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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Rhamnolipid production: effect of oxidative stress on virulence factors and proteome of *Pseudomonas aeruginosa* PA1

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Abstract Under specific environmental conditions, *Pseudomonas aeruginosa* produces a biodegradable surfactant rhamnolipid. Evidences suggest that this biosurfactant is involved in protecting cells against oxidative stress; however, the effects of oxidative stress on its production and other virulence factors are still unclear. Here we show that rhamnolipid production is dependent on the aeration surface when *P. aeruginosa* is cultured in shaken flasks, as well as in production of elastases and alkaline proteases. The production of alginate, lipase, and pyocyanin was not detected in our shaken-flask experiments. *P. aeruginosa* was treated with hydrogen peroxide to trigger its oxidative stress response, and the proteome profile was analyzed. We identified 14 proteins that were expressed differently between samples that were treated and not treated with peroxide; these proteins are potentially involved in the rhamnolipid production/secretion pathway and oxidative stress.

Keywords *Pseudomonas aeruginosa* · Rhamnolipid · Oxidative stress · Virulence factors · Proteomics

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Introduction

In aerobic microorganisms, the respiration process has the inevitable downside of electron leakage from redox enzymes to oxygen, forming reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), the superoxide radical (O_2^-), and the hydroxyl radical (OH·). ROS accumulation results in protein carbonylation, lipid peroxidation, cofactor degradation, and DNA damage (Storz and Imlayt 1999; Imlay 2003).

Pseudomonas aeruginosa typically resists ROS accumulation by expressing several factors such as superoxide dismutases, catalases and peroxidases, and it is hypothesized that rhamnolipid also plays a role in protection from oxidative stress (Sabra et al. 2002). Redox sensing transcription factors tightly regulate the expression of these defense factors as well as a complex physiological response to ROS. The transcription factors OxyR and PerR, from *Escherichia coli* and *Bacillus subtilis*, respectively, are the two best characterized peroxide sensors in bacteria, and orthologs are found in most bacteria (Faulkner and Helmann 2011). Response to H_2O_2 is crucial for bacteria, as demonstrated by *E. coli* lacking both catalases and peroxidases (Seaver and Imlay 2001). This mutant strain is unable to grow under aerobic conditions, even in the absence of exogenous H_2O_2. Response of *P. aeruginosa* against oxidative stress is controlled by the OxyR transcriptional regulator responsible for activating antioxidant genes (Vinckx et al. 2010; Ochsner et al. 2000; Panmanee et al. 2008). In addition, expression of some virulence factors is also under OxyR control (Lau et al. 2005).

P. aeruginosa produces several virulence factors, one of which has received special attention because of its commercial importance. Rhamnolipids are biosurfactants of special interest for different industries such as cosmetics, pharmaceutics and detergent production, and have proven potential in bioremediation and oil recovery (Soberón-Chávez et al.

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2005; Santa Anna et al. 2007). These biosurfactants are biodegradable and have low toxicity, and are comparable to synthetic surfactants in their physical and chemical properties. It was shown that rhamnolipids can be produced using by-products of plants and biodiesel industries, renewable sources such as starch-rich substrates, and waste from cooking oil, dairy, sugar, and lignocellulose industries (Rahman et al. 2002). Therefore, rhamnolipids are potential replacements for synthetic surfactants but with the advantage of being environmentally friendly. However, important obstacles must be overcome in order to achieve competitive industrial production of rhamnolipids. These obstacles include excessive foam formation during cultivation, insufficient rhamnolipid yields and relatively high costs for downstream processing (Banat et al. 2000).

Considerable research effort has been devoted to the development of efficient *P. aeruginosa* cultivation processes for rhamnolipid production, including metabolic strategies to increase rhamnolipid production (Santos et al. 2002), designs for new reactors capable of overcoming the critical operational problem of foaming (Kronemberger et al. 2008) and identification of proteins potentially involved in the rhamnolipid production pathway and its genetic regulation, using proteomic techniques (Reis et al. 2010). Although few studies have demonstrated an influence of oxygen on the rhamnolipid production by these bacteria, some groups have suggested that rhamnolipids participate actively in protection against oxidative stress, preventing the entry of oxygen into solution through the foam layer formed on the surface (Kim et al. 2003; Sabra et al. 2002). Moreover, under conditions of oxidative stress, *P. aeruginosa* secretes large amounts of proteins (Sabra et al. 2002), including some virulence factors such as elastase and alkaline proteases (Kronemberger et al. 2008). This suggests that oxygen supply to the culture broth may be a key factor for *P. aeruginosa* growth and rhamnolipid production.

The present study evaluated *P. aeruginosa* PA1 response against different oxygen transfer rates. We also analyzed the production of virulence factors within a background of high rhamnolipid production. Finally, using a differential proteome we describe new insights concerning the understandings of oxidative stress on *P. aeruginosa* PA1 and its correlation to rhamnolipid production.

Materials and methods

Bacteria and growth media

The *P. aeruginosa* PA1 strain, originally isolated from petroleum wells (Santa Anna et al. 2001), was deposited at the Fiocruz/CMRVS Culture Collection (registered at WDCM with number 575) and identified with the collection number INCQS P4046. The strain was cultured on YPDA (0.3 % yeast extract, 1.5 % peptone, 0.1 % dextrose, and 1.2 % agar) medium at 30 °C for 48 h and transferred to a growth medium as described by Santos et al. (2002). After 24 h in culture, 2 mg of bacteria was transferred to cryotubes containing 25 % glycerol and preserved at −18 °C, here called pre-inoculum. The pre-inoculum was added to 300 mL of growth medium (Santos et al. 2002) in a 1-L conical flask, and incubated at 30 °C for 40 h with agitation (170 rpm). A culture volume of 150 mL was centrifuged at 6,000×*g* for 15 min and the pellet was used as inoculum. Cell density was determined by absorbance at 600 nm in a spectrophotometer, and the cell dry weight concentration (grams per liter) was obtained using a standard curve.

Culturing in different aeration conditions

Cultivations were performed in a 1-L conical flask containing 150, 300, 500, or 700 mL of a medium, previously optimized for rhamnolipid production (Santos et al. 2002), containing 30.0 g/L glycerol, 1.4 g/L $NaNO_3$, 7.0 g/L K_2HPO_4, 3.0 g/L KH_2PO_4, and 0.2 g/L $MgSO_4 \cdot 7H_2O$. Cultivations were maintained at 30 °C, 170 rpm, for 120 h, and appropriate samples were harvested.

Oxidative stress with hydrogen peroxide

P. aeruginosa PA1 growing in exponential (10 h) or stationary (36 h) growth phases were directly exposed to 10 mM hydrogen peroxide (final concentration) and the culture was incubated at 30 °C, 170 rpm, for 1 h.

Lipid peroxidation measurement

Lipid peroxidation was determined by thiobarbituric acid (TBARS), based on the malondialdehyde (MDA) detection (Steels et al. 1994) with modifications. Exponential or stationary bacterial cells (50 mg) were harvested by centrifugation and washed twice with distilled water. The pellets were resuspended in 500 μL of 10 % trichloroacetic acid (TCA) and transferred to a tube containing 1.5 g of glass beads. Cells were lysed by 15 cycles of agitation in a vortex for 20 s and incubation on ice for 20 s, followed by 30 cycles of sonication at 225 W for 10 s, and 10 s of incubation on ice. The supernatants obtained after centrifugation were mixed with 0.1 mL of 0.1 M EDTA, 0.6 mL of 1 %*w*/*v* thiobarbituric acid in 0.05 M NaOH. The reaction mixture was incubated at 100 °C for 15 min and absorbance was measured at 532 nm.

Determination of protein carbonyl groups

Protein carbonyl groups were slot blotted in a polyvinylidene difluoride (PVDF) membrane. Protein samples

(100 μg) were directly applied onto a PVDF membrane by a vacuum system. The PVDF membrane was then incubated with 0.1 mg/mL 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCL for 10 min and washed three times in 5 N HCL to remove free DNPH. Nonspecific protein-binding sites were blocked through incubation of the membrane with PBS containing 5 % milk (*w*/*v*), 0.05 % (*v*/*v*) Tween 20 and 30 μL/mL azide. After 24 h, the membrane was treated with a primary anti-DNP antibody (1:2,500) for 2 h in a blocking solution as described above. At the end of this incubation, the membrane was washed three times with PBS and finally incubated with a peroxidase-conjugated secondary antibody for 1 h in 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl and 5 % milk. Slot blot detection was performed with the chromogenic method (Robinson et al. 1999). Image analysis of the scanned membrane was carried out by using the software Imagem Master 2D Platinum 5. The intensities of immunostained bands are expressed as optical densities, and Student's*t* test (p value<0.05) was applied to appraise the differences in the values of the means.

Rhamnolipid and glycerol quantification

Rhamnolipid quantification was determined indirectly by measuring rhamnose concentration as described by Pham et al. (2004), with modifications. The conversion factor of 2.23 (100 g rhamnolipid/44.89 g rhamnose) was used to determine the total rhamnolipid concentration (Kronemberger et al. 2008). Glycerol concentration was determined in the cell-free samples by the enzymatic/colorimetric method for triglyceride determination (Enzymatic Triglyceride-GPO; Laborlab, Sao Paulo, Brazil).

Virulence factors quantification

Total protein quantification and enzyme activity

Total protein was quantified according to the method described by Lowry et al. (1951). Protease activity was determined according to the method proposed by Charney and Tomarelli (1947), which is based on the formation of azocasein derivatives in alkaline medium due to proteolytic cleavage. A sample of 0.5 mL of the cultivation supernatant was added to 0.5 mL of azocasein solution (50 g azocasein per liter of 0.05 M Tris–HCl buffer, pH 9). The reaction was maintained at 37 °C, and three aliquots of 0.5 mL reaction medium were collected after 5, 10, and 15 min. One unit of proteolytic activity was defined as the amount of enzyme that produces a 0.01 difference in absorbance at 428 nm between the sample and its blank assay, per minute, under the assay conditions.

The elastase activity of the supernatant was measured by degradation of elastin bonded to Congo red (ECR; Sigma, St. Louis, MO, USA), as described by Braga et al. (1994). A 125-μL aliquot of the cultivation supernatant was added to 1 mL of a suspension of elastin-Congo red (1 mg/mL) in 0.05 M Tris–HCl buffer, pH 8. The reaction was maintained at 37 °C for 24 h and insoluble ECR was removed by centrifugation. The absorption of the supernatant was measured at 495 nm, and the supernatant boiled for 10 min was used as control. One unit of elastase activity was defined as the amount of enzyme that produces a difference of one absorbance unit between the sample and its blank, per minute, under the assay conditions.

Lipase activity was determined by the capacity of the enzyme to hydrolyze the substrate p-nitrophenyl laurate (pNPL), which releases p-nitrophenol (pNP), which absorbs at 412 nm at pH 7. A solution of 2.5 mM of pNPL in 25 mM sodium phosphate buffer pH 7 was prepared. The reaction was started by addition of 0.05 mL of enzymatic extract at 30 °C. The progress of the reaction was monitored by absorbance measured at 412 nm. One unit of lipase activity is the amount of enzyme that releases 1 μmol of pNP per minute from pNPL milligram of protein under the conditions mentioned above (Castro-Ochoa et al. 2005, with modifications).

Pyocyanin quantification

Pyocyanin quantification is based on its absorbance at 520 nm in acidic solution (Kurachi 1958). After 72 h of culture (stationary growth phase), a 5-mL sample of culture grown in cultivation medium and in *Pseudomonas* broth (PB; 20 g of Bacto-Peptone (Difco), 1.4 g $MgCl_2$, and 10 g K_2SO_4 per liter) was extracted with 3 mL of chloroform and re-extracted with 1 mL 0.2 N HCl. Pyocyanin concentration was determined by multiplying the absorbance at 520 nm by 17.072. PB medium is optimized to maximize pyocyanin production in liquid culture (Essar et al. 1990) and was used as a positive control for pyocyanin production.

Alginate quantification

Alginate concentration was measured gravimetrically. A 20- or 30-mL sample of culture broth was centrifuged at 10,000 rpm during 15 min and the supernatant was added to 3 volumes of ethanol. The resultant precipitate was dialyzed three times on water for 24 h for removal of small molecules and weighed (May and Chakrabarty 1994).

Proteomics techniques

Protein extraction and quantification

The total protein extract was obtained from 40 mg of cells after 10 and 36 h of culture, to the exponential and stationary growth

phases subjected or not to oxidative stress conditions. Cells were homogenized with the extraction solution [8 M urea, 1 M thiourea, 4 %*w*/*v* CHAPS, 1 %*v*/*v* IPG buffer 4–7, 60 mM DTT, 0.5 %*v*/*v* Triton X-100] and was subjected to five cycles of sonication for 10 s at 10 W and incubation on ice for 1 min, followed by 15 cycles of freezing in liquid nitrogen and thawing at 37 °C. The samples were centrifuged at 14,000×*g* for 15 min, and the soluble fractions were harvested and stored at −80 °C (Reis et al. 2010). Total protein was determined using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis

The isoelectric focusing (IEF) was carried out in the pH range of 4–7 (three gels for each condition). After protein extraction, 700 μg of protein was precipitated with TCA and solubilized for isoelectric focalization (IEF) in 4–7 strips using rehydration solution (8 M urea, 1 M thiourea, 4 %*w*/*v* CHAPS, 0.002 %*w*/*v* Bromophenol blue, 100 mM DTT and 1 %*v*/*v* IPG buffer pH 4–7). The isoelectric focusing was performed in immobilized pH gradient (IPG) gel (pH 4–7) using strips of 18 cm as described by Reis et al. (2010). The strips were rehydrated with the protein sample at 30 V for 12 h at 20 °C and focused at 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 1,000–8,000 V for 30 min and 8,000 V for 5 h. After IEF, the strips were incubated for 15 min with mild agitation in an equilibrium solution (1.5 M Tris–HCl pH 8.8, 6 M urea, 1 %*w*/*v* DTT, 30 %*v*/*v* glycerol, 2 %*w*/*v* SDS, 0.002 %*w*/*v* Bromophenol blue), followed by incubation for 15 min in the same buffer solution, but replacing DTT for 4 %*w*/*v* iodoacetamide. The IPG gel strips were laid on top of the 12 % SDS-PAGE gel (Laemmli 1970) and sealed with agarose. Tris-glycine-SDS buffer (0.025 M Tris base, 0.192 M glycine, and 0.1 %*m*/*v* SDS, pH 8.3) was used at 1X and 2X concentrations. Electrical conditions were 2.5 W/gel for 30 min and 100 W constant until the end of the run. Before staining with colloidal Coomassie G-250, the gels were fixed in 30 %*v*/*v* ethanol and 2 %*v*/*v* phosphoric acid, washed with 2 %*v*/*v* phosphoric acid, and then incubated in 2 %*v*/*v* phosphoric acid, 18 %*v*/*v* ethanol, 15 %*w*/*v* ammonium sulfate and 1 %*v*/*v* of a solution containing 20 g/L of Coomassie blue G-250 for 72 h under moderate shaking. The dye solution was removed, and 1 % *v*/*v* acetic acid was added to avoid fungal contamination (Rabilloud and Charmont 2000).

Analysis of images and identification of proteins

Images of the scanned gels were compared using the software Image Master 2D Platinum 7.0 (GE Healthcare). Spots significantly different (p value<0.01, ANOVA test) between samples subjected or not to oxidative-stress conditions by hydrogen peroxide were excised and trypsinized using a MultiScreen vacuum manifold (Millipore), following the manufacturer's instructions with some modifications. Excised spots were placed on the ZipPlate and incubated with: (1) a solution of 25 mM ammonium bicarbonate/5 % (*v*/*v*) acetonitrile for 30 min, and then twice with an ammonium bicarbonate solution of 25 mM/50 % (*v*/*v*) acetonitrile for 30 min; (2) 100 % acetonitrile for 10 min; and (3) 25 mM ammonium bicarbonate, containing 10 μL of trypsin (100 ng). The plate was incubated at 37 °C in a water bath for 16–20 h. The C18 resin of each well was activated by 10 μL of 100 % acetonitrile for 15 min at 37 °C, washed three times with 1 % (*v*/*v*) formic acid, and the peptides were extracted by incubating twice with 1 % (*v*/*v*) formic acid in 60 % (*v*/*v*) methanol. Samples were concentrated in a SpeedVac to a volume of approximately 5 μL, and stored at −20 °C. Subsequently, 0.3 μL of each peptide sample was applied in the MALDI plate, followed by addition of 0.3 μL of α-cyano-4-hydroxycinnamic acid [10 mg/mL in 50 % (*v*/*v*) acetonitrile 0.3 % (*v*/*v*) trifluoroacetic acid] and dried at room temperature. For samples harvested in the exponential growth phase, the MS spectra were acquired in positive and reflectron modes using 1,250 laser pulses per spot. The eight most abundant ion precursors were selected for fragmentation and analyzed by MS/MS in a MALDI TOF/TOF (Applied Biosystems 4700). Samples from the stationary growth phase were analyzed in a MALDI TOF/TOF 5800 (Applied Biosystems) and the MS1 and MS2 spectra were acquired using 2,040 and 2,000 shots per spectrum, respectively. The ten most abundant ion precursors were selected for fragmentation and analyzed by MS/MS. The fragment masses were searched against the non-redundant National Center for Biotechnology Information database using the MASCOT program (Matrix Science, London, UK) with parameters of two missed cleavages and the following variable changes: carboxyamidomethylation, methionine oxidation, threonine, serine and tyrosine phosphorylation, and N-terminal Pyro-Glu. The parameters used included ±0.8 Da error in MS and ±0.6 Da error in MS/MS, for samples from the exponential growth phase, and ±100 ppm error in MS and ±0.3 Da error in MS/MS, for samples from the stationary growth phase. Search results were analyzed into Scaffold software (Proteome Software) for compilation, normalization, comparison of spectral counts, and assigning probability. Protein identifications were made at the peptide probability greater than 95 %.

Results

Culturing in different aeration conditions

Shake flasks are the most common culture vessels for characterization of the kinetic parameters for liquid cultivation, and the oxygen transfer process in the flask may depend on

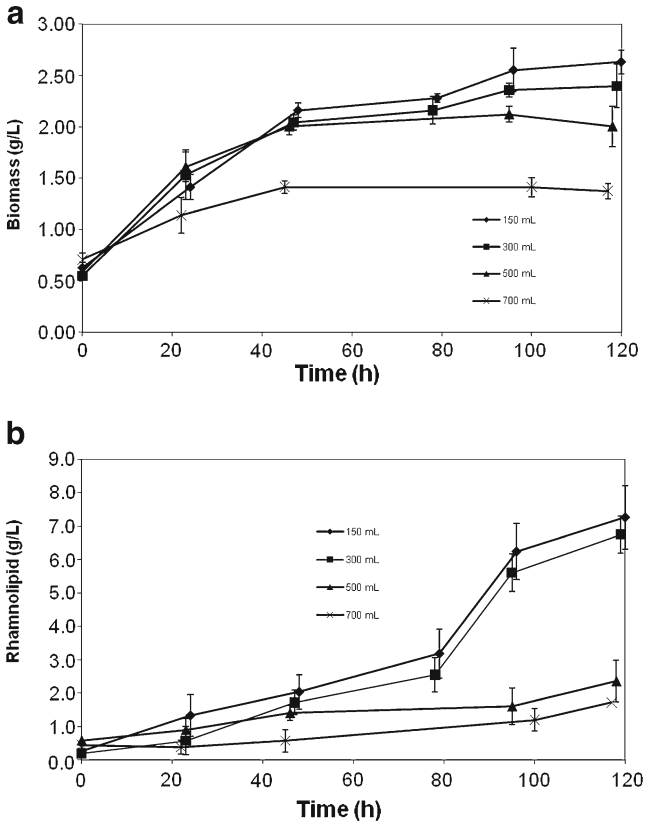

Fig. 1 Profile of biomass and rhamnolipid production of *P. aeruginosa* cultured in different filling volumes at different times. Biomass **(a)** and rhamnolipid **(b)**. Nominal shake flask volume was 1 L

the liquid volume (Maier et al. 2004). The effects of different oxygen transfer rates on the cell growth and rhamnolipid production were investigated. *P. aeruginosa* PA1 was cultured in volumes of 150, 300, 500, and 700 mL of medium using 1-L conical flasks. Samples from each aeration condition were harvested and assayed for production of rhamnolipids and virulence factors as described below.

Effect on cell growth and rhamnolipid production

The cell growth rate (approximated) and biomass decreased with the increase in medium volume, with a marked reduction when cultured in 700 mL of medium (Fig. 1a; Table 1). Interestingly, rhamnolipid production showed a much stronger dependence on aeration condition (Fig. 1b; Table 1). The increase in specific rhamnolipid production rate (Qp) (Table 1) was largely attributed to a significant increase in the yield of the rhamnolipid per cell mass formation (Yp/x) and substrate (Yp/s) in flasks with higher surface aeration rates (150 and 300 mL). The kinetic value of Qp in 150 mL liquid volume was fourfold higher than in flasks containing 700 mL of medium. These results suggest that the medium oxygenation has a more dramatic impact on rhamnolipid production than on cell growth rate and biomass.

Effect on secretion of virulence factors

P. aeruginosa is known to secrete virulence factors, e.g., elastases, proteases, and phospholipases. The activities of alkaline proteases and elastases were higher when *P. aeruginosa* PA1 was cultured in 150 and 300 mL, and considerably lower when cultured in 500 and 700 mL (Fig. 2a, b). Therefore, the different aeration conditions affected the secretion and perhaps the production of proteases, elastases, and rhamnolipid in a similar way. However, the activities of proteases and elastases were high even during the exponential growth phase, whereas rhamnolipid production was detected only during the stationary phase. No lipase activity, alginate or pyocyanin production was detected under the experimental conditions (data not shown). Pyocyanin is a major virulence factor, and its production was further investigated. *P. aeruginosa* PA1 was cultured in a medium optimized for pyocyanin production, samples were harvested after 72 h of culture (stationary growth phase) and the concentration of pyocyanin was determined. The culture medium was green and contained 2.56 μg/mL of pyocyanin, indicating that even in low concentration this pigment is able to significantly alter the color of the culture medium compared to its rhamnolipid-producing counterpart (Fig. 3).

Oxidative stress induced by hydrogen peroxide

P. aeruginosa PA1 was subjected to oxidative stress by hydrogen peroxide after 10 and 36 h of culture, in the exponential and

Table 1 Kinetic parameters of rhamnolipids and biomass production in *P. aeruginosa* cultured in different filling volumes

Volume (mL)	Biomass (g/L)	Rhamnolipid production (g/L)	Yp/x	Yp/s	Qp (mg L^{-1} h^{-1})
150	2.00±0.07	7.26±0.96	3.63±0.21	0.22±0.01	60.52±8.42
300	1.85±0.18	6.75±0.55	4.90±0.19	0.23±0.02	56.75±9.35
500	1.41±0.14	2.36±0.63	1.67±0.17	0.08±0.01	19.98±3.61
700	0.67±0.01	1.72±0.52	2.56±0.15	0.16±0.01	14.70±2.08

Nominal shake flask volume was 1 L. Yp/x refers to rhamnolipid yield coefficient with respect to cell mass. Yp/s refers to rhamnolipid yield with respect to substrate. Qp refers to rhamnolipid productivity

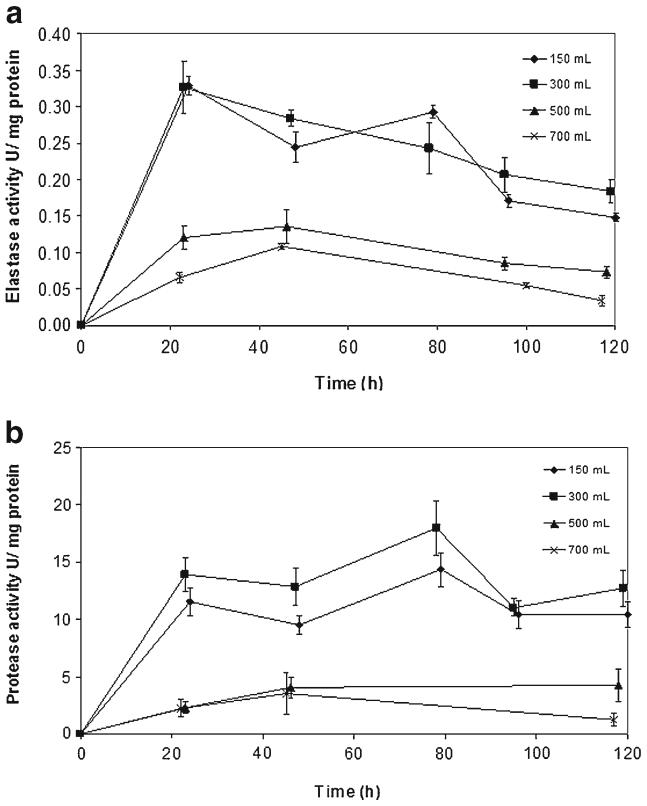

Fig. 2 Profile of elastase and alkaline protease activity of *P. aeruginosa* cultured in different filling volumes at different times. Specific activity of elastase **(a)** and alkaline protease **(b)**. Nominal shake flask volume was 1 L

stationary growth phases, respectively. Samples were harvested after 1 h of treatment and assayed for carbonylated proteins and lipid peroxidation production. The proteome profiles of the samples treated or not treated with hydrogen peroxide were compared.

Lipid peroxidation and protein carbonylation

Lipid peroxidation was slightly affected by the addition of peroxide in both the exponential and stationary growth phases. However, protein carbonylation was approximately three times more intense in the peroxide-treated than in the untreated cells (Table 2). Both the lipid peroxidation and protein carbonylation profiles are expected to be altered in cells under oxidative stress (Cabiscol et al. 2000).

Comparative 2-DE of intracellular proteins

Intracellular proteins from the exponential and stationary phases of *P. aeruginosa* treated or not with peroxide were solubilized and separated using 2-DE gel across the pH range 4–7 (Fig. 4). Gel image and statistical analyses (p value<0.01), considering the mean spot volume from three different gels, were performed to detect differences in protein expression between *P. aeruginosa* treated or not with peroxide (Table 3). A total of 51 differential spots were cut from the gel, and 14 different proteins were identified by mass spectrometry (MALDI-TOF/TOF) and MASCOT MS/MS ion search.

Discussion

Production of rhamnolipids and virulence factors in different aeration conditions

Rhamnolipid production by *P. aeruginosa* was shown to be dependent on oxygen supply when cultured in a bioreactor (Kronemberger et al. 2008). Our results showed that the rhamnolipid production and the bacterium growth rate (approximated) in shake flasks are dependent on the oxygen transfer rate (volume of medium). Rhamnolipids were shown to participate in protection against oxidative stress, acting as dispersers of oxygen molecules and preventing the entry of oxygen into solution through the foam layer formed on the surface (Sabra et al. 2002; Kim et al. 2003). Moreover, the *Pseudomonas* quinolone signal (PQS), the so-called third quorum sensing system in *P. aeruginosa*, is a signal molecule known to regulate the production of QS-dependent factors involved in the stress response, e.g., oxidative stress and UV irradiation resistance (Häussler and Becker 2008). Rhamnolipid production is known to be regulated by PQS, and the results suggest that rhamnolipids solubilize PQS in the extracellular environment, suggesting a positive feedback between rhamnolipids and PQS (Calfee et al. 2005; Reis et al. 2011). Our results showed that high

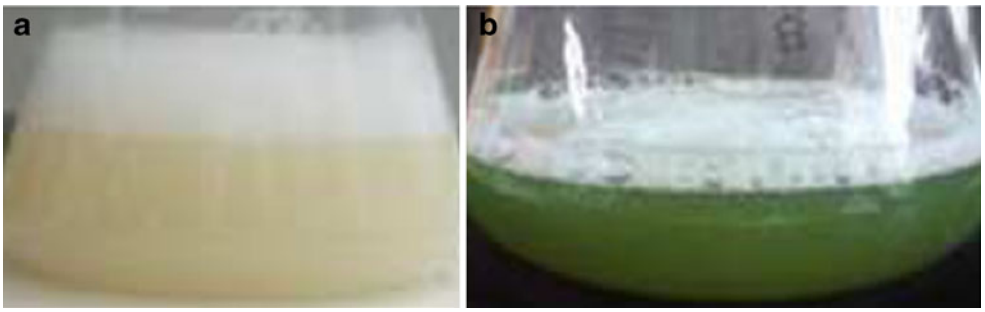

Fig. 3 Comparison of rhamnolipid and pyocyanin-optimized media. *P. aeruginosa* was cultured for 72 h in **a** medium optimized for rhamnolipid production and **b** medium optimized for pyocyanin production. More details in "Materials and methods"

Table 2 Lipid peroxidation and carbonylated proteins at the exponential and stationary growth phases

	Exponential		Stationary	
	Lipid peroxidation (pMol MDA/mg of cells	Protein carbonylation (OD)	Lipid peroxidation (pMol MDA/mg of cells	Protein carbonylation (OD)
Non-treated	505.5±37.9	497.7±135.3	810.7±27.1	561.8±62.6
H_2O_2 treated	637.12±65.8	1,673.7±246.8	896.4±35.5	1,586.5±269.2

MDA malondialdehyde

p value<0.05, by Student's *t* test, significantly different

amounts of rhamnolipids were obtained using smaller volumes of media (probably elevated oxygenation transfer rates), which suggests that rhamnolipids may be involved in protection against oxidative stress directly or indirectly via the PQS quorum sensing system.

The production of rhamnolipids and other virulence factors by *P. aeruginosa* is controlled by the quorum sensing (QS) systems, a cell density dependent process of intercellular communication characterized by the secretion and detection of signal molecules (Williams and Camara 2009). The gene expressions for elastase, alkaline protease, and rhamnolipid are regulated by QS in a similar way (Pearson et al. 1997; Gambello et al. 1993; Deziel et al. 2005). Our results showed the same production profile for these virulence factors, suggesting that rhamnolipids, and secreted proteases are regulated by QS under conditions of varying oxygenation rates.

The absence of lipase activity, pyocyanin, and alginate production suggests that *P. aeruginosa* PA1 is less virulent compared with hospital-isolated strains. Lipase is known to hydrolyze major lipids of the pulmonary surfactant in patients with cystic fibrosis (Henderson and Nataro 2001). However, Gilbert and colleagues (1991) showed reduced lipase activity (<1 μmol min^{-1} mg $cell^{-1}$) when *P. aeruginosa* EF2 was cultured with glycerol as the carbon source. Therefore, our results may also suggest that the undetectable activity of lipase is due to the cultivation medium. The polysaccharide alginate is produced by mucoid *P.*

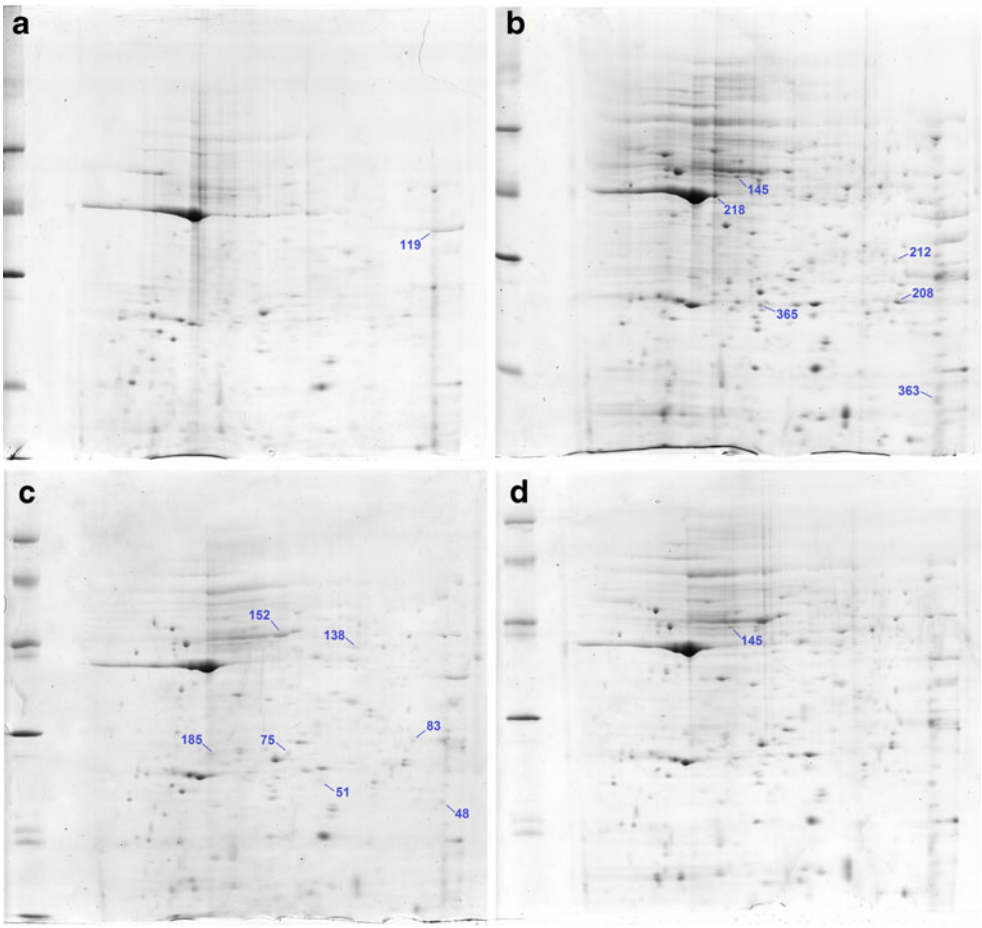

Fig. 4 Intracellular protein expression by *P. aeruginosa* treated with peroxide at the exponential **(a)** and stationary **(b)** growth phases. Gels **c** and **d** represent the control samples at the exponential and stationary growth phases, respectively. Proteins were separated by 2-DE gel, pH 4–7. Proteins identified by peptide de novo sequencing are numbered and listed in Table 3

Table 3 Identification of proteins with altered expression levels in *P. aeruginosa* treated with peroxide

Growth phase	Functional class	Spot no.	Protein	Number of peptides	Sequence coverage (%)	pI pred/exp	MW pred/exp	Expression under oxidative stress[a]
Exponential	Amino acid biosynthesis and metabolism	48	Glutamate *N*-acetyltransferase	2	8	5.39/6.85	41.76/24	Not identified
	Chaperones and heat shock proteins	185	Chaperonin GroEL	2	6	5.04/4.97	57/26.72	Not identified
		51	ATP-dependent Clp protease proteolytic subunit	2	16	5.45/5.82	22.14/24.25	Not identified
	Hypothetical protein	119	Hypothetical protein PSPA7_4504	2	7	9.26/6.94	37.8/20.73	Exclusive
		75	Hypothetical protein PA1733	4	19	5.33/5.56	26.47/27.25	Not identified
	Nucleotide biosynthesis and metabolism	83	Adenylate kinase	2	12	5.98/6.59	23.1/28.85	Not identified
	Translation, post-translational modification, degradation	152	Elongation factor Tu	4	13	5.23/5.47	43.35/48.48	Not identified
		138	Peptide methionine sulfoxide reductase	1	11	5.2/5.4	23.5/27.75	Not identified
Stationary	Adaptation, protection	365	Peroxidase	2	12	5.45/5.82	21.82/25.13	Exclusive
		208	Alkyl hydroperoxide reductase subunit C	3	21	5.89/6.53	20.5/25.28	Exclusive
	Membrane proteins, transport of small molecules	218	Major porin and structural outer membrane porin OprF precursor	3	13	4.98/4.83	37.64/43.58	Exclusive
		145	OprE3	2	5	5.54/5.23	46.83/46.57	Increased
	Putative enzymes, carbon compound and catabolism	212	Enoyl-CoA hydratase	2	9	6.02/6.6	30/31.35	Exclusive
	Translation, post-translational modification, degradation	363	Ecotin precursor	2	13	6.75/6.77	17.31/17.83	Exclusive

Proteins were identified using MALDI/TOF-TOF de novo sequencing. Protein extracts were obtained after 10 and 36 h of culture, in the exponential and stationary growth phases, respectively. Functional class was assigned according to Stover et al. (2000). Spot no. refers to numbers shown in Fig. 4

[a] Protein level in peroxide-treated cells against non-treated cells

aeruginosa especially in cystic fibrosis, and its production is regulated by the transcription regulator AlgR (Ramsey and Wozniak 2005). AlgR is known to suppress rhamnolipid production during biofilm development (Morici et al. 2007), which may suggest that the high production of rhamnolipid in our experiments is enhanced by the absence of alginate production.

Oxidative stress and protein expression

This study showed that when *P. aeruginosa* PA1 was exposed to mild concentrations of peroxide, it underwent an oxidative stress condition that resulted in differential expression of intracellular proteins. Moreover, while peroxide treatment affected lipid peroxidation and, more intensely, protein carbonylation, cell viability was not affected (data not shown). Interestingly, peroxide-treated cells in the exponential growth phase did not express most of the differentially expressed proteins, which includes proteins related to amino acid biosynthesis, protein folding, and nucleotide biosynthesis. However, the cells in the stationary growth phase showed the differentially expressed proteins increased or exclusive to the peroxide-treated condition such as protective/adaptation and membrane proteins. This profile suggests that *P. aeruginosa* was more sensitive to oxidative stress during the stationary growth phase, whereas during the exponential phase the peroxide treatment reduced the expression of growth-related proteins.

Peroxide-treated cells in the stationary growth phase showed increased production of protective proteins such as peroxidase, alkyl hydroperoxide reductase subunit C (AhpC) and ecotin, as well as the membrane proteins OprF and OprE3. Peroxidase is an enzyme that has peroxides (e.g., hydrogen peroxide and organic hydroperoxides) as substrates and is typically involved in defense against peroxide-oxidative stress; therefore, its higher abundance in the peroxide-treated cells may be due to its protective activity. Ecotin is a periplasmic homodimeric protein that

inhibits many serine proteases and was found to protect *P. aeruginosa* against neutrophil elastase (NE) during the infection process (Eggers et al. 2004). Neutrophil elastase was shown to cleave OmpA, an outer membrane protein, leading to increased cell permeability (Belaaouaj et al. 2000) that can result in NE translocation into the periplasm, where ecotin inhibits its proteolytic activity. *P. aeruginosa* is an important pathogen that causes infection in cystic fibrosis patients and is responsible for nosocomial respiratory infections. In this work, ecotin was found to be upregulated in peroxide-treated stationary cells suggesting that *P. aeruginosa* under oxidative stress, either peroxide treatment or infection process (oxygen-rich environment), behaves similarly, to some extent.

The protein AhpC is a well-characterized member of the peroxiredoxin family, a ubiquitous group of cysteine-based peroxidases (Hall et al. 2009). In *P. aeruginosa*, the transcriptional regulator OxyR upregulates the expression of *ahp*C gene in response to peroxide (Ochsner et al. 2000) and also regulates the swarming motility and rhamnolipid production by an unknown mechanism (Vinckx et al. 2010). Therefore, our results indicate that AhpC and rhamnolipid production may be correlated via co-regulation by OxyR under oxidative stress.

The expression of outer membrane proteins is increased under stress conditions in *P. aeruginosa* (Nakajima et al. 1998; Baysse et al. 2005). Both outer membrane proteins identified here, OprF and OprE3, were more abundant in the peroxide-treated cells in stationary growth phase. OprF regulates its permeability according to the growth conditions and was shown to be involved in adaptation to the environment (Jaouen et al. 2004). Recently, OprF was shown to modulate the virulence of *P. aeruginosa*, perhaps through modulation of the QS system (Fito-Boncompte et al. 2011). Moreover, the OprF mutant showed a reduced level of rhlAB mRNA, which encodes for rhamnolipid biosynthetic enzymes, resulting in dramatic reduction of rhamnolipids production in this mutant (Bouffartigues et al. 2011). The rhamnolipid production occurs in the late exponential and throughout the stationary growth phase; therefore, our results suggest that the increased expression of the outer membranes OprF and OprE3 in the stationary phase is due to increased secretion of protective factors, which may include rhamnolipids, and their transcriptional co-regulation.

The results presented here suggest that the oxygen concentration in the culture medium may be a crucial factor in establishing an efficient and economically viable bioprocess, and corroborate previous studies that suggest that rhamnolipids are involved in protecting against oxidative stress. The absence of virulence factors such as lipase, pyocyanin and alginate contributes to the use of the medium containing rhamnolipids in environmental applications. However, elastase, protease, and rhamnolipid production are apparently regulated in a similar way, which makes it necessary to develop molecular strategies that maximize the production of rhamnolipids over other virulence factors. This strategy is especially important to enable the environmental applications of this product by removing the purification step, which usually acts as a limiting factor for the development of economically viable bioprocesses (Banat et al. 2010). Overexpressed proteins identified in oxidative stress conditions, including those related to secretion systems, and adaptation mechanisms potentially related to rhamnolipid production and secretion, will contribute to further studies on understanding of the physiological importance of these proteins within the background of rhamnolipid production. Experiments performed in bioreactors operating under controlled and constant oxygenation conditions will be needed, in order to investigate the extent to which the dissolved oxygen concentration in the medium is capable of positively influencing the production of rhamnolipids, as well as their influence on protein expression by *P. aeruginosa*.

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