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Low-temperature biosynthesis of fluorescent semiconductor nanoparticles (CdS) by oxidative stress resistant Antarctic bacteria

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ABSTRACT

Bacterial biosynthesis of nanoparticles represents a green alternative for the production of nanostructures with novel properties. Recently, the importance of antioxidant molecules on the biosynthesis of semiconductor fluorescent nanoparticles (quantum dots, QDs) by mesophilic bacteria was reported. The objective of this work was the isolation of psychrotolerant, oxidative stress-resistant bacteria from Antarctica to determine their ability for biosynthesizing CdS QDs at low temperatures. QDs biosynthesis at 15 °C was evaluated by determining their spectroscopic properties after exposing oxidative-stress resistant isolates identified as *Pseudomonas* spp. to Cd^{2+} salts. To characterize the QDs biosynthetic process, the effect of metal exposure on bacterial fluorescence was determined at different times. Time-dependent changes in fluorescence color (green to red), characteristic of QDs, were observed. Electron microscopy analysis of fluorescent cells revealed that biosynthesized nanometric structures localize at the cell periphery. QDs were purified from the bacterial isolates and their fluorescence properties were characterized. Emission spectra displayed classical CdS peaks when excited with UV light. Thiol content, peroxidase activity, lipopolysaccharide synthesis, metabolic profiles and sulfide generation were determined in QDs-producing isolates. No relationship between QDs production and cellular thiol content or peroxidase activity was found. However, sulfide production enhanced CdS QDs biosynthesis. In this work, the use of Antarctic psychrotolerant *Pseudomonas* spp. for QDs biosynthesis at low temperature is reported for the first time.

1. Introduction

Semiconductor nanocrystals or quantum dots (QDs) that are composed by elements such as Cd, Te, Se, Pb and As, among others, exhibit unique optical and electronic properties with fluorescence emission wavelengths depending on particle size (Zhou and Ghosh, 2007; Fulekar, 2012; Michalet et al., 2005). Nowadays, the interest in studying QDs has increased because of its use in solar cells, optoelectronics, transistor components, biomedicine and imaging, among many other applications (Gaponik et al., 1999; Huynh et al., 2002; Michalet et al., 2005).

In general, QDs chemical synthesis exhibits unfavorable capital/energy ratios and involves toxic reagents, high temperatures, anaerobic conditions and waste generation (Peng and Peng, 2001; Wang et al., 2009). As a result, obtained nanoparticles display low biocompatibility, thus limiting their applications in biological systems (Schneider et al., 2009; Chang et al., 2012).

During the last years, the use of biological compounds such as peptides, nucleotides, and fusion proteins has been proposed as an alternative to chemical that are routinely utilized for CdS, CdSe and CdTe QDs synthesis (Bao et al., 2006; Aryal and Benson, 2007; Pérez-Donoso et al., 2012). However, nanoparticles generated by these "biomimetic" methods still display biocompatibility problems as well as elevated production costs.

In this context, microbial biosynthesis of nanoparticles has emerged as a cost effective and environmentally friendly green alternative. Biosynthesized nanoparticles display advantages such

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as high stability, water solubility, biocompatibility, high production rates and low cost, among others (Bao et al., 2010a,b). Several microorganisms including *Candida glabrata*, *Schizosaccharomyces pombe*, *Fusarium oxysporum*, *Saccharomyces cerevisiae* and *Escherichia coli* have been employed for biosynthesizing cadmium-based QDs (Dameron et al., 1989; Kowshik et al., 2002; Kumar et al., 2007; Bao et al., 2010a,b; Mi et al., 2011). Most procedures for QDs biosynthesis reported so far are based on biological molecules with antioxidant properties, for instance, metal-binding molecules composed by thiols. In this context, *S. pombe* phytochelatins have been used to synthesize CdS QDs in *E. coli* (Kang et al., 2008). Likewise, phytochelatins have been also utilized in conjunction with metallothioneins for biosynthesizing CdS QDs in recombinant *E. coli* (Park et al., 2010). Recently, the importance of the biological thiol glutathione (GSH) for the intracellular production of CdS and CdTe QDs by *E. coli* was reported (Chen et al., 2009; Monrás et al., 2012). Moreover, it has been observed that cysteine desulfhydrases producing H_2S are responsible for the formation of CdS nanocrystals in *Rhodopseudomonas palustris* (Bai et al., 2009). All these reports relating QDs biosynthesis with antioxidant defenses and the redox status suggest that cellular antioxidants favor intracellular QDs generation.

All reports regarding nanoparticle synthesis, biological or chemical, are developed under different temperature conditions ranging from 35 °C (when using mesophile microorganisms) to 350 °C (chemical synthesis developed). Lower temperatures could improve the chemical process of QDs biosynthesis at molecular level. The chemical reaction involved in bioproduction of QDs could proceed through two pathways; thermodynamic and kinetic control. Thermodynamic control results when the reaction temperature is high and a stable product is synthesized (thermodynamic product). In contrast, kinetic control takes place at low temperatures when products with less activation energy are generated. A simulation of QDs growth shows that after the formation of the initial "seeds" (QDs nucleation points), the size distribution of nanoparticles is "kinetically controlled" (Meixner et al., 2001). Accordingly, at low temperatures smaller seeds (but a higher number) could be obtained when compared to high temperatures (Meixner et al., 2001). Based on this, it is expected that low-temperature QDs synthesis will allow a better control of NPs properties (size and polydispersity, among others) based on a kinetic dependent-nucleation process. On the other hand, if it is possible to biosynthesize QDs at low temperatures with the same yield as compared to high temperature, the decrease on energy costs would be tremendous, favoring the biotechnological application of these green produced fluorescent NPs.

Based on this and due to its unusual mix of extreme conditions including high ultraviolet radiation ad low temperatures, Antarctica represents a perfect environment to isolate psychrotolerant microorganisms capable of producing QDs (Cowan and Tow, 2004). In addition, Chattopadhyay et al. (2011) have reported the low temperature-induced oxidative stress on the Antarctic bacterium *P. fluorescens* MTCC 667. Furthermore, the existence of two Antarctic cyanobacteria strains (*Phormidium murrayi* and *Oscillatoria priestleyi*), displaying high tolerance to UV exposure has been described (Quesada and Vincent, 1997). Finally, QDs biosynthesis at low temperatures could encompass advantages such as lower production costs and the generation of nanoparticles with novel properties.

In this work, five hydrogen peroxide (H_2O_2) and cadmium resistant bacteria were isolated from Antarctic soils. These oxidative stress resistant isolates were identified as *Pseudomonas* spp. and their ability to biosynthesize cadmium based QDs at 15 °C were determined. Intracellularly biosynthesized nanostructures were purified and their spectroscopic properties indicated that they correspond to CdS QDs.

2. Materials and methods

2.1. Sample collection

Samples of soil, sediments and water were collected from King George, Deception, and Southern Shetland Islands during the 48th Chilean Antarctic Expedition (ECA) organized by the Chilean Antarctic Institute (INACH).

2.2. Isolation of bacterial strains

Antarctic samples (100 mg of soil or sediments, or 100 μl of water) were suspended on 1 ml sterile distilled water (final volume) and vigorously stirred by vortexing (30 s). After that, 10 μl of each solution were used to inoculate 990 μl of LB culture medium and incubated at 15 °C. After 24 h, aliquots were seeded on LB agar plates and incubated at the same temperature for two days.

2.3. Selection of oxidative stress resistant bacteria

Resistant bacteria were selected by plating on LB agar plates supplemented with H_2O_2 (12 mM). Bacterial growth at 15 °C was monitored after 24 and 48 h.

2.4. Minimal inhibitory concentration (MIC)

MICs were determined using the protocol previously described by Elías et al. (2012). Solutions containing 200 mM H_2O_2 and 4000 μg/ml $CdCl_2$ solutions were prepared in 200 μl LB medium. Serial dilutions were set in 96-well microplates and inoculated with 5 μl of bacterial cultures grown to $OD_{600} \sim 0.05$. Microplates were incubated at 15 °C and bacterial growth was assessed after 72 h.

2.5. Identification of bacterial isolates

Selected isolates were identified by 16S rDNA sequencing. A single colony of each bacterial isolate was lysed by heating 10 min at 95 °C. 16S rDNA gene amplification was performed using universal primers U515 F and U1492 R (Reysenbach and Pace, 1995; Suzuki and Giovannoni, 1996). PCR was carried out as follows: preheating 5 min at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing 30 s at 55 °C and extension 30 s at 72 °C plus additional incubation for 10 min at 72 °C. Amplified fragments were analyzed by agarose gel electrophoresis and sent to Macrogen Corporation (Korea) for DNA sequencing. Bacterial genres were determined by comparison with sequences deposited in NCBI Gen Bank using BLAST.

2.6. Lipopolysaccharide extraction, purification and visualization

Lipopolysaccharide (LPS) was extracted and purified from each strain using the protocol described by Marolda and Valvano (2007). Briefly, bacterial cells ($OD_{560} \sim 2.0$) were suspended in 150 μl of lysis buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol and 1 M Tris–HCl pH 6.8), heated at 100 °C for 10 min, and incubated with 10 μl of proteinase K (20 mg/ml) at 60 °C overnight. Then, 150 μl of 95% phenol were added, incubated at 70 °C for 15 min and cooled on ice for 10 min. After spinning at 10,000 × *g* for 10 min, the aqueous phase was transferred to another tube and the LPS was precipitated by adding 500 μl of ether–ethanol (2:5) and centrifuged at 10,000 × *g* for 1 min. Precipitated LPS was left at room temperature to remove the remaining ether–ethanol and stored at 4 °C. Resulting LPS samples were fractionated by tricine-SDS-PAGE-Tris (14%, w/v, acrylamide) and subjected to silver staining.

2.7. Metabolic assays

Phenotypic characterization was performed by using nine conventional biochemical tests (Brown, 2009). Each bacterial isolate was evaluated using Christensen urea agar, Simmons citrate agar, lysine iron agar (LIA), triple sugar iron agar (TSI), mobility-indole-ornithine agar (MIO), gelatin hydrolysis test, catalase test, glucose fermentation with Durham tubes and hemolysis in blood agar test.

2.8. Optimal growth temperature

Bacterial cultures were grown at 15 °C overnight and then diluted to $OD_{600} \sim 0.2$. Serial dilutions were prepared in 96-well microplates and 5 μl of each dilution were used to inoculate LB-agar plates. Plates were incubated at 4, 15, 28 and 37 °C and bacterial growth was determined after 24 h. *E. coli* BW25113 and *P. aeruginosa* PAO1 were used as controls.

2.9. CdS nanoparticles biosynthesis

Biosynthesis of nanoparticles was evaluated on selected isolates following the protocol described by Monrás et al. (2012). A bacterial pre-inoculum was diluted on LB medium (1:100) and grown for 24 h at 15 °C. After collecting and resuspending in $CdCl_2$ (10 μg/ml), cells were incubated at the same temperature for different time periods. Samples were then centrifuged at 10,000 × *g* for 5 min, supernatants were discarded and the fluorescence of cell pellets was evaluated after excitation at 360 nm using a short wave UV-transilluminator. For experiments of QDs biosynthesis over time, distilled water, PBS 1× and 100 mM citrate were used and biosynthesis was assessed daily by exposing cell pellets to UV light.

2.10. Purification of biosynthesized nanoparticles

Cell lysis was carried out by adding 1N NaOH to fluorescent bacterial pellets and heating for 5 min at 90 °C. Fluorescent QDs remained in the supernatant and the cell debris was discarded by centrifugation at 10,000 × *g* for 10 min. Fluorescent samples were detected by exposition to UV light as described previously (Monrás et al., 2012). The solution was dialyzed for 1 h against 100 mM Tris–HCl pH 7.0, and incubated overnight at 50 °C with proteinase K (100 μg/ml). The resulting fluorescent solution was used for spectroscopic characterization.

2.11. Fluorescence of purified nanoparticles

Fluorescence spectra of purified nanoparticles were determined by using a multiplate reader, Synergy H1M (Biotek). Emission spectrum of purified nanoparticles was obtained after excitation at 400 nm.

2.12. Transmission electron microscopy (TEM)

Pseudomonas sp. GC01 was grown in QDs biosynthesis conditions (see above). Fluorescent cells were collected by centrifugation, fixed using glutaraldehyde, dehydrated and infiltrated with an epoxy resin. Ultrathin sections (50–100-nm thick) were obtained using an ultramicrotome and placed in copper grids (Formvar carbon 300 mesh, grid hole size: 63 μm). Transmission electron microscopy images were collected using a Phillips Tecnai 12 BioTwin microscope at 80 kV.

2.13. Peroxidase activity assay

Single colonies were inoculated in 1 ml of LB medium and incubated overnight at 15 °C. Then, cultures were diluted 1:100 with fresh LB medium and grown to $OD_{600} \sim 1.5$. Peroxidase activity was determined by the protocol described by Chen et al. (2003), with some modifications. Briefly, 200 μl cell extracts were mixed with 1.8 ml of 0.06% H_2O_2 and the decrease in absorbance at 240 nm after 2 min was determined. To calculate peroxidase activity the concentration of H_2O_2 was determined using the molar coefficient previously reported (Chen et al., 2003). Activity was normalized by the protein concentration (Bradford, 1976).

Table 1
Peroxide and cadmium resistance levels of isolated Antarctic strains. Minimal inhibitory concentrations (MIC) of peroxide and $CdCl_2$ were determined as described in methods.

Strain/MIC	H_2O_2 (mM)	$CdCl_2$ (μg/ml)
GC01	20	325
GC02	40	350
GC03	75	400
GC04	20	270
GC05	30	500

2.14. Total reduced thiol content (RSH)

Pre-inocula of bacterial isolates were grown overnight at 15 °C. Each inoculum was diluted 1:100 in LB medium and grown for 24 h. Cells were centrifuged for 10 min at 5.000 × *g*, washed twice and suspended in 0.1 M Tris–HCl (pH 8.0) that contained 10 μg/ml $CdCl_2$. After 30 min incubation, cells were washed and cell pellets suspended in 1 ml of 0.1 M Tris–HCl (pH 8.0) with 1 mM EDTA. Samples were homogenized by vortexing with glass beads for three cycles of 60 s at 4 M/s in a FastPrep®-24 homogenizer (MP biomedicals). The suspension was centrifuged at 10,000 × *g* and the supernatant was used for thiol quantification. RSH were quantified by mixing 100 μl of supernatant with 10 μl of 1 mM DTNB (5,5′-dithiobis 2-nitrobenzoic acid, Ellman's reagent) and measuring the absorbance at 412 nm after 5 min (Ellman, 1959). RSH concentration was normalized by the total protein content.

2.15. Sulfide detection assay

The generation of hydrogen sulfide was determined using lead acetate soaked papers, as described by Shatalin et al. (2011). The assay was performed using 1 ml of bacterial cultures grown in microcentrifuge tubes to which a lead acetate paper was attached under the cap. Briefly, 10 μl precultures of the Antarctic strains were added to 990 μl M9 minimal medium and grown at 15 °C until $OD_{600} \sim 0.6$. Then, 10 μg/ml $CdCl_2$ and 0.5 mM cysteine were added and the tubes were covered with a paper embedded in lead acetate (100 mM). Controls were performed in the absence of cysteine. Tubes were incubated at 15 °C and sulfide generation was determined after 2 h.

3. Results and discussion

3.1. Isolation of oxidative stress resistant Antarctic bacteria

A total of 217 bacterial isolates able to grow in LB medium at 15 °C were obtained from Antarctic samples. To select peroxide resistant psychrotolerant bacteria, isolates were assayed for H_2O_2 resistance and only five grew in the presence of 12 mM H_2O_2. These five peroxide resistant isolates were arbitrarily named as GC01, GC02, GC03, GC04 and GC05. MICs of H_2O_2 and $CdCl_2$ (the compound used for QDs biosynthesis) were evaluated for these isolates. MICs of hydrogen peroxide and cadmium chloride ranging from 20 to 75 mM and 270 to 500 μg/ml, respectively, were observed for the Antarctic isolates (Table 1). These values are higher than those determined for *P. aeruginosa* PAO1 (1 mM and 100 μg/ml for

H_2O_2 and $CdCl_2$, respectively) and *E. coli* (6 mM and 230 μg/ml for H_2O_2 and $CdCl_2$, respectively). *E. coli* and *P. aeruginosa* are known to posses specific enzymes involved in defenses against H_2O_2 as catalase and glutathione peroxidase (Åslund et al., 1999; Arenas et al., 2010), also, global cellular systems help to deal with ROS and oxidative stress such as the thiol redox system (Storz and Imlay, 1999). Furthermore, cadmium stress is primarily due to thiol depletion and the concomitant production of ROS species (Helbig et al., 2008). The results indicated that selected Antarctic isolates are highly resistant to peroxide and cadmium chloride, thus representing excellent candidates to test for cadmium QDs biosynthesis at low temperatures.

3.2. Characterization of peroxide-resistant isolates

In order to identify the bacterial isolates, a 16S rRNA based identification was carried out. All isolated resistant bacteria correspond to *Pseudomonas* spp with high sequence similarity between all of them (Fig. 1A). Despite some strains posses almost identical 16S rRNA sequences, differences in MIC results suggest that they may not correspond to the same strain. To evaluate this possibility, LPS, metabolic profiles and optimal growth temperature were determined.

All isolates displayed different LPS profiles confirming that they are not identical strains and suggesting that each isolate could represent a different serotype (Fig. 1B). Isolates GC01, GC04 and GC05 have a complete O-antigen region but exhibit differences in their preferential O-antigen chain length which can be consequence of differences on activity and/or expression of the Wzz enzyme. Strains GC02 and GC03 did not display polymerized O-antigen and only the first unit of O-antigen was detectable, suggesting a deficiency on the O-antigen polymerase enzyme Wzy. Since LPS is the major lipid component of the outer membrane and one of the first structures interacting with metal ions in aqueous media, it is considered to confer resistance to positively charged metal ions such as Cd^{2+}. This would occur mainly by a direct interaction with them thus avoiding incorporation into the cell (Pereira et al., 2006). Nevertheless, given that bacterial cadmium resistance does not show a direct correlation with observed LPS profiles, there must be probably other mechanisms determining the high metal resistance observed in these isolates.

Metabolic profiles of selected isolates indicated that strains GC03 and GC04 could ferment glucose. Isolate GC04 was the only displaying motility, indole production and the ability to ferment all sugars in TSI agar. As expected for peroxide resistant bacteria, all isolates were catalase-positive. Also, all strains excepting GC05 were able of using citrate as the carbon source. None of the selected isolates was able to hydrolyze gelatin, induce hemolysis or produce H_2S when using thiosulfate as the only sulfur source. In conclusion, biochemical tests revealed that several differences between isolates confirming that five different peroxide-resistant Antarctic strains belonging to the *Pseudomonas* genre were isolated, each exhibiting different morphological as well as metabolic characteristics.

All isolates were able to grow at temperatures ranging from 4 to 28 °C, optimal growth occurring at 28 °C (Fig. S1). Poor growth of strains GC04 and GC05 was observed at 37 °C in comparison to *P. aeruginosa* PAO1 and *E. coli*. Accordingly, all isolates can be classified as psychrotolerant, a result that is in agreement with the 16S identification indicating that isolates correspond to *Pseudomonas fragi* strains (Morita, 1975; Hebraud et al., 1994).

3.3. CdS quantum dots biosynthesis

Given that Antarctic *Pseudomonas* isolates grew between 4 and 28 °C, the ability to biosynthesize CdS QDs was evaluated at 15 °C (see Section 2). QDs biosynthesis was evaluated at other, different temperatures (8, 28 and 37 °C) and fluorescence was still observed, a somehow surprising result since intracellular QDs biosynthesis under 30 °C has not been reported before (not shown). After 24 h under biosynthetic conditions, bacterial pellets of all isolates exhibited fluorescence when excited with UV light (Fig. 2). This result is evidence of CdS nanoparticles formation since cell fluorescence after Cd^{2+} exposure has been associated to intracellular QDs production by microorganisms (Bao et al., 2010a,b; Monrás et al., 2012). Moreover, at 15 °C, a time-dependent variation of fluorescence color was observed, switching from green to red emission after six days of treatment (Fig. 2A). Changes of fluorescence color confirm the generation of QDs, since evolution of fluorescence emission from green to yellow and then to red is a characteristic property of QDs associated to nanocrystal growth (Rogach, 2008; Bao et al., 2010a,b; Mi et al., 2011; Pérez-Donoso et al., 2012). Also, among the isolates, differences in fluorescence color were observed (Fig. 2B). The differences in QDs-mediated fluorescence observed on the *Pseudomonas* isolates exposed to identical conditions could be consequence of differences in cellular mechanisms to deal with cadmium. Variations in metal uptake kinetics or efflux could generate differences in Cd^{2+} concentration inside cells, thus affecting the crystal nucleation process. Decreasing amounts of the metal substrate could affect the chemical reaction involved in QDs production and also affect the time required to produce it. Differences observed in bacterial resistance to cadmium (Table 1) support this explanation.

On the other hand, molecules present in the solvent could also have an effect on metal uptake, efflux and metal accumulation thus affecting QDs fluorescent properties. It is well known that the uptake of several metal ions such as Cd^{2+}, Mn^{2+} and Zn^{2+} can be modulated by molecules like phosphate or citrate (Keasling, 1997; Krom et al., 2000). Based on this statement, the effect of phosphate and citrate in QDs production was evaluated by determining the fluorescence of *Pseudomonas* isolates after seven days under biosynthetic conditions (Fig. 2B). As shown before, fluorescence colors going from green to orange or red were observed at different exposure times. These results suggest that molecules in solution can affect the speed of the synthesis reaction and/or the QDs fluorescent properties. Cells producing QDs in the presence of PBS exhibited red fluorescence, a result not observed with water or citrate in which most cell pellets displayed yellow fluorescence (Fig. 2B). Phosphates are known to affect metal uptake and accumulation on many organisms, and have been related with bacterial resistance to metals such as cadmium and uranium (Hao et al., 1999; Renninger et al., 2004). Despite citrate has been shown to affect the uptake of bivalent metals in different organisms, no differences in the fluorescence of Cd-treated samples using water or citrate as solvents were observed (Hao et al., 1999; Panfili et al., 2009). This is quite curious, given that citrate can form tridentate complexes with metals such as Cd, Mn and Zn that can be transported inside cells expressing the appropriate specific transporter (Krom et al., 2000; Korithoski et al., 2005; Lensbouer et al., 2008). Results of biochemical tests indicate that all isolated strains excepting GC05 harbor the citrate transporter since they are able to use citrate as the only carbon source (Table 1). However, they probably do not posses the specific metal/citrate-complex transporter, since no differences in cell fluorescence are observed in the presence of citrate as compared to the control (water) (Fig. 2B).

3.4. Transmission electron microscopy

To determine the intracellular location of nanoparticles and to evaluate putative effects on cell morphology of QDs producing bacteria, cells were visualized by TEM. Because of its high efficiency for QDs biosynthesis and growth at 15 °C, the bacterial isolate GC01

A)

Antartic Isolates	Identification	Identitiy (%)
GC01	*Pseudomonas fragi* ATTC 4973	97
GC02	*Pseudomonas fragi* ATTC 4973	96
GC03	*Pseudomonas fragi* ATTC 4971	99
GC04	*Pseudomonas limi* DLE411J	92
GC05	*Pseudomonas fragi* ATTC 4971	99

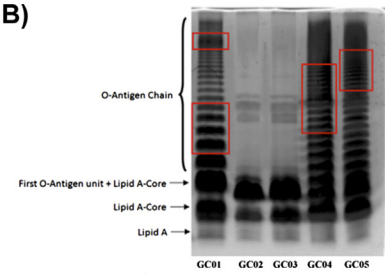

Fig. 1. 16S identification and LPS profiles of Antarctic isolates. (A) Blast-n analysis of 16S rRNA sequences indicating most similar strains. (B) SDS-PAGE of LPS samples purified from Antarctic strains. Red squares indicate the preferential LPS lengths (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

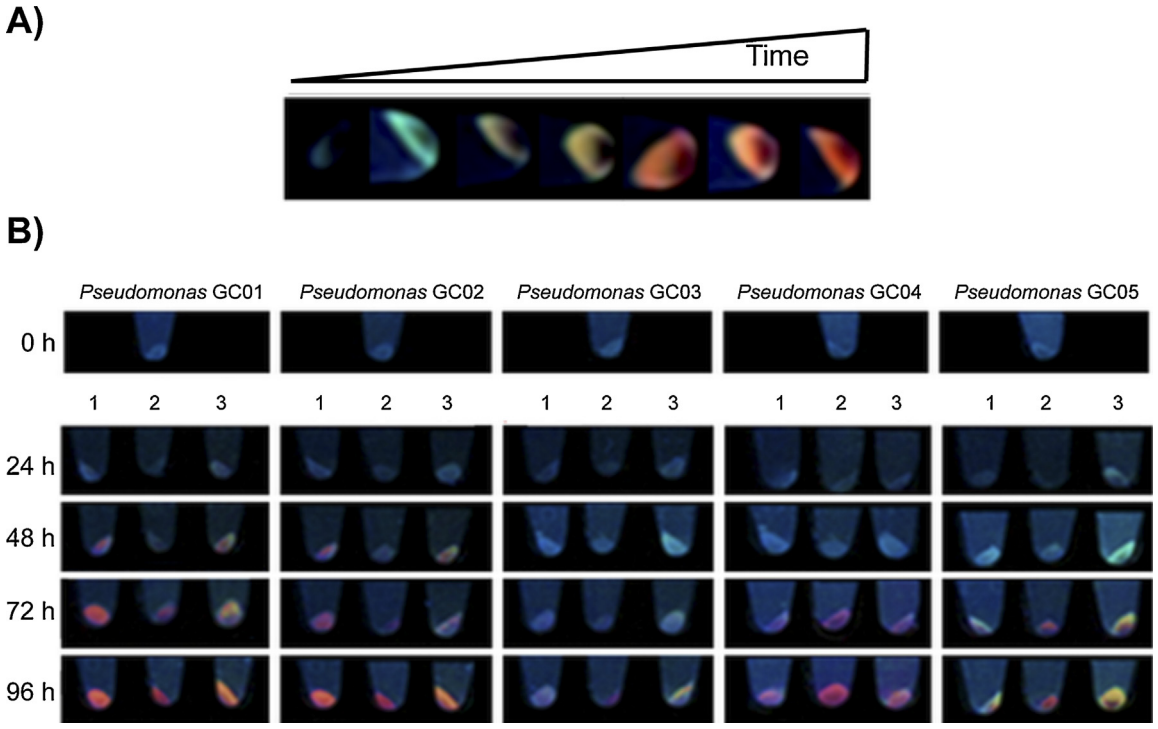

Fig. 2. CdS nanoparticle biosynthesis. (A) *Pseudomonas* GC01 was grown under biosynthesis conditions and the fluorescence when exposed to UV was evaluated every 24 h (7 days total incubation). (B) Bacterial fluorescence under biosynthesis conditions at 15 °C was evaluated daily during 96 h. The effect of three different conditions on QDs biosynthesis was evaluated; (1) distilled water, (2) PBS and (3) citrate.

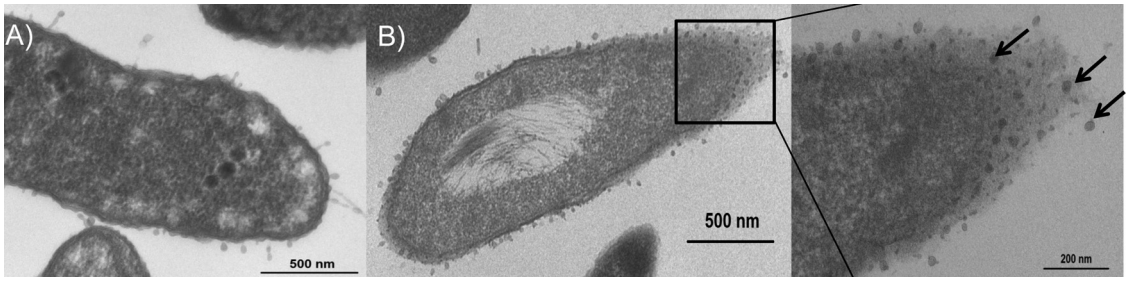

Fig. 3. Morphology of *Pseudomonas* sp. strain in biosynthesis condition. Ultrathin section of bacterial cells exposed for 3 days to nanoparticle biosynthesis conditions. (A) A general view of untreated cells. (B) Cells grown under biosynthesis conditions. The inset shows a bacterial pole in which nanometric structures are accumulated (black arrows).

was used for microscopic studies. Under normal growth conditions, rod shaped cells characteristic of *Pseudomonas* genre were observed (Fig. 3A). Several white granules arranged in clusters and a few electrons dense granules (black spots) were observed in the cytoplasm. These structures most probably correspond to granules in which polymeric materials are accumulated (hidroxybutirate or polyphosphates, among others). Interestingly, some vesicles are visible at the periphery of none-stressed cells. This is an interesting observation that could have implications in metal response and/or nanoparticle localization.

As expected, TEM examination of *Pseudomonas* sp. GC01 cells under QDs biosynthesis conditions revealed an increased number

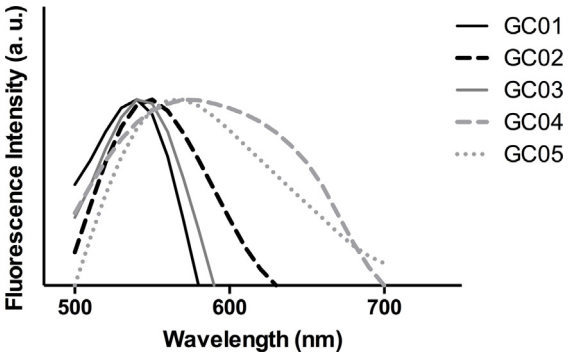

Fig. 4. Spectroscopic properties of CdS nanoparticles purified from *Pseudomonas* isolates. Nanoparticles biosynthesized in the presence of 10 μg/ml $CdCl_2$ were purified and emission spectra after excitation at 400 nm were recorded.

of vesicles at the cell periphery (Fig. 3B). Similar structures have been previously described in *P. aeruginosa* as a normal behavior during the late stationary phase (MacDonald and Kuehn, 2013), which is the same growth phase we used to biosynthesize QDs. In addition, it has been reported that vesicle production is induced in *Pseudomonas* after exposure to H_2O_2 or polymyxin B (MacDonald and Kuehn, 2013). Also, formation of redox reactive vesicles during the cell response against metals has been described for some Gram-negative bacteria such as *Pseudomonas*, *E. coli* and *Serratia marcescens* (Gorby et al., 2008; Mashburn-Warren et al., 2008). Vesicle production could be encouraged by cadmium treatment as consequence of the oxidative stress produced by the metal (Miller et al., 2009; Manara et al., 2012). This phenomenon, along with LPS and some antioxidant activities, could be responsible for the high cadmium tolerance displayed by these Antarctic strain and could also explain, at least in part, its resistance to oxidative stress.

Another interesting observation in QDs producing bacteria was the accumulation at the cell envelope, and close to the cell poles, of electron-dense nanoparticles (not observed in unexposed cells) (Fig. 3B). Most observed nanoparticles displayed spherical morphology with sizes ranging 10–40 nm. Size polydispersity of nanoparticles could be consequence of the binding of macromolecules. This behavior has been previously described for *E. coli* producing CdTe QDs, in which a polar localization of fluorescence and the generation of "vesicle like" structures, most probably corresponding to fragmented cells, were reported (Monrás et al., 2012).

3.5. Characterization of biosynthesized QDs

Fluorescent nanoparticles produced by all the oxidative stress-resistant isolates under study were purified and fluorescence spectra were determined (Fig. 4). Excitation profiles revealed that fluorescence intensity at 500–600 nm do not differ when nanoparticles are excited at 350 or 400 nm (not shown). Since at higher excitation wavelengths the absorption of proteins and peptides decrease, emission spectra of purified samples were obtained by exciting at 400 nm. Fluorescence profile for isolates GC01, GC02 and GC03 displayed an emission peak between 540 and 550 nm, while GC04 and GC05 showed emission peaks near 600 nm. These fluorescence spectra are characteristic of biosynthesized CdS QDs, and the differences in emission suggest the production of different sized QDs (Bao et al., 2010a,b; Mi et al., 2011; Pérez-Donoso et al., 2012).

QDs purified from isolates GC01, GC02 and GC03 displayed thinner emission peaks (near 100 nm) suggesting the generation of nanoparticles of similar size. In general, broad emission spectra observed in biologically synthesized QDs are consequence of organic matter bound to nanoparticles (peptides, proteins or sugars, among others), as has been previously reported (Bao et al., 2010a,b; Monrás et al., 2012).

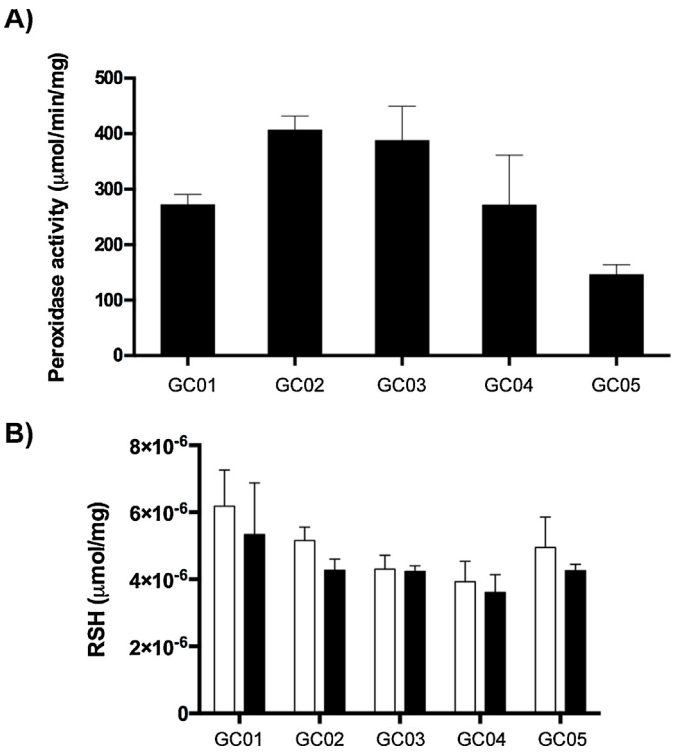

Fig. 5. Total peroxidase activity and reduced thiol (RSH) content of Antarctic isolates. (A) Total peroxidase activity assay was determined in Antarctic *Pseudomonas* isolates grown to stationary phase. Experiments were repeated three times. Values are mean ± SEM. (B) Total reduced thiol (RSH) levels of *Pseudomonas* GC01, GC02, GC03, GC04 and GC05. Cells were exposed to 10 μg/ml $CdCl_2$ for 30 min (black bars). Tris buffer was used as control (white bars) (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

3.6. Peroxidase activity and thiol content

To determine if antioxidant defenses are related with the ability of Antarctic isolates to biosynthesize QDs at low temperatures, peroxidase activity and thiol levels were evaluated. Total peroxidase activity was determined and compared with positive controls that display different H_2O_2-responsive enzymes such as *E. coli* and *P. aeruginosa* PAO1. Selected isolates displayed higher levels of peroxidase activity than *E. coli* but similar to those observed in *P. aeruginosa* PAO1 (Fig. 5A). This result is in agreement with previous reports indicating that *P. aeruginosa* display higher levels of peroxidase activity than *E. coli* (Loewen and Triggs, 1984). The levels of peroxidase activity determined in *E. coli* (10 U/mg) correlates with the activity previously reported for wild type strains (Loewen and Triggs, 1984; Loewen, 1984; Chen et al., 2003). The results indicated that all tested strains display increased levels of peroxidase activity, which could favor CdS QDs cellular production. This could result by allowing cells to better withstand Cd toxicity under biosynthesis conditions or by protecting intracellular QDs from peroxide-mediated degradation. However, and given that similar levels of peroxidase activity were observed in *P. aeruginosa* PAO1 (do not produce QDs), increased peroxidase activity would not determine the ability of Antarctic isolates to biosynthesize QDs.

On the other hand, it has been reported that increased cellular thiol content favors CdS QDs biosynthesis in *E. coli* (Chen et al., 2009;

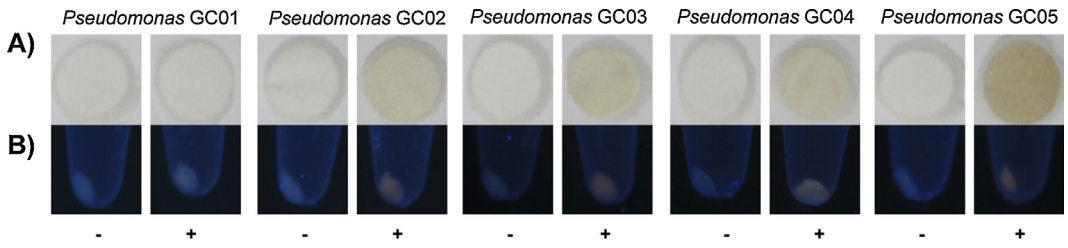

Fig. 6. QDs biosynthesis and hydrogen sulfide production at 15 °C. (A) Lead acetate soaked papers display a gray color as result of the reaction with H_2S produced by culture headspaces (see Section 2). (B) Fluorescence of bacterial pellets after UV exposure. Cells were grown at 15 °C in minimal medium with (+) or without (−) 0.5 mM cysteine. In QDs biosynthesis experiments, cultures were also amended with $CdCl_2$ (10 μg/ml). H_2S production and QDs biosynthesis were evaluated after 2 h (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

Monrás et al., 2012). Based on this statement and the role of cellular thiols in cadmium-resistance, it was decided to evaluate the level of RSH in QDs producing isolates. All strains showed similar reduced thiol content, which seems not to be affected by metal exposure (Fig. 5B). Also, the results indicate that isolates do not produce high RSH levels as compared to *E. coli*, suggesting that these antioxidant molecules are not directly involved in QDs biosynthesis by Antarctic isolates. Under biosynthesis conditions (exposure to 10 μg/ml $CdCl_2$), no major changes in total reduced thiols were determined for all strains. In this context, RSH determinations after metal exposure indicate that Cd^{2+} used for QDs biosynthesis can be handled by the thiol redox homeostasis system without signs of thiol depletion. Interestingly, no decrease of RSH levels was observed when isolates were exposed to a higher cadmium concentration (50 μg/ml $CdCl_2$, data not shown), suggesting that cadmium resistance observed in these isolates could be associated to strong antioxidant defenses that avoid thiol depletion. Since isolated studied here are highly resistant to Cd^{2+} but display normal levels of RSH, it can be speculated that other antioxidant systems can also contribute to cell defense against this metal.

3.7. H_2S production

In order to biosynthesize CdS QDs cells must generate sulfide anions (S_2^-) to react with Cd^{2+}. Hydrogen sulfide production has been recently described as an antioxidant system in bacteria particularly defending cells against H_2O_2 in a mechanism involving desulfhydrase enzymes (Shatalin et al., 2011). However, the sulfide source in the Antarctic strains is puzzling given that biochemical tests revealed that none of them release H_2S when thiosulfate was used as the sulfur source (Table S1). Based on this, H_2S production was assessed under different metabolic conditions. Since bacterial sulfide production has been associated with the activity of cysteine desulfhydrases (Awano et al., 2005), it was decided to determine sulfide generation in the presence of cysteine (the substrate of cysteine desulfhydrases). The generation of black precipitates on lead acetate papers exposed to cultures growing in the presence of cysteine was positive for all *Pseudomonas* spp. isolates, excepting GC01 (Fig. 6).

When bacterial cells were exposed to cysteine under QDs biosynthetic conditions, all strains releasing sulfide biosynthesized CdS nanoparticles after 2 h, excepting GC01 which does not produce H_2S under the conditions tested (Fig. 6). This result is interesting given that under normal biosynthesis conditions reaction takes place approximately in 24 h (Fig. 3). Obtained results demonstrate that sulfide production favors CdS QDs biosynthesis at low temperatures. In this context, the importance of sulfide from cysteine desulfhydrases in CdS QDs biosynthesis at 37 °C has been previously reported in the photosynthetic microorganism *R. palustris* (Bai et al., 2009).

4. Conclusion

This is the first report describing the biosynthesis of CdS QDs at low temperature by Antarctic microorganisms. The importance of antioxidant thiols in QDs biosynthesis has been previously reported; however, no relationship between QDs production and RSH levels was observed here, suggesting that novel mechanisms/molecules could be involved in nanocrystal biosynthesis at low temperature. Sulfide generation enhances CdS QDs biosynthesis, probably by acting as sulfur donor for CdS nanoparticle core formation. In the absence of H_2S, other unidentified cellular moieties such as thiols or small biomolecules must be involved in nanoparticle formation.

Using Antarctic strains for the synthesis of relevant biotechnological nanoparticles represents a novel approach that could allow the green production of QDs with improved properties at low cost. Also, deciphering the molecular mechanism(s) involved in QDs biosynthesis, particularly at low temperatures, represents a unique opportunity that could allow the development of new methods for the *in vitro* synthesis of QDs or to use these microorganisms for industrial production of QDs.

Author contributions

G.C., J.P.M., P.D.O., B.C., S.L.A., D.-T.V., V.F.A., S.C., U.G., and B.D. designed and performed experiments and analyzed data. V.C.C. write the manuscript. J.M.P. directed the study and drafted the manuscript. All authors commented on or contributed to the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.07.017.

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