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ORIGINAL PAPER

Proteomic based investigation of rhamnolipid production by *Pseudomonas chlororaphis* strain NRRL B-30761

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Abstract We recently reported that a strain of the non-pathogenic bacterial species *Pseudomonas chlororaphis* was capable of producing the biosurfactant molecule, rhamnolipids. Previous to this report the organisms known to produce rhamnolipids were almost exclusively pathogens. The newly described *P. chlororaphis* strain produced rhamnolipids at room temperature in static minimal media, as opposed to previous reports of rhamnolipid production which occurred at elevated temperatures with mechanical agitation. The non-pathogenic nature and energy conserving production conditions make the *P. chlororaphis* strain an attractive candidate for commercial rhamnolipid production. However, little characterization of molecular/biochemical processes in *P. chlororaphis* have been reported. In order to achieve a greater understanding of the process by which *P. chlororaphis* produces rhamnolipids, a survey of proteins differentially expressed during rhamnolipid production was performed. Separation and measurement of the bacteria's proteome was achieved using Beckman Coulter's Proteome Lab PF2D packed column-based protein fractionation system. Statistical analysis of the data identified differentially expressed proteins and known orthologues of those proteins were identified using an AB 4700 Proteomics Analyzer mass spectrometer system. A list of proteins differentially expressed by *P. chlororaphis* strain NRRL B-30761 during rhamnolipid production was generated, and confirmed through a repetition of the entire separation process.

Keywords Biosurfactant · Non-pathogenic · Proteomics · *Pseudomonas chlororaphis* · Rhamnolipids

Introduction

Rhamnolipids are microbiologically produced biosurfactants capable of significantly reducing surface tension and interfacial tension between fluid phases. As an example rhamnolipids can reduce the surface tension of water from 76 mN/m to 25–30 mN/m. Such reductions increase the range of water's useful properties, including allowing water to then effectively solubilize hydrocarbons [4]. This biosurfactant activity of rhamnolipids has made them a promising molecule for a range of different industrial applications [1, 3, 12, 13]. These applications include the breakdown and removal of oil spills, as an emulsifying additive to cosmetics and foodstuffs, and antimicrobial agents.

Rhamnolipid structure is composed of a sugar portion consisting of one (mono-rhamnolipid) or two (di-rhamnolipid) molecule(s) glycosolated to a β-hydroxy (3-hydroxy) fatty acid that in most cases is esterified to a second β-hydroxy fatty acid (Fig. 1a, b) [11]. Rhamnolipids were originally described as being produced by the bacteria *Pseudomonas aeruginosa* and later by the bacteria *Burkholderia pseudomallei* [8,

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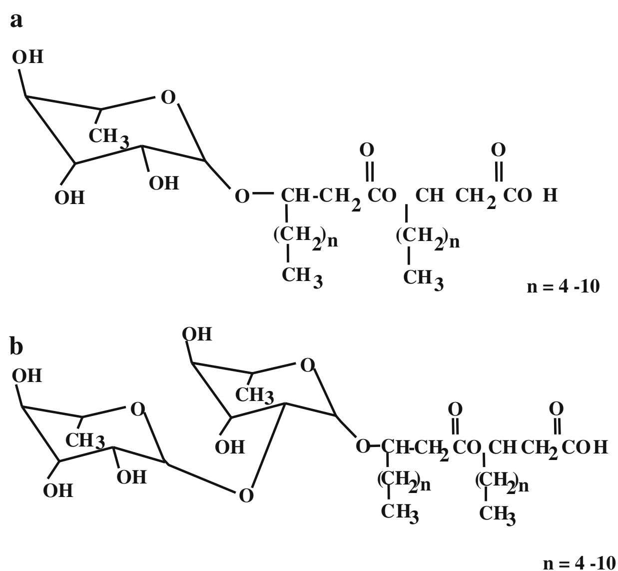

Fig. 1 Chemical structures of mono-rhamnolipid (*a*), and di-rhamnolipid (*b*)

10]. Complicating the process of using rhamnolipids for any commercial application is the pathogenic nature of both of the producer organisms. Attempts to commercially produce rhamnolipids from these organisms would therefore have to address a range of potentially cost prohibitive safety issues. A potential solution to this problem is a recently described strain, NRRL B-30761, of the non-pathogenic bacterium *Pseudomonas chlororaphis* that is capable of producing rhamnolipids [6]. In addition to being non-pathogenic the strain of *P. chlororaphis* has the added bonus of producing rhamnolipids at room temperature under static growth conditions as opposed to the elevated temperatures and mechanical agitation necessary for production of rhamnolipids by the pathogenic organisms. These differences have the potential for significant production cost savings during industrial scale rhamnolipid production.

Unfortunately, almost all of the work done to understand the mechanics and ideal conditions for rhamnolipid production has been done exclusively in *P. aeruginosa* strains [5, 11, 14–16]. Therefore, with the exception of the initial discovery and some research on optimizing conditions for increasing production levels, little research has been done on the microbial mechanics of rhamnolipid production by *P. chlororaphis*. An initial step to rectify this lack of knowledge has been undertaken through a comparative study of protein expression in *P. chlororaphis*. Gel-based two-dimensional protein separation systems are a well-accepted means of separating and comparing the protein profiles. For our research we utilized a relatively new packed column-based two-dimensional separation system with few published studies to date [20, 22]. This study resulted in a list of proteins that were differentially regulated between the conditions when rhamnolipids production was high and when there was little or no production.

Materials and methods

Bacterial strain, growth, and whole cell protein extraction

Pseudomonas chlororaphis strain NRRL B-30761 was maintained on *Pseudomonas* Isolation Agar at 4°C (PIA, DIFCO). Overnight cultures of the *P. chlororaphis* strain were grown in Kay's minimal media (0.3% $NH_4H_2PO_4$, 0.2% K_2HPO_4, 0.2% glucose, 0.5 mg/l $FeSO_4{\cdot}7H_2O$, 0.1% $MgSO_4{\cdot}7H_2O$) with orbital shaking at 250 rpm and 30°C. Aliquots from the overnight cultures were diluted 1:100 into two matched 2 l Erlenmeyer flasks containing 200 ml mineral salts media (per liter: 0.7 g KH_2PO_4, 0.9 g Na_2HPO_4, 2 g $NaNO_3$, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 2 ml of trace elements (per liter: 2 g $FeSO_4{\cdot}7H_2O$, 1.5 g $MnSO_4{\cdot}H_2O$, and 0.6 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$)) supplemented with 2% glucose each [18, 26]. One of the flasks designated as the static flask was incubated at room temperature (20–23°C) under strict static conditions. The other flask designated as the shake flask was incubated at room temperature (20–23°C) with orbital shaking at 250 rpm. Both flasks were incubated at their specific conditions for 72 h.

Next, both cultures are processed in the same manner to extract the entire compliment of proteins being expressed in the individual cultures. Total cell pellets from 50 ml of each culture were isolated by centrifugation (5,000 × *g*). The surface tensions of the resulting supernatants from each growth culture were measured using a DCAT 11 tensiometer (Future Digital Scientific Corp.) to observe for rhamnolipid production. Bacterial pellets were each first washed in 50 ml of 50 mM Tris (pH 8.0) and re-pelleted. Cell pellets were suspended in 0.4 ml of 50 mM Tris (pH 8.0), tubes were placed in an ice bath and sonicated for 30 s using a microtip. The sonication process was then repeated two more times. Next, 1.6 ml of lysis buffer (7.5 M urea, 2.5 M thiourea, 12.5% glycerol, 50 mM Tris, 2.5% N-octylglucoside, 6.25 mM TCEP [Tris–carboxyethyl phosphine hydrocholine], 1.25 mM protease inhibitor) is added to each bacterial suspension. The solution was centrifuged at 14,000 rpm for 60 min and the resulting supernatant was collected. A PD-10 column (Amersham

Biosciences) was then used to exchange the total cellular proteins from the lysis mix into the proprietary Start buffer (Beckman–Coulter). Finally, the total concentration of proteins present in the resulting preparations was measure by means of a BCA protein assay (Pierce Rockford, IL, USA).

2-Dimensional protein separation

Whole cell protein preparations were diluted to a concentration of 5 mg/ml for separation on the Proteolab PF2D system (Beckman–Coulter). First dimension separation utilized a chromatofocusing column (250 × 2.1 mm i.d.) that generated a pH gradient from 8.5 to 4 using the proprietary Start and Elute buffers, this was accomplished over 185 min at a flow rate 0.2 ml/min. Protein separation was monitored by UV absorbance at 280 nm. First dimension fractions were further separated in the second dimension using a C18 reverse-phase column (4.6 × 33 mm; 1.5 μm particle size, non-porous) utilizing an acetonitrile water gradient at a flow rate of 0.75 ml/min over 45 min and monitored by UV absorbance at 214 nm with relative protein concentrations recorded in terms of absorbance units (AU). Fractions were collected using a FC 204 fraction collector (Gilson) in 96 well plates at 30 s intervals between 10 and 30 min. The plates containing the collected fractions were stored at −20°C until processed for identification using mass spectrometry. The ProteoVue™ (Eprogen Inc.) software application was used to convert chromatographic intensities into two-dimensional protein expression maps representative of the contents of the whole cell protein preparations assayed. Differential analysis of individual protein peaks in matched whole cell protein samples was accomplished using the DeltaVue™ (Eprogen Inc.) software application. The concentrations of individual proteins expressed by *P. chlororaphis* NRRL B-30761 grown statically were divided by the concentrations of corresponding individual proteins grown with orbital shaking to produce a ratio of protein expression levels [protein X (static)/protein X (shaken)]. The log of each ratio value was determined and the mean value for all protein ratios in a comparison between whole cell preparations determined. Standard deviations were determined for the mean of all log [ratios] and values that fell outside the standard deviations were recorded as being differentially expressed.

Mass spectrometry sample preparation

Sample fractions were stored at −20°C after collection in 96 deep well plates. Selected fractions for analysis were transferred to 0.5 ml Eppendorf tubes previously washed with 50% acetonitrile followed by 1% trifluoroacetic acid and Milli Q water to remove potential contaminants. Sample volumes were concentrated to approximately 30 μl using a Speedvac concentrator. For tryptic digestion, 10 μl each of NH_4HCO_3 (pH 8.95) and DTT were added to each sample to a final concentration of 100 and 1 mM, respectively, heated upto 60°C for 10 min on a preheated digital dry bath, and allowed to cool at room temperature. One micro liter of Trypsin Gold (Promega, Madison, WI, USA) at a concentration of 50 μg/μl in 50 mM acetic acid was added to each sample and incubated for 4 h at 37°C with gentle agitation. After digestion, samples were treated with 2% TFA to stop the trypsin activity and the resulting peptides were extracted and cleaned using OMIX C18 ZipTips (Pierce, Rockford, IL, USA) following manufacturer recommendations. Briefly, peptides were extracted using a pre-cleaned ZipTip, washed with water containing 0.1% trifluoroacetic acid (TFA), re-extracted with acetonitrile–water (50:50) 0.1% TFA, and mixed with a recrystallized α-cyano-4-hydroxy-cinnamic acid matrix solution (5 mg/ml, acetonitrile–water (50:50) 0.1% TFA) to a final concentration between 100 fmol and 1 pmol/μl. Approximately 0.6–0.7 μl of the peptide-matrix solution was spotted in the mass spectrometer target plate.

Mass spectrometry and protein(s) identification

Matrix-assisted laser desorption/ionization mass spectrometry with automated tandem time of flight fragmentation of selected ions (MALDI-TOF/TOF) of trypsin digested proteins were acquired with a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) in the positive reflectron mode with a 200 Hz Nd-YAG 355 nm laser. Spectra were obtained by averaging 1,000 acquired spectra in the MS mode or 2,500 in the MS/MS mode. Collision induced dissociation (CID) with air as the collision gas at approximately 1×10^{-6} Torr, and a 1 keV acceleration voltage was used for obtaining the MS/MS spectra of selected peptide. Conversion of time of flight to mass (Da) for the monoisotopic ions, $[M + H]^+$, was based on calibration of the instrument with a peptide standard calibration kit (Applied Biosystems) that contained the following peptides: des-Arg^1-bradykinin (*m/z* 904.4681), angiotensin I (*m/z* 1,296.6853), Glu^1-fibrinopeptide B (*m/z* 1,570.6774), ACTH (clip 1–17) (*m/z* 2,903.0867), ACTH (clip 18–39) (*m/z* 2.465.1989), and ACTH (clip 7–38) (*m/z* 3,657.9294). The MS/MS time of flight calibration was obtained from the CID produced fragments of Glu^1-fibrinopeptide B. Peptide

mass fingerprints and MS/MS of selected peptides were combined and queried against primary sequence database using Mascot search engine-associated GPS Explorer program (Applied Biosystems). Reported protein(s) from database searches from putative peptide sequences are within a ≥95% confidence interval.

Results

Protein separation and comparison

Cultures of *P. chlororaphis* strain NRRL B-30761 were grown in one condition statically at room temperature allowing for the production of rhamnolipids and in the other condition with orbital shaking at room temperature which blocks expression of rhamnolipids [6]. The whole cell protein extractions from each of these conditions were separated in two different dimensions using the column-based ProteomeLab PF2D separation system. The system produced two-dimensional maps of the proteins expressed by the individual cultures separating the proteins in the first dimension based on their isoelectric point (p*I*) and in the second dimension by the hydrophobicity of the individual proteins.

The evaluation of the first pair of cultures one making rhamnolipids and one not making rhamnolipids, confirmed by measurement of culture surface tension (data not shown), allowed for the comparison of 111 individual proteins (Fig. 2a). This series of experiments were repeated producing two new cultures one again producing rhamnolipids and the other not producing rhamnolipids. Two-dimensional maps were again constructed from the protein expression data of the two different cultures. In this set of experiments 136 individual proteins were identified for comparison between the two cultures (Fig. 2b). Ratios were constructed by dividing the protein concentrations of the individual proteins produced by the static (positive for rhamnolipids) culture by the concentration of the matching proteins in the shake (negative for rhamnolipids) culture. The log value for the resulting ratio for each protein pair were next plotted on a graph, first for the protein map pair from Fig. 2a (Fig. 3a), and next for the protein map pair from Fig. 2b (Fig. 3b). The mean value for the entire set of log values for each graph were determined and plotted on the graph as a line. The standard deviations from this mean value were next determined for each graph and plotted beside the mean values. The points, representing ratio values, which fall above and below the standard deviation lines were determined to be differentially regulated in regards to the expression of the protein under conditions of rhamnolipid production. Points above the upper standard deviation line represent proteins that were present in a greater concentration in the growth condition where rhamnolipids are produced (static), as compared to the growth conditions where rhamnolipids were not produced (shaken). Conversely, points falling below the lower standard deviation line are present in lesser concentrations in the growth conditions where rhamnolipids were produced (static), as compared to growth conditions where rhamnolipids were not produced (shaken). This statistical evaluation of the original comparison of rhamnolipid growth conditions (Fig. 2a) yielded 15 proteins that appeared to be up regulated and 16 proteins down regulated (Fig. 3a). Applying the same evaluation to the complete repetition of the original experiments (Fig. 2b) resulted in the identification of 13 proteins that were up regulated in cultures where rhamnolipids are produced as well as 22 other proteins that were down regulated (Fig. 3b).

A comparison of the lists generated by the two separate experiments identified five proteins in common between the two sets. The spots representing these proteins are marked on the 2-dimensional maps (Fig. 2a, b), and highlighted on the appropriate data plots (Fig. 3a, b). The appearance of these five proteins in lists generated by completely separate experiments gives us great confidence that these proteins are differentially regulated during conditions resulting in the production of rhamnolipids (Table 1).

Discussion

Rhamnolipids are extremely versatile and useful biosurfactants with the potential for becoming a valuable commercially produced commodity [1, 3, 12, 13]. The major obstacle to commercial production of rhamnolipids is the pathogenic nature of the major microbial producers. This problem has been solved to some extent by the discovery of a strain of the non-pathogenic bacterium *P. chlororaphis* capable of producing rhamnolipids, under conditions requiring little energy investment [6]. Our research employed a relatively new packed column-based system for 2-dimensional protein separations to investigate the proteins differentially regulated by the bacteria under conditions where rhamnolipids are produced [27]. Column-based two-dimensional separation produces data similar in nature to the well established gel-based two-dimensional separation technique with some important differences. The column system is liquid-based and the resulting

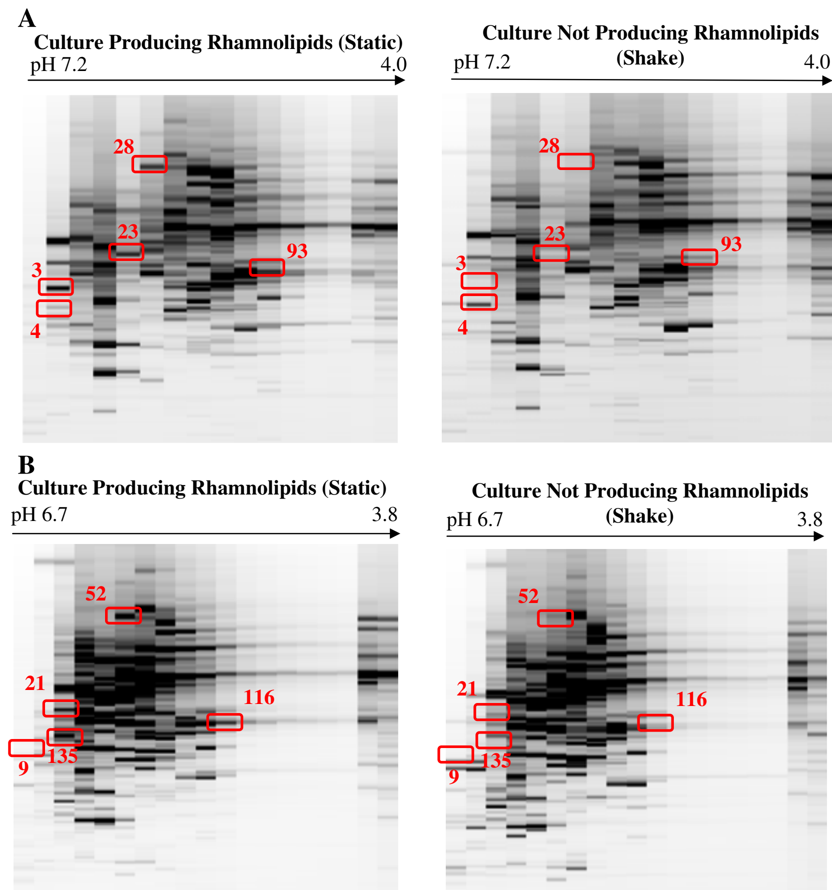

Fig. 2 Two-dimensional protein expression maps of the whole cell protein extracts derived from the various bacterial growth conditions. Proteins are separated in the first dimensions by their isoelectric point (p*I*) and by their hydrophobicity in the second dimension. The first pair of maps represents the differences in protein expression between cultures of *P. chlororaphis* NRRL B-30761 grown under conditions either encouraging or limiting rhamnolipid production (**a**). Differentially expressed protein spots are marked and labeled in the corresponding locations in each map. The second pair of maps represents the differences in protein expression resulting from a complete repetition of the experiments performed to create the first set of maps (**b**). The same protein spots observed to be differentially expressed in the first map pair are again observed to be differentially expressed in the second map. The spots are marked and given new labels

protein fractions are eluted in water/acetonitrile mixtures of various concentrations and do not have to be purified away from a gel matrix, simplifying the preparation of samples for identification with a MALDI-TOF/TOF instrument. In addition the volumes of the first dimension fractions allow for multiple second dimension fractionations without having to prepare an entirely new sample. Finally, our separation system also demonstrated good reproducibility as to relative positions of proteins in the 2-dimensional maps from one experimental sample to the next. In our work the column-based two-dimensional separation system appears to be a good complementary technique to the gel-based separation systems.

The most surprising result of this proteomic survey is the absence of protein orthologues of the *P. aeruginosa* proteins RhlA, RhlB, RhlR and RhlI in the list of differentially expressed proteins. The proteins RhlA,B,R, and I have been shown to be essential in *P. aeruginosa's* production of rhamnolipids [14, 15]. Since our *P. chlororaphis* strain produces rhamnolipids it is reasonable to expect orthologues of these proteins to be found to be up regulated in growth conditions where rhamnolipids are produced as compared to conditions where rhamnolipids are not produced. The absence of these proteins appearing in our survey suggests that either *P. chlororaphis* does not have orthologues of these proteins or that these proteins serve other functions in addition to rhamnolipid production so that they would still be expressed in a bacteria growing under conditions where rhamnolipids were not being produced. The first explanation seems unlikely since we have been able to isolate and sequence, from *P. chlororaphis* strain NRRL B-30761, orthologues of the genes for *rhlA*, *rhlB* and *rhlR* (unpublished data). However, there is some recent research in *P. aeruginosa*

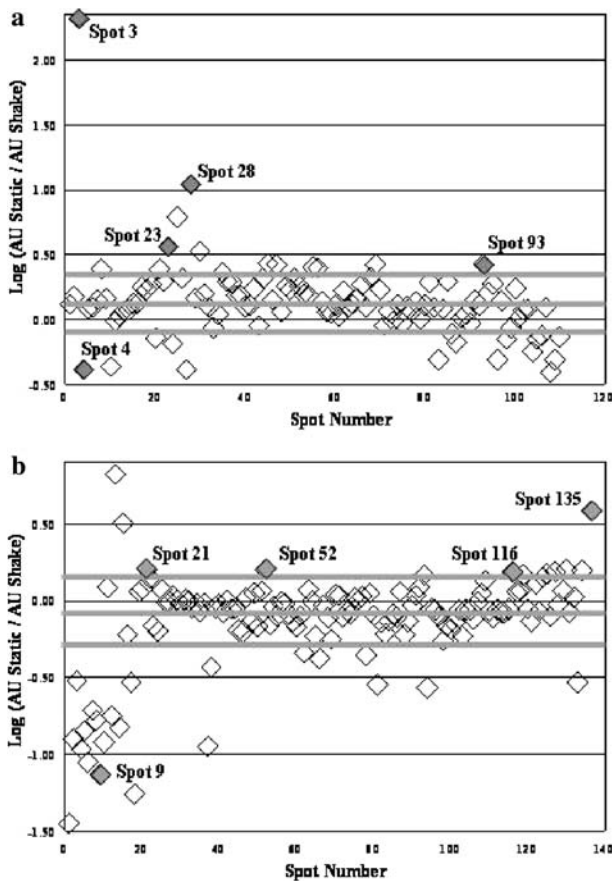

Fig. 3 Plots of the log (AU of protein X from static culture/AU of protein X from shaken culture). Comparison of the matched proteins resulting from the first set of two-dimensional expression maps (**a**) and the subsequent map pair resulting from repeating the previous experiments (**b**). Mean value lines and standard deviation lines (*continuous line*) are included for each protein map comparison. The proteins differentially expressed reliably in each set of experiments are marked (*filled diamond*) on the data plots and labeled with the appropriate experimental number

to suggest multiple roles for at least one of these proteins essential to rhamnolipid production [21].

The ATP synthase F1 alpha subunit is an unexpected protein to be found differentially expressed since the protein is part of the a complex responsible for the extremely essential bacterial process of ATP synthesis. Even more confusing is our observation that the ATP synthase F1 beta subunit is not found to be differentially expressed under the same growth conditions where the alpha subunit is differentially expressed. The genes responsible for the ATP synthase subunits have been shown to be differentially regulated in relation to the growth rate of a bacterial culture [19]. However this differential regulation was observed to be same for the alpha and beta subunits.

Glutamine synthetase type 1 in *P. aeruginosa* has been previously shown to be regulated by RpoN the alternate sigma factor σ^{54} [24]. RpoN mutants were defective in glutamine synthetase production. Similarly RpoN has been shown to affect the expression of genes necessary for rhamnolipid production in *P. aeruginosa* [9, 16, 23]. Since the sigma factor coded for by *rpoN* affects transcription of both the genes for glutamine synthetase as well as rhamnolipids it is expected that we find increased production of glutamine synthetase in the growth condition where rhamnolipids are being produced. The *rpoN* coded σ^{54} has also been shown to affect the transcription of the groups of genes necessary for nitrogen assimilation and biofilm development as well as full virulence in strains of *P. aeruginosa* [23, 24].

Manganese-cofactored superoxide dismutase (Mn-SOD), iron-cofactored superoxide dismutase (Fe-SOD), and the transcription factor MvaT have each been previously identified by other groups to be differentially expressed by *P. aeruginosa* during biofilm formation [2, 7, 17, 25]. Bacteria encased in a biofilm have a great need to be able to deal with reactive oxygen species since they cannot escape their immediate environment. Since *P. chlororaphis* is producing rhamnolipids under conditions leading to biofilm formation it is not surprising that we also see an increase in the production of both superoxide dismutases. Mutations in the *mvaT* gene result in enhanced biofilm formation by

Table 1 MALD/I TOF–TOF identification of orthologues of proteins determined to be differentially expressed under conditions affecting the production of rhamnolipids

Expression condition	Protein spot number	MALD/I identified orthologue (accession #)	pH range	Calculated pI of orthologue	Number of peptides matched	Protein CI (%)
Up regulated	3/135	Mn superoxide dismutase (Q88PA4)	6.93–6.63	5.60	1 / 1	100/96.8
	23/21	Fe superoxide dismutase (AAK14938)	6.93–6.63	5.55	4 / 3	100/99.9
	28/52	ATP synthase F1 α-subunit (Q88BX2)	5.84–5.71	5.38	7 / 5	95.7
	93/116	Glutamine synthetase type 1 (Q88CY3)	4.59–4.29	5.21	13 / 11	99.9/100
Down regulated	4/9	Transciption regulator MVAT, P16 subunit (Q88N50)	6.77–6.47	9.4	5 / 6	99.8/99.0

The protein spot numbers for the identified proteins are included from each of the two separate comparisons. The pH fraction in which the protein is contained is included with the calculated (theoretical) pI of the orthologue. The protein confidence interval is calculated by the MASCOT system, scores above 95% are considered significant and not the result of random matching

P. aeruginosa [25]. This appears consistent with our observation that MvaT is under-expressed by *P. chlororaphis* in static growth conditions where biofilms are produced compared to shake growth condition where biofilm formation is not apparent.

Four of the five proteins identified as being differentially expressed by rhamnolipid-producing *P. chlororaphis* strain NRRL B-30761 have an effect on biofilm formation. *Pseudomonas chlororaphis* strain NRRL B-30761 produces the most significant quantities of rhamnolipids when growing as a biofilm [6]. These observations suggest that this bacterium's mechanisms for controlling rhamnolipid production are also responsible for controlling the formation of biofilm.

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