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Iron-Reversible and Irreversible Inhibition of *Yersinia pseudotuberculosis* by Human Lactoferrin and Transferrin

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ABSTRACT. Iron-free lactoferrin or iron-free transferrin inhibited the growth but did not affect the viability of *Yersinia pseudotuberculosis* cells that were present in iron-complete BHI broth or in BHI broth iron-depleted by $MgCO_3$. This type of inhibition was reversed by the addition of iron-saturated lactoferrin or iron-saturated transferrin, which appeared to be capable of serving as a source of iron in the above iron-depleted medium, or by transfer to iron-complete BHI broth. In contrast, there was a rapid loss of viability for those cells which were suspended in deionized water and were treated by iron-free lactoferrin or iron-free transferrin. The iron-irreversible inhibition which was observed in the absence of medium suggests that both lactoferrin and transferrin have a direct bactericidal effect on *Y. pseudotuberculosis* that cannot be attributed to simple iron-deprivation.

Iron, is an essential element for the survival and growth of bacteria. It is not freely available in the human body because it is mostly bound to the iron-binding proteins such as lactoferrin in secretions and transferrin in blood (Bullen 1981). Lactoferrin and transferrin are iron-chelating agetns which can act by two different mechanisms, depending on their iron-saturation level. Iron-saturated forms have been shown to be used by several species of bacteria to satisfy their iron requirement (Mickelsen and Sparling 1981, Arnold *et al.* 1982, Redhead and Hill 1991, Pickett *et al.* 1992, Salamah 1992, Yang *et al.* 1993, Salamah and Al-Obaidi 1995a). On the other hand iron-free or unsaturated forms of these two iron-chelating proteins, have been shown

Running title: Iron-chelating proteins and *Y. pseudotuberculosis*.

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by several studies (Bullen *et al.* 1971, Bullen 1981, Bullen and Joyce 1982, Finkelstein *et al.* 1983) to inhibit microbial growth through nutritional deprivation of iron. The inhibition observed in most of these studies was bacteriostatic where growth could be readily restored by the addition of exogenous iron in excess of the chelating capacity of lactoferrin or transferrin. In addition to bacteriostatic effect, it has been shown that lactoferrin and transferrin are capable of a direct bactericidal effect on a wide range of microorganisms that is not reversed in the presence of supplemental iron (Arnold *et al.* 1981, Bullen 1981, Bortner *et al.* 1986). Accordingly, several investigators have suggested that lactoferrin and transferrin have other effects against microorganisms such as membrane damage (Arnold *et al.* 1980, Bortner *et al.* 1986, Ellison *et al.* 1988, 1990, Yamauchi *et al.* 1993).

Studies in this laboratory indicated that the growth of *Yersinia pseudotuberculosis* was inhibited by iron-free transferrin (Salamah 1992) and by iron-free lactoferrin (Salamah and Al-Obaidi 1995a). Further, the bacteriacidal activity of these two iron-chelating proteins were affected differently by pH, temperature, and magnesium and calcium ions (Salamah and Al-Obaidi 1995b). The present study aims to investigate the reversible and effects of lactoferrin and transferrin on the *Y. pseudotuberculosis.*

Materials and Methods

Organism and growth

Yersinia pseudotuberculosis ATCC 29833 was obtained from the American Type of Culture Collection (ATCC). Subcultures were maintained at 4° C with monthly transfers on brain heart infusion agar (pH 7.2) slants (Difco, USA). Cells were pre-grown in brain heart infusion broth (BHI) before each experiment harvested at the mid-exponential phase (about 6 h incubation), washed with-and resuspended in sterile 0.85% NaCl, and used for inoculation. The starting inoculum for each experiment was about 10^4 cells/ml.

Preparation of Iron-free and Iron-saturated Lactoferrin and Transferrin

The human lactoferrin and human transferrin were purchased from Sigma Chemical Company (USA). Iron-free lactoferrin and transferrin were prepared by dialysis against 0.2 M sodium acetate – 0.2 M NaH_2PO_4 - 0.4M EDTA, pH 4.0 (Mazurier and Spik 1980). Whereas, iron-saturated lactoferrin and transferrin were prepared by dialysis against 0.1 M ferrous ammonium sulfate. All preparations were filter-sterilized (0.45 μm, millipore filter) and stored in 0.5 ml quantities.

Reversible Inhibition of *Y. pseudotuberculosis*

(a) Inhibited by Iron-free Lactoferrin or Iron-free Transferrin

Sterile iron-free lactoferrin and iron-free transferrin were added to separate 25 ml flasks (each contains 15 ml BHI broth) at a concentration of 1 mg/ml of BHI broth, pH 7.2. Flasks containing BHI broth without transferrin or lactoferrin were used as a control. All flasks were inoculated and shaked at 37° C and 120 RPM. Samples were removed at zero time and then at 4 h intrvals, centrifuged at 400 x g for 5 min., diluted with a sterile 0.85% NaCl, spread on blood agar plates (0.1 ml per plate) and counted after 48 h incubation at 37° C. The reversibility of inhibition was tested after 8 h and 12 h by adding iron-saturated lactoferrin or iron-saturated transferrin (3 mg/ml) to the appropriate flasks.

(b) Inhibited by $MgCO_3$

$MgCO_3$ was added to BHI broth at a concentration of 80 mg/ml. The iron was precipitated from the broth with continuous stirring at mild temperature followed by centrifugation at 9000 x g for 20 min. The supernatant was sterilized by autoclaving and used as an iron-free medium. Sterile $FeSO_4$, iron-saturated lactoferrin, and iron-saturated transferrin were added to separate flasks with 15 ml of iron-free medium per each flask at a concentration of 4 mg, 3 mg, and 3 mg/ml, respectively. Three flasks containing iron-free medium were used as a control. Iron-saturated lactoferrin and iron-saturated transferrin were added to other flasks at 8 h and 12 h post inoculation. All flasks were shaked at 35°C and 120 RPM. Samples were removed at zero time and then at 4 h intervals up to 20 h, diluted, plated on blood agar plates, and counted after 48 h incubation at 37° C.

Inhibited by $MgCO_3$ and Treatment of Cells by Iron-free Lactoferrin or Iron-free Transferrin for 12 h.

Iron-depleted BHI broth was prepared as described above using $MgCO_3$ and was divided into 3 sets of flasks; iron-free lactoferrin was added to the first set and iron-free transferrin was added to the second set at a concentration of 1 mg/ml of broth. The third set was kept as a control. The flasks were inoculated, shaked for 12 h at 37° C, and then centrifuged. The bacterial cells were resuspended in iron-complete BHI broth and shaked at 37° C. Samples were removed at zero time and then at 4 h intervals up to 20 h, diluted, plated on blood agar plates, and counted after 48 h incubation at 37° C.

Irreversible Inhibition of *Y. pseudotuberculosis*

An exponentially growing bacterial cells were suspended in deionized water and divided into three sets of flasks; iron-free lactoferrin was added to the first set and

iron-free transferrin was added to the second set at a concentration of 1 mg/ml of broth. The third set was kept as a control. The flasks were shaked for 2 h at 37° C and 120 RPM and then centrifuged at 1300 x g for 10 min. The bacterial cells were resuspended in iron-complete BHI broth and shaked at 37 °C. Samples were removed at zero time and then at 4 h intervals up to 20 h, diluted, plated on blood agar plates, and counted after 48 h incubation at 37° C.

Results

Reversible Inhibition

Iron-free lactoferrin and iron-free transferrin inhibited the growth of *Y. pseudotuberculosis* in BHI broth (Fig. 1). However, aliquots removed at various intervals of inhibition indicated no loss in viability. Further, the inhibition was reversed by the addition of iron-saturated lactoferrin or iron-saturated transferrin at 8 h and 12 h of inhibition.

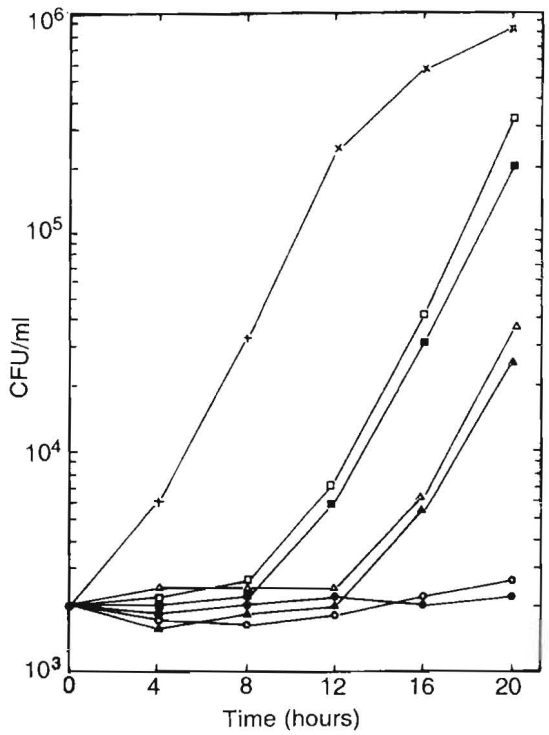

Fig. 1. Growth curves of *Y. pseudotuberculosis* in iron-complete BHI broth (x); or in BHI broth iron-depleted with iron-free lactorferrin (O) + iron-saturated lactoferrin at 8 h (□) and 12 h (△); or in BHI broth iron-depleted with iron-free transferrin (●) + iron saturated transferrin at 8 h (■) and 12 h (▲).

Y. pseudotuberculosis was not able to grow in BHI broth that was iron-depleted by $MgCO_3$ (Fig. 2). There was no loss of viability during this inhibition. Further, the inhibition was reversed by the addition of $FeSO_4$ at zero time; or by the addition of iron-saturated lactoferrin, or iron-saturated transferrin at zero time, 8 h, and 12 h of inhibition.

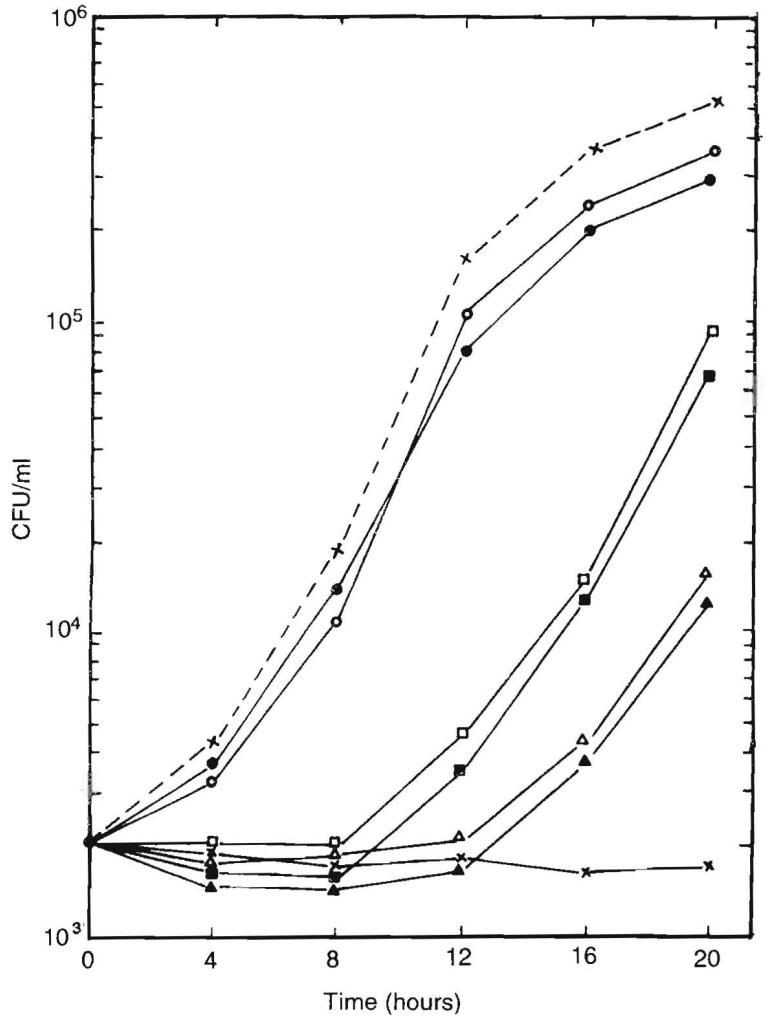

Fig. 2. Growth curves of *Y. pseudotuberculosis* in BHI broth iron-depleted with $MgCO_3$: without additions (x; continuous line) with $FeSO_4$ (x; broken line); with iron-saturated lactoferrin (open symbols, O, □, Δ) or with iron-saturated transferrin (closed symbols, ●, ■, ▲) at zero time (circles), 8 h (squares), and 12 h (triangles).

Y. pseudotuberculosis cells that were treated by iron-free lactoferrin or iron-free transferrin in BHI broth that was iron-depleted by $MgCO_3$ were able to maintain their viability during 12 h of double iron-starvation by either lactoferrin or transferrin and $MgCO_3$. Further, they were able to grow upon their transfer to an iron-complete (normal) BHI broth (Fig. 3).

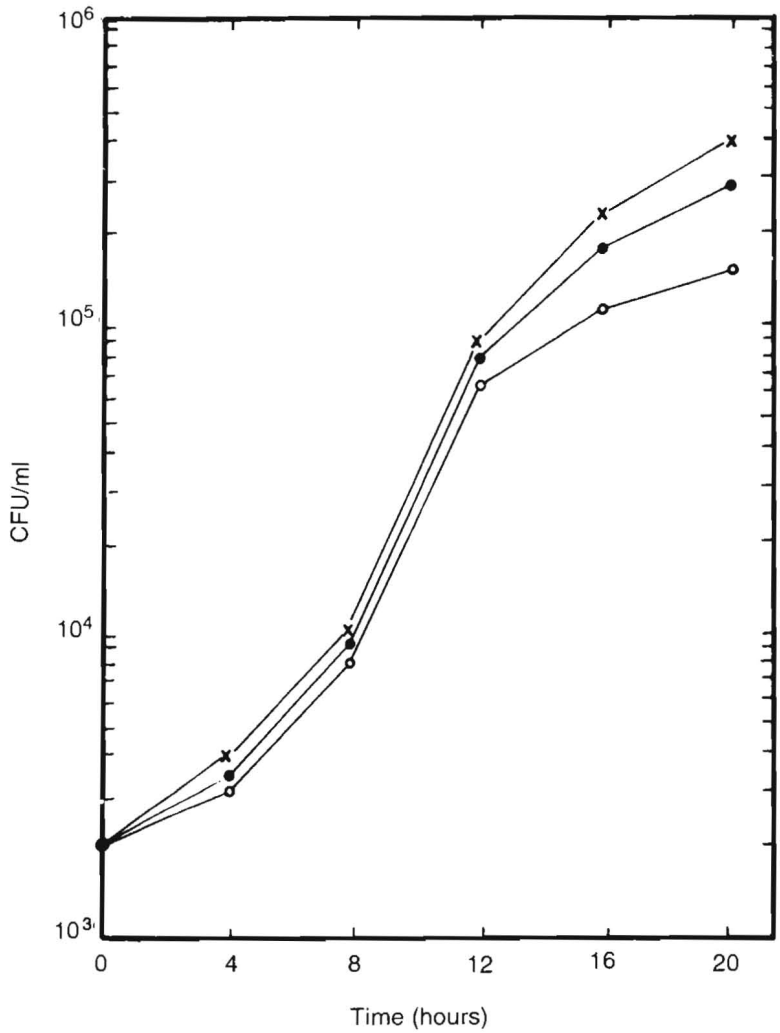

Fig. 3. Growth curves of *Y. pseudotuberculosis* cells which were transfered to an iron-complete BHI broth after they were incubated for 12 h in: BHI broth iron-depleted by $MgCO_3$ (x); BHI broth iron-depleted by $MgCO_3$ + iron-free lactoferrin (o) or iron-free transferrin (•).

Irreversible Inhibition

In contrast to the above reversibility results, *Y. pseudotuberculosis* cells that were treated by iron-free lactoferrin or iron-free transferrin in deionized water for 2 h, were not able to grow after their transfer to an iron-complete BHI broth (Fig. 4).

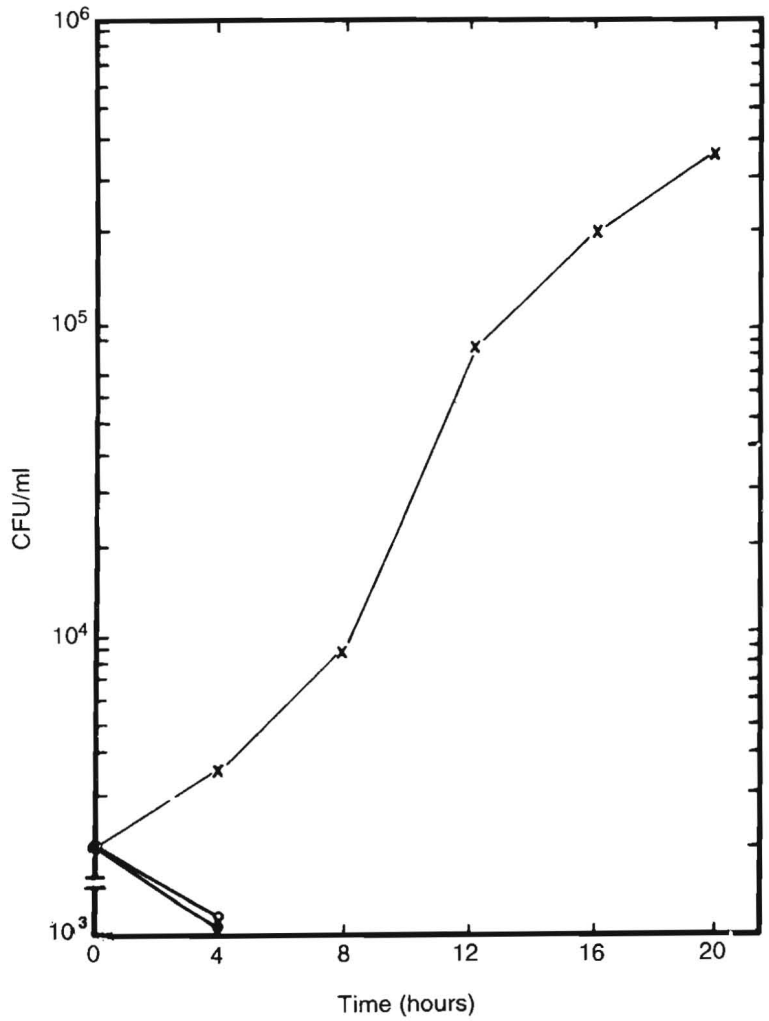

Fig. 4. Growth curves of *Y. pseudotuberculosis* cells which were transfered to an iron-completed BHI broth after they were incubated for 12 h in: deionized water (x); deionized water + iron-free lactoferrin (o) or iron-free transferrin (•).

Discussion

Pathogenic bacteria require iron for their growth (Weinberg 1978). Accordingly, they can be inhibited by the iron-chelating proteins such as lactoferrin and transferrin. If lactoferrin and transferrin, however, are saturated with iron or if iron compounds are available to bacteria, the inhibition properties of these proteins are abolished (Bullen *et al.* 1971, Bullen 1981, Bortner *et al.* 1986, Ward *et al.* 1986, Salamah 1992, Salamah and Al-Obaidi 1995a).

On results showed that the inhibition can be either reversible or irreversible. The effect of iron-free lactoferrin or iron-free transferrin on *Y. pseudotuberculosis* cells that were present in an iron-free medium was only bacteriostatic, *i.e.* the inhibition was reversed by the addition of an excess iron-saturated lactoferrin or iron-saturated transferrin or by the transfer of cells to a BHI broth with its normal iron-concentration. In contrast, when cells were incubated directly with iron-free lactoferrin or iron-free transferrin in the absence of medium, there was an iron-irreversible inhibition in subsequent growth.

The treatment of cells by iron-free lactoferrin or iron-free transferrin in a BHI broth iron-depleted with $MgCO_3$ did not effect their viability. This result was unexpected, since the iron-free environments provided by lactoferrin in polymorphs (Bullen and Wallis 1977) or transferrin in blood (Finkelstein *et al.* 1983) were essential for the bactericidal activity of polymorphs or blood. However, the absence of killing noted above in the iron-depleted medium is probably due to several factors such as the indirect interaction between iron-free lactoferrin or iron-free transferrin and bacteria, the ability of these proteins to bind with the residual iron or with other metals in medium (Bullen *et al.* 1978), and eventually the differences between *in vivo* and *in vitro* environmental circumstances. Some studies have shown that the lactoferrin and transferrin attach to an appropriate bacterial cell surface receptors (Lee and Schryvers 1989, Schryvers 1989, Tigyi *et al.* 1992, Ala'Aldeen *et al.* 1993, Morton *et al.* 1993). Accordingly, they must reach the cell wall to induce their killing. This attachment was supported by the findings that the capsulated bacteria are more resistant to lactoferrin (Arnold *et al.* 1980).

The irreversible inhibition and rapid loss in viability within 2 h incubation period, observed with iron-free lactoferrin or iron-free transferrin in deionized water, suggest that iron-free lactoferrin and iron-free transferrin have a direct bactericidal effect on *Y. pseudotuberculosis* that cann't be attributed to simple iron deprivation. Arnold *et al.* (1980) found that the irreversible inhibition by lactoferrin cann't be reversed even after lactoferrin removal. Further, Arnold *et al.* (1982) and Ellison

et al. (1988, 1990) have indicated, that the lactoferrin and transferrin can interact directly with the outer membrane of gram-negative bacteria and damage it, subsequently, causing the release of lipopolysaccharides and cell death. The above findings abolished the possibility that lactoferrin blocks the channels that are required for the transport of iron or other nutritional elements.

To conclude, the *in vitro* inhibition of *Y. pseudotuberculosis* by iron-free lactoferrin or iron-free transferrin can be either reversible or irreversible depends on the presence or absence of medium, respectively. On the other hand, *Y. pseudotuberculosis* appears to be capable of utilizing lactoferrin- or transferrin-bound iron as a source of iron. The *in vivo* effect of lactoferrin and transferrin, however, is governed by a large variety of circumstances all of which could make an important contribution to what have been termed as nutritional immunity (Weinberg 1978) or to the pathogenicity of bacterial infections (Ward *et al.* 1986).

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التثبيط الحديدي - القابل وغير القابل للانعكاس - للبكتيريا *Yersinia pseudotuberculosis* بواسطة اللاكتوفرين والترانسفرين الانساني

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ص .ب (٢٤٥٥) - الرياض ١١٤٥١ - المملكة العربية السعودية

اللاكتوفرين (في الافرازات) والترانسفرين (في الدم) عبارة عن مركبين بروتينيين رابطين للحديد ، ونظرا لأن الحديد عنصر مهم لنمو البكتيريا ، فان قدرة البكتيريا على انتزاع الحديد من هذين المركبين عامل مهم في استراتيجيتها الامراضية .

أوضح الدراسة أن اللاكتوفرين والترانسفرين الخاليان من الحديد ثبطا نمو خلايا البكتيريا *Yersinia pseudotuberculosis* الموجودة في بيئة Brain heart infusion (BHI) الغنية بالحديد أو في بيئة BHI المنزوعة الحديد بواسطة كربونات المغنيسيوم ، بدون أن يؤثرا على حيويتها ، وهذا النوع من تثبيط النمو تم التغلب عليه باضافة اللاكتوفرين والترانسفرين المشبعين بالحديد والقادرين على أن يعملا كمصدرين للحديد في البيئة المنزوعة الحديد أعلاه ، أو بنقل الخلايا إلى بيئة BHI الغنية بالحديد .

على النقيض من ذلك ، كان هناك تناقص سريع في حيوية الخلايا التي تم تعليقها في الماء المقطر وعوملت باللاكتوفرين أو الترانسفرين الخاليين من الحديد ، وهذا النوع من تثبيط النمو والذي تم في عدم وجود البيئة الغذائية

يوحي بأن اللاكتوفرين والترانسفرين لهما تأثيراً قاتلاً مباشراً على *Y.pseudotuberculosis* لا يمكن أن يعزى فقط إلى نقص الحديد .