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ORIGINAL ARTICLE

Inhibition of quorum-sensing-dependent virulence factors and biofilm formation of clinical and environmental *Pseudomonas aeruginosa* strains by ZnO nanoparticles

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Significance and Impact of the Study: Virulence inhibition by quorum quenchers in *Pseudomonas aeruginosa* is usually tested in laboratory strains and studies of their effects in relevant clinical and environmental strains are scarce. This study is significant as the effects of ZnO nanoparticles in QS-dependent virulence factor production were tested in six clinical strains from cystic fibrosis patients, a C-30 resistant clinical strain from urine, two PA14 gallium resistant mutants, a PA14 C-30 resistant mutant, and four environmental isolates. ZnO nanoparticles decreased elastase, pyocyanin, and biofilms for most of the strains; indicating they have broad spectrum and may be an alternative to treat *Ps. aeruginosa* infections.

Keywords
antimicrobials, pseudomonads, quorum quenching, quorum sensing, virulence.

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Abstract

Quorum quenching decreases *Pseudomonas aeruginosa* virulence factors and biofilm formation, alleviating infections in animal models. Nevertheless, it is usually performed in laboratory strains such as PAO1 and PA14, and studies involving clinical or environmental isolates are scarce. In this work, the effects of ZnO nanoparticles, a potent quorum and virulence quencher for the PAO1 strain, were tested in six clinical strains from cystic fibrosis patients, a furanone C-30 resistant clinical strain from urine, two PA14 gallium resistant mutants, a PA14 C-30 resistant mutant and four environmental isolates. ZnO nanoparticles effectively decreased elastase, pyocyanin, and biofilm formation for most of the strains; regardless their origin or their resistance against the canonical quorum quencher C-30 or the novel antimicrobial gallium. The data indicate ZnO nanoparticles may have a broad spectrum for the quorum quenching of relevant strains and that may be an alternative to treat *Ps. aeruginosa* recalcitrant infections.

Introduction

Quorum quenchers (QQ) are compounds that interfere with bacterial cell–cell communication systems attenuating the expression of several virulence factors like proteases, toxins, adhesins, siderophores, swarming motility, and biofilm formation (Antunes *et al.* 2010). Ideally, these compounds act exclusively as virulence inhibitors without interfering with growth and hence it was expected that no resistance would be developed against them (Defoirdt *et al.* 2010; Rasko and Sperandio 2010). However, recently resistance against one of the better characterized QQ, the brominated furanone C-30 was found. This compound quenches communication via homoserine lactone autoinducers and resistance against it was first reported in *Pseudomonas aeruginosa* mutants selected by growing them in adenosine as sole carbon source, because its catabolism is positively controlled by the Las system (Maeda *et al.* 2012).

In addition, clinical strains resistant to C-30 had also been identified. Interestingly some of them are also resistant against another QQ, 5-fluorouracil (5-FU) (Maeda *et al.* 2012; García-Contreras *et al.* 2013b,c). These findings highlight the importance to test QQ not only in laboratory model strains like PAO1 and PA14 but also in clinical isolates that potentially could have the ability to neutralize their effects. In this regard, ZnO nanoparticles had been recently identified to be effective QQ for the laboratory strain PAO1, attenuating its production of several virulence factors such as: pyocyanin, PQS signal, pyochelin, and its haemolytic activity without a high growth inhibitory effect (Lee *et al.* 2014).

ZnO nanoparticles decrease the surface hydrophobicity of PAO1, and induce the zinc cation efflux pump *czc* operon, requiring the *czc* regulator CzcR to be present to function (Lee *et al.* 2014). To test the ability of ZnO nanoparticles to attenuate the virulence factor production of *Ps. aeruginosa* relevant clinical and environmental strains, we selected a panel of six clinical strains isolated from paediatric patients with cystic fibrosis, including three strains resistant against furanone C-30 (García-Contreras *et al.* 2015) and the PA14 derived C-30 resistant mutant *mexR* as a control (Maeda *et al.* 2012), one clinical strain isolated from the urine of a child which is also resistant against C-30 and 5-FU (García-Contreras *et al.* 2013c), and four environmental strains, isolated from the Indian ocean (Manwar *et al.* 2004), a rotten coconut (Palmeros *et al.* 1994), dolphin gastric juice, and a basin in Cuatro Cienegas, Mexico (Grosso-Becerra *et al.* 2014), two PA14 derived mutants resistant to the novel antimicrobial gallium nitrate (García-Contreras *et al.* 2013a) and the laboratory strains PAO1 and PA14 as positive controls (Table S1).

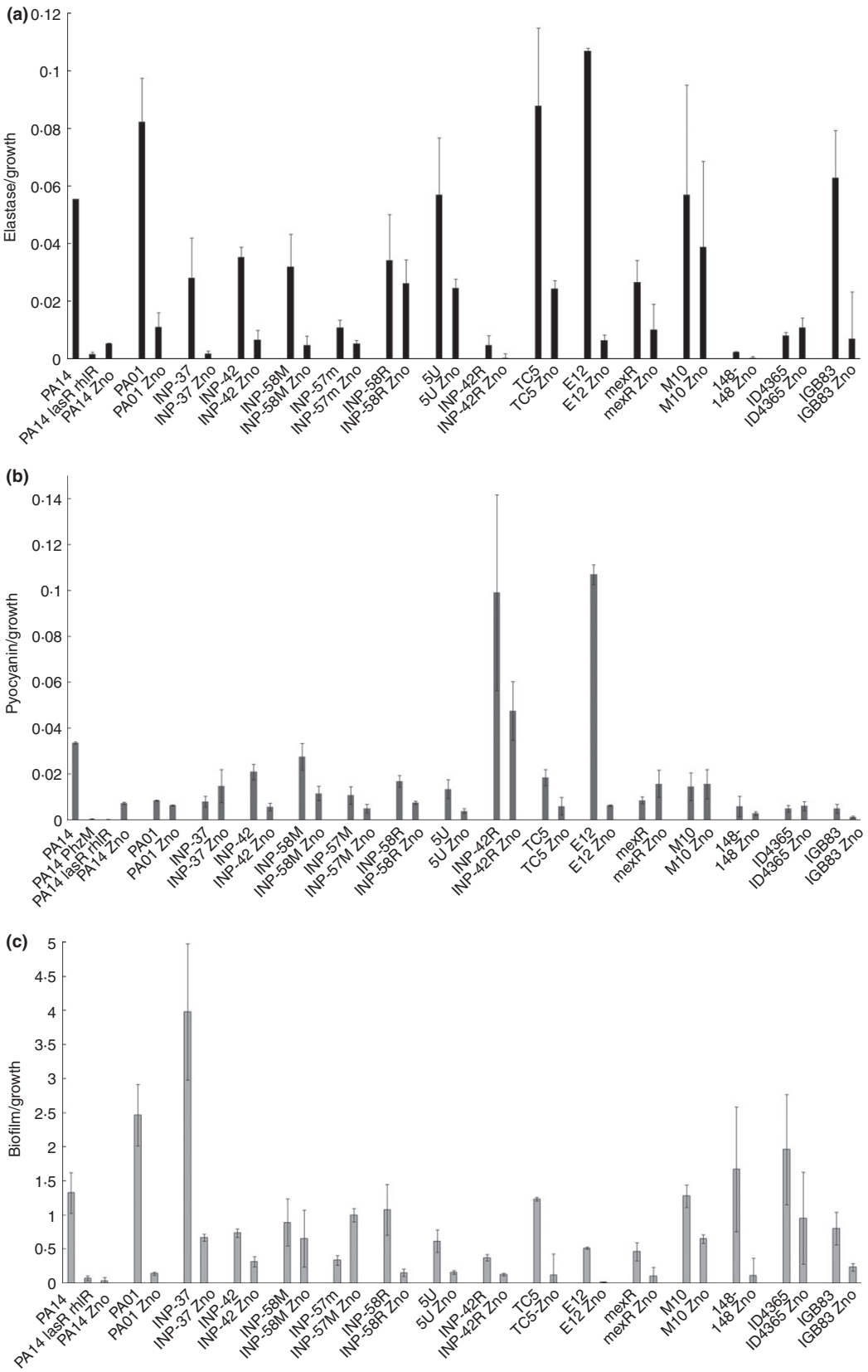
The effect of ZnO nanoparticles on the production of the virulence factors: pyocyanin, elastase, biofilm formation was evaluated. In general, ZnO nanoparticles inhibited all virulence factors for most of the strains indicating this novel QQ has a broad effect to attenuate the virulence of several strains regardless their ability to resist other quenchers like C-30 and 5-FU, and to resist metal-based antimicrobials like Ga containing compounds. Nevertheless, the exceptions (phenotypes that were not attenuated by ZnO in some strains) also indicate that as previously found for C-30 and 5-FU, QQ are likely not universally effective against all the strains producing infections in patients.

Results and discussion

Dynamic light scattering analysis shows the surface charge of $-11{\cdot}1 \pm 1{\cdot}65$ mV as zeta potential value for ZnO nanoparticles. Figure S2 shows the Transmission Electron Microscopy (TEM), and High-Resolution Transmission Electron Microscopy (HR-TEM) of inverse fast Fourier transform (IFFT) images of ZnO nanoparticles. From the TEM images, the average size distribution of the ZnO nanoparticle was calculated to be 65 ± 17 nm which corroborates the high surface area of the nanoparticles as described by the manufacturer, Sigma (St. Louis, MO, USA). Due to less negative surface charge and increased surface area, ZnO nanoparticles are more favourably utilized by the bacterial cells.

To confirm the ability of ZnO nanoparticles to reduce the expression of *Ps. aeruginosa* virulence factor production, the strain PAO1 was first used as the ZnO QQ effects were originally described in this strain (Lee *et al.* 2014). In addition, a second laboratory strain, PA14, also widely used for QS and QQ studies was analysed. As expected, ZnO administered at 1 mmol l^{-1} displayed strong inhibition of all virulence factors for both the laboratory strains (inhibiting the elastase of both strains ~ 90%, pyocyanin 75 and 25% for PA14 and PAO1, respectively, and biofilm 97 and 94% respectively) (Fig. 1a,b). The effect of ZnO in the virulence factors of clinical and environmental strains was then tested. For the clinical strains ZnO inhibited the production of elastase for all of them (from 23% to ~100%) except for the environmental strain ID4365, which elastase was slightly increased by the ZnO addition. The production of pyocyanin was inhibited for all strains (from ~50% to 95%) except the clinical strain INP-37, the PA14-derived mutant *mexR* and the environmental isolates M10 and ID365, demonstrating ZnO it is an effective QQ. Similarly, biofilm formation of all strains except for INP-57M was inhibited from 26% to ~100% in microtitre plates without shaking, and similar results were obtained in biofilms grown in glass tubes with 70 rev min^{-1} shaking (Fig. 1a,b and Figure S1 respectively). Interestingly, ZnO nanoparticles were able to inhibit most virulence factors of the majority of the strains, regardless their very high variability in their basal

Figure 1 Effect of the treatment with ZnO nanoparticles administered at 1 mmol l^{-1} on the production of: (a) Elastase all differences were significant in a 1-tailed *T*-test $P < 0{\cdot}05$, except for the strain M10), (b) Pyocyanin (all differences were significant in a 1-tailed *T*-test $P < 0{\cdot}05$, except for strains 42R, mexR, M10, 148, and ID4365), and (c) Biofilm formation in 96-wells plates (all differences were significant in a 1-tailed *T*-test $P < 0{\cdot}05$, except for strains 58M and ID4365). Phenotypes were measured in at least three independent cultures and the average of the virulence factor normalized by growth $\pm$ SD is shown.


(a)
Elastase/growth
0·12
0·1
0·08
0·06
0·04
0·02
0
PA14
PA14 lasR rhlR
PA14 Zno
PA01
PA01 Zno
INP-37
INP-37 Zno
INP-42
INP-42 Zno
INP-58M
INP-58M Zno
INP-57m
INP-57m Zno
INP-58R
INP-58R Zno
5U
5U Zno
INP-42R
INP-42R Zno
TC5
TC5 Zno
E12
E12 Zno
mexR
mexR Zno
M10
M10 Zno
148-
148 Zno
ID4365
ID4365 Zno
IGB83
IGB83 Zno
(b)
Pyocyanin/growth
0·14
0·12
0·1
0·08
0·06
0·04
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PA14
PA14 PhzM
PA14 lasR rhlR
PA14 Zno
PA01
PA01 Zno
INP-37
INP-37 Zno
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INP-42 Zno
INP-58M
INP-58M Zno
INP-57M
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INP-58R
INP-58R Zno
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5U Zno
INP-42R
INP-42R Zno
TC5
TC5 Zno
E12
E12 Zno
mexR
mexR Zno
M10
M10 Zno
148-
148 Zno
ID4365
ID4365 Zno
IGB83
IGB83 Zno
(c)
Biofilm/growth
5
4·5
4
3·5
3
2·5
2
1·5
1
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PA14
PA14 lasR rhlR
PA14 Zno
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PA01 Zno
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INP-37 Zno
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INP-42 Zno
INP-58M
INP-58M Zno
INP-57m
INP-57M Zno
INP-58R
INP-58R Zno
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levels of pyocyanin, elastase and biofilms. Such high variability in QS-dependant virulence factor production was expected, as it was also found in respiratory clinical isolates from ventilator-associated pneumonia (Le Berre *et al.* 2008), in environmental strains (Grosso-Becerra *et al.* 2014), and it is in general observed in most QS bacterial systems (Defoirdt *et al.* 2010).

It is also important to note that for all the tested strains, the addition of ZnO nanoparticles had no or only a slight inhibitory effect of <20% in the bacterial growth as it was previously observed for the PAO1 strain (Lee *et al.* 2014). In addition, concentrations of ZnO nanoparticles able to inhibit 50% of the growth (MIC50) of the related environmental species *Pseudomonas putida* are between 1·2 mmol l^{-1} and >2·4 mmol l^{-1} (Mallevre *et al.* 2014), while for effectively inhibit *Pseudomonas alcaligenes* growth, concentrations in the range of 4-7 mmol l^{-1} are needed (Raghupathi *et al.* 2011). This evidence indicates ZnO nanoparticles are not very toxic to bacteria belonging to the *Pseudomonas* genus.

The presented data indicate that the inhibition of the virulence factor production and biofilms was related to the quorum quenching effect of the nanoparticles and not to bacteriostatic or bactericide effects. To investigate if biofilm inhibition by ZnO nanoparticles (and perhaps elastase and pyocyanin) was correlated with a decrease in surface hydrophobicity in the clinical and environmental strains, as was demonstrated previously for PAO1(Lee *et al.* 2014), changes in this phenotype were evaluated. Our results indicate changes in hydrophobicity are indeed observed in the laboratory strain PAO1, however, in contrast with previous results in our conditions PAO1 hydrophobicity increased rather than decreased after the ZnO treatment, and the same was found with PA14, the other laboratory strain used, and in some isolates like INP-58R and ID4365, in contrast other strains do not have any significant change in this parameter, and strains like INP-37 decrease instead of increase their hydrophobicity after ZnO treatment (Fig. 2), indicating that changes in this surface property are not strictly correlated with the inhibition of biofilm and QS-dependent virulence factors.

Our results (Figs 1 and 2, and Table S2) show that ZnO nanoparticles may have a broad spectrum quenching the quorum and attenuating the virulence of relevant clinical and environmental strains that could also be relevant for human health since they are highly virulent in an acute mice model infection (Grosso-Becerra *et al.* 2014), regardless their ability to resist other quorum quenchers such as furanone C-30, and 5-FU, and metal based antimicrobials such as gallium. Although further research is necessary to understand better the mechanism of virulence inhibition by the ZnO nanoparticles, at least for the reference PAO1 strain, it is known that the presence of the two-component response regulator CzcR is needed for the inhibition of biofilm and pyocyanin production by ZnO nanoparticles which also repress the production of the PQS autoinducer (Lee *et al.* 2014). In addition, it is also known that CzcR is required for the full expression of QS 3-oxo-C12-HSL and C4-HSL autoinducers (Dieppois *et al.* 2012). The CzcR regulator could be a suitable target for the development of

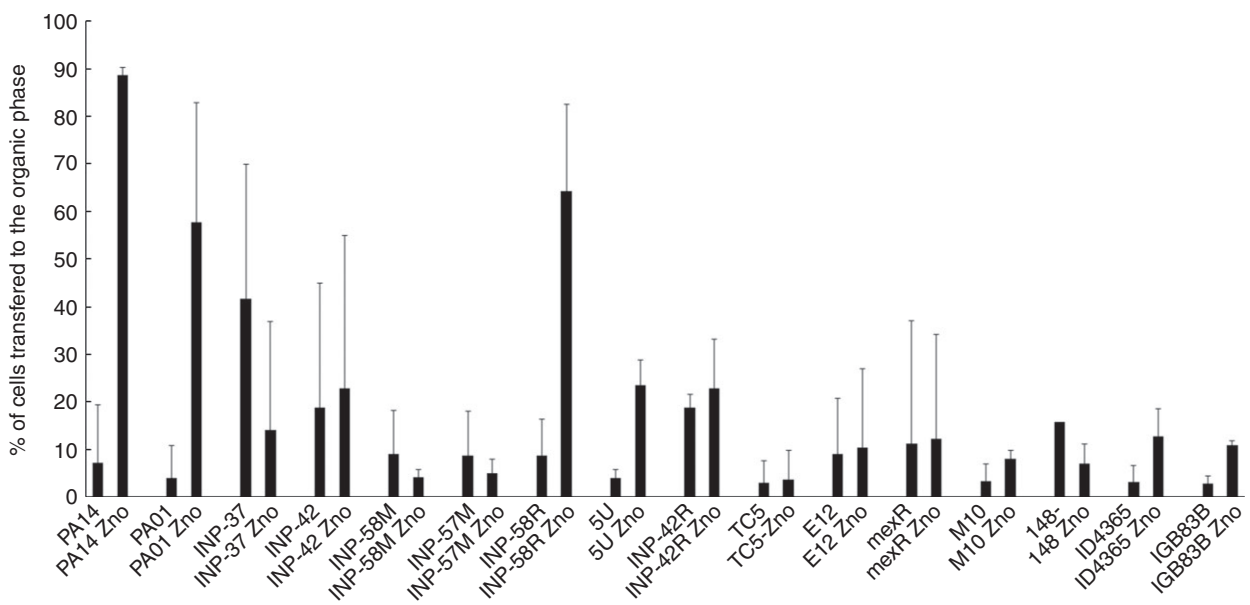

Figure 2 Effect of the ZnO nanoparticles over surface hydrophobicity. The percentage of cells transferred from the aqueous to the organic (hexadecane) phase ± SD is shown. The experiments were carried out in triplicate.

new anti-virulence therapies. Our results suggest that this response regulator may be functional in most of the clinical strains, as ZnO nanoparticles are able to inhibit their pyocyanin and biofilm formation, however, the complex genetic mechanisms underlying QS and virulence gene expression, present in these strains may be responsible for the exceptions observed, particularly regarding the strain INP-37 since its pyocyanin was not inhibited by ZnO but slightly increased, while its biofilm is severely decreased, and the strain INP-57M which shows the opposite behaviour (pyocyanin inhibition and biofilm activation) Fig. 1b, c and Table S2. Also interestingly, while the biofilm of all the four environmental strains was decreased by ZnO, no effect of the particles in the pyocyanin production of two of them (M10 and ID4365) was found (Fig. 1b,c and Table S2). In addition, it would be also interesting to discriminate to what extend their effects are due the nanoparticles themselves or by any free Zn ions released from the particles (as at least for PAO1 Zn ions also inhibit the virulence factor production (Lee *et al.* 2014) and to test their effects *in vivo*, our study suggests that the evaluated nanoparticles could be a promising start point to the design of effective quorum quenchers of relevant strains found in nature including nosocomial strains. For this work, the main criterion for choosing the strains to test the activity of ZnO nanoparticles was their susceptibility for the canonical QQ furanone C-30 and the new antimicrobial gallium nitrate. However, more systematic future studies are needed to corroborate its effects. One approach to perform such studies would be simply to test a higher number of strains either from the same of different kind of infections or environments, nevertheless, in the light of previous experimental efforts to achieve universal quality standards for testing new anti-pathogenic products, the virulence factor production, antibiotic resistance patterns and genetic characterization of large collections of strains may be analysed to define association patterns and create strain clusters (Fonseca *et al.* 2007) for which representative strains then may be used to test the ZnO nanoparticles or any other anti-virulence compounds. In the other hand, the standardization of simple methods such as the evaluation of biofilm formation in microtitre plates for *Ps. aeruginosa* strains is also advisable, taking into account the current standardization of such methods for Gram (+) bacteria (Stepanovic *et al.* 2007; Extremina *et al.* 2011).

In addition, since biofilm formation is a complex process influenced by several environmental factors such as osmolarity, pH, the presence of stress, growth phase etc. (Prigent-Combaret *et al.* 2001; Corona-Izquierdo and Membrillo-Hernandez 2002; Zhang *et al.* 2007; Kavamura and de Melo 2014), either systems that better mimic the natural conditions and environments in which clinically important biofilms are formed, such as microfluidics devices (Karimi *et al.* 2014) or biofilm infection models in animals (Kadurugamuwa *et al.* 2003) would be ideal for the further test of ZnO nanoparticle anti-biofilm activity.

Also critically, the stabilization of the particles (Vielkind *et al.* 2013) as well as the effect of several ZnO nanoparticles parameters such as size, physicochemical properties, particle dissolution to ionic zinc, particle-induced generation of ROS (Raghupathi *et al.* 2011; Ma *et al.* 2012), etc. over the bacterial QS and growth and in the toxicity toward the host should be tested to optimize the nanoparticle properties which could enable their application in the clinic as an effective alternative to treat *Ps. aeruginosa* recalcitrant infections.

Materials and methods

Characterization of ZnO nanoparticles

Size, polydispersity index and zeta potential of the ZnO nanoparticles were measured using dynamic light scattering method at 25°C using Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK). Morphologies of ZnO nanoparticles were studied using a high-resolution transmission electron microscopy (HR-TEM, Tecnai G2 TF 30ST; FEI, Hillsboro, OR). The average particle size distributions and standard deviations of the ZnO nanoparticles were calculated by averaging 200 particles from the TEM images using IMAGE J software (developed by National Institutes of Health, Bethesda, MD, USA).

Strains and culture conditions

The strains used in this work are listed in the Table S1. For each strain, precultures were initiated from single colonies grown on LB plates at 37°C and incubated aerobically in 5 ml of LB medium at 37°C with shaking at 200 rev min^{-1} for approx. 16 h, to test the effect of ZnO nanoparticles (<50 nm diameter (determined by TEM), and a surface area >10·8 m^2 g^{-1}, according to the provider Sigma catalogue number 677450) in the virulence factor production. For this work, more detailed physicochemical properties of the ZnO nanoparticles were determined and are available in the results and discussion section and in the Fig. S2. The nanoparticles suspended in sterile water were added at 1 mmol l^{-1} in 5 ml of LB media from the beginning of each culture, and cultures with and without ZnO were incubated under the same conditions than the precultures. After 24 h of incubation, growth was determined by measuring the optical density at 600nm and subsamples of 2 ml for each culture were taken, cells were then centrifuged at 7 500 ***g*** for 2 min and the cell-free supernatants were used to determine the production of the virulence factors pyocyanin and elastase.

Pyocyanin and elastase determination

Pyocyanin production was assayed according to the protocol described in (Essar *et al.* 1990), briefly it was determined spectrophotometrically after the extraction of the supernatants with chloroform and HCl (0·2 N). For the estimation of the pyocyanin concentration, absorbance at 520 nm was used with a ε of 2·46 mmol l^{-1} cm^{-1}. The production of the elastase was assayed by the protocol of (Ohman *et al.* 1980). Briefly, it was determined spectrophotometrically by the Elastin-Congo-red (Sigma) assay at 495 nm. The reaction medium contained 20 mmol l^{-1} Tris-HCl, pH 7·5 and 1 mmol l^{-1} $CaCl_2$. To report the pyocyanin and elastase produced relative to the number of cells in the cultures their production was normalized by growth (divided by the OD600nm of the cultures).

Biofilm formation

Biofilm formation was evaluated in polystyrene 96-well plates or in 15 ml glass tubes. Each well (300 μl) or tube (2 ml) was inoculated by precultures to an initial OD600nm ~ 0·05. Wells or tubes contained: 0, or 1 mmol l^{-1} of ZnO nanoparticles, the plates were incubated without shaking at 37°C during 24 h, while tubes were incubated 48 h at 37°C with 70 rev min^{-1} shaking, after that, growth was recorded (OD600nm) and the plates/tubes were washed three times with distilled water, dried and stained for 20 min with 300 μl (wells) or 2 ml (tubes) of 0·1% (w/v in water) crystal violet, thereafter crystal violet was removed and the plates washed three times with distilled water, dried and finally the die attached at the well walls or bottom was dissolved with absolute ethanol and the biofilm quantified by recording the absorbance of each well at 595 nm (O'Toole and Kolter 1998). The formation of biofilms was normalized by growth (divided by the OD600nm of the cultures).

Surface hydrophobicity determination

It was determined by estimating the percentage of cells transferred from an aqueous phase to an organic one, following the protocol described in (Zhang *et al.* 2007). Briefly, cultures with and without 1 mmol l^{-1} ZnO nanoparticles were grown in LB medium and incubated until they reached a turbidity of OD600nm ~1·0. One millilitre of cells were taken, centrifuged at 13 000 rpm for 2 min and the cell pellet was washed twice with PUM buffer (22·2 g of potassium phosphate trihydrate, 7·26 g of monobasic potassium phosphate, 1·8 g of urea, and 0·2 g of magnesium sulphate heptahydrate/litre (pH 7·1)), after that cells were resuspended in 1 ml of PUM buffer, 100 μl were taken to determine the initial cell density in the aqueous phase and 400 μl of hexadecane (Sigma) were added to the 900 μl of remaining PUM suspended cells, the mixtures were then vortexed thoroughly for 2 min, after 5 min at room temperature for phase separation, the aqueous phase was removed and the OD600nm was measured to determine the cell density that remained in the aqueous phase.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

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

Figure S1. Biofilm formation in glass tubes

Figure S2. ZnO nanoparticle characterization.

Table S1. List of strains used in this work.

Table S2. Virulence factor inhibition by 1 mM of ZnO nanoparticles.