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Effect of Titanium Ion and Resistance Encoding Plasmid of *Pseudomonas aeruginosa* ATCC 10145

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Titanium and its alloys are technically superior and cost-effective materials, with a wide variety of aerospace, industrial, marine, and commercial applications. In this study, the effects of titanium ions on bacterial growth were evaluated. Six strains of bacteria known to be resistant to both metal ions and antibiotics were used in this study. Two strains, *Escherichia coli* ATCC 15489, and *Pseudomonas aeruginosa* ATCC 10145, proved to be resistant to titanium ions. Plasmid-cured *P. aeruginosa* resulted in the loss of one or more resistance markers, indicating plasmid-encoded resistance. The plasmid profile of *P. aeruginosa* revealed the presence of a 23-kb plasmid. The plasmid was isolated and transformed into DH5α. Interestingly, the untransformed DH5α did not grow in 300 mg/l titanium ions, but the transformed DH5α grew quite well under such conditions. The survival rate of the transformed DH5α also increased more than 3-fold compared to that of untransformed DH5α.

Keywords: titanium ion, resistance, *Escherichia coli*, *Pseudomonas aeruginosa*, bacteriostatic effect

Environmental contamination with certain metal compounds is causing serious health problems in humans, including genetic disorders. Metals such as mercury (Gadd and Griffiths, 1978), cadmium (Gadd and Griffiths, 1978; Yu *et al.*, 1986; Yu *et al.*, 1990), iron (Demerec *et al.*, 1951), manganese (Demerec *et al.*, 1951), and lead (Muro and Goyer, 1969; Rho and Kim, 2002) have been reported to have a negative influence on microorganisms, affecting their growth, morphology, mutagenicity, and biochemical activity.

Titanium is widely distributed in the earth's crust; it is the eighth most abundant chemical element. Titanium-containing compounds have a wide variety of commercial applications. Titanium tetrachloride is a colorless to pale yellow liquid, which gives off fumes with a strong odor. It is not a naturally occurring substance in the environment, but is made from minerals that contain metallic titanium. Titanium tetrachloride is used to produce titanium metal and other titanium-containing compounds. Titanium dioxide is used extensively as a white pigment in paint, plastics, paper filling and other hardware products, and also as an intermediate in the production of other chemicals, such as ceramics. It is also used as a coloring agent in the food and cosmetics industries and the production of iridescent glass. Also, titanium dioxide, along with several other titanium compounds, is used as a catalyst in a variety of chemical reactions. Ferrotitanium is widely used in the steel industry. Titanium exists largely in the +3 and +4 oxidation states. As a result, titanium ions usually have neither donor electrons nor a closed-shell configuration, which in turn precludes biochemically relevant redox chemistry. Titanium thus also has many biological applications.

Titanium alloys are increasingly used in dental implants due to their biocompatibility (Breme, 1989; Elagli *et al.*, 1989), corrosion resistance (Breme and Wadewitz, 1989; Elagli *et al.*, 1992), and bio-functionality (Breme, 1989). Metallic titanium has no bacteriostatic effect on oral bacteria of different morphologies, namely the respiratory and sugar fermentation types (Elagli *et al.*, 1992; Joshi and Eley, 1998).

However, metal ions, including titanium, can inhibit apatite formation and growth (Blumenthal and Cosma, 1989), cell proliferation (Poleo and Huh, 1995), extracellular mineralization (Thompson and Puleo, 1995), and specific cellular functions, such as ALP (alkaline phosphatase) activity (Thompson and Puleo,

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1996). Some *in vivo* studies have discovered titanium pigmentation in the soft tissues surrounding titanium implants (Rosenberg *et al.*, 1993; Kim *et al.*, 1997). Titanium dioxide levels of up to 0.1 M seem to have some effect (Elo *et al.*, 1972). Metallic titanium can selectively accumulate in the lung and adjacent lymph nodes when a person is acutely exposed to a high concentration of titanium tetrachloride. Chronic exposure to low doses can lead to pulmonary injury via bioaccumulation (Elo *et al.*, 1972). Specifically, chronic exposure to titanium may cause pulmonary granulomatous disease (Redline *et al.*, 1986). Titanium ions might slowly diffuse into surrounding tissues, where they are transported into the serum and urine (Woodman *et al.*, 1984).

Although bulk titanium is stable and biocompatible *in vivo* (Dorr *et al.*, 1990), measured titanium levels of up to 21 mg/l have been found in fibrous membranes surrounding titanium-based implants (Dorr *et al.*, 1990). Large pieces of titanium used in implants normally have an insulating oxide layer, but solutions and fine particles of titanium may influence its local composition. Also, tumor necrosis factor-α (TNF-α) levels can be increased as a response to titanium particles larger than 0.45 μm (Ryuchiro *et al.*, 2002).

These findings show that titanium materials are not 100% safe for human use. Also, our preliminary investigations showed that titanium ions exhibited bacteriostatic effects on several bacterial species. In this present study, we investigated the effect of titanium ions on bacterial strains that known to exhibit resistance to metal ions and antibiotics and discovered their mechanisms of titanium ion resistance.

Materials and Methods

Bacterial strains, chemicals and plasmid DNA

The six bacterial strains were purchased from the ATCC (American Type Culture Collection) and the KCCM (Korean Culture Collection of Microorganisms): *Escherichia coli* ATCC 15489, *Pseudomonas aeruginosa* ATCC 10145, *Pseudomonas fluorescens* KCCM 40223, *Pseudomonas stutzeri* KCCM 12540, *Bacillus subtilis* KCCM 12148, and *Staphylococcus aureus* subsp. *aureus* KCCM 40050. All strains were known to exhibit resistance to metal ions and antibiotics. The titanium ion solution (998 mg/l of Ti in H_2O) was purchased from Aldrich (USA) and all other chemical reagents were of ultra-pure grade. Competent cells (F^-, ø80d*lacZ*ΔM15, Δ(*lacZYA-argF*) U169, *deoR, recA1, λ-endA1, hsdR17* (r_k^- m_k^+), *phoA*, *supE*44, *thi-1*, *lacI* ^{q}, *relA1*) (Carsten *et al.*, 2000) were prepared by the method of Inoue *et al.* (1990).

Growth conditions

All strains were incubated overnight at 26 (*P. fluorescens*), 30 (*B. subtilis*) and 37°C (*E. coli, P. aeruginosa, P. stutizeri,* and *S. aureus*) with agitation at 180 rpm in LB medium (1% pancreatic digestion of casein, 0.5% yeast extract, 1% NaCl, pH 7.0, purchased from Difco, USA). After autoclaving the medium, the titanium ions were added to the LB medium, and the pH was adjusted by the addition of sterilized NaOH solution (Lee *et al.*, 2000) used a 0.2 μm syringe filter (Corning, Germany). The cultured medium was reinoculated into fresh medium containing 0 to 300 mg/l titanium ions and then incubated for 48 h at 26, 30 and 37°C with agitation at 180 rpm. Titanium ion-resistant strains were selected by their absorbance at 660 nm (Specgen, England).

Bacteriostatic effect of titanium ion

The agar diffusion method was used to ascertain the bacteriostatic effect of titanium ions. All strains were inoculated on solid LB medium. Then we put metal cups (Ø 0.8 mm) on the medium and added different concentrations of titanium ion (0 to 900 mg/l) to each metal cup. All solid LB plates were incubated overnight at 26, 30 and 37°C.

Physiological change by titanium ion

An API kit (BioMerieux, France) was used according to the manufacturer's instructions to assess the physiological changes in the titanium ion-resistant strains due to the various titanium ion concentrations. *E. coli* and *P. aeruginosa* were incubated 48 h in LB medium, either with or without 300 mg/l titanium ions, at 37°C, with agitation at 180 rpm. Colonies were obtained from the solid LB medium after incubation at 37°C.

Plasmid-curing

Plasmid-curing of *E. coli* and *P. aeruginosa* was carried out by treatment with mitomycin C, N-methyl-N′-nitro-N-nitrosoguanidine (NTG), gentamycin C, kanamycin, oxacillin, penicillin G, ampicillin (Sigma, USA) and erythromycin (Fluka, Switzerland).

Overnight cultured broth was centrifuged at 10,000 rpm then removed supernatant. Washed with distilled water twice and added plasmid-curing reagents 0.1 to 10% (mitomycin C, gentamycin C, kanamycin, oxacillin, penicillin G, ampicillin), 0.1 to 1% (NTG), and 0.1 to 5% (erythromycin). After treatment, strains were incubated in 5 ml LB broth for a day then incubated on solid LB agar broth. Colonies that grew on the plate inoculated 5 ml LB broth checked their plasmid DNA.

Growth of plasmid-curing strains, competent cell, and transformed competent cell

To ascertain whether the obtained plasmids conferred resistance to titanium ions, plasmid-cured *E. coli* and *P. aeruginosa* were incubated for 48 h in LB medium containing 300 mg/l titanium ions, at 37°C, with agitation at 180 rpm. The cell growth was measured by the absorbance at 660 nm. Competent cells and transformed competent cells were grown under identical conditions to assess their growth in LB medium containing titanium ions. Transformation was performed by the standard method described by Sambrook *et al.* (1989).

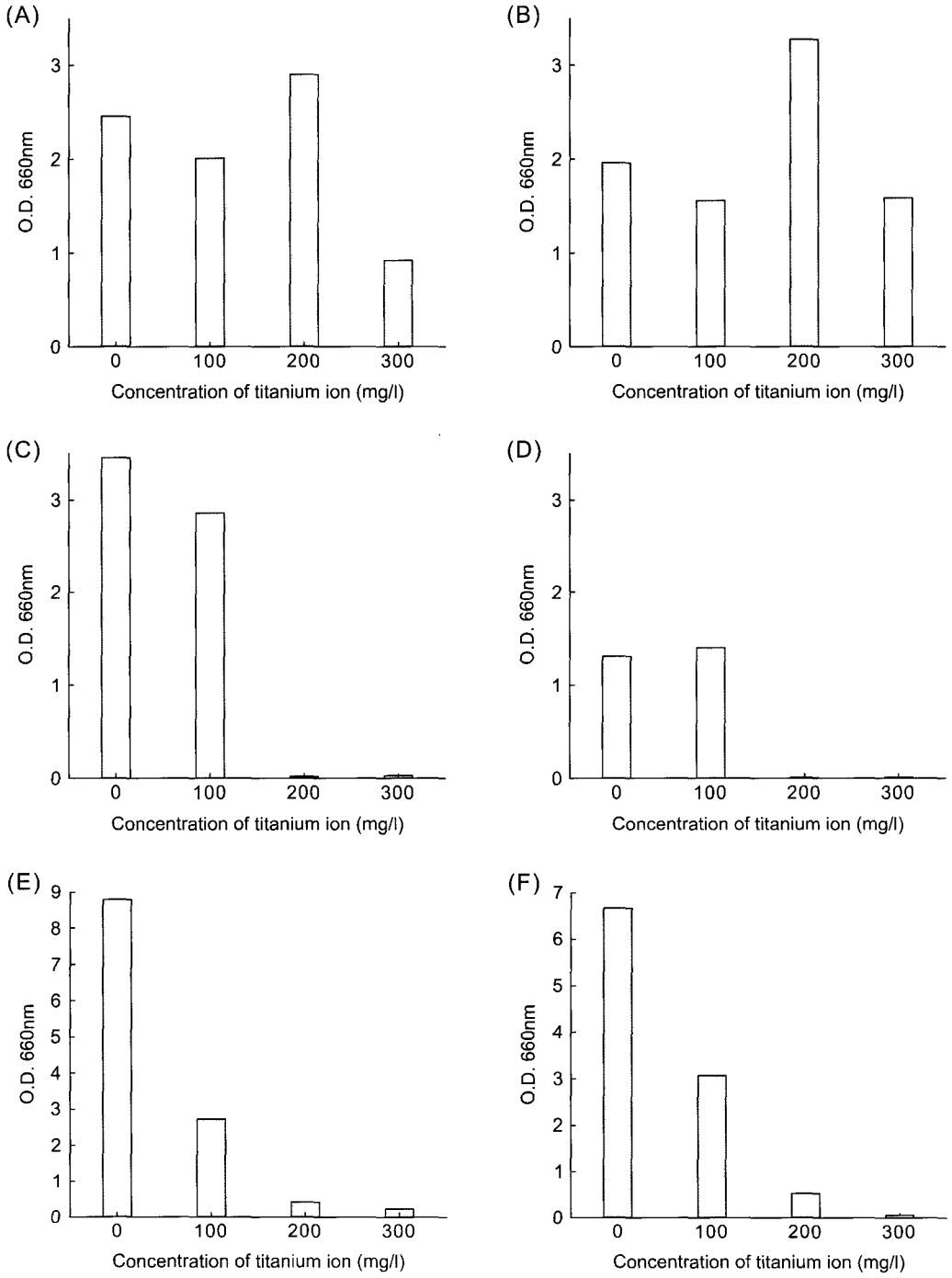

Fig. 1. Effect of different concentrations of Ti ions on growth of 6 strains. (A) *E. coli*, (B) *P. aeruginosa*, (C) *P. fluorescens*, (D) *P. stutzeri*, (E) *B. subtilis*, (F) *S. aureus*.

Electrophoresis

For electrophoresis, the total plasmid DNA of *E. coli*, *P. aeruginosa*, and plasmid-cured *E. coli* and *P. aeruginosa* was prepared by the procedure described earlier in this section. The plasmid DNA was fractionated on a 0.8% agarose gel and transformed following the standard procedure described by Sambrook *et al.* (1989). Gels were stained using 0.5 μg ethidium bromide per ml (Jung *et al.*, 2000) and photographed through a transilluminator (Vilber Lourmat, France).

Results and Discussion

Titanium ion resistant strains

The titanium ion resistance, as evidenced by the growth of the six strains, is illustrated in Fig. 1. *P. fluorescens* KCCM 40233 and *P. stutzeri* KCCM 12540 grew in medium containing 100 mg/l titanium, but not in medium containing over 200 mg/l titanium. Although the growth of *P. stutzeri* actually increased in medium containing 100 mg/l titanium, an investigation of this phenomenon was considered to be beyond the scope of this study. *B. subtilis* KCCM 12148 and *S. aureus* KCCM 40050 also grew in medium containing 200 mg/l titanium, but not in medium containing 300 mg/l titanium. *E. coli* ATCC 15489 and *P. aeruginosa* ATCC 10145 grew in medium containing 300 mg/l titanium, apparently uninhibited. *P. aeruginosa* exhibited better growth characteristics in medium containing 300 mg/l titanium than *E. coli*. Although *E. coli* and *P. aeruginosa* were observed to grow in medium containing 400 mg/l titanium, the absorbance of the *E. coli* was 0.23, which constitutes almost no growth, whereas that of *P. aeruginosa* ($O.D._{660}$ = 1.06) indicated satisfactory growth (Table 1). Attempts were made to grow *P. aeruginosa* in medium containing 500 mg/l titanium, but this proved to be very difficult, since the titanium ions may have denatured or bound to some of the compounds within the medium (when 500 mg/l titanium were added, the medium became muddy). The growth rates of both *E. coli* and *P. aeruginosa* increased in medium containing 200 mg/l titanium, but decreased in concentrations of 100 and 300 mg/l, which was checked in several experiments with similar results. However, further

Table 1. Effect of various titanium ion concentrations on growth of *E.coli* and *P. aeruginosa*

Concentration of Titanium ion (mg/l)	Growth (O.D. 660 nm)	
	E. coli	*P. aeruginosa*
0	2.45	1.96
100	2.01	1.56
200	2.90	3.27
300	0.92	1.59
400	0.23	1.06

E. coli and *P. aeruginosa* were incubated 180 rpm, at 37°C for 48 h.

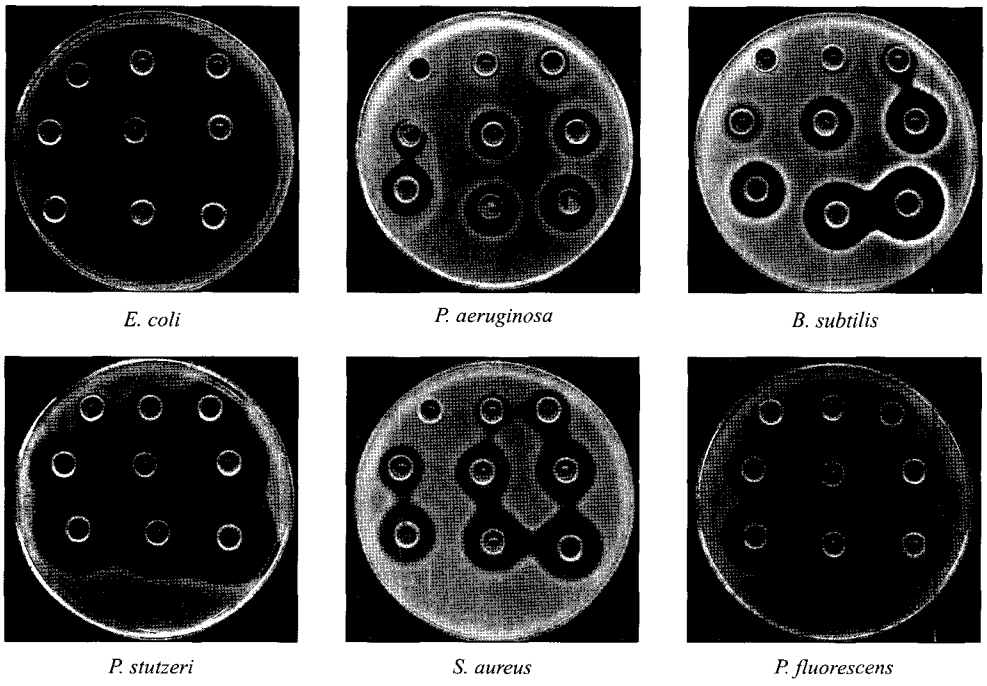

Fig. 2. Bacteriostatic effect of titanium ions (100 to 900 mg/l) on various bacteria resistant to metal ions and antibiotics.

experiments will be needed to study this strange effect. The agar diffusion method was used to show the bacteriostatic effect of titanium ions on the 6 bacteria, and the results are shown in Fig. 2. Solid LB medium was used for the incubation of the bacteria at 26, 30 or 37°C for 24 h. *P. stutzeri* exhibited the strongest inhibition with all concentrations of titanium ions and was therefore the least resistant to titantium. *B. subtilis*, *S. aureus*, *P. fluorescens*, *E. coli* and *P. aeruginosa* also exhibited inhibition, but not in the medium containing 100 mg/l titanium ion.

Plasmid DNA extraction of the E. coli and P. aeruginosa

Plasmid DNA was extracted from *E. coli* using the alkaline lysis method (Sambrook *et al.*, 1989) of a standard plasmid DNA extraction kit, and the presence of one 23 kb plasmid was discovered. *P. aeruginosa* plasmid DNA was extracted with TNE buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, pH 8.0), and one 22 kb plasmid was found. Plasmid-curing strains of *E. coli* and *P. aeruginosa* were also extracted as described for the original strains. Upon comparison of the presence of the plasmid DNAs between the original and plasmid-cured strains, it was apparent that plasmid DNA was being isolated from the original but not the cured strains (data not shown).

Physiological changes in the resistant strain resulting on 300 mg/l titanium ion

Four strains, *B. subtilis*, *P. stutzeri*, *P. fluorescens* and *S. aureus*, did not grow in medium containing 300 mg/l titanium. The two titanium ion resistant strains were grown for 48 h in LB medium either without (the control) or with 300 mg/l titanium ions at 37°C and agitation at 180 rpm and were then inoculated into solid medium via the dilution method. A colony from each solid medium was extracted and added to suspension medium. After inoculation in the tray, 5 ml distilled water were added to the tray to maintain the moisture content. The trays were incubated at 37°C for 24 h after which TDA (BioMerieux, ref. 70 402), JAMES (BioMerieux, ref. 70 542), VP 1, 2 (BioMerieux, ref. 70 422) and NIT 1, 2 (BioMerieux, ref. 70 442) reagents were added using the methods described in the manufacturer's manual. The results are shown in Table 2. *P. aeruginosa* was detected equally in all tests, but *E. coli* was detected at different levels in the test for the reduction to N_2 gas. *E. coli* tested positive for reduction to N_2 gas, but the plasmid-cured *E. coli* tested negative. This means that plasmid-cured *E. coli* had lost the ability to reduce N_2 gas in medium containing 300 mg/l titanium. It was

Table 2. Physiological differences of *E.* coli (A) and *P. aeruginosa* (B) grown in medium containing titanium

(A) *E. coli*

Test	Control	300 mg/l
Beta-galactosidase	+	+
Arginine dihydrolase	+	+
Lysine decarboxylase	+	+
Ornithine decarboxylase	-	-
Citrate utilization	+	+
H_2S production	-	-
Urease	-	-
Tryptophane deaminase	-	-
Indole production	+	+
Acetoin production	-	-
Gelatinase	-	-
Glucose fermentation	+	+
Mannitol fermentation	+	+
Inositol fermentation	-	-
Sorbitol fermentation	+	+
Rhamnose fermentation	+	+
Sucrose fermentation	-	-
Melibiose fermentation	-	-
Amygdalin fermentation	-	-
Arabinose fermentation	+	+
NO_2 production	+	+
Reduction to N_2 gas	+	-

(+) : positive, (-) : negative

(B) *P. aeruginosa*

Test	Control	300 mg/l
Reduction of nitrates to nitrites	-	-
Indole production	-	-
Acidification	-	-
Arginine didrolase	+	+
Urease	-	-
Hydrolysis (β-glucosidase)	-	-
Hydrolysis (protease)	+	+
Beta-galactosidase	-	-
Glucose assimilation	+	+
Arabinose assimilation	-	-
Mannose assimilation	-	-
Mannitol assimilation	+	+
N-acetyl-glucosamine assimilation	+	+
Maltose assimilation	-	-
Gluconate assimilation	+	+
Caprate assimilation	+	+
Adipate assimilation	+	+
Malate assimilation	+	+
Citrate assimilation	+	+
Phenyl-acetate assimilation	-	-

(+) : positive, (-) : negative

observed that the titanium ions not only inhibited the growth rate of the strains but also disrupted some components of the metabolism of *E. coli*.

Plasmid-curing of E. coli and P. aeruginosa

E. coli and *P. aeruginosa* were treated with various curing reagents and subjected to electrophoresis as described in Materials and Methods. The original strains of both *E. coli* and *P. aeruginoa* contained a single plasmid species, and mitomycin C resulted in the loss of these plasmids. Both the original and cured strains of *E. coli* and *P. aeruginosa* were inoculated for 48 h in medium containing 300 mg/l titanium at 37°C with agitation at 180 rpm. The growth of original *E. coli* was 0.75 at O.D.$_{660}$ in medium containing 300 mg/l titanium, and growth of plasmid-cured *E. coli* was 0.68 at O.D.$_{660}$. This indicates that the titanium resistance of *E. coli* was not dependent on its plasmid DNA, since the growth rates were roughly equal (Yu, 2003). The growth rate of original *P. aeruginosa* was 1.52 at O.D.$_{660}$, whereas the plasmid-cured *P. aeruginosa* did not grow in medium containing over 200 mg/l titanium. This result shows that the titanium ion resistance of *P. aeruginosa* is in fact dependent on its plasmid (Fig. 3).

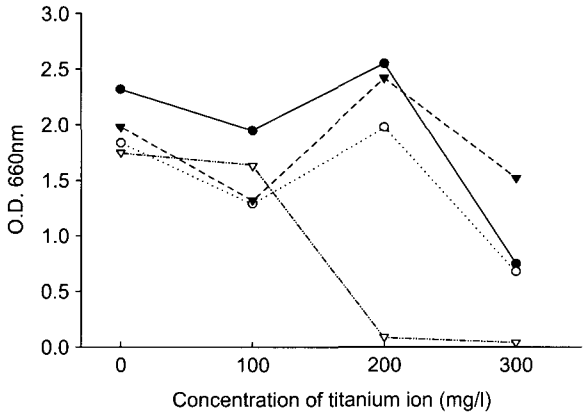

Fig. 3. Growth rate of original *E. coli*, *P. aeruginosa*, plasmid-cured *E. coli* and *P. aeruginosa*. Growth was measured by the absorbance at 660 nm. Symbols: *E. coli* (original) •, *E. coli* (plasmid-cured) ○, *P. aeruginosa* (original) ▼and *P. aeruginosa* (plasmid-cured) ▽.

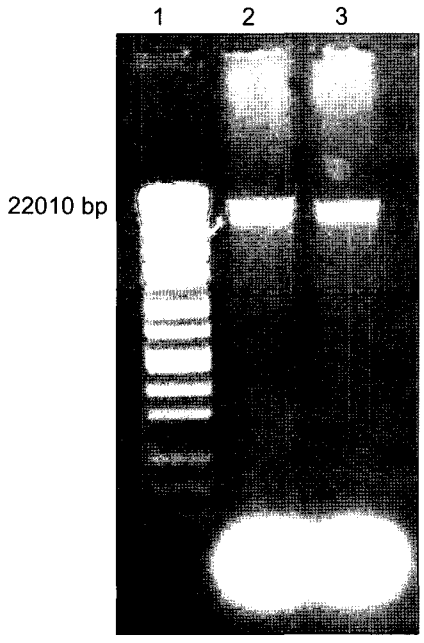

Fig. 4. Electrophoresis of plasmid DNA isolated from *P. aeruginosa* and transformed DH5α. Lanes: 1, *Eco*T14I/*Bgl*II marker; 2, *P. aeruginosa* (original); 3, DH5α (transformant).

Transformation and titanium ion resistant of P. aeruginosa (transformant)

The plasmid of *P. aeruginosa* was transformed into DH5α competent cells (Fig. 4), which were placed into an e-tube and incubated on ice for 10 min. Less than 10 μl of the DNA was placed into the tube and mixed gently, incubated on ice for 30 min and heat-shocked in a water-bath at 42°C for 90 sec, and then rapidly put on ice for 2 min. 800 μl of SOC medium (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 20 mM glucose) were added. The sample was then incubated 60 min at 37°C with gentle agitation. The preparation was then plated on to SOB agar (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 1.5% agar) plates containing 300 mg/l titanium and further incubated overnight at 37°C (Glover and Hames, 1996).

Untransformed DH5α cells grew in medium containing 200 mg/l titanium (to O.D.$_{660}$= 0.66), but not in medium containing 300 mg/l. Untransformed DH5α and DH5α transformant were incubated for 48 h in medium containing 0 to 300 mg/l titanium at 37°C with agitation at 180 rpm. The growth of the DH5α and DH5α transformant in the medium containing 300 mg/l titanium was measured at 1.05×10^7 /ml and 1.11×10^8 /ml, respectively, after plating on the solid LB medium. The DH5α transformant thus grew over 10-fold more than the original DH5α.

The growth of the *P. aeruginosa* (original) in the control and 300 mg/l titanium-containing media was measured at 1.94×10^9 /ml and 1.08×10^9 /ml, respectively. The relative growth of DH5α was 1.3% in medium containing 300 mg/l titanium, but that of the DH5α transformant was 40%. Thus, the growth of the DH5α transformant increased by over 30-fold relative to untransformed DH5α (Table 3) even though no physiological changes were demonstrated

Table 3. Growth of *P. aeruginosa* (original) and DH5α (transformant) in medium containing 300 mg/l titanium

Strains	Conc. of titanium ion (mg/l)	Growth (O.D. 660 nm)	Relative growth (%)
P. aeruginosa (original)	0	2.19	100
	300	1.85	84.5
P. aeruginosa (plasmid-cured)	0	1.75	100
	300	0.04	2.29
DH5α	0	1.81	100
	300	0.37	20.4
DH5α (transformant)	0	1.96	100
	300	1.28	65.3

All strains were incubated 180 rpm, at 37°C for 48 h.

between DH5α and the DH5α transformant (data was not shown).

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