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THE EFFECT OF THE TI (IV)-CITRATE COMPLEX ON *STAPHYLOCOCCUS AUREUS* GROWTH AND BIOFILM FORMATION

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Abstract: The primary objective of this study was to investigate the influence of the Ti (IV)-citrate complex on growth dynamics and biofilm formation of *S. aureus*. Speciation analysis was performed in order to estimate the structure of the Ti complex existing in citrate solutions at near-physiological pH. It is estimated that the fully deprotonated tris(citrate) titanate ion $[Ti(C_6H_4O_7)_3]^{8-}$ predominates in solution at pH 6.46-7.44, and that this is most probably the biologically active form of Ti(IV)-citrate. In *in vitro* experiments, increasing concentrations of citric acid solutions (0.05, 0.005, 0.0005 M), served as positive controls, while the effects of respective concentrations of Ti(IV)-citrate were examined. The obtained results indicate that citrate decreased *S. aureus* 48 growth at all studied concentrations, whereas *S. aureus* 44 growth was decreased only by high concentrations of citrate (0.05M). Incubation of *S. aureus* culture with Ti(IV)-citrate significantly potentiated citrate-induced effects. Ti(IV)-citrate significantly altered specific bacterial growth rate in a similar manner. The most significant growth reduction was observed at the initial period of bacterial growth. At the same time, the opposite effect was detected in investigations of the effect of citrate and Ti(IV)-citrate on *S. aureus* biofilm formation. Citric acid suppressed *S. aureus* biofilm formation, whereas Ti(IV)-citrate displayed a significant stimulatory effect. Our findings suggest that Ti(IV)-citrate possesses a more pronounced biological effect than citrate. The proposed mechanism of this action is activation of complex transport into the cell and induction of oxidative stress. However, the exact mechanism of Ti(IV)-citrate biological action on bacterial cultures remains unknown.

Key words: Titanium (Ti); citric acid; *Staphylococcus aureus*; bacterial growth; biofilm formation

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INTRODUCTION

Ti is widely used for the production of medical implants (Buser, 2001; Elias et al., 2008; Mishnaevsky et al., 2014) as well as cosmetics (Glowczyk-Zubek, 2004; Onoda and Yamaguchi, 2013). Despite its inertness, metal ions are released into the internal environment of an organism (Hanawa, 2004). This may lead to titanic ion interaction with biomolecules. In particular, citrate is able to interact with Ti due to the relatively high concentration of citric acid in biological fluids (Collins et al., 2005). At the same time, citrate-dependent release of Ti ions from alloys should be kept in mind (Ducheyene et al., 1984). These factors can lead to the formation of Ti-citrate complexes with different structures.

Numerous studies on Ti-microorganism interaction exist. Most of them are devoted to Ti dioxide (Chen et al., 2012; Chung et al., 2009; Roy et al., 2010;

Tang et al., 2011). Synthetic Ti compounds are also widely studied (Jose et al., 2005). At the same time, the influence of Ti complexes with organic acids and citric acid in particular is insufficiently studied. In single studies examining this problem, the influence of Ti (III)-citrate complex on bacterial cell dynamics was studied (Wachenheim and Hespell, 1984), while there is no data clarifying the biological effect of Ti(IV) citrates on bacteria.

S. aureus is a pathogenic microorganism involved in the development of a wide spectrum of infectious pathologies, including diseases of skin, teeth, viscera, musculoskeletal system, etc (Higaki et al., 2000; Nair et al., 2000; Zecconi and Scali, 2013). Taking into account the areas of application of Ti preparations and production (cosmetics, implants), investigation of the influence of Ti on *S. aureus* is of great interest. Therefore, the primary objective of this study was to investigate the effect of the Ti (IV)-citrate complex on the growth dynamics and biofilm formation of *S. aureus*.

MATERIALS AND METHODS

pH-dependent speciation of aqueous Ti(iv)-citrate complexes

In order to specify the structure of the complex existing in citrate solutions in near-physiological pH ranges, we studied the equilibrium in the Ti(IV)-citric acid system. The metal:ligand ratio was chosen to be 1:3 due to a relatively high concentration of citrate in intracellular fluid. All solutions were prepared using carbonate-free double-distilled water. The stock solutions of NaOH and citric acid ($C_6H_8O_7$) were prepared from crystalline material. Fe(III)-free titanyl sulfate was prepared from Ti(IV) tetrachloride (Puriss., Fluka) in accordance with the existing method (Ehrlich, 1965). Titanyl sulfate was prepared using 1 mol L^{-1} H_2SO_4 in order to prevent further hydrolysis of the compound. Final Ti(IV) ion concentration was assessed titrimetrically using 0.1% xylenol orange, hydrogen peroxide and 0.05 mol L^{-1} solution of tetrasodium salt of ethylenediaminetetraacetic acid. The exact concentration of citric acid in the solution was estimated with standard NaOH solution using 0.1% thymol blue. 0.1-0.5 mol L^{-1} carbonate-free NaOH solutions were made from 18 mol L^{-1} stock solutions. The actual concentration of NaOH in the solution was estimated by acidimetric titration using 0.1% methyl red in 95% ethanol as an indicator. 0.1 N H_2SO_4 was used as a titrated solution in acidimetry.

0.0041-0.0630 mol L^{-1} solutions of citric acid and 0.0013-0.0200M solutions of Ti(IV) with a 1:3 metal:ligand ratio were used for pH-metric titration. The investigation of complex formation in the Ti-citric acid system was performed in argon medium. The complexation equilibrium was studied in the absence of base electrolyte. This approach allowed minimization of the influence of foreign ions on the complex formation scheme in the solution. Potentiometric analysis was performed in a thermostat-cell maintained at 25°C under permanent stirring. pH-213 (Hanna Instruments, USA) was used for pH-metric analysis.

Table 1. Ti(IV)-citrate accumulation in aqueous solution at near-physiological pH values (%).

pH	$[Ti(C_6H_5O_7)_2(C_6H_4O_7)]^{6-}$	$[Ti(C_6H_5O_7)(C_6H_4O_7)_2]^{7-}$	$[Ti(C_6H_4O_7)_3]^{8-}$
6.46	5	27	51
6.67	2	21	62
6.89	1	14	70
7.24	<1	7	76
7.44	<1	4	79

Data on $[Ti(C_6H_6O_7)_3]^{2-}$, $[Ti(C_6H_6O_7)_2(C_6H_5O_7)]^{3-}$, $[Ti(C_6H_6O_7)(C_6H_5O_7)_2]^{4-}$, $[Ti(C_6H_5O_7)_3]^{5-}$ accumulation were not significant under current conditions (<1% at all pH points) and are not shown.

Stability constants and the percent of accumulation of the complex forms in the solution were estimated using pH-metric analysis data. Data processing was carried out using the least-squares method on the CPESSP software (Sal'nikov et al., 1989). The determination of complexation scheme and stability constants calculation of all forms in the system was held by solution of the inverse problem based on different experimental physicochemical data. The result of the current calculation is the equilibrium concentrations and percent parts of all complex forms. The number of forms is determined by the size of the stoichiometric matrix. The percent of the complex accumulation in the solution analyzed was calculated as a function of the concentration using the following formula:

$$\alpha = [c(complex)/c(Ti(IV))]\cdot 100\%,$$

where α – the portion of complex ion accumulation solution; c(complex) – concentration of titan (IV)-citrate complex in the solution (mol L^{-1}); c(Ti(IV)) – concentration of Ti(IV) ions in the solution.

Based on the potentiometric titration data the curves of dependence of generation functions on pH were constructed. The generation function in the current method is the mean value of ligand titration ñ (Bjerrum function). The latter is equal to the amount of the titrated protons (H^+) on 1 mol of citric acid (data not shown). Based on the pH data, the calculation of stoichiometric data of the existing complexes was performed.

Equilibrium modeling in the excess of the ligand was started from the simple scheme based on the literature data (Collins et al., 2005; Deng et al., 2007; Kefalas et al., 2005). According to the earlier data in the excessive ligand concentration, the most characteristic complexes formed in the system Ti(IV)-citric acid are compounds with a 1:3 metal:ligand ratio: $[Ti(C_6H_6O7)_3]^{2-}$, $[Ti(C_6H_6O_7)(C_6H_5O_7)_2]^{4-}$, $[Ti(C_6H_5O_7)_2(C_6H_4O_7)]^{6-}$, $[Ti(C_6H_4O_7)_3]^{8-}$.

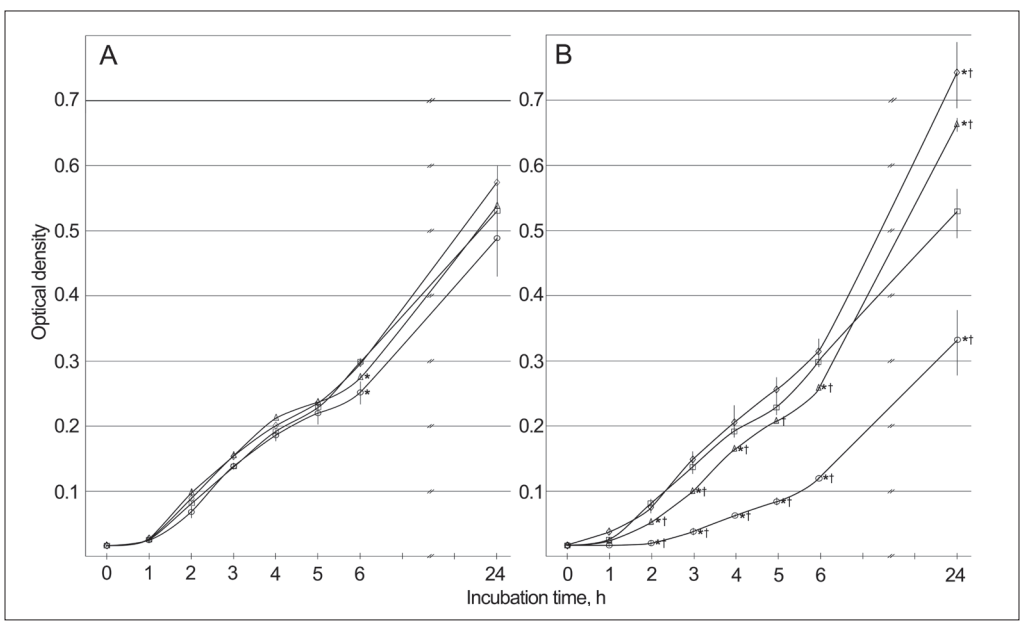

Fig. 1 Influence of citrate (A) and Ti(IV)-citrate (B) on *S. aureus* 44 growth. Graph represents mean values ±SD. *Significant difference in comparison to the control culture ($p < 0.05$). †Significant difference in comparison to the respective concentrations of citrate ($p < 0.05$). The studied chemicals' concentrations are signed as follows: □ – Control (0); ○ – 0.05 mol L^{-1}; Δ - 0.005 mol L^{-1}; ◊ - 0.0005 mol L^{-1}. In the case of complexes, ligand concentrations are indicated. Metal concentrations for the studied complex are 0.015, 0.0015, and 0.00015 mol L^{-1}, respectively.

Preparation of $Na_8[Ti(C_6H_4O_7)_3]$ and citric acid stock solutions for *in vitro* investigation

Regarding speciation analysis, all solutions for *in vitro* investigation were prepared in carbonate-free double-distilled water. For stock solution preparation, 5.3 g $C_6H_8O_7 \cdot H_2O$ (ACS reagent, Sigma-Aldrich) were diluted in 50 ml of water. Consequently, 1.5 g $TiOSO_4 \cdot 2H_2O$ was added under permanent stirring. Salt dissolution occurred in a period of several hours at room temperature. Further, 18 mol L^{-1} NaOH (ACS reagent, Sigma-Aldrich) solution was slowly added until pH 6.96. Thus, the final concentration of chemicals in the stock solution of Ti(IV)-citrate complex was 0.15 mol L^{-1} for Ti and 0.5 mol L^{-1} for citric acid (metal:ligand ratio = 1:3; pH = 6.96). Throughout the manuscript the concentrations of the ligand are indicated for the complex (0.05; 0.005; 0.0005 M). The metal concentrations for these ligand concentrations in the studied complex were 0.00015, 0.0015 and 0.015 mol L^{-1}, respectively.

The stock solution of citric acid was prepared in a similar manner. Citric acid concentration in the stock solution was 0.5 mol L^{-1}. The pH was adjusted to 6.93 using an 18 mol L^{-1} NaOH solution.

Evaluation of bacterial growth dynamics

Two bacterial cultures, *S. aureus* 44 and *S. aureus* 48, were used for *in vitro* investigation. Both cultures were isolated from festering wounds of two diabetic patients. *S. aureus* 44 possessed significant hemolytic activity, whereas *S. aureus* 48 was not hemolytic. After identification, the cultures were stored in the museum collection of cultures at the Institution of Cellular and Intracellular Symbiosis of the Ural Branch of the Russian Academy of Sciences until use.

The influence of citrate and Ti(IV)-citrate on *S. aureus* 44 and 48 growth was estimated after incubation of the culture in microtiter plates in the presence of the investigated solutions for 24 h. Briefly, 25 μL

Table 2. Influence of citrate and Ti(IV)-citrate on *S. aureus* 44 specific growth rate

T_1-T_2 (h)	Control	Citrate (mol L^{-1})			Ti(IV)-citrate (mol L^{-1})§		
		0.0005	0.005	0.05	0.0005	0.005	0.05
0-1	0.411± 0.210	0.428± 0.076	0.409± 0.086	0.436± 0.071	1.007± 0.278*†	0.242± 0.064*†	0.041± 0.005*†
1-2	1.115± 0.164	1.052± 0.080	1.136± 0.053	0.950± 0.419	0.826± 0.337	0.702± 0.038*†	0.069± 0.098*†
2-3	0.512± 0.032	0.527± 0.037	0.442± 0.158	0.734± 0.335	0.735± 0.113	0.610± 0.024	0.503± 0.035†
3-4	0.341± 0.060	0.253± 0.000*	0.310± 0.081	0.299± 0.137	0.328± 0.104	0.480± 0.005*†	0.446± 0.196
4-5	0.175± 0.007	0.159± 0.009	0.108± 0.010*	0.166± 0.046	0.231± 0.052*†	0.232± 0.035*†	0.262± 0.106
5-6	0.266± 0.046	0.229± 0.006*	0.153± 0.039*	0.136± 0.032*	0.214± 0.068	0.210± 0.008†	0.336± 0.098†
6-24	0.032± 0.009	0.036± 0.006	0.041± 0.003	0.036± 0.006	0.048± 0.002*†	0.052± 0.003*†	0.054± 0.017

Data are presented as mean±SD; * – significant difference in comparison to control; † – significant difference in comparison to the respective concentrations of citrate; § – ligand concentrations as indicated. Metal concentrations for the studied complex are 0.00015, 0.0015, and 0.015 mol L^{-1}, respectively.

of bacterial culture ($5x10^8$ colony-forming units per ml) and 25 µL of the investigated solution (citrate/Ti(IV)-citrate) were added to every well of the plate containing 200 µL of beef-extract broth. Afterwards the plates were incubated at 37°C. Bacterial growth was estimated by analyzing the optical density (OD) of the culture at 540 nm, using a Multiscan Accent microplate reader (Thermo Labsystems, Finland). In the current study, we evaluated the optical density of the bacterial culture at 0 (initial), 1, 2, 3, 4, 5, 6 and 24 h of incubation. Each experiment was repeated 5 times.

In order to estimate the dynamics of bacterial growth, we calculated the specific bacterial growth rate according to the following formula (Berney et al., 2006):

$$\mu = (\ln OD t_2 - \ln OD t_1)/(T_2 - T_1),$$

where µ is the specific bacterial growth rate, ODt_2 and ODt_1 are values of optical density at times T_2 and T_1, respectively. Specific bacterial rate was estimated at 0-1, 1-2, 2-4, 3-4, 4-6 and 6-24 h.

Estimation of biofilm formation

Analysis of biofilm formation was performed after 24 h of *S. aureus* incubation at 37°C in the presence of different concentrations of citrate and Ti (IV)-citrate. Determination of biofilms was performed using crystal violet aqueous solution with subsequent measurement of optical density at 540 nm (Merritt et al., 2005) with a Multiscan Accent microplate reader (Thermo Labsystems, Finland).

Statistical analysis

The obtained data were expressed as mean values of 5 measurements and the respective standard deviations (M±δ). Mann-Whitney U-test was used for group comparison at a significance level $p<0.05$ (*). All statistical analyses were performed using Statistica 10 software (StatSoft Inc., 2011).

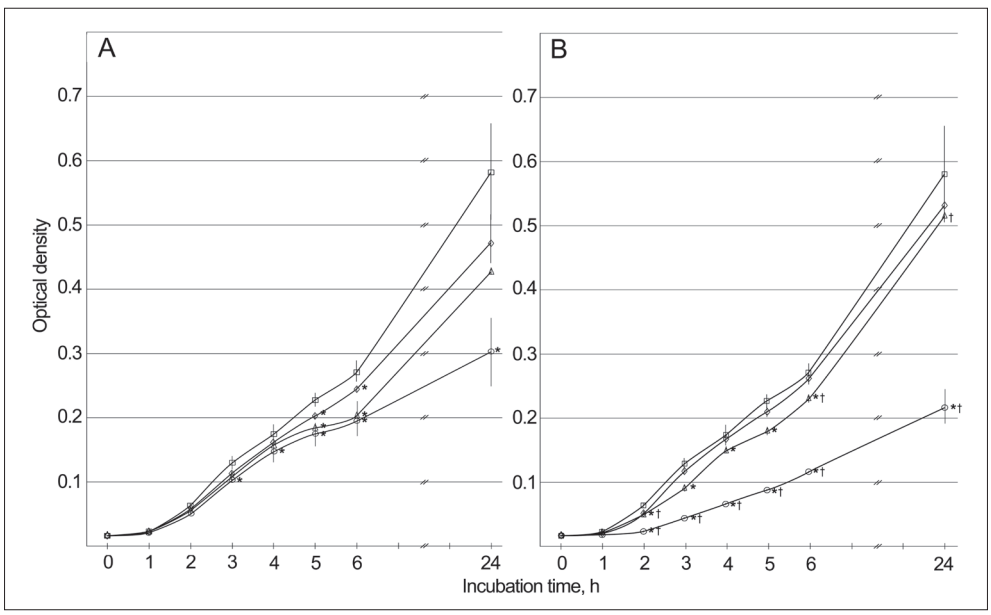

Fig. 2 Influence of citrate (A) and Ti(IV)-citrate (B) on *S. aureus* 48 growth. Graph represents mean values ±SD. *Significant difference in comparison to the control culture ($p < 0.05$). †Significant difference in comparison to the respective concentrations of citrate ($p < 0.05$). The studied chemicals' concentrations are signed as follows: □ – Control (0); ○ – 0.05 mol L^{-1}; Δ - 0.005 mol L^{-1}; ◊ - 0.0005 mol L^{-1}. In the case of complexes, ligand concentrations are indicated. Metal concentrations for the studied complex are 0.015, 0.0015 and 0.00015 mol L^{-1}, respectively.

RESULTS

Metal-ligand stoichiometry.

Ti(IV) complexation with citric acid in aqueous solutions in the presence of excess ligand is described by a simple model, based on the proposed existence of the following complexes: $[Ti(C_6H_6O_7)_3]^{2-}$, $[Ti(C_6H_6O_7)_2(C_6H_5O_7)]^{3-}$, $[Ti(C_6H_6O_7)(C_6H_5O_7)_2]^{4-}$, $[Ti(C_6H_5O_7)_3]^{5-}$, $[Ti(C_6H_5O_7)_2(C_6H_4O_7)]^{6-}$, $[Ti(C_6H_5O_7)(C_6H_4O_7)_2]^{7-}$, $[Ti(C_6H_4O_7)_3]^{8-}$, which is in accordance with our earlier data (Bezryadin et al., 2013). Fully deprotonated tris(citrate)titanate ion $[Ti(C_6H_4O_7)_3]^{8-}$ dominated in the solution at the pH range from 6.46 to 7.44. At the same time, in a slightly acidic media (pH <7), the ion $Ti(C_6H_4O_7)_3]^{8-}$ exists in equilibrium with $[Ti(C_6H_5O_7)_2(C_6H_4O_7)]^{6-}$ and $[Ti(C_6H_5O_7)(C_6H_4O_7)_2]^{7-}$ forms. However, the percent of accumulation of the latter in the solution did not exceed 5 and 30%, respectively. Further increase in pH results in decreased accumulation of $[Ti(C_6H_5O_7)_2(C_6H_4O_7)]^{6-}$ and $[Ti(C_6H_5O_7)(C_6H_4O_7)_2]^{7-}$ forms (Table 1). Other forms of the Ti(IV)-complex were not significant under current conditions.

Influence of citrate and Ti(IV)-citrate on S. aureus 44 growth

Our data (Fig. 1) demonstrate that after 1 h of *S. aureus* 44 incubation in the presence of different concentrations of citrate no significant differences in bacterial growth were observed. A 2-h incubation with 0.05 mol L^{-1} citrate decreased *S. aureus* 44 growth by 17% when compared to the control values. At the same time, administration of citrate in final concentrations of 0.005 and 0.0005 mol L^{-1} into the nutrient medium had a slight stimulating effect, increasing bacterial growth by 24 and 14%, respectively. However, the observed changes were not significant. This tendency was observed up to 5 h of incubation. After a 5-h incubation of *S. aureus* 44 with 0.05 mol L^{-1} citrate, a non-significant 5% decrease in bacterial growth was observed when compared to the control values. At the same time, the presence of 0.005 and 0.0005 mol L^{-1} citrate in the incubation medium increased the optical density of the bacterial culture by 5%. After a 6-h incubation in the presence of 0.05 and 0.005 mol L^{-1} citrate, significant decreases in the amount of *S. aureus* 44 (by 16 and 7%, respectively) were detected. At the same time, treatment with 0.0005 mol L^{-1} citrate did not alter bacterial growth significantly. Finally, after 24 h of incubation, 0.05 mol L^{-1} citrate decreased *S. aureus* 44 growth by 8% in comparison to the control culture, whereas 0.005M citrate did not affect bacterial quantity. At the same time, treatment of *S. aureus* 44 with 0.0005 mol L^{-1} citrate resulted in a 9% increase in growth.

Our research data indicate that all studied concentrations of Ti(IV)-citrate did not alter *S. aureus* 44 growth significantly after a 1-h incubation. A 2-h incubation of *S. aureus* 44 in 0.05, 0.005 and 0.0005 mol L^{-1} Ti(IV)-citrate-containing media decreased the optical density of the bacterial culture by 67, 31 and 16% in comparison to the control values, respectively. It is important to note that this tendency changed after 3 h of incubation. Particularly, 0.05 and 0.005 mol L^{-1} Ti(IV)-citrate decreased *S. aureus* 44 growth by 67 and 25%, respectively. At the same time, incubation of bacteria in the 0.0005 mol L^{-1} Ti(IV)-citrate-containing media resulted in a slight stimulation of growth. This tendency remained relatively stable up to 6 h of incubation. Thus, after a 6-h incubation, a significant 58 and 13% decrease in bacterial quantity was observed in the 0.05 and 0.005 mol L^{-1} Ti(IV)-citrate-treated cultures in comparison to the control one, respectively. The presence of 0.0005 mol L^{-1} Ti(IV)-citrate in the nutrient medium stimulated bacterial growth by 3%. Another situation was observed after 24 h of incubation. Namely, 0.05 mol L^{-1} Ti(IV)-citrate significantly decreased *S. aureus* 44 growth by 36% when compared to untreated control culture. At the same time, the presence of 0.005 and 0.0005 mol L^{-1} Ti(IV)-citrate in the nutrient medium resulted in a 26 and 39% increase in the optical density of the bacterial culture, respectively.

Influence of citrate and Ti(IV)-citrate on *S. aureus* 48 growth

As observed for *S. aureus* 44, incubation of *S. aureus* 48 in the citrate-containing medium did not affect

bacterial growth significantly (Fig. 2). After a 2-h incubation, the presence of 0.05 mol L^{-1} citrate in the nutrient medium resulted in a 15% decrease in the bacterial quantity, as compared with the control culture. At the 3rd hour of observation, 18, 15 and 12% decreases in *S. aureus* 48 growth were detected in 0.05, 0.005 and 0.0005 mol L^{-1} citrate-containing media, respectively. This tendency was observed over the whole period of incubation. Finally, after 24 h of treatment with 0.05, 0.005 and 0.0005 mol L^{-1} citrate, a significant 48, 26 and 19% decrease in the optical density of the bacterial culture was observed, respectively.

Investigation of Ti(IV)-citrate effect on *S. aureus* 48 growth indicated that after a 1-h incubation a minor reduction in bacterial growth was detected. The observed effect increased in a time-dependent manner. Namely, after a 2-h incubation in the presence of 0.05, 0.005 and 0.0005 mol L^{-1} Ti(IV)-citrate, significant 68, 26, and 26% decreases, respectively, in *S. aureus* 48 growth were observed. At the later periods of observation, the inhibitory action of Ti(IV)-citrate on bacteria was dose-dependent. In particular, treatment with 0.05, 0.005 and 0.0005 mol L^{-1} Ti(IV)-citrate resulted in a 58, 16 and 5% growth inhibition, respectively. At the end of the observation (24 h), the studied concentrations of metal-citrate complex decreased the amount of bacteria by 63, 12 and 9% when compared to the control culture, respectively.

Influence of citrate and Ti(IV)-citrate on S. aureus 44 specific growth rate

Table 2 shows that the studied concentrations of citrate failed to affect specific bacterial growth rates during the three hours of observation. In the period between 3 and 4 h, a tendency for decrease in specific bacterial growth rate was noted in 0.005 and 0.05 mol L^{-1}-citrate-treated cultures. However, a significant 26% decrease in *S. aureus* 44 specific growth rate was detected only after incubation with 0.0005M citrate. In the period of 4-5 h, only 0.005 mol L^{-1} citrate significantly lowered specific growth rate by 38%. In the later interval (5-6 h), the presence of 0.0005, 0.005 and 0.05 mol L^{-1} citrate in the incubation medium resulted in a significant decrease in bacterial growth rate by 14, 42 and 49%, respectively. It is notable that no significant difference was observed between the values of specific bacterial

Table 3. Influence of citrate and Ti(IV)-citrate on *S. aureus* 48 specific growth rate

T_1-T_2 (h)	Control	Citrate (mol L^{-1})			Ti(IV)-citrate (mol L^{-1})§		
		0.0005	0.005	0.05	0.0005	0.005	0.05
0-1	0.356± 0.097	0.397± 0.048	0.331± 0.008	0.243± 0.149	0.227± 0.085	0.306± 0.098	0.144± 0.015*†
1-2	1.061± 0.071	0.956± 0.028	0.913± 0.237	0.825± 0.210	1.149± 0.162	0.969± 0.060	0.279± 0.119*†
2-3	0.760± 0.246	0.691± 0.209	0.654± 0.055	0.653± 0.146	0.892± 0.023	0.636± 0.036	0.728± 0.283
3-4	0.304± 0.011	0.363± 0.118	0.381± 0.082	0.384± 0.147	0.378± 0.014	0.513± 0.049*	0.430± 0.114
4-5	0.273± 0.069	0.228± 0.049	0.156± 0.019*	0.174± 0.026*	0.232± 0.027	0.191± 0.012*	0.294± 0.087†
5-6	0.178± 0.017	0.192± 0.017	0.103± 0.058*	0.104± 0.050*	0.224± 0.044	0.253± 0.023*†	0.287± 0.048*†
6-24	0.042± 0.010	0.036± 0.009	0.053± 0.006	0.022± 0.010	0.040± 0.000	0.045± 0.002	0.035± 0.013

Data are presented as mean±SD; * – significant difference in comparison to control; † – significant difference in comparison to the respective concentrations of citrate; § – ligand concentrations as indicated. Metal concentrations for the studied complex are 0.00015, 0.0015, and 0.015 mol L^{-1}, respectively.

growth rate of citrate-treated and control cultures in the period between 6 and 24 h.

The influence of Ti(IV)-citrate on the *S. aureus* 44 specific growth rate was more apparent than that for citrate. During the 0-1 h period, the presence of 0.0005 mol L^{-1} Ti(IV)-citrate in the incubation medium increased the specific growth rate more than 2-fold when compared both to the control and 0.0005M citrate-treated cultures. At the same time, incubation of bacteria with 0.005 and 0.05 mol L^{-1} Ti(IV)-citrate resulted in a nearly 2- and 10-fold decrease in growth rate values in comparison to the control and citrate-treated cultures, respectively. In the period of 1-2 h, *S. aureus* 44 incubation in the presence of 0.0005 and 0.005 mol L^{-1} Ti(IV)-citrate resulted in a 26 and 37% decrease in specific bacterial growth rate, as compared with the control and citrate-treated cultures, respectively. During this period of observation, 0.05 mol L^{-1} Ti(IV)-citrate decreased the specific growth rate more than 16- and 13-fold in comparison to the control and citrate-treated cultures, respectively. Ti(IV)-citrate-dependent modification of *S. aureus* 44 specific growth rate in further periods was variable, displaying a tendency to increase. The presence of 0.0005 and 0.005 mol L^{-1} Ti(IV)-citrate in the incubation medium resulted in 44 and 32% increases in specific bacterial growth rate, respectively, when compared to matching control values during the 4-5 h period. It is notable that values obtained for 0.0005 and 0.005 mol L^{-1} Ti(IV)-citrate-treated cultures exceeded the respective ones observed for citrate-treated bacteria by 45 and 11%. In the period of 5-6 h, incubation of *S. aureus* 44 in the presence of Ti(IV)-citrate did not affect specific bacterial growth rate in comparison to the control values. At the same time, 0.05 M Ti(IV)-citrate significantly exceeded the specific *S. aureus* 44 growth rate in comparison to that obtained in 0.05 mol L^{-1} citrate medium. Finally, during the 6-24 h period, a Ti(IV)-citrate-induced increase in specific bacterial growth rate was observed.

Influence of citrate and Ti(IV)-citrate on *S. aureus* 48 specific growth rate

The results presented in Table 3 indicate that citrate did not significantly affect the specific growth rate of *S. aureus* 48 in the period of 0-1 h. At the same time, a weak inhibitory effect was observed for 0.005 and 0.05 mol L^{-1} citrate. During the 2-3 h period, the specific growth rates in 0.0005, 0.005 and 0.05 mol L^{-1} citrate-containing media were decreased by 9, 14, and 14% in comparison to the control culture, respectively. It is notable that in the interval from 3-4 h, all studied concentrations increased the specific bacterial growth rate by 19, 25 and 26%, respectively. However, these differences were not significant in both cases. The slight stimulation was changed by a subsequent significant deceleration of specific bacterial growth rate in citrate-treated cultures. Namely, during the 4-5 h incubation period, in the presence of 0.005 and 0.05 mol L^{-1} citrate, 42 and 36% decreases in specific growth rates, respectively, as compared to matching control values, were observed. During the 5 to 6 h interval, the presence of 0.005 and 0.05 mol L^{-1} citrate significantly decreased specific bacterial growth rate by 42%. At the same time, citrate-induced changes in the final period of observation (6-24 h) were not significant.

As indicated for *S. aureus* 44, Ti(IV)-citrate significantly affected the specific growth rate of *S. aureus* 48 at the initial stages of observation. Particularly, the presence of Ti(IV)-citrate in the studied concentrations (0.0005, 0.005 and 0.05 mol L^{-1}) in the nutrient medium significantly decreased specific growth rate by 36, 14 and 60%, as compared to the control values in the period of 0-1 h. It is also notable that 0.05 mol L^{-1} Ti(IV)-citrate treatment resulted in a 41% reduction in the analyzed parameter in comparison to the values obtained for the respective citrate concentration. During the 1-2 h interval, the presence of 0.0005 and 0.005 mol L^{-1} Ti(IV)-citrate did not alter the specific growth rate of *S. aureus* 48 in comparison to the control and citrate-treated cultures. At the same time, 0.05 mol L^{-1} Ti(IV)-citrate caused significant 3.8-fold and 3-fold decreases in specific growth rates when compared to the respective values of the control and 0.05M citrate-treated microorganisms. The observed tendency changed during the later periods of incubation. Namely, during the 3-4 h period, the presence of increasing Ti(IV)-citrate concentrations resulted in 24, 69 and 41% elevations of *S. aureus* 48 specific growth

rate in comparison to the control culture. It is also notable that the values of specific growth rate obtained for Ti(IV)-citrate-treated cultures exceeded those obtained for citrate-treated cocci. In the following observation period (5-6 h), the specific growth rate of the cultures treated with 0.0005, 0.005 and 0.05 mol L^{-1} Ti(IV)-citrate exceeded the control values by 26, 42 and 61%, respectively. It is also important to note that 0.005 and 0.05 mol L^{-1} Ti(IV)-citrate exhibited a 2-fold greater effect when compared to the respective concentrations of citrate. During the final, 6-24 h period, no significant Ti(IV)-citrate-induced changes in specific growth rates were observed in comparison to the control and citrate-treated-cultures, respectively.

Influence of citrate and Ti(IV)-citrate on biofilm formation

The investigation of *S. aureus* 44 biofilm formation (Fig. 3A) indicated that 0.005 and 0.0005 mol L^{-1} citrate significantly 2-fold decreased biofilm formation when compared to the control values. At the same time, the presence of 0.05, 0.005, and 0.0005 mol L^{-1} Ti(IV)-citrate significantly elevated biofilm formation by 38 53 and 37%, respectively. It is also notable that Ti(IV)-citrate-induced biofilm formation exceeded the values obtained after the respective citrate concentrations' treatment by 4-, 2- and 2-fold, respectively.

The presence of 0.05 and 0.005 M citrate in the incubation media decreased *S. aureus* 48 biofilm formation by 34 and 62% as compared to the control values, respectively. The tendency of Ti(IV)-citrate to influence *S. aureus* 48 biofilm formation remained the same as for *S. aureus* 44. Namely, incubation of bacteria with 0.05; 0.005 and 0.0005 mol L^{-1} Ti(IV)-citrate resulted in a 7-, 3- and 2-fold increases in biofilm formation, respectively. The results of biofilm formation obtained for 0.05 and 0.005 mol L^{-1} Ti(IV)-citrate-treated bacteria (10- and 7-fold, respectively) exceeded the values obtained for citrate-treated *S. aureus* 48. At the same time, the intensity of biofilm formation after 0.0005 mol L^{-1} Ti(IV)-citrate treatment was 66% higher in comparison to the values obtained for 0.0005 mol L^{-1} citrate.

DISCUSSION

The results of the speciation analysis show that in aqueous solutions at near-physiological pH the predominant complex form is tris(citrate)titanate ion $[Ti(C_6H_4O_7)_3]^{8-}$. It is proposed that the tris(citrate)ti-

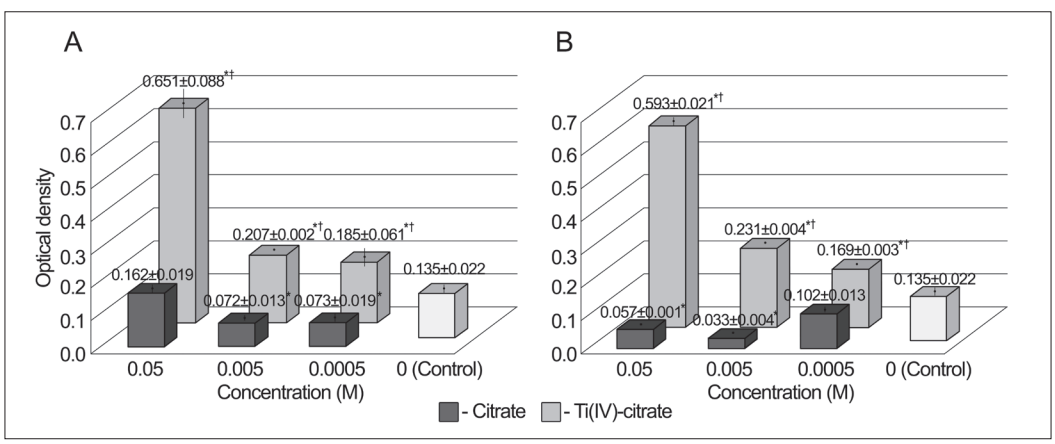

Fig. 3 Influence of citrate and Ti(IV)-citrate on *S. aureus* 44 (A) and *S. aureus* 48 (B) biofilm formation. Graph represents mean values ±SD. *Significant difference in comparison to the control culture ($p < 0.05$). †Significant difference in comparison to the respective concentrations of citrate ($p < 0.05$). In the case of complexes, ligand concentrations are indicated. Metal concentrations for the studied complex are 0.015, 0.0015 and 0.00015 mol L^{-1}, respectively.

tanate ion was biologically active under the current research conditions and consequently caused most of the observed effects. The present complex was synthesized earlier and was characterized by bidentate ligands (Collins et al., 2005).

Our data indicate an inhibitory action of high concentrations (0.05 mol L^{-1}) of Ti(IV)-citrate on bacterial growth when compared to the control and citrate-treated *S. aureus* cultures. However, a number of differences were observed in *S. aureus* 44 and 48 responses to Ti(IV)-citrate. Both citrate and Ti(IV)-citrate delayed *S. aureus* 48 growth in a dose-dependent manner. At the same time, citrate did not affect *S. aureus* 44 growth significantly. Moreover, the lowest concentration of citrate (0.0005 mol L^{-1}) possessed a slight stimulatory effect on *S. aureus* 44 growth. These changes were more pronounced after the Ti(IV)-citrate treatment. Generally, the obtained data indicate the potentiation of citrate-induced changes after administration of Ti(IV)-citrate complex.

The observed bacterial growth dynamics were in accordance with the values of specific bacterial growth rate. The highest concentration of Ti(IV)-citrate led to a significant decrease in *S. aureus* 44 and 48 specific growth rate in the initial periods of bacterial growth, which are critical for population development. It is also notable that the Ti(IV)-citrate-induced increase in specific growth rate in later periods did not compensate the initial reduction. The data obtained also indicate that Ti(IV)-citrate induces a significant dose-dependent increase in *S. aureus* biofilm formation. Taking into account the character of citrate-induced decrease in biofilm formation, the observed effect is proposed to be the consequence of the biological action of Ti.

It can be assumed that the more expressed effect of Ti(IV)-citrate occurs due to the complex transport into the bacterial cell. Previously, a secondary metal-citrate transport system CitMHS was described (Lensbouer and Doyle, 2010). The presence of Fe^{3+}-citrate transporters in this CitMHS family points to the possibility of Ti-citrate transport into the cell in view of the physicochemical similarity of Ti(IV) and Fe(III), as has been suggested by the works of Tinoco and Valentine (2005) and Parker Siburt et al. (2010). Moreover, earlier studies indicated that *Pseudomonas fluorescens* is able to transport only bidentate citrate complexes, particularly with Fe(III), into the cell (Joshi-Tope and Francis, 1995). The studied complex $[Ti(C_6H_4O_7)_3]^{8-}$ is also bidentate, which indirectly confirms our assumption about Ti(IV)-citrate transport into the bacterial cell. Finally, citrate is a specific siderophore in *E. coli* (Yue et al., 2003). Taking into account a possibility of siderophore-mediated transport of a wide variety of non-ferric metals (Schalk et al., 2011), it can be supposed that citrate may also take part in Ti transport into the bacterial cell. However, the proposed assumption may have several limitations, in particular as in *S. aureus*, citrate is a structural part of the more complex siderophore family of staphyloferrins (Hammer and Skaar, 2011). However, the abovementioned hypothesis needs to be studied further.

It is also notable that citrate transport into *Streptococcus mutans* is stimulated by Fe(III) (Korithoski et al., 2005). Taking into account the observed potentiation of citrate-induced effects after administration of Ti(IV)-citrate, it can be supposed that Ti also stimulates citrate transport into the cell.

Our research points to the inhibitory effect of high concentrations (0.05 mol L^{-1}) of citrate on bacterial growth. This observation corresponds to earlier data (Rammel, 1962; Lee et al., 2001; Abu-Ghazaleh, 2013). It was also noted that low concentrations of citrate (0.0005 mol L^{-1}) possessed a slight stimulatory effect on *S. aureus* 44; this is in agreement with the previous observation of growth-stimulating activity of citrate in *Streptococcus mutans* (Terleckyj et al., 1975). The role of citrate in energetic homeostasis via the citric acid cycle may be the possible basis for the observed action.

A number of observed effects cannot be explained only by the effect of the citric ion, which suggests the direct action of Ti ion in bacteria. The most likely

mechanism of this action is oxidative stress. The role of Ti compounds in the induction of oxidative stress was demonstrated in eukaryotic cells (Park et al., 2008; Liu et al., 2010; Hernandez et al., 2010; Jaeger et al., 2012; Huerta-Garcia et al., 2014; Faria et al., 2014). At the same time, such data obtained from experiments with prokaryotes seem to be absent. However, in the case of high concentrations of Ti(IV)-complex, Ti-induced oxidative damage to the bacterial cell may occur, as it was demonstrated for other metal complexes (Paez et al., 2013). Significant activation of biofilm formation in Ti(IV)-citrate-treated *S. aureus* may also result from oxidative stress. It has been shown that under stress conditions *S. aureus* induced oxidative stress-dependent biofilm formation (Kulkarni et al., 2012).

The obtained results show that: (i) citrate decreased *S. aureus* 48 growth at all studied concentrations whereas *S. aureus* 44 growth was reduced only by high concentrations of citrate in the incubation medium; (ii) Ti significantly potentiated the citrate-induced effects, and (iii) citric acid suppressed *S. aureus* biofilm formation, whereas Ti(IV)-citrate possesses a significant stimulatory effect.

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