO and Liu method | Present | Present | |
| Siderophore production on CAS agar | Positive | Positive | Tf mutant produces much more than wild type |
| API20 test | *Salmonella* spp. | *Salmonella* spp. | *Salmonella* spp. : excellent identification |
| Ferrichrome utilization | Yes | Yes | |
| Colony type on LB agar | Round, dome, smooth edge | Irregular edge, dome, slightly brown color | |

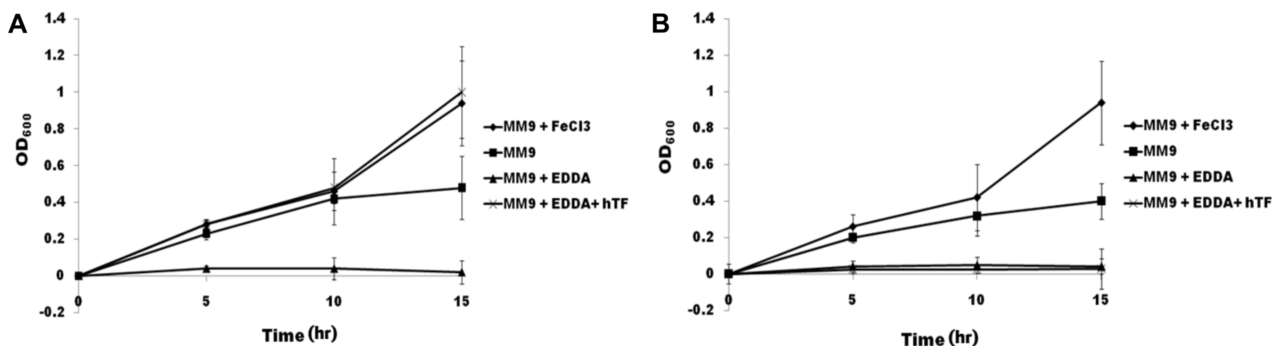


Fig. 4. Growth curves of hTf mutant in the M9 media containing various sources of iron. Growth curves of wild type and hTf mutant in the M9 media containing various sources of iron were indicated by A and B, respectively. Open circle is results grown in M9 media complemented with EDDA. Remained marks were indicated by the same symbols as shown in Fig. 1.

Streptonigrin enrichment cultures were performed by M9 liquid media with EDDA, hTf, Km, and Sn for 12 hr at 37℃. The results showed that 2800 CFU (colony forming unit) was observed by treatment of 4 µg/ml streptonigrin and 16 CFU by 8 µg/ml (Table 1). The next isolated 500 colonies were patched onto CM agar plates containing Km with and without EDDA/hTf. One of 500 patched colonies was unable to grow on CM agar with Km, EDDA, and hTf. The analyses of growth of mutant were performed by each factor for demonstration of mutant as shown in Table 2. The mutant didn't grow under existence of hTf for indicated times (Fig. 4). As shown in Table 2, biochemical characterizations were the same as wild type *S*. Typhimurium SL1344. However, nucleotide sequencing results revealed that insertion site of Tn *phoA* is located on completely unrelated gene with iron transport system (data not shown). Therefore, it was assumed that the hTf mutant was obtained by accumulation of stress followed treatment of streptonigrin. Although we didn't know to bind at any places, these results demonstrated that hTf-bound iron is possible of utilization through directly interaction with *S*. Typhimurium without extracellularly intermediated carriers such as siderophores.

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## 초록 : *Salmonella* Typhimurium SL1344의 사람의 트렌스페린(hTf)에 부착된 철 이용에 관한 연구

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*S.* Typhimurium SL1344는 성장을 위한 보조인자로 무기철이 요구된다. 철 킬레이트제인 ethylenediamine di-*o*-hydroxyphenylactic acid (EDDA)가 첨가 된 M9 최소배지에서 *S.* Typhimurium은 성장에 있어 철 이용이 완전하게 억제된다. 하지만, 세균이나 곰팡이와 같은 미생물들은 철이 부족한 환경에서 제한된 철을 이용하기 위해 사이드로포어를 생산한다. 사람에서 유래한 트랜스페린(hTf)-철 복합체를 M9 배지에 첨가한 조건에서 *S.* Typhimurium의 사이드로포어 생산은 완전하게 중단되었다. 반면, *S.* Typhimurium의 성장은 염화철($FeCl_3$)을 첨가한 조건과 동일한 수준으로 유지되었다. 이 결과는 사이드로포어의 생산 없이도 *S.* Typhimurium이 hTf에 부착된 철을 직접적으로 이용할 수 있다는 것을 알 수 있다. 돌연변이주의 구축과 이를 이용한 분석을 통하여 우리는 세균이 hTf-철 복합체를 직접적으로 이용할 수 있다는 것을 확인할 수 있었다.