Journal of Life Science 2005, Vol. 15. No. 4. 641~646 ©JLS

Effect of Growth Conditions in the Attachment of *Salmonella typhimurium* to the Host Cells

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Received June 30, 2005 / Accepted August 8, 2005

An approximately 10-fold higher level of adherence of *Salmonella typhimurium* strain TML to Int-407 cells was observed with organisms grown in Luria broth or in high-iron containing medium than those grown in low-iron containing medium. Iron specifically enhanced adherence, while other cations such as calcium, cobalt, copper, potassium, magnesium and manganese did not. It was suggested that iron did not act as a passive ligand - probably it stimulated production of bacterial factors necessary for adherence. A similar pattern of iron modulation of adhesiveness was also seen in *Salmonella* mutants with single or different combinations of multiple mutations in genes encoding the mannose sensitive hemagglutinin (type 1 fimbriae), mannose resistant hemagglutinin and flagellum. The adhesiveness of an isogenic *fur* mutant was modulated by iron in a manner similar to the wild-type strain, suggesting that iron modulation of adherence is independent of the *fur* gene product.

Key words – iron replete medium, *S. typhimurium*, Int-407 cell, adherence

Salmonellosis represents a major communicable bacterial disease problem throughout the world. Although many serovars of *Salmonella* have known to cause gastroenteritis in human beings, *S. typhimurium* is one of the most common etiologic agents of such infections.

Evidences abound which point to the fact that iron is present in abundance in the gastrointestinal contents. First, of the total 15~20 mg of iron taken in an average daily diet only 0.5 to 1 mg is absorbed in the duodenum and jejunum to replenish body stores. The rest of the iron remains in the lumen to be excreted[3]. Second, a daily loss of 0.26 mg, 0.1 mg and 0.4 mg of iron in bile, within shed intestinal epithelial cells and in blood, respectively, has been estimated to occur through the gastrointestinal tract[10].

Iron is an essential element required by bacteria for growth and survival. Consequently, organisms have evolved elaborate methods to acquire iron under limiting conditions as for example the production of high-affinity iron chelators called siderophores. *Salmonella* synthesize the cathechol siderophore enterobactin[18] and some strains also synthesize the hydroxamate siderophore aerobactin[17]. Regulation of the genes for the components of high-affinity iron uptake systems in *S. typhimurium* is mediated by the protein product of the fur locus which acts as a co-repressor with ferrous iron[6].

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Acquisition of iron by *Salmonella* has been studied to some extent, but the role of iron in the expression of virulence determinants remains to be explored. Previous studies have provided evidence that oral or parenteral administration of iron increased virulence of *S. typhimurium* in parenterally inoculated mice[14,19] where the bacteria produce typhoid-like systemic infection. Also iron deficiency either dietary or chelator induced, decreased the severity of disease caused by *S. typhimurium* administered parenterally in mice[14,19]. The most obvious mechanism by which excess iron enhances the systemic infection may be demonstrated by serving as a nutrient for the invading microbes[22].

The first step in the interaction of enteropathogens with their hosts is attachment to the intestinal cells, which play a role for the natural portal of entry. However, little information is available about the modulation by iron of the factors involved in the attachment and penetration of the cells by enteropathogens. The present study commences a definition of the effects of iron in the initial interaction between *Salmonella* and host cells.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in the adherence and colonization studies are listed in Table 1. The constructions of isogenic *fur*, *fimA*, *fli* and *inv* mutants of strain TML are given in Table 1. Transductions between *S. typhimurium* strains

Table 1. *S. typhimurium* strains.

Strains	Genotype	Source
TML, LT7	wild type	Lab stock
χ3376	*fli-8007*::Tn10 derivative of LT2	Lab stock
χ4439	*fimA*-10::Cm derivative of SL1344	Lab stock
SL4720	MRHA::Tn5 derivative of TML	Lab stock
JF2043	*fur-1 zbf5123*::Tn10 derivative of LT2	Lab stock
χ4846	*fur-1 zbf5123*::Tn10 P22HT*int* on TML	This work
χ4929	*fil-8007*:: Tn10 P22HT*int* on TML	〃
χ4930	*fimA-10*::Cm P22HTP22HT*int* on TML	〃
χ4931	*fim-10*::Cm, *fli-8007*::Tn10	〃
χ4932	*fimA-10*::Cm, MRHA::Tn5	〃
χ4933	MRHA::Tn5, *fli-8007*::Tn10	〃
χ4934	*fim-10*::Cm, MRHA::Tn5, *fli-8007*::Tn10	〃
χ8003	*invA*::kan on TML	〃
χ8004	*invB*::kan on TML	〃
χ8005	*invC*::Tn*phoA*-50 on TML	〃
χ8006	*invD*::Tn*phoA*-11 on TML	〃
χ8007	*invH*::*aph*, Km^R on TML	〃

were via bacteriophage P22HT*int*[20]. The *fimA* mutant was confirmed by absence of mannose-sensitive agglutination of guinea-pig RBC and absence of agglutination with antiserum specific for *S. typhimurium* type 1 fimbriae. The *fli* mutant was confirmed by absence of diffused growth in semisolid agar. The *fur* mutant was confirmed by inability to utilize citrate, succinate and acetate[1].

Media

In the iron modulation experiments Luria broth was routinely used as the control medium. Low-iron medium was prepared by adding 0.3 mM dipyridyl (Sigma) to Luria broth and high-iron medium was prepared by addition of 0.25 mM ferrous sulfate (Sigma) to the low-iron medium. For the Int-407 cell adherence assays MacConkey agar (Difco) was used, and for the intestinal colonization experiments *Salmonella-Shigella* agar (Difco) was used to recover organisms. Antibiotics were used at the following concentrations per ml of media: ampicillin 100 μg, chloramphenicol 25 μg, cycloheximide 200 μg, kanamycin 60 μg, and nalidixic acid 75 μg, tetracycline 15 μg, etc.

Tissue culture

Int-407 human embryonic intestinal cells (ATCC CCL6) were routinely grown in minimum essential medium (MEM; GibcoBRL) supplemented with glutamine (2 mM) and fetal calf serum (10% v/v) which was designated to tissue culture medium (Tcm). Benzyl penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) to Tcm were added to produce Tcm-A.

Adherence assay

Preparation of monolayers, bacterial challenge and recovery of organisms following experiments were performed as described before[5] with appropriate modifications. Briefly, each well in a 24-well tissue-culture tray was seeded with approximately 2×10^5 Int-407 cells in 1 ml of Tcm-A. The monolayers were used at 80% confluency. Just before inoculation of the wells with bacteria, the spent Tcm-A was removed, and the monolayers were washed once with adequate Hank's balanced salt solution (Gibco) to ensure complete removal of the antibiotics. To maintain growth conditions as much as possible for the cells and for the bacteria a 1:1 mixture of Tcm with either (a) Luria broth (control) or (b) low iron media (low) or (c) high-iron media (high) was used during the assays. One ml of the mixture was added to each well. Organisms grown statically for 3 h to log phase at 37℃ were added in 10 μl volumes, containing around 2×10^6 cfu, per well. The trays were incubated at 37℃ for 30 min in a 5% CO_2 incubator. The supernatants were removed, and monolayers were washed twice with cold PBC. One ml of Triton X-100 (1% in PBC) was used to lyse the monolayer as described by Douce *et al.*[5] and then bacterial counts adhered to Int-407 cells were estimated on MacConkey agar plates. In control experiments, organisms grown in iron-restricted conditions were found not to be more sensitive to 1% Triton X-100 than organisms grown in iron-replete conditions. Adherence was assessed as a percentage of adhered bacteria to the initial inoculum in each experiment.

RESULTS

Standardization of the growth conditions for *Salmonella*

To investigate growth inhibition of *S. typhimurium* TML by dipyridyl concentration, viable bacterial counts were made over a period of 4 h after suspension of *S. typhimurium* TML in Luria broth with increasing concentrations of dipyridyl and static incubation at 37℃. When compared with control, *S. typhimurim* TML showed growth similar to control down to 0.3 mM dipyridyl, whereas it inhibited growth up to this concentration. Therefore, the concentration of 0.3 mM of dipyridyl was selected to prepare the designated low-iron medium (data not shown). To determine appropriate ferrous sulfate concentration for using media, ferrous sulfate was added to the low-iron medium to pre-

pare a series of media with increasing concentrations of iron. The adhesiveness of the bacteria increased with the rising concentration of iron and at the concentration of 0.25 mM the adhesiveness of the bacteria was similar to that of the control (data not shown). The concentration of 0.25 mM ferrous sulfate was chosen to prepare high-iron medium for the subsequent experiments.

Iron mediated modulation for adherence of *S. typhimurium* TML to tissue culture cells

The effect of iron-replete versus iron-deficient conditions on the adhesive property of *Salmonella* was assessed. The adhesiveness of organisms grown in control to high-iron medium was observed at high-iron media about 10% higher than that of control (Fig. 1). Since the growth rate of bacteria in low-iron medium was similar to the growth in either control or high-iron media, the reduced adherence did not assume due to death of organisms in low-iron medium. The reduced adherence may be also not due to any effect of iron restriction on Int-407 cells, since similar levels of association of organisms grown in Luria broth were observed with Int-407 cells when the cell-monolayers were either (i) used without any pretreatment or (ii) pretreated for 30 min with iron-restricted medium [(i) 100%; (ii) 104± 11%]. Iron induces anaerobiosis and anaerobiosis has been shown to increase invasiveness of *Salmonella*[7]. Whether the increased adhesiveness of organisms grown in high-iron media was due to iron induced anaerobiosis was tested. Inocula for adhesion assays were prepared by growing organisms in high-iron media both (i) statically and (ii) with aeration. The levels of adherence with both types of inocula were similar [(i) 87.8±11.0%; (ii) 83.5±9.0%]. Therefore, these results revealed that increase of adherence is specific phenomena of *S. typhimurium* TML grown in iron-replete media.

Iron-modulation is only specific for adherence

A number of cationic salts were used (at a concentration of 0.25 mM) to supplement the low-iron medium instead of iron. The analysis of adhesiveness of *S. typhimuirum* strain TML in these various media reveals that cationic salts except for iron failed to restore adherence (Fig. 2). However, all the different supplemented media supported the viability of the organism in a similar manner to that of the control medium (data not shown). This result was in contrast to the finding with iron-supplemented medium.

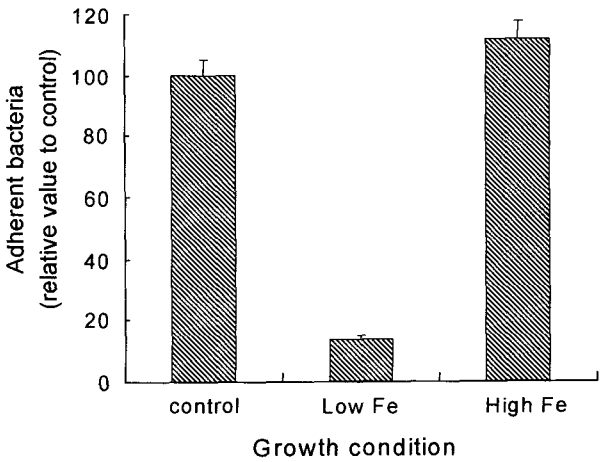

Fig. 1. Modulation by iron of adherence of *S. typhimurium* TML to Int-407 cells. Organisms were grown to log phase in Luria broth (control), and low-iron (low) and high-iron (high) media. Assays were carried out for 30 min in a 1:1 mixture of Tcm and i) Luria broth, ii) low-iron and iii) high-iron media. The bars represent standard error of the means from three wells tested on one day. Data are representative of several experiments.

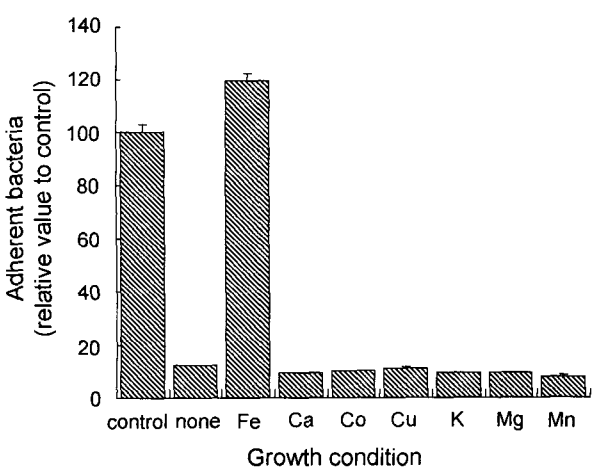

Fig. 2. Specificity of iron-modulation of adherence of *S. typhimurium*. For the adherence assays organisms were pre-grown in either control media or low-iron media (none) or low-iron media supplemented with different cationic salts at a concentration of 0.25 mM: ferrous sulfate (Fe), calcium chloride (Ca), cobalt chloride (Co), cupric sulfate (Cu), potassium chloride (K), magnesium chloride (Mg) or manganese chloride (Mn). The bars represent standard error of the means from three wells tested on one day. Data are representative of three experiments.

Iron on common bacterial appendages didn't have any effects

Since iron modulated adherence of *Salmonella*, it was necessary to analyse the effect of iron on some of the com-

mon appendages which are likely candidates for binding *Salmonella* to host cells. A number of mutants of strain TML were generated with single or different combinations of multiple mutations in the genes encoding cell appendages such as type 1 fimbriae (Fim$^-$), mannose resistant hemagglutinin (MRHA$^-$) and flagella (Fla$^-$). The adhesiveness of these mutants to wild-type was compared and shown in Table 2. A similar pattern of iron modulation of adhesiveness as seen in the wild-type was also seen in these isogenic mutants (Fig. 3). However, the more pronounced modulation by iron was seen only in the mutants with intact flagella, i.e. χ4930 (Fim$^-$), SL4720 (MRHA$^-$) and χ4932 (Fim$^-$ MRHA$^-$) (adhesiveness of low-iron grown organisms was approximately 15% of control). Lesser degrees of modulation were seen in mutants which lacked flagella, i.e. χ4929 (Fla$^-$), χ4931 (Fim$^-$ Fla$^-$), χ4933 (MRHA$^-$ Fla$^-$) and χ4934 (Fla$^-$ Fim$^-$ MRHA$^-$) (adhesiveness of low-iron grown organisms was approximately 40% of control). These results indicate that Fim, MRHA, and Fla deduced to play roles as adhesins don't exhibit any effects for adhesiveness of *S. typhimurium* TML to Int-407 by iron conditions.

The *inv* mutants didn't change adherence to iron modulation

The *inv* genes have been found to be important in the interaction between *Salmonella* and host cells[9]. We used nonpolar *invA* (χ8003), *invB* (χ8004), *invC* (χ8005), *invD* (χ 8006) and *invH* (χ8007) mutants of S. *typhimurium* TML to evaluate whether the *inv* genes were involved in iron mediated adhesiveness. Fig. 4 shows that the adhesiveness of the isogenic *inv* mutants of strain TML were less than 16% of the control when they were grown in low-iron medium. Although the *invC* (χ8005) mutant showed adherence less than other mutants in high iron condition, the *inv* mutants exhibited no effect for adherence to Int-407 cells.

Table 2. Iron-modulation of adherence of mutants of *S. typhimurium* TML to Int-407 cells.

Strain	Control[a]	Low Fe	High Fe
Wild-type	100.0[b]	10.4	108
Fim-	96.2	13.2	108
MRHA-	52.8	8.1	54.7
Fla-	28.3	11.5	32.1
Fim-Fla-	30.2	12.5	34.0
MRHA-Fla-	39.6	15.1	39.6
Fim-MRHA-	32.1	5.1	30.2
Fim-Fla-MRHA-	12.8	4.3	13.4

[a]Grown in Luria broth

[b]The mean adhesiveness of wild-type parent (5.3%) was set to 100

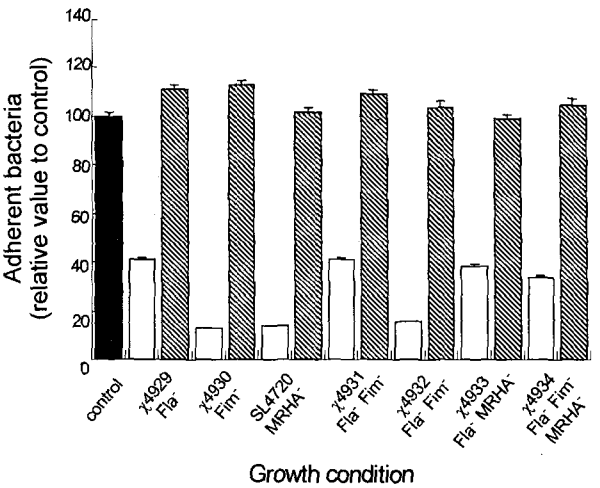

Fig. 3. Modulation by iron of adherence of mutants of *S. typhimurium* TML with single or different combinations of multiple mutations in genes for common bacterial appendages. The adhesiveness of each mutant grown in low-iron (open box) or high-iron (hatched box) media was compared to the adhesiveness of the same mutant grown in control (filled box) medium (Luria broth). The bars represent standard error of the means from three wells tested on one day. Data are representative of three experiments.

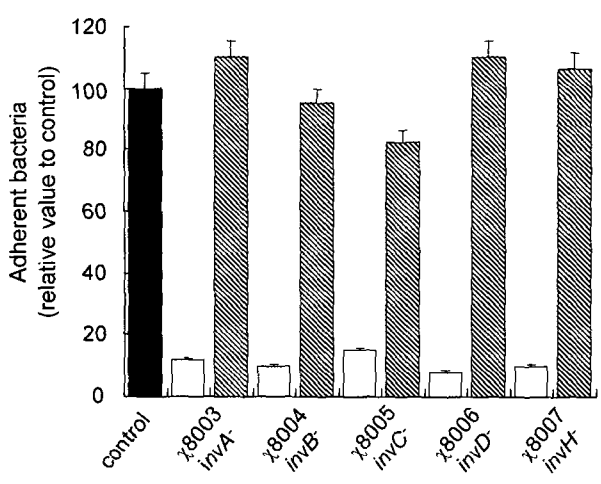

Fig. 4. Modulation by iron of adherence of *inv* mutants of *S. typhimurium* TML to Int-407 cells. The adhesiveness of each mutant grown in low-iron (open box) or high-iron (hatched box) media was compared to the adhesiveness of the same mutant grown in control (filled box) medium (Luria broth). The bars represent standard error of the means from three wells tested on one day. Data are representative of three experiments.

Iron modulation for adherence is independent of Fur.

Fur plays a central role in the regulation of the high af

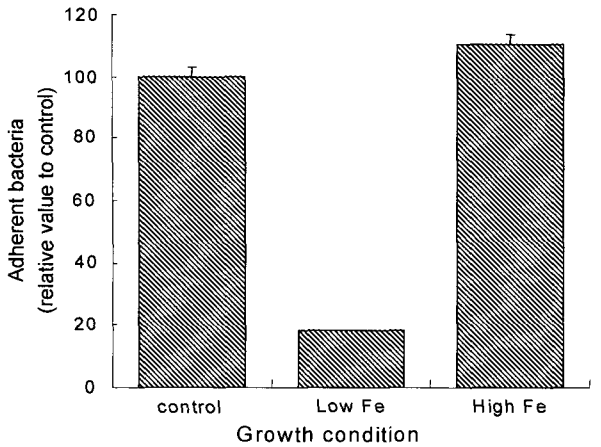

Fig. 5. Modulation by iron of adherence of *fur* mutant of *S. typhimurium* TML to Int-407 cells. The bars represent standard error of the means from three wells tested on one day. Data are representative of several experiments.

finity iron-uptake system in *Salmonella*. Whether it has any effect on iron modulation of adherence was tested. An isogenic fur mutant χ4846 exhibited a similar degree of adherence to Int-407 cells as the parent *S. typhimurium* TML (TML: 100%; fur mutant: 92.9±2.2%). As seen with the wild-type, the adhesiveness of the mutant when grown in low-iron medium was reduced to approximately 18% of the control (Fig. 5). Therefore, it appears that iron modulation of adhesiveness is independent of Fur.

DISCUSSION

Salmonella undergoes differential gene expression depending on iron concentration and availability like many other microorganisms. While production of proteins which function in the uptake of iron has been shown to provide survival advantages to the bacteria in iron-limiting conditions, the effect of iron-replete conditions remains largely unknown. This study represents an effort to extend our understanding for the feature involving iron in the modulation for adherence of *Salmonella* to intestinal cells. The data presented in this report indicate that adherence levels *in vitro* and *in vivo* were approximately 10-fold higher for *S. typhimurium* grown in iron-supplemented medium than that in low-iron medium.

The production of fimbriae and flagella was not impaired when wild-type organisms were grown in low-iron medium. The adhesions of various mutants with single or different combinations of multiple mutations in the genes of type 1 fimbriae, MRHA and flagella were exhibited with the level of iron in growth media similar to the findings with the wild-type strain. These effects could be due to differential expression of some factors (which we will refer to as iron induced adhesions, *lia*) rather than these common bacterial appendages in low-or high-iron conditions.

In the pathogenesis of enteric infection with *Salmonella* it is presumed that the bacterial adherence to the epithelium of the intestine is a prerequisite for invasion, but the precise nature of this adherence is unclear yet. *S. typhimurium* produces a variety of surface organelles that are likely candidates for mediating colonization of the intestine. The best known of these are type 1 fimbriae which mediate a mannose-sensitive agglutination to guinea-pig erythrocytes and attachment to a variety of epithelial cells in culture[12,16]. However, the lack of these fimbriae was found to have no effect on the colonization *in vivo*[16]. Flagella may be important in the pathogenicity of *S. typhimurium*. Motility could increase the number of productive interaction achieved by luminal bacteria with host mucosa promoting the frequency of adherence, or flagella themselves may be adhesins. Defect in flagella and motility reduced the ability of *S. typhimurium* to invade tissue culture cells[11,15] but did not have significant effect in the colonization of mouse intestine. The role of other adhesins including long polar fimbriae [2], bundle forming pili[21], thin aggregative fimbriae/curli [4], plasmid encoded fimbriae[8,13] in the colonization of host niches remains to be studied.

Acknowledgements

This work was supported by Korea Research Foundation Grant (KRF-2003-003-E00036). Y. H. Kim is the recipient of a graduate student fellowship supported by the Brain Busan 21 project.

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초록 : 생육조건에 따른 *Salmonella typhimurium*의 숙주세포 부착성

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Salmonella typhimurium TML이 Int-407 숙주세포에 부착하는 정도는 *S. typhimurium*이 낮은 농도의 철이 함유된 배지에서 배양되었을 때보다 LB 액체배지 또는 높은 농도의 철이 함유된 배지에서 생육한 것에서 약 10배 정도의 높은 수준으로 관찰되었다. 고농도의 철이 포함된 배양조건이 살모넬라가 숙주세포에 부착시키는 정도를 향상시키는데 반해, 칼슘, 코발트, 구리, 인산, 마그네슘 그리고 망간과 같은 다른 양이온은 그렇지 않다. 이것은 아마도 철이 *Salmonella*가 부착에 필요한 요소들의 발현을 활성화하는 역할을 하는 것으로 추정된다. 철 농도에 따른 부착정도의 차이들은 type 1 fimbriae, mannose resistant hemagglutinin과 flagellum 등을 생성하지 않는 다양한 *S. typhimurium* 돌연변이주들에서도 관찰되는 것으로 미루어보아 이들 구조체들과 상관이 없는 밝혀지지 않은 어떤 인자가 부착성 증가에 관여하는 것으로 사료된다. *fur* 유전자가 불활성화된 *S. typhimurium* 돌연변이주의 부착성이 야생형 *Salmonella*와 유사한 방식으로 철에 의해 조절되었는데, 이는 철 농도에 따른 부착성의 변화에 관여하는 잠재적인자의 발현이 Fur 단백질과 독립적으로 이루어진다는 것을 나타낸다.