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ARTICLE

Effects of Oxytetracycline and Sulfachloropyridazine Residues on the Reductive Activity of *Shewanella decolorationis* S12

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Effects of oxytetracycline (OTC) and sulfachloropyridazine (SCP), two of the widely used antibiotics in livestock production, on beneficial environmental microorganisms were studied. *Shewanella decolorationis* S12 was selected as the target bacteria for the role in reduction of Fe(III) and dye under anaerobic conditions. The results showed that the antibiotics significantly inhibited Fe(III) reduction and dye decoloration in the reduction system. The rates of Fe(II) formed ($-r$) were 3.6 and 0.2 mg/L/day for the OTC concentrations of 0−1 mg/L and 1−50 mg/L, respectively, with 1 mg/L as the turning point of the inhibition effect. The turning point of inhibition effect was much higher for SCP treatments, at 4 mg/L. The results also showed higher production values for adsorbed Fe(II) than soluble Fe(II) in OTC treatments, but the reverse occurred in the SCP treatments. The difference between the treatments could be due to higher sorption coefficients of OTC as compared to SCP. Transmission electron micrographs showed changes in cell structures of *S. decolorationis* S12 grown in medium with OTC. Detached cell walls and large vacuoles in internal cell contents were found in OTC-treated cells. The results of the present study indicated that the inhibition of antibiotic on the reduction activity of *S. decolorationis* S12 may be due to a decrease in live *S. decolorationis* S12 population and/or damages of their cell structure in this reduction system.

KEYWORDS: Oxytetracycline; sulfachloropyridazine; *Shewanella decolorationis* S12; antibiotics; reductive activity

INTRODUCTION

Antibiotics are widely used in livestock farming as growth promoters and/or to treat infectious diseases (*1*). Tetracyclines and sulphonamides are some of the most important antibiotics because of their effectiveness and low price (*2*). It has been reported that over 10,000 tons of antibiotics were used for the prevention and treatment of animal diseases with an annual consumption of 95 tonnes for livestock farming in European countries, of which 78 tonnes were for pig production (*3*). Since 2006, the EU countries have prohibited the use of antibiotics as growth promoters for livestock production, but the use of such drugs for prevention and treatment of diseases is still allowed (*4*). The actual consumption of veterinary drugs in most developing countries including China and many southeast Asian countries, such as Malaysia, is not known. Intensive livestock farming, particularly pig and poultry, uses large amounts of these drugs (*2*).

Many of the antibiotics are poorly absorbed by the animals. About 25 to 75% of the antibiotics are excreted via feces or urine as the parent compounds or their metabolites and are released into the environment either directly by speading as manure or after collection and storage in the form of sludge (*3*). When present in the environment, these chemicals may have detrimental effects on terrestrial and aquatic organisms as well as play a role in the development of antimicrobial resistance. Currently, the environmental hazards of veterinary antibiotics have received a great deal of attention (*2*,*4*). Tetracyclines and sulphonamides are broad-spectrum bacteriostatic antibiotics, which inhibit dihydropteroate synthesis in the folic acid pathway (*4*), and reduce the reproductive functions of bacteria. Residual concentrations of antibiotics have been observed in soils and surface and groundwater (*5*−*7*). They may affect the beneficial bacterial communities in the environments. The fate of antibiotics in the soil and aquatic environments such as sorption and fixation, mobility and transport, eco-toxicity, resistance, and degradation are well documented (*1*, *8*−*14*), but information on the effect of antibiotics on beneficial bacteria in the environment is scanty.

Shewanella decolorationis S12 is a member of the genus *Shewanella*, which includes beneficial bacteria widely distributed in the natural soil−water environment. A notable feature of members of this genus is their ability to use a variety of different electron acceptors such as manganese, iron oxides, uranium, thiosulfate, and elemental sulfur (*15*−*17*). *Shewanella decolorationis* S12 elicits more than 90% decolorization activity of dyes such as Fast Acid Red GR and Reactive Brilliant Blue within

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12 h, and is able to reduce nitrate, nitrite, ferric compounds, and thiosulfate with lactate or acetate as the electron donor (*18*). It is also able to reduce various azo dyes in a defined medium with formate, lactate, and pyruvate or H_2 as the electron donor under anaerobic conditions (*18*). The objectives of this study were to investigate the effects of oxytetracycline and sulfachloropyridazine on the reduction of Fe(III) oxides and dye decoloration by *S. decolorationis* S12, with a view to evaluate the possible effects of these antibiotics on environmental bacteria.

MATERIALS AND METHODS

Chemicals. Oxytetracycline (OTC) and sulfachloropyridazine (SCP) (Sigma-Aldrich, USA) stock solutions were prepared at a concentration of 50 and 200 mg/L, respectively, in 0.8% salt solution and then further diluted with the same salt solution to the experimental concentration standards in mobile phase to construct a standard calibration curve. Other chemicals with analytical grade were from Guangzhou Chemical Co. (China).

Preparation and Characterization of Goethite. Goethite (α-FeOOH; 60.2 m^2/g) was prepared as reported by Schwertmamm and Cornell (*19*). The prepared goethite was washed with distilled water to remove soluble salts. The purity of goethite was determined by X-ray diffraction spectroscopy (XRD) on Rigaku D/Max-III. The diffractometer was set at room temperature and operated at 30 kV and 30 mA using Cu Kα radiation (λ = 0.15418 nm). The phases were identified by comparing diffraction patterns with those on the standard powder XRD cards compiled by the Joint Committee on Powder Diffraction Standards (*20*). The total surface area and total pore volume of the prepared sample were measured by the Brunauer−Emmett−Teller (BET) method in which the N_2 adsorption at 77 K was applied, and a Carlo Erba Sorptometer was used (*21*).

Pure α-FeOOH was obtained because the peaks of (110), (130), (111), (121), (221), and (151) were attributable to the characteristic peaks of α-FeOOH as reported previously (*22*). The crystal size of α-FeOOH was 41.9 nm, which was deduced from Sherrer's formula (*22*) with the corresponding strongest XRD peak, while the specific surface area measured by the BET-BJH method was 120.93 m^2/g, and the total pore volume was 0.16 cm^3/g for α-FeOOH.

Microbial Strain and Culture Conditions. *Shewanella decolorationis* S12 was isolated from an activated sludge of a textile-printing wastewater treatment plant in Guangzhou, China (*23*). It is a facultative bacterium growing under both aerobic and anaerobic conditions. It can grow in a pH range of between 7.0 to 10.0, with an optimum pH of 8.0, and temperatures between 4 and 40 °C, with an optimum temperature range of 20 to 30 °C. The starter culture of *S. decolorationis* S12 was prepared by growing a single colony in a 500-mL conical flask containing 200 mL of nutrient medium at 30 °C for 16 h in an incubation shaker (180 rpm). The aerobically grown cells were harvested at the end of the exponential growth phase by centrifugation (10,000 rpm for 10 min at 10 °C), washed twice, and cultivated under anaerobic conditions in 100 mL of defined medium (pH 7.2) containing 7.4 mM $NaHCO_3$, 1.2 mM NH_4Cl, 1.1 mM $NaH_2PO_4 \cdot 2H_2O$, 0.34 mM KCl, vitamin, and trace element solution.

Bacteria Reduction Experiments. *Batch Cultures for Fe(III) Reduction and Dye Degradation.* Goethite is the source of Fe(III) for the batch experiments. Single batch reduction experiments were performed with 10^8 CFU (colony forming units)/mL of *S. decolorationis* S12 cells in the fresh defined medium (initial pH 7.2) containing 8 mM α-FeOOH as electron acceptors and 10 mM lactic acid as electron donors. For the experiment on the effect of different antibiotic concentrations on the reduction of Fe(III) by *S. decolorationis* S12, 0, 0.2, 0.4, 1, 2, 4, 10, 20, 30, and 50 mg/L OTC and 0, 1, 2, 4, 10, 20, 50, 100, 150, and 200 mg/L SCP were used as initial antibiotic concentrations. The Fe(II) productions were analyzed after 3 days of incubation. For the experiment of different antibiotic reaction times on the reduction of Fe(III) by *S. decolorationis* S12, 0, 1, 10, 30, and 50 mg/L OTC and 0, 4, 20, 100, and 200 mg/L SCP were used as initial antibiotic concentrations. The Fe(II) productions were analyzed on days 3, 4, 5, 6, 7, and 8, and the populations of *S. decolorationis* were also estimated on days 1, 4, and 8. The populations were estimated using the colony counting method. In the dye decoloration experiment, orange II and rhodamine were used as model dyes to determine their degradation by *S. decolorationis* S12 under two different concentrations of SCP (10 mg/L and 100 mg/L). The treatments were different combinations of live *S. decolorationis* S12, iron oxide, and SCP as follows: (i) without iron oxide, SCP, and live cells (blank), (ii) with live cells only, (iii) with iron oxide and live cells (control), (iv) with live cells, iron oxide, and 10 mg/L SCP, and (v) with live cells, iron oxide, and 100 mg/L SCP. The concentrations of dye were analyzed at 0, 30, 60, 180, and 300 min using a UV−visible spectrophotometer (TU-1800PC).

Different concentrations of OTC, SCP, and dye solutions were filtered sterilized (0.22 μm filter) before flushing with O_2-free nitrogen gas for 15 min to remove traces of oxygen. All batch experiments were conducted in 100-mL serum bottles. At initial time, 10^8 CFU/mL *S. decolorationis* S12 was added, and the serum bottles were sealed with butyl rubber and aluminum stoppers. Bottles inoculated with heat-killed cells acted as controls. All cultures were replicated 3 times, incubated at 30 °C with shaking at 180 rpm, and sampled at the designated times as described above for the various experiments.

Analysis of Fe(II). Total Fe(II) in the samples was extracted using 0.5 mM HCl for 1.5 h (*24*). Extracted total Fe(II) and soluble Fe(II) were filtered through 0.22-μm filters to remove particles in the samples, and 1,10-phenanthroline was used as the chromogenic agent (*24*). The concentrations of total Fe(II) and soluble Fe(II) were determined by colorimetric method using a UV−visible spectrophotometer (TU-1800PC). Adsorbed Fe(II) was calculated as the difference between total Fe(II) and soluble Fe(II).

Cell Structure Experiment. The cell structure of *S. decolorationis* S12 after 8 days of incubation with and without 20 mg/L OTC was studied using transmission electron microscopy (TEM). The cells were incubated in borosilicate glass tubes and harvested by centrifugation (10,000 rpm for 10 min at 10 °C). They were then resuspended in 10 mL of 0.1 M KNO_3 solution containing 1% (v/v) OsO_4, centrifuged again (10000 rpm for 10 min at 10 °C), and washed with 0.1 M KNO_3. The pellet was immersed in 1 mL of OsO_4 (1% in 0.1 M KNO_3) overnight, then washed in 0.1 M KNO_3, and mixed with 0.5 mL of 2% agar. The conventional embedding and sectioning procedures for thin sections were performed, and the sections were examined with a transmission electron microscope (Zeiss E.M.95, 60 kV).

Statistical Analysis. The data were analyzed by using Statistical Analysis System (SAS) (*25*). Duncan's Multiple Range Test was used to compare the treatment means, and 0.05 level of probability was used to identify differences.

RESULTS AND DISCUSSION

Effects of OTC and SCP on Goethite Reduction. Concentrations of total Fe(II) (0.5 M HCl extractable) produced from Fe(III) (goethite) as a function of *S. decolorationis* S12 activity in various concentrations of OTC and SCP are presented in **Figure 1**. Total concentrations of Fe(II) produced from Fe(III) decreased with increased concentrations of OTC or SCP. This indicated that these two antibiotics inhibited goethite reduction activity.

The inhibition on goethite reduction activity was more sensitive in lower concentrations of OTC and SCP than in higher concentrations. As shown in **Figure 1**, the turning point of the inhibition effect (i.e., the point at which rapid inhibition ended and a more gradual inhibition began) was at 1 and 4 mg/L for OTC and SCP, respectively. When the concentration of OTC increased from 0 to 1 mg/L, the total Fe(II) produced decreased from 19.7 to 16.1 mg/L at a rate ($-r$ value) of 3.6 mg/L/day, but when the concentration of OTC increased from 1 to 50 mg/L, the total Fe(II) formed decreased at a slower rate, from 16.1 to 6.6 mg/L, with a $-r$ value of 0.2 mg/L/day. Similarly, when the concentration of SCP increased from 0 to 4 mg/L, the $-r$ value was 0.8 mg/L/day, but when the concentration of SCP increased from 4 to 200 mg/L, the corresponding $-r$ value was only 0.03 mg/L/day. These results showed a clear inhibition of OTC and SCP on Fe(III) reduction induced by *S. decolorationis* S12, and the inhibition effects of OTC and SCP were more obvious at a

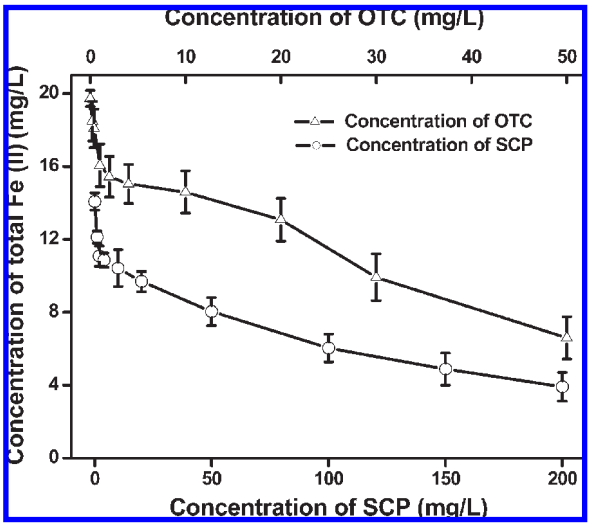

Figure 1. Effects of OTC and SCP concentrations on total Fe(II) (0.5 M HCl extractable) produced after 3 days of incubation in the system containing 8 mM α-FeOOH, 10 mM lactic acid, and 10^8 CFU/mL of *S. decolorationis* S12 cells. (Each data point represents the average of three tests, and the error bars represent the standard errors of the mean.)

range of less than 1 and 4 mg/L, respectively. When the total Fe(II) production decreased to 6.6 mg/L, the OTC and SCP concentrations were 50 and 80 mg/L, respectively (**Figure 1**). This indicated that a lower concentration of OTC than SCP was required for the same inhibition effect on total Fe(II) production. This is probably due to the higher sorption coefficient (K_d) of OTC when compared to SCP. The K_d of OTC have been reported to be 417 L/kg in sandy soil and 1026 L/kg in sandy loam (*26*), while the K_d values of SCP have been found to be 0.9 to 6.41 L/kg in soil and soil−slurry mixtures (*27*, *28*). A higher sorption coefficient implies a stronger inhibition effect on Fe(III) oxide reduction.

Effects of OTC and SCP reaction time on Fe(II) production caused by *S. decolorationis* S12 are shown in **Figure 2**, **Figure 3**, and **Table 1**. The total Fe(II) production was initially rapid and then slowed down with time in the control treatment. There were significant differences in total Fe(II) production rates (*r* value) between the control and antibiotic treatments (**Table 1**). The *r* values were 6.64, 4.44, 4.34, 3.91, and 3.73 mg/L/day when the concentrations of OTC were 0, 1, 10, 30, and 50 mg/L, respectively. The corresponding *r* values were 8.30, 7.50, 5.74, 4.72, and 3.74 mg/L/day when concentrations of SCP were 0, 4, 20, 100, and 200 mg/L. The *r* values of Fe(II) production were significantly different at lower concentrations of OTC (between 0 and 1 mg/L) and SCP (between 0 and 4 mg/L) treatments. The results from the present study also showed higher values for adsorbed Fe(II) (**Figure 2c**) than soluble Fe(II) (**Figure 2b**) in the OTC treatments. However, higher soluble Fe(II) (**Figure 3b**) and lower adsorbed Fe(II) (**Figure 3c**) were observed in the SCP treatments. The relationship between the adsorbed Fe(II) and the sorption coefficient is still unknown and requires further studies.

Effects of SCP on Dye Degradation. The effects of SCP on the degradation of rhodamine and orange II dye (10 mg/L) by *S. decolorationis* S12 are shown in **Figure 4**. The results showed that the decolorization rate decreased with increased SCP concentrations. For the control (without SCP), the decolorizing efficiency of *S. decolorationis* S12 was 30% and 81% after 300 h of reaction time for rhodamine and orange II, respectively. In comparison, the decolorization efficiencies in 10 and 100 mg/L SCP treatments after 300 h of reaction time were only 21% and 19%, respectively,

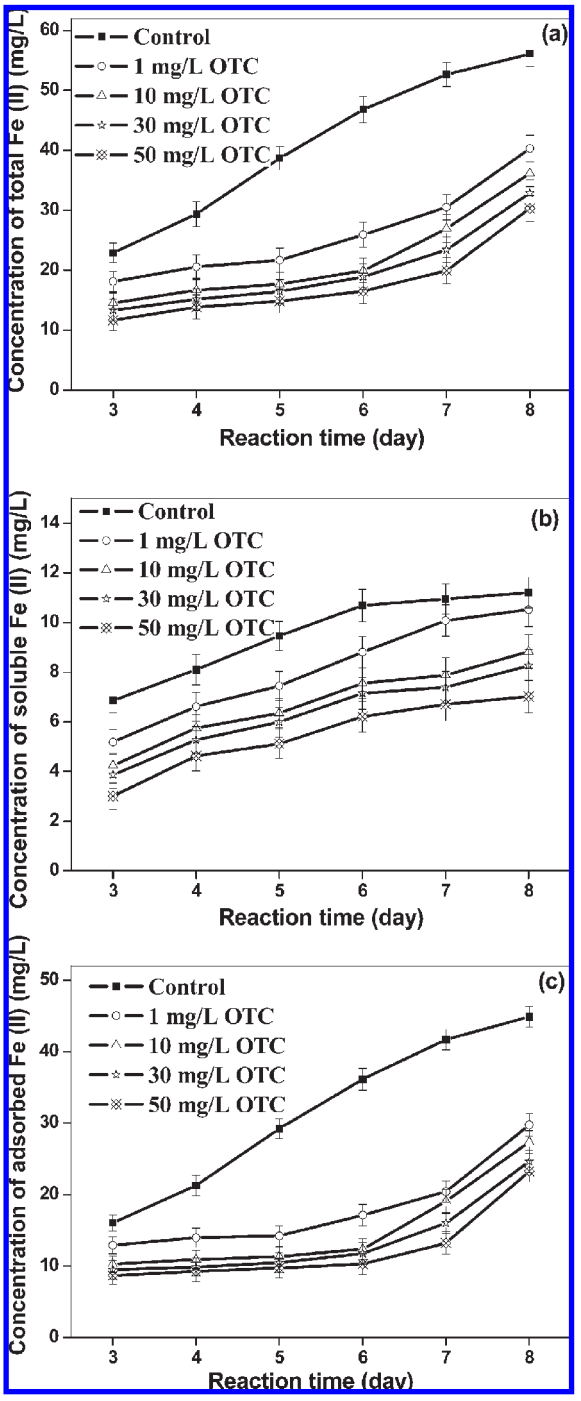

Figure 2. Effect of OTC reaction time on Fe(II) produced in the system containing 8 mM α-FeOOH, 10 mM lactic acid, and 10^8 CFU/mL of *S. decolorationis* S12 cells. (**a**) Total Fe(II); (**b**) soluble Fe(II); (**c**) adsorbed Fe(II).

for rhodamine (**Figure 4a**), and 70% and 67%, respectively, for orange II (**Figure 4b**). In the blank, the color remained almost unchanged. Decolorizing efficiency of *S. decolorationis* S12 was significantly different between rhodamine and orange II (**Figure 4**), with better efficiency for orange II than rhodamine. This may be because the reduction ability of *S. decolorationis* S12 differs in different types of dyes (*29*). Orange II used in this study is a typical azo dye, and *S. decolorationis* S12 has been shown to have a high decolorizing efficiency for azo dyes (*18*, *29*). The results also showed that without iron oxide, the rate of dye degradation was slow, which indicated that the formed Fe(II)

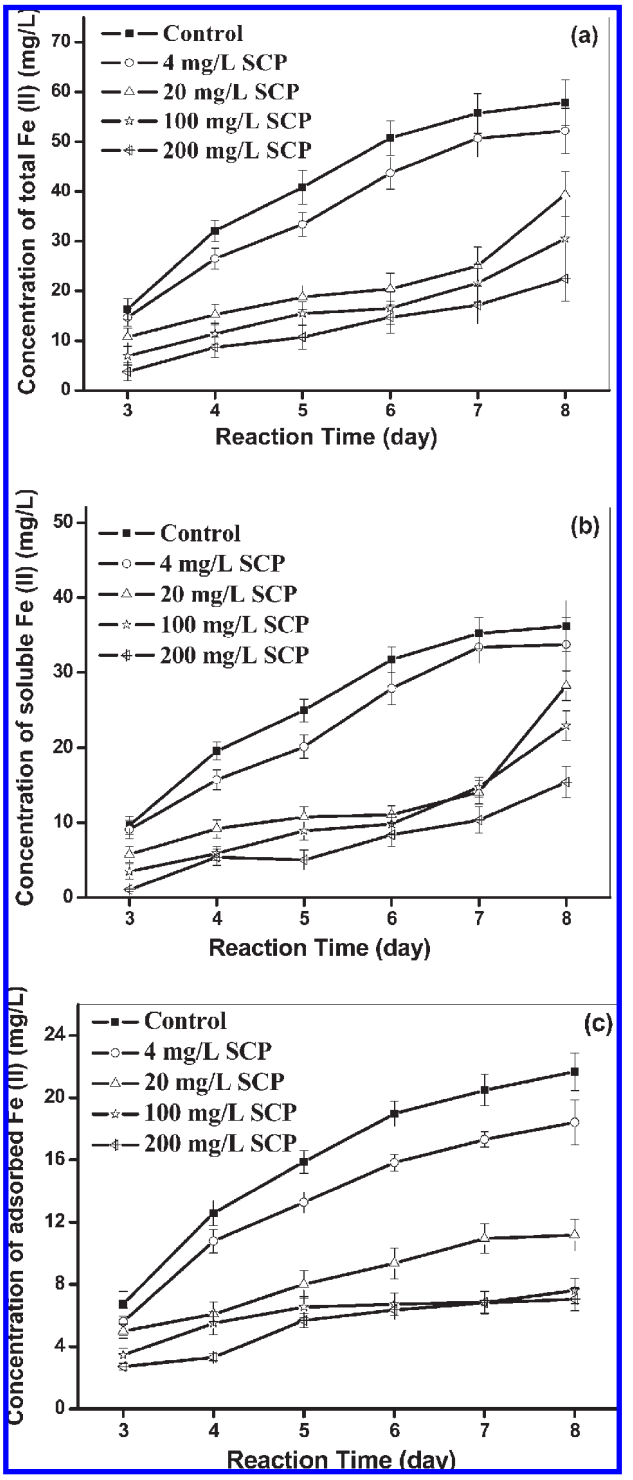

Figure 3. Effect of SCP reaction time on Fe(II) produced in the system containing 8 mM α-FeOOH, 10 mM lactic acid, and 10^8 CFU/mL of *S. decolorationis* S12 cells. (**a**) Total Fe(II); (**b**) soluble Fe(II); (**c**) adsorbed Fe(II).

probably played a role in the degradation of the dye. However, further studies are needed to validate this.

Effect of OTC and SCP on the Population of *S. decolorationis* S12. The populations of live *S. decolorationis* S12 cells in the Fe(III) reduction experiment under different OTC and SCP concentrations are shown in **Table 2**. The population of live *S. decolorationis* S12 decreased with time and increasing concentrations of OTC (1−50 mg/L) and SCP (4−200 mg/L). At day 8 of incubation, the populations of *S. decolorationis* S12 in 4 mg/L SCP and 1 mg/L OCT had decreased to 2.0×10^5 and 2.4×10^5 CFU/mL, respectively; when compared to the control of 1.6×10^6 CFU/mL and at 200 mg/L SCP and 50 mg/L OCT, the

Table 1. *r* Values (mg/L/day) in the Experiment of OTC and SCP Reaction Time (8 days) on Total Fe(II) Production

OTC concentration	*r* value	SCP concentration	*r* value
without OTC (control)	6.64 ± 0.30 a[a]	without SCP (control)	8.30 ± 0.33 a
1 mg/L OTC	4.44 ± 0.27 b	4 mg/L SCP	7.50 ± 0.35 b
10 mg/L OTC	4.34 ± 0.24 b	20 mg/L SCP	5.74 ± 0.34 c
30 mg/L OTC	3.91 ± 0.19 c	100 mg/L SCP	4.72 ± 0.36 d
50 mg/L OTC	3.73 ± 0.23 d	200 mg/L SCP	3.74 ± 0.36 e

[a] Mean with different letters in the same column differed significantly ($P < 0.05$). $r = (C_t - C_0)/t$ where, C_t is the concentration of Fe(II) produced at *t* days, and C_0 is the concentration of Fe(II) produced at the beginning. *t* is the time (day).

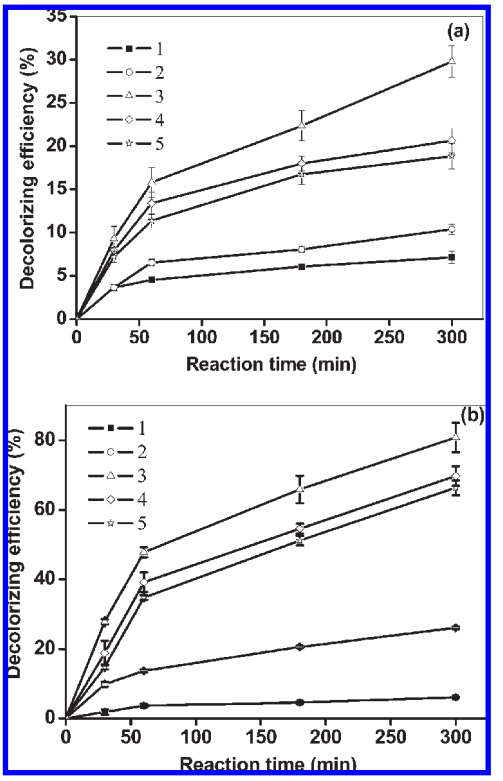

Figure 4. Effect of SCP on the degradation of 10 mg/L dye by 10^8 CFU/mL of *S. decolorationis* S12 cells in the system containing 8 mM α-FeOOH and 10 mM lactic acid. (**a**) Rhodamine; (**b**) orange II (1, without iron oxide, live S12 cells and antibiotic (blank); 2, with live S12 cells only; 3, with live S12 cells and iron oxide (control); 4, with live S12 cells, iron oxide, and 10 mg/L SCP; and 5, with live S12 cells, iron oxide, and 100 mg/L SCP).

Table 2. Effects of SCP and OTC Concentrations on the Population of *S. decolorationis* S12

	population of *S. decolorationis* S12 (CFU/mL)		
treatment	day 1	day 4	day 8
control	6.2×10^7	5.0×10^6	1.6×10^6
4 mg/L SCP	1.2×10^7	1.6×10^6	2.0×10^5
20 mg/L SCP	1.0×10^7	1.0×10^6	5.6×10^4
100 mg/L SCP	2.5×10^6	2.0×10^5	1.5×10^3
200 mg/L SCP	1.3×10^6	1.6×10^5	3.0×10^2
1 mg/L OTC	5.1×10^7	4.8×10^6	2.4×10^5
10 mg/L OTC	3.9×10^7	3.7×10^6	2.9×10^4
30 mg/L OTC	2.7×10^7	6.7×10^5	5.8×10^3
50 mg/L OTC	2.3×10^7	3.3×10^5	2.3×10^3

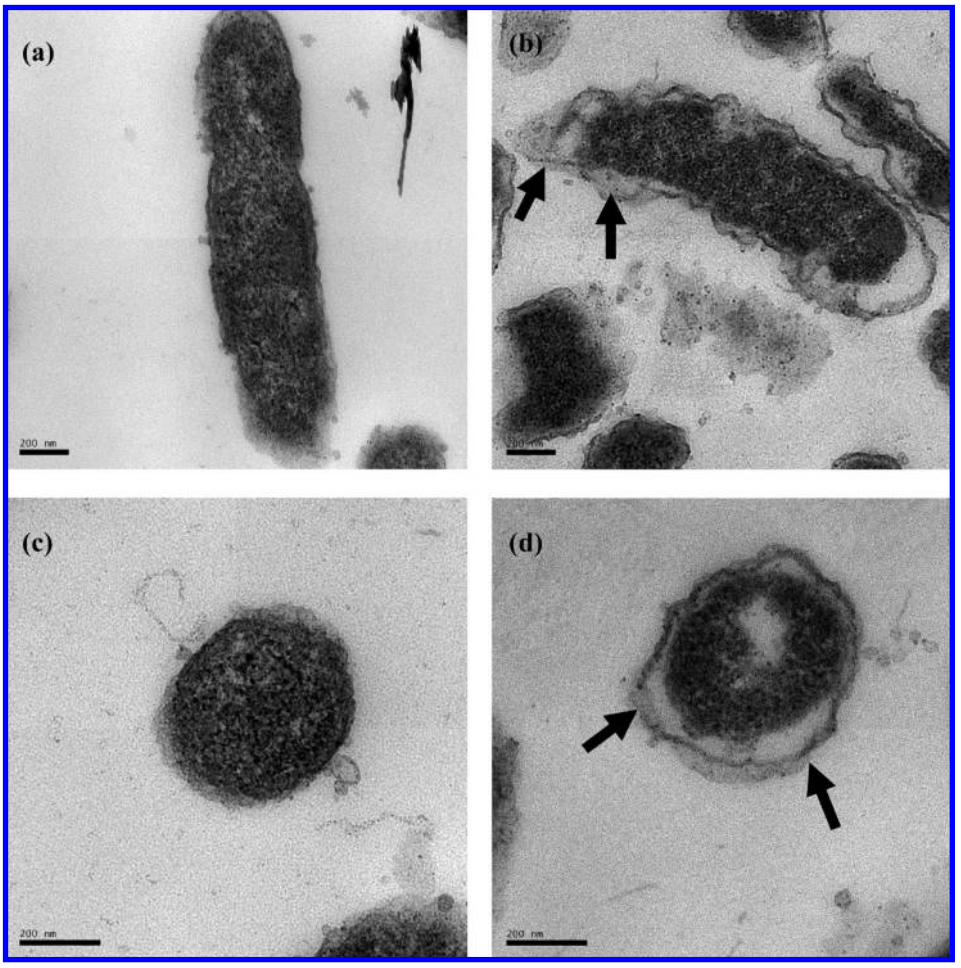

Figure 5. Transmission electron micrographs of *S. decolorationis* S12 at day 8 of incubation. (**a** and **c**) Normal cells of *S. decolorationis* S12 grown in culture medium without OTC. (**a**) Random section; (**c**) oblique cross-section. (**b** and **d**) *S. decolorationis* S12 cells grown in medium with 20 mg/L OTC. (**b**) Random section; (**d**) Oblique cross-section. Note that the outer membrane of cells grown in medium with OTC (**b** and **d**) is irregular and detached with some parts damaged or disintegrating (arrows), resulting in an overall deformation of the cell structure as compared to the normal cells grown in medium without OTC.

populations were drastically reduced to 3.0×10^2 and 2.3×10^3, respectively. The results of this study clearly showed that both OCT and SCP had inhibitory effects on the growth of *S. decolorationis*.

Effect of OTC on the Cell Structure of *S. decolorationis* S12. The TEM study showed differences in the cell structures of *S. decolorationis* S12 grown in media with and without OTC. Transmission electron micrographs of normal *S. decolorationis* S12 cells are shown in **Figure 5a** (random section) and **c** (oblique cross-section). The characteristics of a normal cell (grown in medium without OTC) were a well-defined cell wall and a uniformly dense cell interior, which corresponded to the presence of protein and DNA (*30*). There were significant changes in the cell structure of *S. decolorationis* S12 grown in the OTC medium (**Figure 5b** and **d**). Compare to the normal cells, the outer membranes of the cells from the OTC treatment group were irregular and detached from their interior cell contents. Some parts of the detached cell wall were damaged or disintegrating, leading to a leakage of the internal cell contents and creating a large vacuole in the interior of the cell (**Figure 5d**). Biogenic nanoparticles of metal pollutant have been reported to be present in the periplasmic space of the bacterial cells (*31*), but in this study, no biogenic nanoparticles were detected probably because of the low solubility of OTC and the absence of metal ions in the OTC solution.

Reasons for the Inhibition of *S. decolorationis* S12 Reduction Efficiency by Antibiotics. It has been reported that *S. decolorationis* S12 has high efficacy not only for Fe(III) reduction but also for dye decolorization (*18*, *29*, *32*). Our results demonstrated that OTC and SCP, two of the widely used antibiotics in livestock production, have an adverse effect on *S. decolorationis* S12 and its Fe(III) reduction and dye decolorization ability. Bonneville et al. (*33*) have reported that microbial reduction of Fe(III) follows a two-step mechanism: initial attachment of Fe(III) colloids to the cell surface followed by reduction of Fe(III). It is important to note that although live and dead cells have equal opportunity to be attached to the Fe(III) colloids in this system, only the live ones have the capability to reduce Fe(III). Therefore, treatment groups that had fewer live cells would result in lower Fe(III) reduction. The lower populations of live *S. decolorationis* S12 cells in the OTC and SCP treatments thus resulted in the lower reduction of Fe(III) to Fe(II) when compared to that in their respective control treatments. From these results, we suggest that one of the possible ways in which OTC and SCP adversely affect the reduction of goethite and degradation of the dye by *S. decolorationis* S12 in this study is by inhibiting the growth of the bacteria and thus decreasing their reductive efficiency.

Xu, et al. (*29*) had reported that the decreased reduction of Fe(III) in dye decoloration by *S. decolorationis* S12 could be caused by an effect on the activity of *S. decolorationis* S12. However, the actual mechanism of how the activity of bacteria was affected is still unclear. The present study suggested that antibiotics could damage the cell structure of the bacteria

by causing the outer cell wall to detach from the inner cell contents, disintegrating in some areas, leading to possible leakage of the inner cell contents (**Figure 5b** and **d**), which subsequently could result in some physiological changes within the cell and thus affect its reductive efficiency.

In conclusion, results of this study showed that the two antibiotics, OTC and SCP, inhibited the ability of *S. decolorationis* S12 in Fe(III) reduction and dye degradation. The two possible explanations for the inhibition are a decreased live bacteria population and damage in the ultrastructure of *S. decolorationis* S12 cells in the reduction systems copresent with antibiotics.

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