RESEARCH ARTICLE

Nanoparticles affect the survival of bacteria on leaf surfaces

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Abstract

The increasing presence of nanomaterial and nanoproducts makes it imperative to learn more about the associated impacts of these materials on human health and the environment. In this study, the effect of the nanomaterial TiO_2 on the phyllosphere microbial community was investigated. Analysis results by PCR-denaturing gradient gel electrophoresis revealed a TiO_2-induced change in the community structure of microorganisms. An epiphytic bacterium, *Bacillus cereus* 905, was chosen to study the role of the superoxide dismutase-encoding genes, *sod*A-1 and *sod*A-2, and its survival from TiO_2 photocatalysis. Our results showed that the expression of *sod*A-1 and *sod*A-2 was induced by photocatalytic oxidation, with a higher induction observed in *sod*A-2. In addition, compared with wild-type *B. cereus* 905, a reduced bacterial population was observed in a *sod*A-1 and *sod*A-2 double deletion mutant strain KOS on a cucumber leaf surface sprayed with TiO_2. Because the phyllosphere is considered as one of the major habitats for microorganisms, and substantial areas of the earth are covered with leaves, the results of this work provides information of the potential impact of photocatalytic nanomaterial in the environment.

Introduction

Nanotechnology presents new opportunities to create materials and products with unique electronic and biological properties. Nanoengineered materials have found their way into different products, including coatings, computers, clothing, cosmetics, sports equipment, medical devices, and intermediate materials for industrial products (Environmental Law Institute, 2005; Environmental Protection Agency, 2007). The growing presence of nanomaterials and nanoproducts necessitates learning more about their impacts on human health and the environment. In addition, the technological applications of semiconductor-based photocatalysis have expanded rapidly in recent years. Along with several other metal oxide semiconductors (ZnO, Fe_2O_3, and WO_3), nano-TiO_2 has received considerable attention for its capability to eliminate the recalcitrant contaminants, or microorganisms, by generating light-induced reactive oxygen species (Hoffmann *et al.*, 1995; Mills & LeHunte, 1997; Cho *et al.*, 2005; Liu *et al.*, 2005; McCullagh *et al.*, 2007). In addition, it has also been reported that photocatalytic phytopathogen inactivation has a potential for plant disease control by spraying TiO_2 suspensions, using TiO_2 thin films under sunlight or UV lamps (Hur *et al.*, 2005; Lu *et al.*, 2006; Maneerat & Hayata, 2006; Sichel *et al.*, 2007; Yao *et al.*, 2007).

The phyllosphere is defined as a microbial habitat on the leaf surface, and the zone immediately below the leaf surface (Beattie & Lindow, 1995; Hirano & Upper, 2000). Because of the vast magnitude of plant vegetation on our planet, the phyllosphere is considered as an important habitant for microorganisms. This habitat provides a reservoir for bacterial survival and may contribute considerably to the dissemination and spread of pathogens among hosts of plants, animals, and humans (Beattie & Lindow, 1995; Yang *et al.*, 2001; Lindow & Brandl, 2003; Lambais *et al.*, 2006). Leaf-associated bacteria have evolved diverse adaptations in order to establish and colonize themselves in the phyllosphere (Beattie & Lindow, 1995). With wide usages of nanomaterials in the future, a significant amount of these nanoparticles can be potentially deposited on the leaf surface through air, rain, and water by exhaust systems, chimneys, or smoke stacks. However, it is not clear as to

how the photocatalytic nanomaterials may affect phyllosphere microorganisms if the leaf surface is deposited with these substances.

It was reported that TiO_2 particles damaged *Escherichia coli* cells by reducing the intracellular activity of superoxide dismutases (SODs, EC 1.15.1.1) (Koizumi *et al*., 2002). Mechanisms scavenging reactive oxygen species are required when escaping from photocatalytic TiO_2. SODs have been considered as the key enzymes defending against oxidative stress by catalyzing and eliminating $O_2^{\bullet-}$ species in several microorganisms (Wang *et al*., 2007). SOD is reported to reduce the TiO_2 photocatalytic rate of quinoline disappearance, suppress the formation of 2-aminobenzaldehyde, and decrease the amount of 4-quinolinone (Cermenati *et al*., 1997). However, the role of SOD in the survival of the microorganism on the phyllosphere under a photocatalytic condition is unclear. In this study, we examine the effect of TiO_2 on foliar bacterial communities. In addition, by combining a green fluorescent protein (GFP) reporter with flow cytometry (Leveau & Lindow, 2002; Peng *et al*., 2006), the expression of *sod*A in an epiphytic bacterium, *Bacillus cereus*, from the TiO_2-treated leaf surface was examined. Finally, the role of SOD in the survival of *B. cereus* on the surfaces of plant leaves and its interactions with TiO_2 was investigated.

Materials and methods

Bacterial strains, plasmids, and nanoparticles

The bacterial strains, vectors, and primers used in this study are listed in Table 1. All strains were stored at $-80\,^{\circ}C$ in 15% glycerol and grown in Luria–Bertani (LB) medium. The viable population size was determined by counting the *B. cereus* CFU after plating serially diluted cultures with 0.1 M potassium phosphate buffer (pH 7.0) on LB agar grown at $32\,^{\circ}C$ for 8 h. Plasmids isolated from *E. coli* DH5α were transformed into *B. cereus* strains by electroporation using a Micro Pulser Electroporation system (Bio-Rad). Erythromycin was used to select transformants with concentrations of $100\,\mu g\,mL^{-1}$ for *E. coli* and $5\,\mu g\,mL^{-1}$ for *B. cereus*.

AEROXIDE® TiO_2 P25 (Degussa, Germany) was used in this research. Because of its high photoactivity, AEROXIDE® TiO_2 P25 is often recognized as the 'gold standard' in photocatalysis (Mills & Lee, 2002). AEROXIDE® TiO_2 P25 is a highly dispersed titanium dioxide, and the average particle size is 21 nm.

Molecular biology techniques

To extract DNA from phyllosphere bacteria, the young leaves (30 g per replication) were placed into sterile plastic

Table 1. Bacterial strains, plasmids, and DNA primers used in this study

	Characteristics*	References or origin
Strains		
Bacillus cereus		
905	Wild-type strain	Wang *et al*. (2007)
KOS	*sod*A-1 *sod*A-2 deletion mutant of 905	Wang *et al*. (2007)
CS1	KOS was homologous recombined with pEBS-CS1, Em^R	This work
CS2	KOS was homologous recombined with pEBS-CS2, Em^R	This work
905 sodA-1 (atg) ::gfp-pEBS	905 was homologous recombined with pEBS-sodA1:gfp, Em^R	This work
905 sodA-2 (atg)::gfp-pEBS	905 was homologous recombined with pEBS-sodA2:gfp, Em^R	This work
Escherichia coli	F^- Δ(*lac-arg*F)*U169 rec*A-1 *end*A-1 *hsd*R (r_K^- m_K^+) *sup*E-44 *gyr*A-1 *rel*A-1 *deo*R *thi-1* (Φ80d*lac*-ZΔM15)	Strategene
DH5α		
Plasmids		
pAD4412	9.5 kb, carrying *gfp*mut3a, Ap^R, Cm^R	Dunn & Handelsman (1999)
pEBS	4.2 kb, Em^R	Wang *et al*. (2007)
pHP14	4.2 kb, bifunctional replicon, Em^R	Smith *et al*. (1987)
pEBS-sodA1gfp	pEBS with the promoter of *sod*A-1and *gfp* report gene, Em^R	This work
pEBS-sodA2gfp	pEBS with the promoter of *sod*A-2 and *gfp* report gene, Em^R	This work
pEBS-CS1	pEBS with the entire *sod*A-1 gene and flanking sequence, Em^R	This work
pEBS-CS2	pEBS with the entire *sod*A-2 gene and flanking sequence, Em^R	This work
Primers		
P1	5′-ACAGAGCTCGGTACACTAAGAATCC-3′	This work
P2	5′-ACAGGATCCACAACTTCATTCTACC-3′	This work
P3	5′-ACAGAGCTCTCTGTTCCCATATACC-3′	This work
P4	5′-ACAGGATCCAACATTACCTCCTTTC-3′	This work
C2	5′-ACAGGATCCC CCCTAGCTTT TTCTA-3′	This work
C4	5′-ACAGGATCCA GGATTTCGAC AATAC-3′	This work

*Em^R, erythromycin; Ap^R, ampicillin; Cm^R, chloramphenicol.

bags and submerged with washing buffer (0.1 M potassium phosphate buffer, pH 7.0, and 0.1% Bacto peptone) and were sonicated for 10 min in an ultrasonic cleaning bath (KQ3200B, Kunshan Shumei Ultrasonic Inc., China) (Kinkel *et al.*, 1996). The bacterial suspension was centrifuged at 30 000 ***g*** for 30 min at 4 °C. The pellet was washed with 2 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) three times. Then the bacteria were resuspended in 500 μL of TE buffer containing 10 mg mL^{-1} lysozyme (Amresco Inc.) and placed at 37 °C for 1 h. The cell lysis solution was further used for extracting DNA from phyllosphere microorganisms using the cetyltrimethyl ammonium bromide method (Ausubel *et al.*, 1999).

For PCR-denaturing gradient gel electrophoresis (DGGE) analyses, the DNA fragment in the V3 region of the small subunit rRNA gene from the phyllosphere community was amplified as described previously (Yang *et al.*, 2001). The 16S rRNA gene fragments were separated by DGGE in 8% (w/v) acrylamide : bisacrylamide (37.5 : 1, m : m) gels containing a linear chemical gradient ranging from 30% to 60% denaturant [100% denaturing solution contained 40% (v/v) formamide and 7 M urea]. DGGE was performed using a Dcode Universal Mutation Detection System (Bio-Rad). After running for 7 h at 130 V and 60 °C in 1 × TAE electrophoresis buffer, the gels were stained with ethidium bromide and photographed with an AlphaImager (Alpha Innotech).

Construction of mutants

The KOS, a *sod*A-1 and *sod*A-2 deletion mutant of wild-type 905, was constructed previously (Wang *et al.*, 2007). To complement the *sod*A-1 gene in KOS, plasmid pEBS-CS1 was constructed (Table 1). A 1.7-kb DNA fragment containing the upstream region of *sod*A-1 and the entire *sod*A-1 gene was cloned into pEBS, yielding the plasmid pEBS-CS1. Similarly, a DNA fragment containing the entire *sod*A-2 gene and the flanking sequence was inserted into the pEBS, yielding plasmid pEBS-CS2 (Table 1). These pEBS-derivative plasmids containing *sod*A-1 and *sod*A-2 were confirmed by DNA sequencing. The pEBS-CS1 and pEBS-CS2 were transformed into *B. cereus* KOS by electroporation. Transformants of single crossovers were selected on erythromycin plates. DNA sequencing and SOD activity determination (Supporting Information, Fig. S1) were performed to validate the complemented strains (Wang *et al.*, 2007).

Plant material and plant inoculation

The cucumber seed (*Cucumis sativus* cv. Qiupeng Erhao) was used in this study (Yunong Institute of Vegetable and Horticulture, Beijing, China). All experiments were conducted with 2-week-old, greenhouse-grown cucumber plants. An artist's airbrush (HD-470, Yehong Air Tools Co., China) that did not wet the leaf surface was utilized with the aim to avoid spatial displacement of microorganisms (Monier & Lindow, 2005). *Bacillus cereus* 905, KOS, CS1, or CS2 strains were applied separately to these leaves by spraying the bacterial suspension (10^8 CFU mL^{-1} in H_2O) using the artist's airbrush.

Effect of TiO_2 concentrations on bacterial populations

The 2-week-old cucumber plants were grown in a greenhouse covered with a PEP-UV2702AD plastic film (Shanghai HiTeC Plastics Co., China). Solar UV-A and UV-B radiation to the leaves was monitored by a UV sensor (YK-34UV, Taiwan Taina Co., China). The plot in the greenhouse was 60 m long, 8 m wide, and was divided into fifteen separated blocks. The experimental design was a randomized, complete block with three replications for each of the five TiO_2 treatments tested. The treatments were devised according to the dosage of TiO_2 (0, 0.002, 0.02, 0.2, and 2 mg mL^{-1}). TiO_2 in water was sonicated for 20 min before the experiment so that the particles could be uniformly dispersed. To simulate a durative deposit with TiO_2 on the leaf surface, newly expanded leaves of each plant were sprayed with various concentrations of the TiO_2 suspensions once a week from the early growth stage. Eighty plants were treated with a 100-mL suspension per block, each time. The experiment lasted 30 days and sampling was performed a day after the last TiO_2 treatment. The trials were conducted in the greenhouse located at Shangzhuang Agricultural Experimental Station, China Agricultural University, Beijing, China.

To collect leaf samples, fully expanded young leaves (30 g per replication) were chosen randomly from the plant canopy on July 30, 2007, using sterile scissors to nip individual leaves into sterile plastic bags containing washing buffer. These bags were transported to the laboratory on ice and sonicated for 10 min in an ultrasonic cleaning bath. The resulting bacterial suspension was centrifuged for DNA extraction or plated for viable count. Viable counts of the total population size of leaf-associated bacteria were determined by serial dilution of cultures and plating on nutrient agar (1% w/v). Results are representative of at least three independent experiments.

Dynamics of bacterial population densities on leaves

Bacillus cereus 905 was chosen in this study because the *sod*A activities play unique roles in bacterial colonization on the surface of plants (Wang *et al.*, 2007). A series of experiments were conducted with 2-week-old, potted cucumber plants. An inoculum of *B. cereus* strains (10^8 CFU mL^{-1} in H_2O) was sprayed onto the cucumber leaves, respectively, until the

upper sides of the leaf surfaces were uniformly covered with inoculum. Such an inoculation procedure yielded *c.* 10^7 bacteria per leaf. One hour later, the plants were treated with the TiO_2 suspensions (0.02 mg mL^{-1}). The plants were then placed in the phytotron with a 90% relative humidity at 24 °C with a 12-h photoperiod. A bank of 20 W black light fluorescent lamps (Yahong, Shanghai Xinxin Lighting Inc., China) was held above the leaves, which simulated the UV-A portion of sunlight. The vertical distance between the leaf surface and the lamp was 10 cm, and the UV light intensity (I) was 0.2 mW cm^{-2}. At each sampling time, to ensure that these cucumber leaves were in similar age, the first fully expanded leaf of each plant was harvested for the recovery of bacteria. The total and internal population sizes of the bacterium were examined, respectively. Each treatment was replicated with six pots of plants (four plants per pot). Three independent experiments were conducted for each strain.

In this study, the total population size of leaf-associated bacteria was determined by washing leaves individually in the ultrasonic cleaning bath to dislodge the cells from leaves. The internal population sizes of the bacterium were determined by homogenizing leaves that had been surface sterilized with 15% H_2O_2 for 5 min as described previously (Wilson *et al.*, 1999). Serial dilutions of leaf homogenates or washes were plated onto LB agar with erythromycin. The mean population sizes were calculated using samples consisting of 12 leaves for each treatment.

Expression of *sod*A genes during phyllosphere colonization

The *gfp* reporters were constructed to study the expression of SOD of *B. cereus* 905 in the phyllosphere. The promoterless *gfp* gene was digested with XbaI and HindIII from pAD4412 (Dunn & Handelsman, 1999). The digested DNA fragment was ligated into pHP14 to create the plasmid pHP14-gfp. DNA fragments of *sod*A-1 (GenBank accession no. EF075931) and *sod*A-2 (GenBank accession nos. EF075932) promoter regions were PCR amplified from *B. cereus* 905 chromosomal DNA with the primers P1/P2 and P3/P4, respectively (Table 1). The PCR-amplified products from primers P1/P2 and P3/P4 were ligated into pHP14-gfp, respectively, and were further cloned into pEBS to produce pEBS-sodA1gfp and pEBS-sodA2gfp. The pEBS-sodA1gfp and pEBS-sodA2gfp were introduced into the host bacterial cells and erythromycin antibiotic selection was performed, which forced these constructs to integrate into the genome via a single crossover recombination at the homologous region. This created *B. cereus* 905 sodA-1 (atg)::gfp-pEBS and *B. cereus* 905 sodA-2 (atg)::gfp-pEBS. These bacteria were confirmed by DNA sequencing and the fluorescent phenotype was verified.

The expression of *sod*A-1 and *sod*A-2 was measured by an FACS Caliber flow cytometer (BD Biosciences, San Jose, CA). The wild-type *B. cereus* 905, 905 sodA-1 (atg)::gfp-pEBS or 905 sodA-2 (atg)::gfp-pEBS were grown on LB medium at 32 °C. The bacterial cells were harvested from plates and resuspended in the LB broth to an $OD_{600\,nm}$ of 0.1. After mixing with TiO_2 particles, the bacterial suspension was seeded into 24-well plates (1 mL per well). The plates were then irradiated from the upper side with black light fluorescent lamps at 20 ± 1 °C in a thermostatic chamber. The light intensity was 0.2 mW cm^{-2}. Cells from three stochastic wells were collected per treatment.

The cucumber leaves that had just expanded were harvested from 2-week-old greenhouse-grown plants. Cucumber plants were used to investigate the effect of TiO_2 on expression of *sod*A-1 and *sod*A-2 on the phyllosphere. *Bacillus cereus* 905, 905 sodA-1 (atg)::gfp-pEBS or 905 sodA-2 (atg)::gfp-pEBS were inoculated on these leaves by immersing the upper portion of the leaves into the bacterial suspension (10^8 CFU mL^{-1} in H_2O) for 5 s. After inoculation, these leaves were placed in a 6-well plate (Cellstar, Greiner bio-one, Germany) that had been paved with a sterile filter paper disk, moistened with 500 μL sterile-distilled water at the well bottom. The cut end of the leaf petiole was covered by a piece of sterile absorbent cotton submerged with 100 μL sterile-distilled water to retain the moisture (Fig. S2). The leaves were then sprayed with a TiO_2 suspension, 2 mL per plate. The plates were placed in a thermostatic chamber at 20 ± 1 °C with irradiation of the black light fluorescent lamps (Yahong, Shanghai Xinxin Lighting Inc.) until sampling. Bacterial cells of leaf washings (six leaves per treatment) were further harvested and washed by centrifugation at 1000 ***g*** for 4 min. The cells were then run through the flow cytometer. Samples were subjected to flow cytometry until 25 000 fluorescent particles had been examined. The results were analyzed by CELL QUESTPRO software (Peng *et al.*, 2006). All of the experiments were repeated in triplicate.

Statistical analyses

The GELCOMPAR 2.0 software package was used to cluster analysis and bootstrap analysis of the DGGE bands (Vauterin & Vauterin, 1992). For the survival of bacteria on the leaf surface, all calculations are based on Tukey's honestly significant different test using SPSS (SPSS Inc., Chicago, IL).

Results

Effect of TiO_2 on the foliar bacterial community of cucumber

DGGE profiles (Fig. 1) and cluster analysis (Fig. 2) showed that similar community structures were observed in the

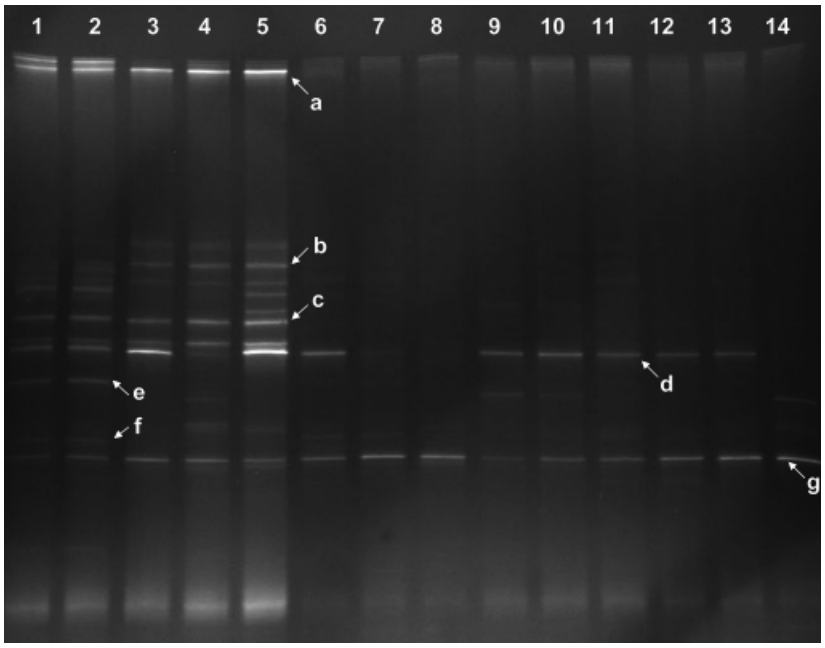

Fig. 1. PCR-DGGE 16S rRNA banding profiles of microorganisms from the cucumber phyllosphere sprayed with different concentrations of TiO_2. Lanes: 1, 2, 0 mg mL^{-1} TiO_2; 3–5, 0.002 mg mL^{-1} TiO_2; 6–8, 0.02 mg mL^{-1} TiO_2; 9–11, 0.2 mg mL^{-1} TiO_2; 12–14, 2 mg mL^{-1} TiO_2. DGGE bands designated as a, b, c, d, e, f and g were selected to isolate, clone and sequence (Table 2).

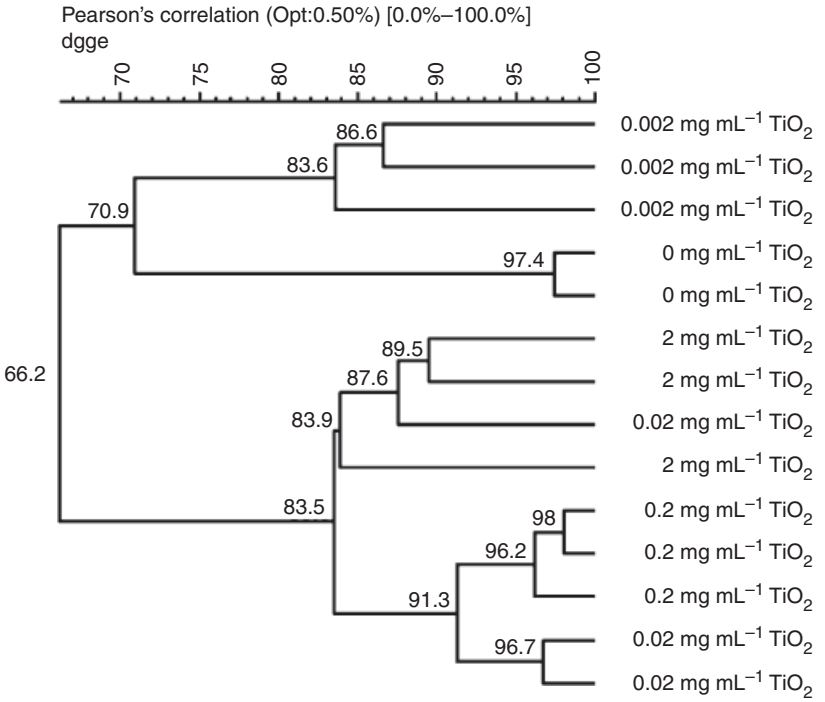

Fig. 2. Cluster analysis of 16S rRNA banding profiles for bacteria from the cucumber phyllosphere sprayed with different concentrations TiO_2. Bootstrap (confidence) values are shown at the branches.

control (0 mg mL^{-1} TiO_2) and low-level TiO_2 treatment (0.002 mg mL^{-1} TiO_2). However, an obvious TiO_2-induced change in the community structure was observed at a TiO_2 concentration of 0.02 mg mL^{-1} and above, which had only 15% similarity in comparison with the control treatment. In addition, the total culturable leaf surface population sizes in the phyllosphere community were significantly reduced with an increase in the TiO_2 concentration to 0.02 mg mL^{-1} ($P < 0.05$), which also indicated that the TiO_2 affected the foliar bacterial community (Fig. 3).

To further understand the differences between various treatments, dominant 16S rRNA DGGE bands obtained from microorganisms on cucumber leaves were isolated, cloned, and sequenced (Yang *et al.*, 2001). Seven DGGE

bands designated a, b, c, d, e, f, and g were selected (Fig. 1). The nucleotide sequences of six individual clones were obtained from each of these bands (Fig. 1, Table 2). Sequence data from 16S rRNA bands suggested that more than one organism was present in some of the bands sequenced in this study (Table 2). The a, b, c, e, and f bands were no longer visible with increasing TiO_2 concentration, which suggested that the catalytic oxidation of TiO_2 reduced or eliminated the bacteria identified in these 16S rRNA bands in the PCR-DGGE assay. The bacteria identified from bands d (*Pantoea* sp. and *Pseudomonas* sp.) and g (uncultured bacterium clone ARDRA0157) showed resistance to catalytic oxidation of TiO_2. The fact that the appearance of band g was not affected by the TiO_2 supplemented on the leaf surface implied that this microorganism was highly resistant to the oxidative stress produced by TiO_2.

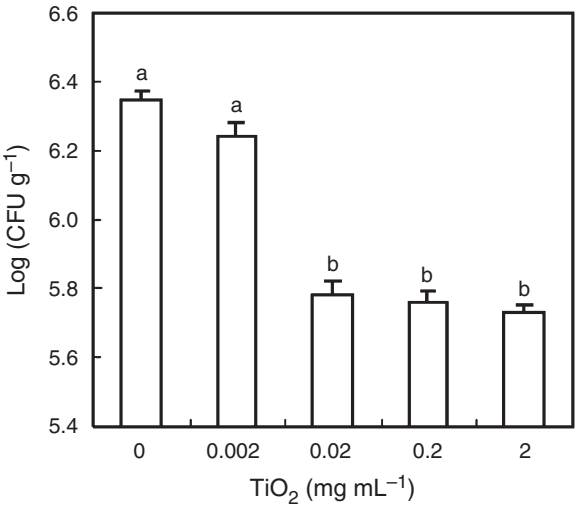

Fig. 3. The total culturable phyllosphere population sizes from the cucumber phyllosphere sprayed with different concentrations of TiO_2. Data are the means of three determinations and the error bars indicate SD. Values are representative of three independent experiments. Three replicates were used in this experiment. Different letters indicate significant differences based on Tukey's honestly significantly different test using SPSS ($P < 0.05$).

Survival of *B. cereus* during photocatalytic oxidation on leaves

Because SOD plays a role in resistance to oxidative stress in bacteria, the survival of *B. cereus* 905 and *sod*A mutant KOS under TiO_2 photocatalysis was investigated. Bacterial cells were sprayed onto cucumber leaves 1 h before TiO_2 treatment (0.02 mg mL^{-1}). Plants were then exposed to UV-A irradiation under high relative humidity conditions (90%). No significant difference in the bacterial population was observed among leaf surfaces treated with H_2O or TiO_2 when bacterial cells were examined immediately after inoculation ($P < 0.05$) (Fig. 4a). A similar population size of the wild-type *B. cereus* 905 was observed on the leaf surface sprayed with a H_2O or a TiO_2 suspension at 8 h after inoculation (Fig. 4b). In addition, similar bacterial populations were observed between *B. cereus* 905 and *sod* mutant *B. cereus* KOS on leaf surfaces sprayed with H_2O at 8 h after inoculation. Compared with mutant KOS on the leaf surface sprayed with H_2O, an order magnitude reduction of the bacterial population of mutant KOS was observed on the leaf surface sprayed with the TiO_2 suspension ($P < 0.05$) (Fig. 4b). The bacterial population of CS2 on the leaf surface treated with TiO_2 was complemented to wild-type levels, but CS1 was only partly complemented to the level of the wild-type bacterium (Fig. 4b). No significant difference was observed among bacterial population sizes in the protected or the inner sites of the leaf (Fig. 4). In summary, SOD of *B. cereus* plays a role in the bacterial survival in the TiO_2-treated leaf surface. The *sod*A-2, but not *sod*A-1, contributes to protection of the bacterial cells from oxidative stress caused by photoexcited TiO_2.

To study the colonization of wild-type *B. cereus* 905, *sod*A mutant KOS, and complementary strains CS1 and CS2 on cucumber leaves, the total population kinetics of these bacterial strains were examined on the phyllosphere between

Table 2. BLAST results of predominant 16S rRNA DGGE bands obtained from the phyllosphere of cucumber leaves

Gel position	Database match (GenBank accession no.)	DDBJ accession no.	Sequence similarity (%)
Band a	*Pseudomonas* sp. Bsi20397 16S rRNA gene (EU330375.1)	AB377180	94
Band a	*Sphingomonas* sp. ATCC 31555 16S rRNA gene (AF503280.1)	AB377181	94
Band a	*Providencia* sp. SBS1 16S rRNA gene (EU195872.1)	AB377182	99
Band b	*Pseudomonas* sp. m33 16S rRNA gene (EU375658.1)	AB377183	100
Band c	Uncultured *Exiguobacterium* sp. clone AV 4R-S-O05 16S rRNA gene (EU341181.1)	AB377184	100
Band c	Uncultured bacterium clone 2-3A 16S rRNA gene (EU289492.1)	AB377185	100
Band d	*Pantoea* sp. DAP18 16S rRNA gene (EU302843.1)	AB377186	100
Band d	*Pseudomonas* sp. SYW1D 16S rRNA gene (EU293151.1)	AB377187	100
Band e	Uncultured *Providencia* sp. small subunit rRNA gene (DQ533898.1)	AB377188	100
Band f	Uncultured bacterium clone 6224 16S rRNA gene (EU368385.1)	AB377189	100
Band g	Uncultured bacterium clone ARDRA0157 16S rRNA gene (EF050118.1)	AB377190	100

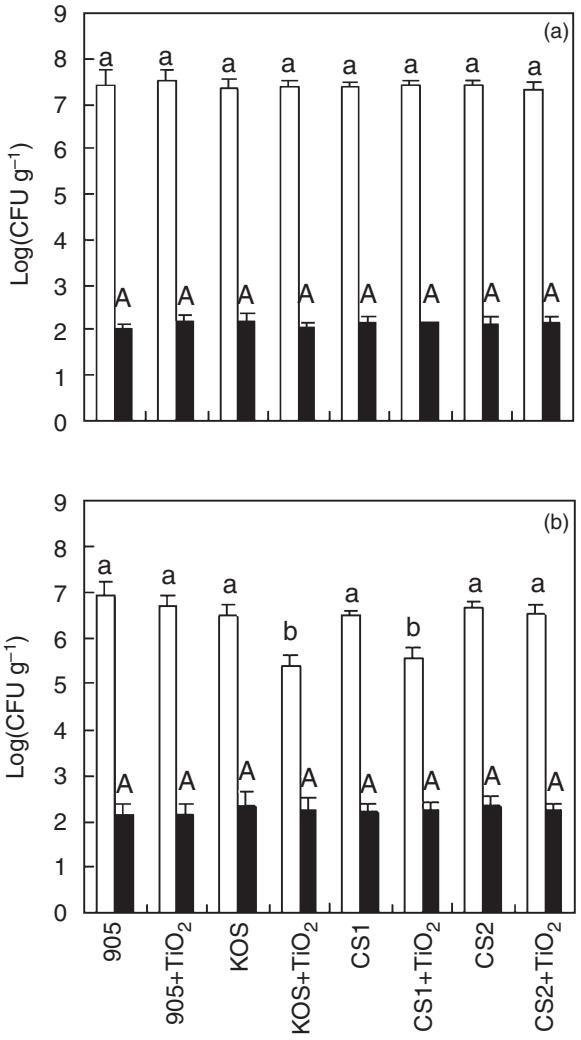

Fig. 4. Survival of wild-type *Bacillus cereus* 905, *sod* mutant KOS, and complementary strains CS1 and CS2 from the cucumber leaves subjected to photocatalytic oxidation stress. Leaves of cucumber were inoculated with bacterial suspension and further sprayed with TiO_2 (0.02 mg mL^{-1}) or H_2O (for control), then were placed in a chamber with UVA irradiation (I = 0.2 mW cm^{-2}). Data are the means of three determinations and the error bars indicate SD. (a) Immediately after inoculation. (b) After 8 h of inoculation under high relative humidity. White bars, total leaf-associated bacterial populations sizes; black bars, bacterial population sizes in the protected or inner sites of the leaf. Values are representative of three independent experiments. Three replicates were used in this experiment. Different capital or lower case letters indicate significant differences based on Tukey's honestly significant different test using SPSS ($P < 0.05$).

0 and 72 h at an interval of 8 h (Fig. S3). No significant differences among bacterial populations were observed between *B. cereus* 905 and CS2 during the 72-h period ($P > 0.05$). Compared with *B. cereus* 905 and CS2, in general, a lower population of CS1 and KOS was observed after 32 h ($P < 0.05$) (Fig. S3).

Expression of *sod*A in *B. cereus* 905 under oxidation stress

Bacillus cereus 905 sodA-1 (atg)::gfp-pEBS and *B. cereus* 905 sodA-2 (atg)::gfp-pEBS were used to investigate the expression of *sod*A-1 and *sod*A-2 in medium or on the cucumber phyllosphere under the photocatalysis of TiO_2. Compared with *B. cereus* 905 sodA-2 (atg)::gfp-pEBS in LB alone, $>$ 2.5-fold induction of *sod*A-2 was observed in LB supplemented with TiO_2 after 1 h of growth ($P < 0.05$) (Fig. 5a). Compared with *B. cereus* 905 sodA-1 (atg)::gfp-pEBS in LB alone, around a 1.4-fold induction of *sod*A-1 was observed in LB supplemented with TiO_2 ($P < 0.05$).

The expression of *sod*A-1 and *sod*A-2 of *B. cereus* was also measured on the phyllosphere. A higher expression of *sod*A-2 was observed in the bacterial cells sprayed on the cucumber surface in comparison with *B. cereus* 905 grown in LB (Fig. 5a and b). Compared with *B. cereus* 905 sodA-1 (atg)::gfp-pEBS on the phyllosphere alone, around a 1.4-fold induction of *sod*A-1 was observed on the phyllosphere supplemented with TiO_2 ($P < 0.05$). However, compared with *B. cereus* 905 sodA-2 (atg)::gfp-pEBS on the leaf surface alone, about a 3.4-fold induction of *sod*A-2 ($P < 0.05$) was observed on the leaf surface sprayed with TiO_2 (Fig. 5b), suggesting that *sod*A-2 exhibited higher levels of induction than *sod*A-1 by TiO_2 photocatalysis. These results demonstrate that the expression of *sod*A-1 and *sod*A-2 can be induced by photoexcited TiO_2 with a higher induction of *sod*A-2 when exposed to this photocatalyst.

Discussion

In this paper, we observed a TiO_2-induced change of the bacterial community structure at a TiO_2 concentration of 0.02 mg mL^{-1} and above using culture-independent DGGE-PCR analysis. In addition, a reduced bacterial population was observed in TiO_2-treated leaf samples by culturing bacterial samples on medium plates. This suggests that TiO_2 not only alters the complexity of the epiphytes but also reduces the bacterial population in the phyllosphere. The 16S rRNA band g identified from the phyllosphere bacteria appeared to be more resistant to oxidative stress produced by TiO_2 (Fig. 2). Band g is homologous to the DNA fragment from an uncultured bacterium clone ARDRA0157 isolated from rural aerosol (Table 2). Future experiments will be needed to identify this bacterium and study the mechanisms for its resistance to TiO_2 photocatalysis. It will also be of interest to determine the minimum TiO_2 dose required to induce a shift in bacterial community structures for nanoparticle exposure. Finally, our study suggests that the TiO_2 at a concentration of 0.02 mg mL^{-1} (about 3.57–4.17 $\times$ 10^{-5} mg deposition of TiO_2 particles per cm^2 cucumber leaf) and above may have a significant impact in altering community structures and reducing bacterial sizes

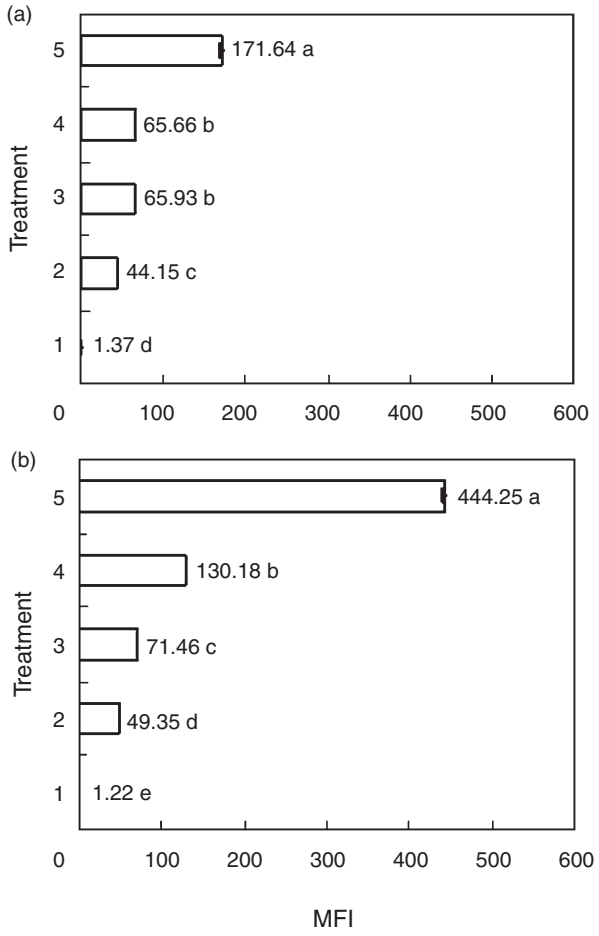

Fig. 5. Expression of the *sod*A-1 and *sod*A-2 in *Bacillus cereus* 905 during photocatalytic oxidation stress. UVA irradiation was 0.2 mW cm^{-2}. The concentration of TiO_2 was 0.02 mg mL^{-1}. Mean fluorescence Intensity (MFI) means average GFP fluorescence intensity of total bacterial cells examined (Peng *et al.*, 2006). Data are the means of three determinations and the error bars indicate SD. (a) In LB medium after UVA irradiation for 1 h. (b) In cucumber phyllosphere after UVA irradiation for 1 h. Treatment 1, *B. cereus* 905 and TiO_2. Treatment 2, *B. cereus* 905 *sod*A-1(atg)::*gfp*-pEBS. Treatment 3, *B. cereus* 905 *sod*A-1(atg)::*gfp*-pEBS and TiO_2. Treatment 4, *B. cereus* 905 *sod*A-2 (atg)::*gfp*-pEBS . Treatment 5, *B. cereus* 905 *sod*A-2 (atg)::*gfp*-pEBS and TiO_2. Values are a representative of three independent experiments. Three replicates were used in this experiment. Different letters indicate significant differences based Tukey's honestly significantly different test using SPSS ($P < 0.05$).

in phyllosphere if the leaf surface is contaminated with this photoexcited material.

The fluctuation in the environmental conditions plays a decisive role in microbial community structure (van Hannen *et al.*, 1999). Kadivar and colleagues also reported that although the maize seeds were from the same ear, the bacterial species from the maize phyllosphere found in the greenhouse and in the field are quite different. It is suggested that the bacteria on the phyllosphere are deposited from the environment, and influenced by environmental factors rather than from where the bacteria were originally carried (e.g. from seed coat) (Kadivar & Stapleton, 2003). We compared our sequence list (Table 2) with previous sequence data of culture-independent bacterial groups collected from terrestrial plant phyllospheres (Yang *et al.*, 2001; Kadivar & Stapleton, 2003; Lambais *et al.*, 2006); only the sequence of AB377186 had 100% similarity to DQ221385 or DQ221384 from *Trichillia clausenii* (Lambais *et al.*, 2006). This very limited parallelism suggests that there are few bacterial species common to various plant samples from different places, and plant species are likely to be important determinants of phyllosphere bacterial community composition (Yang *et al.*, 2001; Lambais *et al.*, 2006). Finally, the trend toward decreased diversity in TiO_2-treated samples implied that bacterial species sensitive to oxidative stress are being eliminated and a distinct bacterial community is formed after the phyllosphere is exposed to TiO_2.

An epiphyte *B. cereus* 905 was used in this study to elucidate the role of *sod*A in TiO_2 photocatalysis on the phyllosphere. In agreement with that in rhizosphere colonization (Wang *et al.*, 2007), the *sod*A activities play unique roles in *B. cereus* 905 colonizing the surface of leaves (Fig. S3). Our study showed that an increased expression of *sod*A-1 and *sod*A-2 of *B. cereus* 905 was observed when bacterial cells were treated with TiO_2. In addition, a reduced bacterial population of *sod*A mutant *B. cereus* KOS was observed on the phyllosphere applied with TiO_2, suggesting that superoxide dismutase plays a role in the bacterial survival under photocatalytic stress. Similar bacterial population sizes were observed among bacterial strains treated with H_2O or TiO_2 in the internal site of leaves (Fig. 4). A possible explanation may be that limited photocatalytic activity of TiO_2 is able to reach the internal sites of the leaves occupied by the bacterium. As a consequence, a less oxidative environment was present in the internal areas of the leaves treated with TiO_2 in comparison with the leaf surface. Koizumi *et al.* (2002) postulated that SOD might play a role in protecting *E. coli* cells against oxidative stress through quenching catalysis produced by TiO_2 (Koizumi *et al.*, 2002). In this study, we demonstrate that the community structures of the phyllosphere are disturbed by active oxidant species. Our data further suggest that *sod*A genes of *B. cereus* 905 are induced by photoexcited TiO_2 and these gene products play an essential role in the protection of bacterial cells from excess oxidative stress arising from TiO_2.

A significant amount of the earth is covered with leaves, which harbor hundreds of species of bacteria, fungi, and yeast (Lindow & Brandl, 2003). The leaf surfaces are subjected to high-intensity visible/UV light and airborne pollutants, which create a unique, but harsh environment for epiphytes to adapt (Beattie & Lindow, 1995; Yang *et al.*, 2001; Lindow & Brandl, 2003; Lambais *et al.*, 2006; Sandhu

et al., 2007). With the wide utilization of nanomaterials, the consequent effect on the environment can be potentially significant. An effect of TiO_2 photocatalysis on phyllosphere microbial complexity and population was observed in this work. In addition, our study suggests that SOD may play a role in bacterial survival on the phyllosphere under oxidative stress. Finally, further research is needed in the future to identify the other impacts of these nanomaterials in the microbial ecological system, and to find out how to minimize these adverse impacts.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Detection of SOD activity in *Bacillus cereus* KOS and its *sod*A complementations on nondenaturing polyacrylamide gels with negative staining.

Fig. S2. The just-expanded cucumber leaves were placed in a six-well plate.

Fig. S3. Colonization of wild-type *Bacillus cereus* 905, *sod* mutant KOS, and complementary strain CS1, CS2 on cucumber leaves.

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