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THE EXPLORATION OF THE ANTIBACTERIAL MECHANISM OF FE^{3+} AGAINST BACTERIA

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ABSTRACT

This study demonstrated that the bacteria could adsorb Fe^{3+} and reduce Fe^{3+} to Fe^{2+}. Iron had significant bacteriostatic effects, which were directly proportional to the iron concentration and under the influence of pH and chelator. It presumed that the inhibition of Fe^{3+} acts through the formation of hydroxyl free radicals.

Key words: antibacterial activity, chelator, hydroxyl radical, ferric ion, siderophore

Iron is a nutritionally essential trace element and has important functions in the metabolic processes of aerobic organisms, such as photosynthesis, respiration, oxygen transport, gene regulation and DNA biosynthesis (1, 3). Under physiological conditions, iron mainly exists in two readily reversible redox states, the reduced Fe^{2+} form and the oxidized Fe^{3+} form. Although abundant in nature, iron tends to form highly insoluble hydroxides in the aerobic neutral pH environment. The concentration of free ferric ion in solution at biological pH is probably 10^{-9}-10^{-18} mol l^{-1} (2, 5, 9), a concentration too low to allow growth by aerobic microorganisms. Microorganisms have evolved a range of strategies to acquire iron. The major strategies include production and utilization of siderophores, utilization of host iron proteins such as transferrin, and lactoferrin, and reduction of Fe^{3+} to Fe^{2+} with subsequent transport of Fe^{2+} (4). On the other hand, iron is a devastating metal. Fe^{2+} reacts with H_2O_2 to form a hydroxyl radical (6). HO· is known to be a highly reactive, indiscriminate oxidizing agent, which can damage proteins and nuclear acids. Thus, aerobic organisms have a dilemma: they need a scavenging system that is effective in accumulating iron, without allowing too much to accumulate. Much attention has been given to the response of bacteria to iron-limited conditions, but not to how the bacteria respond to adequate or excess iron supply. The growth of bacteria usually requires 10^{-6} mol l^{-1} of iron, and the proper amount of iron can stimulate the growth of bacteria, but under excessive concentrations, the microorganism's growth will be inhibited.

In this article, we studied the adsorption and reduction of 2 Gram-negative bacteria (*Escherichia coli* CVCC 249 and *Ralstonia solanacearum*) and 2 Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis*) to Fe^{3+} under excess Fe^{3+} conditions. The antibacterial effects of Fe^{3+} were investigated and we conducted a preliminary analysis of the inhibition mechanism of Fe^{3+}.

Each bacterial strain was inoculated into 100 ml LB broth and incubated at 37°C with shaking at 170 rpm for 12 h. The culture was centrifuged and washed twice with sterile physiological saline, then the pellet was resuspended in appropriate amount of sterile physiological saline and the turbidity of the samples was measured at 600 nm using a UNICO 2000 UV-Vis spectrophotometer, made the turbidity

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values were 2.0. A sample volume of 5 ml of bacteria suspension was centrifuged and the cell was resuspended into 5 ml of $FeCl_3$ solution (1 mmol l^{-1}, containing 2.5 mmol l^{-1} HCl) and then incubated at 37°C, with shaking at 170 rpm for 2 h. Similarly, the 5 ml bacteria suspension containing 5 ml $FeCl_3$ solution (1, 0.5 and 0.25 mmol l^{-1}, containing 2.5 mmol l^{-1} HCl, respectively) was incubated at room temperature with shaking for 0-45 min at every 5 min interval and then it was centrifuged the supernatant was collected. The ferrous and ferric ion concentrations in the supernatant were measured using 1, 10-phenanthroline spectrophotometry at 510 nm and 364 nm, respectively (8, 12). A volume of 1 ml hydroxylamine hydrochloride (w/v 5%) was added to 1 ml of supernatant, incubated 15 min, and then mixed with 1 ml 1, 10-phenanthroline (m/v, 0.25%), and 1 ml sodium acetate buffer (0.5 mol l^{-1}, pH 4.5), and then added to deionized water to make a total volume of 5 ml. The mixture was further incubated at room temperature for about 30 min, after which the absorbance at 510 nm was measured to determine the total iron content in the supernatant. The hydroxylamine hydrochloride was replaced with the same amount of deionized water and the absorbance at 510 nm and 364 nm were measured to characterize the Fe^{2+} and Fe^{3+} ion content, respectively. A solution of 1 mmol l^{-1} $FeCl_3$ was used as a control. The results showed that all four bacteria could adsorb Fe^{3+}. *R. solanacearum* had the strongest adsorptive capacity for Fe^{3+} at about 50%, followed by *B. subtilis* and *S. aureus* ATCC 25923 which retained about 30% while the absorption capacity of *E. coli* CVCC 249 for Fe^{3+} was the weakest at close to 15%. As can be seen from Figure 1, the iron absorption of the 4 bacteria reached equilibrium in 5-10 min and thus, the adsorption was an extremely rapid process. The four bacteria had similar absorption dynamics curves of total iron and Fe^{3+}. For *E. coli* CVCC 249, *S. aureus* ATCC 25923 and *B. subtilis* bacteria, the Fe^{3+} adsorption could achieve saturation when the Fe^{3+} concentration was 0.25 mmol l^{-1}, but for *R. solanacearum*, saturation could only be reached when the concentration increased to 0.5 mmol l^{-1}. This indicated that *R. solanacearum* had the strongest Fe^{3+} adsorptive capacity. The results also indicated that the decrease of the total iron and Fe^{3+} content was accompanied by the generation of Fe^{2+}. The dynamics curve of the Fe^{2+} production was similar for the 4 bacteria. Moreover, the Fe^{2+} production of *R. solanacearum* and *B. subtilis* was higher than *E. coli* CVCC 249 and *S. aureus* ATCC 25923, which corresponded directly with their Fe^{3+} adsorptive capacity.

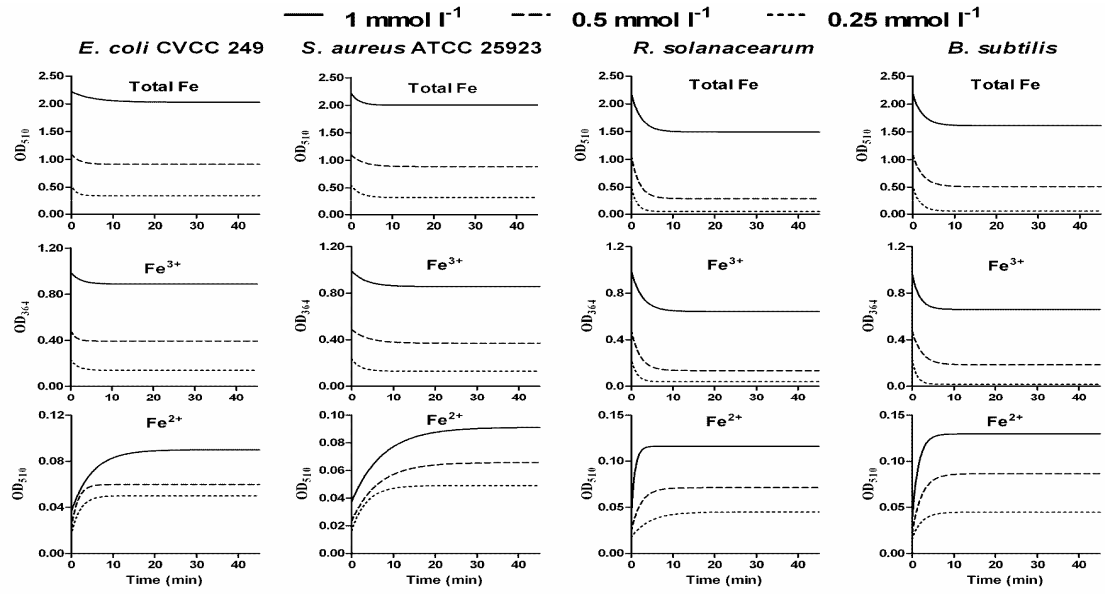

Figure 1. Effects of Fe^{3+} concentration and treatment time on Fe^{3+} absorption and reduction. All results were repeated for three times, the coefficient of variation (CV) was less than 5%.

To investigate the antibacterial effects of high concentrations of ferric iron, the appropriate amount of $FeCl_3$ was sterilized using ultraviolet radiation and then it was combined with sodium hydrogen phosphate - citric acid buffer solutions (pH 4.0, 5.0, 6.0, and 7.0, prepared using 10 mmol l^{-1} sodium hydrogen phosphate and 5 mmol l^{-1} citric acid, separately and then sterilized at 121°C for 30 min.) or sterile water to compound the $FeCl_3$ solutions separately. It was prepared just before use and diluted to the necessary concentrations. The bacterial culture was appropriately diluted with sterile saline and then mixed with different concentrations and pHs of $FeCl_3$ solution at room temperature for 10, 20, 30, and 40 min, respectively. A volume of 100 µl of the solution was spread onto a LB plate. Plates were incubated at 37°C overnight, and colonies on the plates were counted. Samples were plated in triplicate and sterile saline was used as a negative control. The results illustrates that the different concentrations of $FeCl_3$ aqueous solutions (without buffer) had definite antibacterial activities. The bactericidal capacity of Fe^{3+} increased with the increasing concentration of Fe^{3+}. Additionally, the inhibitory effect was an extremely rapid process. In 10 min, 70% of *E. coli* CVCC 249 bacteria were inhibited by 1 mmol l^{-1} Fe^{3+}, and 90% of S. aureus ATCC 25923 were inhibited at the same concentration. Both *R. solanacearum* and *B. subtilis* were completely inhibited when the Fe^{3+} concentration was only 250 µmol l^{-1} and 62.5 µmol l^{-1}, respectively. The antibacterial results of four pH Fe^{3+} solutions on the growth of the bacteria. The four different pH buffer solutions did not obviously inhibited *E. coli* CVCC 249 and *S. aureus* ATCC 25923. However, for *R. solanacearum* and *B. subtilis*, they had varying degrees of inhibition while a solution with a pH value of 4.0 could suppress their growth completely. For solutions with pH values of 4.0, 5.0, and 6.0, 1 mmol l^{-1} Fe^{3+} could suppress the growth of *E. coli* CVCC 249, while at 0.5 mmol l^{-1} Fe^{3+} no inhibitory effects were observed. For the pH 7.0 solution, only the 2 mmol l^{-1} Fe^{3+} displayed inhibitory effects. Under all pH conditions, only 2 mmol l^{-1} Fe^{3+} could obviously inhibit the growth of *S. aureus* ATCC 25923. For solutions with pH values of 5.0 and 6.0, 500 µmol l^{-1} and 250 µmol l^{-1} Fe^{3+} had obvious inhibitory effects on the growth of *R. solanacearum* and the *B. subtilis*, respectively. When the pH value was 7.0, the inhibitory effects weakened. By comparison of these results, it was determined that the antibacterial effects of the buffered Fe^{3+} solutions were weaker than those of the Fe^{3+} aqueous solutions. Thus, it is necessary that higher concentrations of $FeCl_3$ were needed in buffered solutions in order to achieve the same antibacterial efficiency. During the preparation of the fresh $FeCl_3$ solution, the hydrolysis state of ferric ion changes continuously. Depending on the pH of the sodium hydrogen phosphate - citric acid buffer solution, the Fe^{3+} will be in different hydrolysis forms which have correspondingly different relative antibacterial capacities. At pH 7, the concentration of free ferric ion is 1.4×10^{-9} mol l^{-1} in the aqueous solution and the main species is $Fe(OH)_2^+$. In the presence of 40 mmol l^{-1} phosphate, the concentration of free ferric ion is decreased to 3.3×10^{-12} mol l^{-1} because of the formation of ferric phosphate (Chipperfield and Ratledge 2000). Depending on the different pH conditions, the hydrolysis of Fe^{3+} and the formation of ferric phosphate complexes results in a decline in the free Fe^{3+} content, which make the antibacterial activity of the iron weaker. The sensitivities of the various bacteria to the ferric iron were different, but the bacteriostasis dynamics curves were the same. This indicates that the antibacterial effect of Fe^{3+} is likely a non-selective mechanism.

The $FeCl_3$ solution was mixed with three different chelator solutions, acetohydroxamic acid (ICN Biomedicals), 2, 3-dihydroxybenzoic acid (Acros Organics) or a siderophore which produced by *Aspergillus niger* An76 (11) for 2 min, and then added into a bacterial suspension at room temperature for 10 min, respectively. In addition, the $FeCl_3$ solution was added into the bacterial suspension at room temperature for 5 min and then mixed with the above three chelator solutions for 5 min, respectively. Results of the comparison between the antibacterial activity of Fe^{3+} and chelators are shown in Figure 2. Solutions of Fe^{3+} alone displayed remarkable bacteriostasis. The inhibitory rates of 1 mmol l^{-1} Fe^{3+} for *E. coli* CVCC 249 and *S. aureus* ATCC 25923 were approximate 80% within a 10

min treatment time, and 83 μmol l^{-1} Fe^{3+} could completely suppress the growth of *R. solanacearum* and *B. subtilis*. All chelators had no obvious inhibitory effects on *E. coli* CVCC 249, but did inhibit *S. aureus* ATCC 25923. 2, 3-Dihydroxybenzoic acid could strongly inhibit the growth of *B. subtilis*. Acetohydroxamic acid showed a certain extent inhibitory effect for *S. aureus* ATCC 25923, but for other three bacteria did not obvious inhibitory action. *Aspergillus niger* An76 siderophore can completely inhibit *R. solanacearum* and *B. subtilis* growth. When 2, 3-dihydroxybenzoic acid or acetohydroxamic acid was combined with Fe^{3+} together, it was determined that both of the chelators could enhance the Fe^{3+} inhibition of *E. coli* CVCC 249 and *S. aureus* ATCC 25923 regardless of whether they were added before or after the Fe^{3+} solutions were mixed with the bacterial suspension. But the An76 siderophore could reduce the Fe^{3+} inhibition of *E. coli* CVCC 249 and *S. aureus* ATCC 25923.

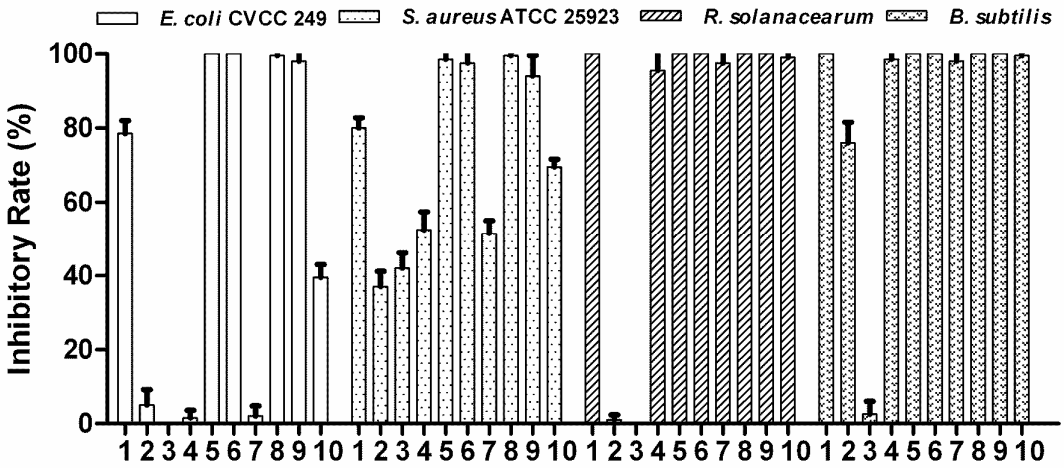

Figure 2. Comparison of the antibacterial effects of Fe^{3+} and chelators. **Notes:** 1: $FeCl_3$; 2: 2, 3-dihydroxybenzoic acid; 3: acetohydroxamic acid; 4: *A. niger* An76 siderophore; 5, 6, 7: First, $FeCl_3$ with 2, 3-dihydroxybenzoic acid or acetohydroxamic acid or *A. niger* An76 siderophore were mixed for 2 min, respectively; 8, 9, 10: After $FeCl_3$ treatment for 5 min, it was mixed with 2, 3-dihydroxybenzoic acid or acetohydroxamic acid or *A. niger* An76 siderophore for 5 min, respectively. The final concentrations of $FeCl_3$, 2, 3-dihydroxybenzoic acid, and acetohydroxamic acid were 1 mmol l^{-1} for *E. coli* CVCC 249 and *S. aureus* ATCC 25923, and those were 83 μmol l^{-1} for *R. solanacearum* and *B. subtilis*. The final concentrations of *A. niger* An76 siderophore were 1 mg/ml for *E. coli* CVCC 249 and *S. aureus* ATCC 25923, and those were 100 μg/ml for *R. solanacearum* and *B. subtilis*.

Acetohydroxamic acid and 2, 3-dihydroxybenzoic acid can form complexes with Fe^{3+}, which cause the hydrolysis of ferric ion to be suppressed. One molecule of Fe^{3+} can bind to the 2, 3-dihydroxybenzoic acid between the acid group and a neighboring hydroxyl group and form an iron complex that subsequently reduces an additional Fe^{3+} (13). The 2, 3-dihydroxybenzoic acid – Fe^{3+} complex can catalyze the generation of hydroxyl free radicals which reach a maximum at about a 1:1 ratio (10). The Fenton reagent, which is a complex of hydrogen peroxide and an iron catalyst, has a strong sterilization action, which has been proven to work through HO·. In our study, 4 bacteria were able to rapidly reduce Fe^{3+} to Fe^{2+}. Moreover, H_2O_2 is a normal metabolite in aerobic and facultative aerobic organisms (7). Our previous research demonstrated that the siderohpre produced by *Aspergillus niger* An76 has significantly scavenging activity of hydroxyl free

radicals (11). Therefore, it is presumed that pure Fe^{3+} does not have the antibacterial effect alone, but instead, it becomes effective as an inhibitor after the first reduction of Fe^{3+} to Fe^{2+}, and further oxidation with oxidized factors, such as superoxide radical and hydrogen peroxide, with the ultimate formation of hydroxyl free radicals which have a strong non-selective sterilization ability.

The formation of drug resistant microorganisms remains a difficult medical problem. Non-selective sterilization would be a useful technique to combat drug resistance and the information provided herein should provide knowledge towards making a disinfectant using iron chelators and iron. However, the interaction of iron ions with chelating agents and their inhibitory mechanism still needs more in-depth research.

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