Understanding the antimicrobial mechanism of TiO$_2$-based nanocomposite films in a pathogenic bacterium

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Titania (TiO$_2$)-based nanocomposites subjected to light excitation are remarkably effective in eliciting microbial death. However, the mechanism by which these materials induce microbial death and the effects that they have on microbes are poorly understood. Here, we assess the low dose radical-mediated TiO$_2$ photocatalytic action of such nanocomposites and evaluate the genome/proteome-wide expression profiles of Pseudomonas aeruginosa PAO1 cells after two minutes of intervention. The results indicate that the impact on the gene-wide flux distribution and metabolism is moderate in the analysed time span. Rather, the photocatalytic action triggers the decreased expression of a large array of genes/proteins specific for regulatory, signalling and growth functions in parallel with subsequent selective effects on ion homeostasis, coenzyme-independent respiration and cell wall structure. The present work provides the first solid foundation for the biocidal action of titania and may have an impact on the design of highly active photobiocidal nanomaterials.

The incorporation of biocidal agents into engineered polymer-based nanocomposites has led to the development of versatile antimicrobial materials that are useful for a wide variety of packaging, biomedical and general use applications$^1$-$^5$. The development of such materials is difficult because thermodynamic and kinetic barriers inhibit the dispersal of inorganic, often hydrophilic, nanoparticles in hydrophobic polymer matrixes. Antimicrobial nanocomposites based on titania (TiO$_2$) have been actively investigated in recent years. Upon photoactivation of the oxide component, the biocidal action is a result of the modulation of charge (electron-hole) carriers at the interface of the external surface of the material, yielding potent and long-lasting capabilities when the dispersion of the inorganic phase and organic-inorganic interfacial contact are optimally achieved$^6$-$^{12}$. Titania has substantial advantages over both chemical (NO, H$_2$O$_2$, small organic molecules) and metal (typically Ag)-based systems$^13$,$^{14}$. First, titania nanoparticles have a broad spectrum of activity against microorganisms, including Gram-negative and positive bacteria and fungi, which is of particular importance for multiple drug resistant strains$^13$,$^{14}$. Second, and more importantly, titania-polymer nanocomposites are intrinsically environmentally friendly and exert a non-contact biocidal action. Therefore, no release of potentially toxic nanoparticles (with unpredictable effects on human health) to the media is required to achieve disinfection capabilities$^6$,$^{10}$,$^{11}$,$^{15}$,$^{16}$.

A critical point for addressing the biocidal capabilities of titania-based nanomaterials concerns the mechanism by which cell viability is lost and cell death occurs. While this has been addressed to some degree with Ag-based biocidal materials$^{15}$-$^{18}$, reports on titania-based biocides are scarce$^{19}$,$^{20}$. Little is known about the mechanisms of titania-induced biocidal activity beyond the relatively well-studied initial oxidative attack on the outer/inner cell membrane of the microorganism$^{16}$,$^{20}$ as well as alterations of Coenzyme A-dependent enzyme activities$^{21}$ and damage to DNA via hydroxyl radicals$^{22}$. Therefore, there is a need for basic knowledge linking the action of photocatalytic-induced biocidal entities with their biological effects; this knowledge would allow us to achieve the
full disinfection power of TiO₂-based systems. Such studies would
define the nature (random vs. non-random) of the photocatalytic
reaction, the cell systems involved in the response and the degree
of reversibility of the action of TiO₂.

In this paper we aimed to
i) analyse and quantify the radical
species released from the biocidal films and
ii) assess their role in
cell damage and death. Using a joint chemical and biological
approach, we attempt to address the current limitations in exploring
the link between the photobiocidal action of titania and the metabolic
and transport properties of the microorganism. The investigation
was carried out using a clinically relevant bacterium, Pseudomonas
aeruginosa strain PAO1. We found that the cells attempted to
counteract the TiO₂ (or other photoactive nanomaterials)-mediated
action by triggering a broad array of regulatory responses without
substantial loss of metabolic function within the tested time scale.
Moreover, the study provides clear indications about the selective
effect of TiO₂-photo catalysis in specific, cell-critical systems.

Results
Nanocomposites: charged carriers with biocidal action. Fig. 1
displays the X-ray diffraction (XRD) and Raman spectra of the
bare TiO₂ component, which showed an anatase structure with a
primary particle size of ca. 9 nm and a Brunauer Emmett Teller
(BET) area of ca. 104 m² g⁻¹. The dispersion into the ethylene-vinyl
alcohol (EVOH) copolymer matrix to a concentration of 2 wt.%
(previously found to provide optimum killing rates) was carried
out by a simple but effective melting compounding method. This
process generated aggregates with an average diameter of ca. 90 nm,
as evidenced by the transmission electron microscopy (TEM) image
included in Fig. 2. Micro-Raman studies (also presented in Fig. 1)
showed that both the oxide (inset at left) and the polymer (main
Raman plot at right) materials were essentially unaltered after the
process of incorporation of titania into the polymer matrix. The
physicochemical properties of these materials were therefore
preserved in the nanocomposite material. The high resolution
TEM (HR-TEM) study presented in Fig. 2 provides evidence that
anatase aggregates are composed of oxide nanoparticles interwoven
with the organic component at a nanometre scale. A detailed view of
the inorganic-organic interface is also presented in Fig. 2. The crystal
planes of the inorganic nanoparticles are observed together with the
more disordered (essentially amorphous, according to the electron
diffraction patterns) polymer matrix surrounding the oxide
nanoparticles. The oriented, parallel preferential stacking of both
components at the interface layers is direct evidence of the perfect
matching between the components in our nanocomposite film.

The effectiveness of this system in eliminating a series of micro-
organisms, including two Gram-negative (Escherichia coli and P.
aeruginosa) and two Gram-positive (Staphylococcus aureus and
Enterococcus faecalis) bacterial species and a yeast species (Pichia
jadinii), was previously investigated and is summarised in
Supplementary Table S1. The system is particularly effective with
P. aeruginosa strain PAO1, but there is no obvious correlation
between the cell wall characteristics of the bacteria and the killing
capacity as high and low death rates are observed for specific micro-
organisms of Gram-positive or Gram-negative bacteria. Concerning
P. aeruginosa, we can highlight the exceptional biocidal action of the
nanocomposite, with the rates and time response competing quite

Figure 1 | Right: X-ray Diffraction (main view) and Raman spectra (inset) of TiO₂ alone or at the nanocomposite (e.g., polymer contribution subtracted). Left: Raman spectra of the TiO₂-EVOH nanocomposite material and the EVOH polymer reference.

Figure 2 | Slide views of the TiO₂-EVOH nanocomposite film. (a) TEM general view (darker zones, TiO₂; lighter zones, EVOH); (b) HR-TEM detail showing a TiO₂ aggregate; and (c) HR-TEM view of the organic-
inorganic interface (interference fringes are evident in the TiO₂ component while an amorphous state of the EVOH polymer is observed at the interface).
favourably with those of chemicals or Ag-based disinfection technologies\(^1\)\(^{-3}\)\(^,\)\(^15\)\(^{-16}\).

To ascertain the mechanism of action of the composite upon photocatalysis, we first analyzed the specific charge carrier species involved in the system using electron paramagnetic resonance (EPR). Using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a trapping molecule in water and ethanol, we tracked the formation of reactive species after light excitation\(^4\),\(^6\),\(^12\),\(^19\),\(^20\). Representative spectra are presented in Supplementary Fig. S1, which shows the exclusive formation of OH\(^-\) radicals in water and the absence of other signals related to oxygen species, even after 20 min of prolonged light treatment. This result clearly demonstrates that only reactive species reached the surface of the polymer-TiO\(_2\) film and subsequently interacted with the neighbouring media and microorganisms. Moreover, nano-composite films also ensure the absence of cell damage/death induced by phagocytosis, a situation typically observed for powder TiO\(_2\) samples\(^19\),\(^20\).

From detailed measurements (see the Methods section), we estimated an initial rate of OH\(^-\) radical formation of 1.0 \(\times\) 10\(^-8\) mol-radical s\(^{-1}\) mg\(^{-1}\). In our experimental assay we used 20 ng of nanomaterial per 10\(^9\) cells, which means that an initial rate of 2 \(\times\) 10\(^{-7}\) nmol OH\(^-\) radical s\(^{-1}\) for 10\(^9\) cells and a total amount of \(\sim\)0.24 nmol OH\(^-\) radicals were produced in our 2-min assays. This amount is comparable to that found \textit{in vivo} when cells are exposed to stress conditions favouring oxidative damage\(^1\),\(^2\),\(^3\),\(^22\); however, we should note that the type of cells and experimental conditions just mentioned are not comparable to those described in the present study.

### Genome- and proteome-wide responses of \textit{P. aeruginosa} PAO1 to TiO\(_2\)-photocatalysis

No statistically significant differences (\(p \leq 0.001\)) in transcription patterns were discernible in samples of PAO1 cell suspensions irradiated with UV light (UV-alone) in the presence or absence of EVOH (UV + EVOH) reference films (not shown), which is in agreement with the fact that in both control conditions (e.g., UV-alone and UV + EVOH), no differences in the biocidal activity were observed after two minutes of intervention\(^7\). Accordingly, the expression values in the presence of UV + EVOH were considered hereafter for comparative purposes. In contrast, the levels of 165 transcripts (approximately 3.0% of the total transcripts) increased from 5.5 to 224-fold and those of 151 transcripts (approximately 2.7% of the total transcripts) decreased from 5.4 to 117-fold in samples of PAO1 cell suspensions containing TiO\(_2\)-coated EVOH particles (Supplementary Table S2).

Integration of the expression data (Supplementary Table S2) can be performed to further constrain the available PAO1 genome-scale constraint-based model and to develop context-specific models\(^25\),\(^26\). This integration organises the data in terms of the underlying knowledge embedded in the model structure and might help to better understand the on-going biological process. However, post-transcriptional and post-translational regulation and modifications, as well as allosteric effects, prevent the direct use of transcript levels as a proxy for reaction fluxes in the available model. Here we used the integration algorithm iMAT to find a flux distribution resembling, as close as possible, the measured gene expression levels, while allowing for biomass production\(^27\). In this case, the predicted biomass production compatible with the expression data indicates that the impact on the gene-wide flux distribution and the metabolism itself is moderate (approximately below 17%) in this time span.

The limited reduction in the maximum growth rate points to the fact that the main factors leading to a decreased survival rate of \textit{P. aeruginosa} (when confronted to TiO\(_2\)-based biocidal materials)\(^8\) are not directly caused by the loss of metabolic robustness. The differentially expressed genes identified on the arrays (Supplementary Table S2) were classified according to biological categories for interpreting the data. As shown in Fig. 3, fourteen general biological categories were defined, with the number of differentially expressed genes assigned to 11 out of 14 categories being extensively altered as a consequence of TiO\(_2\)-UV treatment. Fig. 4 further summarises the average expression levels of the genes assigned to each of the corresponding biological categories.

To confirm the expression levels and the roles of key relevant genes as indicated by the array analysis (see details below), the protein expression profiles of bacterial cells on control (UV + EVOH) and TiO\(_2\)-coated EVOH particles with UV activation were evaluated. Statistical comparison was performed between triplicates. Across all experiments, a total of 1137 proteins were identified, and among them 722 proteins were quantified with at least two peptides, representing 13.4% of the theoretical PAO1 proteome. Compared to the control (UV + EVOH) condition, only a minor number of proteins

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**Figure 3 | Distribution by function of genes expressed at higher (light grey colour) and lower (dark grey colour) transcript level in response to low doses of TiO\(_2\)-UV treatment compared to the control (UV + EVOH) condition.** The number of genes assigned to each category was calculated on the basis of the gene expression levels specifically shown in Supplementary Table S2. Abbreviation as follows: CMR, central metabolic reactions. For the cell wall biogenesis pathway, the number of under-expressed genes encoding proteins involved in lipopolysaccharide and peptidoglycan metabolisms, pilus biosynthesis and protein insertion are specifically shown (inset at the right down corner).
were differentially detected at a statistically significant level \((p \leq 0.1)\) in cells treated with TiO\(_2\)-coated EVOH particles (Fig. 5 and Supplementary Table S3). First, it should be noted that there is still a need to improve the proteomic approaches applied to studies on the biocidal effects of titania for assessing statistical significance, as titania exhibited a profound biocidal action under the conditions of our short-term assay (2-min intervention). In spite of this, the examination of the expression levels of those proteins confirms many of the effects caused by the titania at the gene level. We describe each of these effects below.

**TiO\(_2\) treatment selectively affects cell structure components.** Altered expression of a number of genes encoding enzymes involved in the cell wall and cell membrane composition and integrity was found (Figs. 3 and 4). In total, 14 genes encoding enzymes for the metabolism of lipids essential for cell membrane structure were found to be over-expressed (from 5.6 to 23.0-fold), whereas only two were expressed at a lower level (from 5.5 to 7.4-fold). The over-expressed genes include those encoding an acetyl-CoA synthetase, two CoA transferases, five (hydroxyl) acyl-CoA dehydrogenases, two (keto) acyl-CoA thiolases, a 2,4-dienoyl-CoA reductase, an acetyl-CoA acetyl transferase, a short-chain dehydrogenase and a fatty acid oxidation complex subunit alpha (Supplementary Table S2). In contrast to the above observations, the genes essential for cell wall structure, including those encoding proteins involved in lipopolysaccharide and peptidoglycan metabolism, pilus biosynthesis and...
protein insertion (13 genes in total), were expressed at significantly lower levels (from 5.4 to 15.1-fold) (Figs. 3 and 4). These genes include i) a 4-amino-4-deoxy-L-arabinose transferase, a glycosyl transferase family 1 protein, a nucleotide sugar dehydrogenase, an ADP-L-glycerol-D-manno-heptose-6-epimerase and a membrane protein, possibly involved in lipopolysaccharide metabolism; ii) a cell shape-determining protein and a membrane-bound lytic murein transglycosylase, possibly involved in peptidoglycan metabolism; iii) two pili assembly proteins, involved in pilus biosynthesis; and iv) two outer membrane proteins, a membrane translocase and a cell wall peptidase, possibly involved in cell wall integrity (Supplementary Table S2). Lower transcription levels of the pmrAB regulatory genes (7.5- and 6.7-fold, respectively), which are known to be involved in regulating proteins involved in cell wall composition and resistance7, provided further strong evidence for possible deficiencies at the level of the cell wall.

The expression of certain key genes was also confirmed by shotgun proteomics. Acetyl-CoA acetyltransferase was significantly over-expressed (PA2001, approximately 3.4-fold) whereas the peptidoglycan-associated lipoprotein (OppL; PA0973) was found to be expressed at a significantly lower level (approximately 4.7-fold). Taken together, the results unambiguously demonstrate that after the initiation of the intervention, cells may have significant deficiencies in the cell wall components, including lipopolysaccharides, peptidoglycan, pili and proteins. To compensate for these deficiencies, cells seem to react by activating the set of genes and proteins that produce lipids for the cell membrane found immediately below the cell wall. Clearly, radicals disrupt the first cell defence barrier (the cell wall), which provides strength and rigidity, while the cells react by activating a second defence barrier (the cell membrane), which provides support for the cytoskeleton.

Cells react to TiO2-UV treatment by increasing the one-carbon pool through non-oxidative pathways. Within the amino acid metabolism pathway, genes encoding proteins for glycine production from serine (glyA2, 50.5-fold) and its utilisation via the glycine cleavage system H protein (gcvH2, 10.0-fold), glycine dehydrogenase (gcvP2, 39.1-fold) and glycine cleavage system aminomethyltransferase (gcvT2, 7.7-fold) were significantly over-expressed, whereas those of the glycine betaine to sarcosine pathway (gcbA, 19.5-fold; gcbB, 17-fold; dgbB, 7.8-fold) and the sarcosine to glycine pathway (soxABD, 13.2-, 11.0- and 11.7-fold, respectively) were expressed at significantly lower levels than the control. This is of particular significance because reactions supported by glycA2, gcvT2 and gcvH2 are known to provide the largest part of the one-carbon units to the cells through tetrahydrofolate and 5,10-methylenetetrahydrofolate32.

Accordingly, it is plausible that when confronted with rather short treatment times and modest radical levels, cells react by activating the catalytic fate of glycine to the one-carbon pool for synthetic purposes (Supplementary Fig. S2). Alternatively, the fact that 5,10-methylenetetrahydrofolate acts as a chromophore in enzymes that repair UV-B-induced DNA lesions in a light-dependent manner53 might also explain the activation of reactions producing such molecules. The attenuation of the glycine betaine to glycine pathway may be explained by the fact that SoxABD sarcosine oxidases produce H2O2 as a subproduct during the oxidative demethylation of sarcosine54, which could potentially be harmful to cells under the strongly oxidative stress conditions induced by TiO2 photocatalysis. To investigate this possibility, we monitored the relative concentrations of some of the most representative metabolites involved in this pathway (Supplementary Fig. S2). As shown in Table 1, we found that dimethylglycine (5.3-fold) and sarcosine (4.1-fold), from the pathway that converts glycine betaine to glycine, were less abundant in cells treated with TiO2-coated EVOH particles with UV activation compared to control cells (UV + EVOH), in agreement with the lower expression levels of the gbcAB, dgbB and soxABBC genes (see above). In contrast, serine (5.2-fold) and tetrahydrofolate (15.5-fold), from the pathway that converts glycine to serine, were significantly more abundant in the cells treated with TiO2-coated EVOH particles with UV activation, which also agrees with the higher expression level of the glyA2 gene (see above).

TiO2-UV treatment decreases the ability of cells to assimilate and transport iron and inorganic phosphate (Pi). With regards to the functions related to ion biogenesis (including proteins with roles in transport, binding and catalysis), major differences were found in the
expression of genes related to iron homeostasis (7 genes) and Pi uptake (10 genes), which were expressed at significantly lower levels compared to the control (Supplementary Table S2 and Figs. 3 and 4). These include the complete set of genes constituting the Pho regulon: three regulatory proteins (phoBRU; from 24.6 to 15.6-fold), four transporters and porins (pslASCB, from 71.0 to 5.8-fold; oprO, 9.2-fold) and an alkaline phosphatase (phaÅ, 6.7-fold). These data suggest that P. aeruginosa will be highly deficient in phosphorus uptake and metabolism. It should also be noted that the Pho regulon has been reported to influence biofilm formation capacity and pathogenicity.

The lower ability of cells to assimilate and transport iron was further evidenced by the decreased expression of twelve siderophore pyoverdine synthesis genes (pvdAEPHGNQO, opmQ, PA2411, PA2393, from 48.5 to 6.4-fold), three pyoverdine/transferin/lactoferrin receptors (phuR, 8.0-fold; vreÅ, 8.3-fold; PA4675, 6.6-fold), three iron transport proteins (icsPM, PA1673, PA4358; from 57.1 to 8.0-fold) and a haem degradation protein (PA4709, 6.9-fold), which catalyses the final step in heme degradation in vitro. Additionally, an oxygen-independent coproporphyrinogen III oxidase (hemN), also involved in the biosynthesis of heme groups, was expressed at significantly lower levels (34.6-fold). This suggests that TiO$_2$ photocatalysis most likely decreases the ability to produce siderophores and thus decreases the ability to assimilate and transport iron, an essential ion for cell growth and survival. It should be recalled that pyoverdine has also been demonstrated to be important for bacterial virulence and biofilm development.

In addition, it is plausible that TiO$_2$ photocatalysis lowers the cell capacity to synthesise and degrade heme components required for the correct activity of a number of heme-proteins and to acquire iron ions. This possibility was further demonstrated by examining the shifts in the protein expression patterns in cells treated with TiO$_2$-coated EVOH particles with UV activation compared to control cells (UV + EVOH). Most notably, there was a significant reduction in the expression of pyoverdine biosynthesis-related proteins including PvdO (PA2395; approximately 5.2-fold) and PvdN (PA2394; ~3.0-fold), the ferripyoverdine receptors FpvA (PA2398; approximately 4.4-fold) and FpvB (PA4168; approximately 3.5-fold), a TonB-dependent haemoglobin/transferin/lactoferrin receptor family protein PhuR (PA4710; approximately 4.5-fold) and a heme oxygenase (PA0672; approximately 5.2-fold).

**TiO$_2$-UV treatment decreases cell-to-cell communication.** The spermidine biosynthesis pathway was significantly attenuated in the 2-min time span due to the decreased expression of an S-adenosylmethionine decarboxylase (speD, 9.6-fold) and a spermidine synthase (speE, 8.5-fold) (Supplementary Table S2 and Figs. 3 and 4). Recently, new functions for spermidine on the cell surface have been proposed. Namely, spermidine produces polyamines as an organic polycation to bind lipopolysaccharides (LPS) and to stabilise and protect the cell wall against oxidative damage. This suggests that TiO$_2$ photocatalysis lowers the production capacity not only of cell wall components (see above) but also of molecules involved in their binding. Recent results also strongly suggest an important role for spermidine as a signalling regulator, leading us to suggest that TiO$_2$ photocatalysis affects cell signalling. This hypothesis was supported by the significantly decreased expression of genes involved in the synthesis of quorum-sensing signal molecules (Supplementary Table S2 and Figs. 3 and 4), such as homoserine lactone and pyocyanin, which have been shown to play a role in diverse functions such as pathogenesis, biofilm development, stress resistance and cell survival.

**Table 1 | Metabolomic target analysis of key chemical species participating in the one-carbon pool pathway.** The separation and quantification was performed as described in the Methods section. The area of the peak (calculated on the basis of appropriate standards) as determined in triplicate assays (with standard deviation) by CE-TOF-MS is given.

| Metabolite            | UV + TiO$_2$-coated EVOH | UV + EVOH |
|-----------------------|--------------------------|-----------|
| L-Serine              | 9134893 ± 3081018        | 1759253 ± 446069 |
| Saccharose            | 20606119 ± 275529        | 83962799 ± 4251813 |
| Dimethyl glycine      | 28050873 ± 901772        | 1.48e$^6$ ± 7147982 |
| Tetrahydrofolate      | 19764 ± 1072             | 1272 ± 159 |

**TiO$_2$-UV treatment affects growth-essential genes and operon-linked regulatory genes.** Within the carbohydrate metabolism pathway, a set of nine genes was over-expressed, whereas only one displayed decreased expression (Supplementary Table S2 and Figs. 3 and 4). In contrast, a gene encoding a poly(3-hydroxyalkanoic acid) synthase (phaC2) was expressed at lower level (6.8-fold); this enzyme is involved in the production of polyhydroxyalkanoates and is produced by *Pseudomonas* species to store carbon and energy sources. This might suggest that cells tend to react to TiO$_2$-UV treatment by decreasing their capacity to store carbon sources as cells demand the mobilisation of carbon sources rather than carbon storage under stress conditions. This hypothesis was further supported by proteomic data demonstrating that a polyhydroxyalkanoate synthesis protein PhaF (PA5060) was more highly expressed (~5.0-fold) in cells treated with TiO$_2$-coated EVOH particles with UV activation than control cells (UV + EVOH). This protein has been shown to behave as a negative regulator of the phaC gene (i.e., the expression of phaF gene decreases the expression rate of the phaC$^T$). Accordingly, the higher expression level of the PhaF protein agrees with the significantly lower expression (6.8-fold) of phaC2 as evidenced by transcriptome data.

Notably, the expression of a pyruvate dehydrogenase (PA3416) was also found to be significantly higher (10.1-fold) in TiO$_2$-UV treated samples, which suggests that PAO1 cells also have a higher capacity to transform pyruvate into acetyl-CoA, thus linking the glycolysis pathway to the citric acid cycle and releasing energy via NADH. Furthermore, in silico analyses, as well as genome-scale transposon mutant studies, have shown that the acetyl-CoA carboxylase subunit accD is essential for growth. Therefore, lower expression (5.5-fold) of acetyl-CoA under TiO$_2$ photocatalysis might
also be linked to increased cell death. Moreover, the expression of a number of regulatory genes potentially involved in morphogenesis and virulence was significantly decreased including *hpaR* (8.0-fold) and *gmrR* (11.2-fold)58. Finally, a set of three genes (PA0622, 5.9-fold; PA0623, 6.5-fold; PA0627, 8.8-fold) encoding proteins presumptively involved in genomic prophage-mediated cell death and lysis, as well as a *prfN* gene (7.8-fold) that encodes a related transcriptional activator51, were expressed at significantly higher levels as a consequence of TiO$_2$ photocatalysis. Our interpretation of this result is that prophage-mediated cell lysis is rapidly induced after the initiation of TiO$_2$-UV treatment, as previously reported during H$_2$O$_2$-induced oxidative stress58.

Finally, the fact that 72 over- and 54 under-expressed genes encoded uncharacterised proteins suggests that these proteins may play yet unknown roles in radial defence and metabolic alterations. Interestingly, 48 highly expressed genes were components of 14 operons (containing at least 3 genes each) whose expression, according to ProdonNet (http://www.prodonnet.tu-bs.de/query.php), is controlled by the transcriptional regulatory genes *anr*, PA4493 and PA5344, none of which were found to be differentially expressed in our experimental conditions. In contrast, genes whose expression levels were lowered by the TiO$_2$ photocatalysis were mostly under the control of 25 different genes encoding transcriptional regulators. Notably, of these 25 genes, the haem biosynthesis and denitrification enzyme regulator Dnr, the ferric uptake regulator PvdS and the quorum-sensing regulator RhlR showed significant decrease in their transcript levels (14.8-, 48.5- and 6.7-fold, respectively). This finding is of special significance because these genes regulate operons consisting of 20, 41 and 16 genes (http://www.prodonnet.tu-bs.de/query.php), thus indicating a rapid and concerted regulatory deactivation as a direct consequence of TiO$_2$ photocatalysis.

**Discussion**

Recent studies using silver- or zinc-containing nanomaterials have recently suggested that the toxicity of these materials partially originates from membrane damage and disruption of ion homeostasis52,53. Here, by integrating transcription profiling with genome-scale constraint-based modelling, we found that the impact of radical-mediated TiO$_2$ photocatalysis on genome-wide flux distribution and the metabolism itself is limited and thus does not directly affect PA01 cell activity in the time frame measured. Rather, gene chip experiments and shotgun proteomics in combination with chemical species identification and quantification support the notion that PA01 cells respond by attempting to increase their capacity to protect and repair DNA and proteins in addition to promoting the coenzyme-dependent respiratory chain, mobilisation of carbon resources and one-carbon pools, as well as the cycle of fatty acid β-oxidation (the latter being essential for maintenance of membrane integrity). Furthermore, cells react to TiO$_2$ photocatalysis by improving membrane integrity to compensate for the strong alterations caused in the cell wall that covers the cell membrane. Additionally, we observed the activation of metabolic pathways involving production and utilisation of antioxidant coenzymes, such as (ubiquinol (used by cytochrome oxidases) and 5,10-methenyltetrahydrofolate (produced by GlyA2 and GcvT2 proteins), with the corresponding attenuation of those producing oxidative sub-products, such as H$_2$O$_2$ (produced by Sox enzymes), most likely to compensate for the strong radical stress pressure inside the cell. Supplementary Fig. S3 summarises the major effects in *P. aeruginosa* cells exposed to TiO$_2$ photocatalysis.

This study provides evidences that cells exposed to TiO$_2$ photocatalysis exhibit i) rapid cell inactivation at the regulatory and signalling levels, ii) a strong decrease of the coenzyme-independent respiratory chains, iii) a lower ability to assimilate and transport iron and phosphorous and iv) a lower capacity for the biosynthesis and degradation of heme (Fe-S cluster) groups. These activities, together with the extensive cell wall modifications, are the main factors that explain the high biocidal performance of TiO$_2$-based nanomaterials. However, it is not known which of these responses precipitate subsequent events. Future dynamic profiling assays are needed to further study these responses. Nonetheless, the fact that titania confers profound biocidal action, even at extremely low doses, under our temporally short assay conditions (2-min intervention), causes dynamic profiling at shorter and longer assay times to be technically challenging due to technical difficulties and extensive cell death and damage, respectively. It should noted that TiO$_2$-based materials possess a high affinity for phospho-proteins and phospho-peptides54,55, and that phosphorylation of proteins plays a role in the regulation of signalling pathways and the control of enzymatic activities by an “on/off” switch mechanism56. Therefore, the fact that after the initiation of TiO$_2$-UV treatment cell lysis is rapidly induced, suggests that this material could be used in situ to bind and enrich phosphoproteins to further explore the level of post-transcriptional protein modification at given times, as a molecular measure of altered protein activities. Whatever the case, the broad-spectrum action at the genetic level illustrated herein allows an investigation of the most significant properties of the photocatalytic disinfection process: universality (i.e., the absence of weakness against any type of microorganisms), and irreversibility (i.e., avoiding cell repairation opportunities and slowing down regulatory/signalling networks). In addition, the high efficiency demonstrated against a clinically relevant pathogen indicates the adequacy of TiO$_2$-based polymer films for a series of technological applications, such as hospital tools and furniture, but also food preservation or wastewater treatment.

**Methods**

**Synthesis of nanocomposite films and basic characterization.** The TiO$_2$ component was prepared using a microemulsion synthetic route and calcined at 723 K for 2 h. A commercially available EVOH (Solvay), containing a nominal 71 mol% vinyl alcohol content, was used as polymeric matrix in the preparation of EVOH-TiO$_2$ nanocomposite films with an inorganic content of 2 wt%. These novel materials were prepared by melt blending in a Haake Minilab twin-screw extruder. An optimal processing window was selected to optimise the final dispersion of nanoparticles and to avoid EVOH degradation (temperature at 195°C, a rotor speed of 100 rpm and a mixing time of 15 min). Prior to this stage, nanoparticles and the EVOH copolymer were sonicated (Sonics VC505) and stirred to homogenise the batch. After this processing, the specimens were compression-moulded in a Collin press between hot plates at 210°C and at a pressure of 1.5 MPa for 5 min to obtain films of approximately 100 microns. A quench was applied to the different films from the melt to room temperature.

X-ray diffraction (XRD) patterns were recorded with a Bruker D8 Advance apparatus using a Cu Kα radiation and a 20-step program of ca. 0.02 degrees per second. Micro-Raman spectra were acquired with an inVia Raman microscope using a near-IR diode laser (785 nm) with a nominal 2-nm resolution. Transmission electron microscopy experiments were also carried out at room temperature with a 200 Kv JEM-2000 FX JEOL microscope. The samples were embedded in Spurr resin and cut into sections of 100 μm using a thick sectioning knife (Diatome, USA) and then polished with a Buehler model 1500 grinder. The thin sections were observed using a Philips CM200 operated at an accelerating voltage of 180 kV and equipped with energy-dispersive X-ray spectrometry (EDS).

Electron paramagnetic resonance experiments.** The EPR measurements were performed with a Bruker ER200D spectrometer operating in the X-band and calibrated with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) standard. For the DMPO spin trapping EPR experiments, the nanocomposite samples were immersed in an aqueous or methanolic solution of 0.01 M DPPH spin trap (supplied by Fluka–Aldrich–Sigma Chemical Co.; St. Louis, MO, USA) and kept on ice during the whole set of experiments. Blank experiments were also performed over mixtures of 100 μl of the DMPO solution and water or methanol mixed in an EPR flat quartz cell under atmospheric air and irradiated at different times through a filter with a cut-off at ca. 220 nm with a UV-B (280 nm) light and then immediately transferred to the spectrometer cavity for EPR analysis. A small decay in radical concentration (of ca. 5% on average) was observed in the dark during the course of spectrum recording. The spectra were obtained at 20°C at ca. 9.75 GHz microwave frequency, 19.5 mW microwave power, 100 kHz modulation frequency, 2 G modulation amplitude and 2–4 × 10$^{-4}$ spectrometer gain; no significant signal saturation was observed under these conditions. Blank experiments were also performed over mixtures of 100 μl of the DMPO solution and 100 μl of water or ethanol to confirm the absence of radical formation in the absence of the nanocomposite film under the employed conditions.
Bacterial strains, media and chemicals. TiO2–EVOH composites and the P. aeruginosa PA01 strain were used in the present study. The PA01 strain was maintained at 37 °C on Luria Bertani (LB) agar plates. Overnight cultures were grown with agitation in 250-ml Erlenmeyer flasks at 37 °C in LB and used to inoculate 10-ml portions of pre-warmed LB (10 times diluted) in 250-ml Erlenmeyer flasks to obtain an initial optical density at 600 nm (OD600) of 0.05. The resulting cultures were grown at 37 °C on the exponential phase (OD600 0.3) and subsequently used for the photochemical cell assays. An appropriate amount of this culture was diluted in pre-warmed LB (10 times diluted) to achieve a final cell concentration of 10^8 CFU ml^{-1}. The oxide-cell slurry (3 ml final volume) was placed in the UV-B spectrometer chamber (UVKON 990) and irradiated with a UV-B light of 280 nm. Identical cultures treated with either non-composite EVOH lacking TiO2, or UV-B alone served as the controls. After 2 min of irradiation, three aliquots of 1000 ml were immediately collected and subjected to RNA, protein and metabolite isolation as described below. For each experiment, the culturing of bacteria, exposure to the TiO2-nanocomposite and subsequent RNA, protein and metabolite isolation was performed in triplicate on the same day.

Gene chip experiments. All samples for the RNA measurements were stabilised with RNAProtect bacterial reagent (Qiagen GmbH, Hilden, Germany). The samples were mixed with this reagent, incubated for 5–15 min at room temperature and centrifuged for 15 min at 4000 × g and 4 °C. The supernatant was discarded, and the pellet frozen in −70 °C. In a deep freezer, the RNA was stored until use. Immediately, 1.2 ml milli-Q water was added to the cell pellet, and, after re-suspension, the samples were sonicated for 30 s (10 watts) on ice (5 cycles × 0.5 min). After sonication, the pellet was removed by centrifugation at 16,000 × g for 20 min at 4 °C and the methanol solution was stored at −80 °C until use. Immediately, 1.2 ml milli-Q H2O was added to the cell pellet, and, after re-suspension, the samples were sonicated for 30 s (10 watts) on ice (5 cycles × 0.5 min). After sonication, the pellet was removed by centrifugation at 16,000 × g for 20 min at 4 °C and the water solution was separated. Finally, equal volumes (1 ml) of each of the methanol and H2O solutions were mixed and stored at −80 °C until analysis. The full description of the methods used for CE-TOF-MS analysis is available in the Supplementary Methods section.

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Author contributions

M.F., M.F.-G. and A.K. designed the project. M.F. and M.F.-G. wrote the main manuscript draft. A.K. and M.F. carried out photo-killing test and prepare samples serving as raw materials for omic tests. S.C., I.Z. and J.P.A. performed and interpreted the proteomic data. D.R. and C.B. performed and interpreted the chemical analysis. All authors participated in the discussion and commented on the paper.

Additional information

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