Changes in protein expression in *Burkholderia vietnamiensis* PR1₃₀₁ at pH 5 and 7 with and without nickel

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Burkholderia vietnamiensis PR1₃₀₁ (PR1) exhibits pH-dependent nickel (Ni) tolerance, with lower Ni toxicity observed at pH 5 than at pH 7. The Ni tolerance mechanism in PR1 is currently unknown, and traditional mechanisms of Ni resistance do not appear to be present. Therefore, 2D gel electrophoresis was used to examine changes in protein expression in PR1 with and without Ni (3.4 mM) at pH 5 and 7. Proteins with both a statistically significant and at least a twofold difference in expression level between conditions (pH, Ni) were selected and identified using MALDI-TOF-MS or LC-MS. Results showed increased expression of proteins involved in cell shape and membrane composition at pH 5 compared with pH 7. Scanning electron microscopy indicated elongated cells at pH 5 and 6 compared with pH 7 in the absence of Ni. Fatty acid methyl ester analysis showed a statistically significant difference in the percentages of long- and short-chain fatty acids at pH 5 and 7. These findings suggest that changes in membrane structure and function may be involved in the ability of PR1 to grow at higher concentrations of Ni at pH 5 than at pH 7.

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INTRODUCTION

Nickel (Ni) is a common co-contaminant in soils and sediments due to anthropogenic pollution (e.g. mining, refining, metal plating) and natural emissions (e.g. volcanic activity and dust) (Nriagu, 1980; Adriano, 2001). High Ni concentrations also occur naturally in ultramafic

(serpentine) soils and sediments (Proctor & Woodell, 1975). These higher concentrations of Ni (micro- and millimolar concentrations) can be toxic to micro-organisms through the displacement of other divalent cations (e.g. Mg²⁺, Zn²⁺, Fe²⁺) from key enzymes (Hausinger, 1993). In micromolar quantities, Ni has also been shown to inhibit DNA replication, translation and transcription by limiting the supply of ATP (Guha & Mookerjee, 1979) and can cause oxidative damage in cells as a result of the formation of oxygen radicals (Sigler *et al.*, 1999). Because of its frequent occurrence at US Environmental Protection Agency National Priority List sites and its toxicity, Ni is ranked 53rd on the 2007 CERCLA priority list of hazardous substances (<http://www.atsdr.cdc.gov/cercla/07list.html>).

Exposure of bacteria to stressors, such as toxic metals or pH changes, can induce altered protein expression either

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Abbreviations: ACP, acyl carrier protein; AHL, acylhomoserine lactone; FAME, fatty acid methyl ester; PHA, polyhydroxyalkanoate; PR1, *Burkholderia vietnamiensis* PR1₃₀₁; SEM, scanning electron microscopy; SOD, superoxide dismutase; TFE, 2,2,2-trifluoroethanol.

Representative growth curves and additional 2D gels, details of proteins identified by MALDI-TOF-TOF and LC-MS/MS and further details of differential expression are available as supplementary material with the online version of this paper.

from damage done by the stressor or as a protective mechanism against the stressor. A transient increase in SOS, heat shock and oxidation stress proteins was observed in *Escherichia coli* after Cd exposure (Ferianc *et al.*, 1998). Similarly, *Synechococcus* sp. showed increased expression of the chaperonin protein GroEL when exposed to Cd, Zn or Cu (Ybarra & Webb, 1998). *Streptococcus oralis* displayed increased expression of ATP synthase and superoxide dismutase (SOD) when grown at pH 5.2 compared with growth at pH 7 (Wilkins *et al.*, 2001). Identification of proteins with altered expression levels when bacteria are exposed to stressors may lead to a better understanding of the stressor's target as well as the mechanisms of adaptation that allow survival and growth under these adverse conditions.

Environmental pH alone has been shown to be a strong predictor of microbial diversity and composition in soils (Fierer & Jackson, 2006). Many metal-contaminated sites are acidic due to co-disposal of acids or the natural acidity of the soils and sediments. Therefore, it is important to understand the effect of pH on micro-organisms and on metal toxicity. It has been shown previously that pH affects Ni toxicity to *Burkholderia vietnamiensis* PR1₃₀₁ (PR1), with higher toxicity observed at pH 7 compared with pH 5 and 6 (Van Nostrand *et al.*, 2005, 2007b). This pH-dependent Ni toxicity has also been observed in other, Gram-positive micro-organisms (Van Nostrand *et al.*, 2007a). This observed trend in Ni toxicity is not explained by Ni speciation predicted from thermodynamic modelling, suggesting that the mechanism of Ni resistance in PR1 is influenced by pH (Van Nostrand *et al.*, 2005). In addition, genes for known Ni efflux transporters (*cnr*, *ncc*, *nre*) were not detected in PR1 using primers designed based on their sequences deposited in GenBank (Van Nostrand *et al.*, 2005) or using a comprehensive 50mer functional gene microarray containing probes for *cnr*, *ncc*, *nre* and *ncr* (J. D. Van Nostrand, unpublished data). Therefore, a proteomics approach, using 2D gel electrophoresis, was utilized to examine protein expression in PR1 in the presence of Ni at pH 5 and 7 to help identify potential mechanisms of Ni tolerance.

METHODS

***Burkholderia vietnamiensis* PR1₃₀₁.** PR1 was provided by Dr Malcolm Shields (Idaho State University) and was grown in a modified minimum mineral medium (4M) designed to reduce metal complexation with medium components by use of a non-phosphate-containing buffer and glycerophosphate as an organic phosphate source (Van Nostrand *et al.*, 2005). Stocks of PR1 culture were stored and inoculum was prepared as described previously (Van Nostrand *et al.*, 2005). Freezer stocks were prepared prior to the start of proteomics experiments and a fresh freezer stock was used for each experiment to avoid freeze-thawing. Briefly, aliquots of freezer stocks were plated onto LB agar to confirm purity and then colonies were transferred to 4M and incubated for 24 h at 30 °C. An aliquot of this culture was transferred to fresh 4M and incubated for 12–14 h at 30 °C and centrifuged. The pellet was resuspended in fresh 4M to an

OD₆₁₀ of 2.2–2.4 (determined using a Beckman Coulter DU650 spectrophotometer) and used as inoculum.

Nickel exposure at pH 5 or 7. All glassware was acid-washed in 25 % HCl and rinsed with distilled water prior to use in experiments. Nickel stock solutions (62.5 mg ml⁻¹) were prepared using Ni(NO₃)₂·6H₂O (J. T. Baker) in deionized water and filter-sterilized (0.2 µm nylon; Fisher Scientific). Erlenmeyer flasks (125 ml) with Teflon-lined screw caps containing 24.75 ml 4M (pH 5 or 7) were inoculated with 250 µl microbial culture as described previously (Van Nostrand *et al.*, 2005). Cultures were shaken (200 r.p.m.) at 30 °C in the dark. Every 2–3 h, aliquots (600 µl) were removed and the OD₆₁₀ was measured to monitor growth. PR1 was grown to mid-exponential phase (4.5 h). Cultures were centrifuged (5468 g, 10 min) and the pellet was resuspended in 23.2 ml (25 ml starting volume minus 600 µl at each time point) fresh medium (4M, pH 5 or 7). Ni (final concentration of 3.41 mM) or the same volume of deionized water (74.2 µl) was added to the cultures and incubated for 3 h at 30 °C in the dark with shaking (200 r.p.m.). The cells were harvested by centrifugation (5468 g, 10 min) and the pellet was frozen in liquid N₂ and stored at –80 °C. Experiments were performed twice in triplicate to obtain six replicates under each condition (pH 5 and 7, with and without Ni). Because PR1 was grown to mid-exponential phase before Ni exposure, similar concentrations of protein were obtained at pH 5 and 7 even in samples with Ni (Supplementary Fig. S1; Van Nostrand *et al.*, 2005).

Exposure time to Ni was chosen based on a preliminary examination of the growth response of PR1. In these preliminary experiments, PR1 was exposed to 0, 0.85, 3.41 or 8.52 mM Ni. At higher concentrations of Ni (8.52 mM, pH 5; 0.85 mM, pH 7), growth inhibition occurred immediately following exposure and continued for 1–2 h afterwards before recovering and resuming growth at 3 h. Based on the hypothesis that proteins expressed during growth inhibition are the result of toxic effects of the metal while those proteins expressed following growth recovery are involved in metal resistance (Ferianc *et al.*, 1998), a 3 h Ni exposure was chosen for PR1. The Ni concentration examined in this study (3.41 mM Ni) resulted in different growth levels for PR1 at pH 5 and 7 (Van Nostrand *et al.*, 2005).

Protein extraction. Proteins were extracted from pellets using sonication and a mild detergent buffer (Molloy *et al.*, 1998; Deshusses *et al.*, 2003). Briefly, each pellet (obtained as described above) was suspended in 200 µl lysis buffer [11.6 mM Tris/HCl, 5.8 M urea, 2.3 M thiourea and 2.3 % CHAPS in 50 % 2,2,2-trifluoroethanol (TFE)]. These solubilization conditions were designed to extract both aqueous (cytosolic) proteins and less soluble proteins, including some membrane proteins (Molloy *et al.*, 1998). The co-solvent TFE was used to maintain the solubility of hydrophobic proteins during separation, thereby improving the quality of protein separation (Deshusses *et al.*, 2003). The samples were vortexed for 5 min and then placed into a room-temperature water-bath sonicator for 10 min. Vortexing and sonication were repeated twice. Samples were centrifuged (20 000 g, 15 min) and the supernatant was then transferred to a fresh microcentrifuge tube. Concentrations of total protein were determined by the method of Bradford (1976). Protein extracts were frozen in liquid N₂ and stored at –80 °C.

Two-dimensional gel electrophoresis. The protein concentration was chosen to optimize protein resolution on the gels (data not shown). Aliquots (64.8 µg) of extracted protein were incubated at room temperature for 30 min with 50 U Benzonase (Sigma) per 100 µl to degrade any DNA present in the sample. Samples were then brought up to 175 µl with TFE rehydration buffer (10 mM Tris/HCl, 5 M urea, 2 M thiourea and 2 % CHAPS in 50 % TFE). Aliquots of 40 % Bio-Lyte ampholytes (3/10; Bio-Rad) (1 µl, final concentration

0.2 %) and freshly prepared 1 M DTT (9 µl) were added and vortexed briefly. Samples were then centrifuged (100 000 g, 30 min) to remove any precipitates (e.g. proteins) that may have formed. No visible precipitate was present in any sample after centrifugation. The supernatant was removed and placed into a rehydration tray (Bio-Rad). IEF strips (pH gradients of 4–7, 11 cm, ReadyStrip IPG strips; Bio-Rad) were placed in the tray and incubated at room temperature for 1 h. The strips were covered with mineral oil and rehydrated overnight and then focused using a Protean IEF cell (Bio-Rad). Focusing was performed using linear increases in voltage from 0 to 250 V over 2 h, from 250 to 5000 V over 1 h and from 5000 to 8000 V over 2.5 h. This final voltage was held for 80 000 Vh. A maximum current of 50 µA per strip was used.

Once focused, the strips were equilibrated in buffer (0.375 M Tris/HCl, pH 8.8, 6 M urea, 2 % SDS, 20 % glycerol in deionized water) with 2 % DTT for 15 min and again in the same buffer with 2.5 % iodoacetamide for 15 min. The strips were run in the second dimension with either pre-poured gels (8–16 % acrylamide gradient, 13.3 × 8.7 cm, 1 mm thick, Criterion; Bio-Rad) or hand-poured gels using Criterion cassettes (4 % stacking, 12 % resolving gel; Bio-Rad protocol 4110106 Rev B). All gels were run at 100 V for 15 min and then at 200 V for approximately 1 h, until the dye front ran off the bottom of the gel.

Proteins were visualized using SYPRO Ruby (Bio-Rad) fluorescent staining (pre-poured gels) or with Coomassie blue staining (hand-poured gels). Gel cases were cracked open and the gels were rinsed twice with deionized water. All subsequent steps were performed on a rocker. Gels to be stained with SYPRO Ruby were fixed with a solution of 10 % methanol and 7 % acetic acid for 30 min and then rinsed twice with deionized water. SYPRO Ruby stain was added and incubated in the dark for at least 3 h and then destained in 10 % methanol and 7 % acetic acid for 30 min. Gels were then rapidly rinsed three times in deionized water and a final time for 10 min. Gels to be stained with Coomassie blue were incubated in filtered 40 % methanol, 10 % acetic acid and 0.035 % Coomassie brilliant blue R-250 for at least 6 h. The gels were then destained with several passages through destainer (40 % methanol, 10 % acetic acid).

Gel imaging and analysis. Gels were scanned with an FX Pro Plus fluorescent imager (Bio-Rad) and digitally analysed using PDQuest software (version 8.0; Bio-Rad). Spot intensities were normalized within PDQuest based on the total quantity of all valid spots on the gel. Protein expression from replicate ($n=6$) SYPRO Ruby-stained gels was compared at pH 5 and 7 with and without Ni and at 3 h exposure. Significant differences in protein expression were determined based on several criteria. First, a quantitative analysis was performed where proteins were chosen with at least a twofold difference in level of expression between conditions (i.e. pH, presence of Ni). Next, a statistical analysis was performed and proteins with a statistically significant (Student's *t*-test, $P<0.05$) difference were chosen. A Boolean analysis then chose proteins that showed both a quantitative and statistically significant change in expression between conditions. Those protein spots chosen based on the Boolean analysis were visually confirmed to be the same on each replicate gel.

Protein identification. Twenty-seven protein spots on the SYPRO Ruby-stained 2D gels met our predetermined criteria, outlined above, and were chosen for identification by MALDI-TOF-TOF or LC-MS-MS. One of the protein extracts from PR1 (pH 7, 0 mM Ni) containing all 27 protein spots was run on two hand-poured gels and stained with Coomassie blue. Since Coomassie blue is a less sensitive stain (10–15 ng protein detection limit) (Brush, 1998; Kang *et al.*, 2002) than SYPRO Ruby (1–2 ng protein) (Berggren *et al.*, 2000), only more abundant proteins were selected for further identification. Lanne & Panfilov (2005) found that Coomassie blue staining resulted in better MS results (higher signal-to-noise in spectra, more reliable

identifications and larger numbers of matching peptides) than SYPRO Ruby staining. Of the 27 protein spots chosen for identification, 17 were visible on the Coomassie blue-stained gels and were matched to the SYPRO Ruby-stained gels. These 17 proteins were excised using the ProteomeWorks spot cutter (Bio-Rad) and plugs representing the same protein spot were combined.

Excised proteins were digested in-gel with trypsin. Gel plugs were washed with 100 mM NH_4HCO_3 and then destained twice with 50 mM NH_4HCO_3 in 50 % acetonitrile for 10 min with vortexing. The gel plugs were washed for an additional 5 min with fresh 50 mM NH_4HCO_3 in 50 % acetonitrile and then dehydrated for 5 min in 100 % acetonitrile. The supernatant was removed and the plugs were allowed to dry for 10 min at room temperature. Aliquots of trypsin (10 µl of 12.5 ng trypsin μl^{-1} in 50 mM NH_4HCO_3 ; Promega) were added and incubated for 30 min at 4 °C to allow the gel pieces to absorb the trypsin and then the gel plugs and trypsin were transferred to 37 °C and incubated overnight. Supernatants were transferred to a fresh tube and the gel plugs were vortexed for 20 min in 10 µl 50 % acetonitrile and 2 % formic acid followed by water-bath sonication for 5 min at room temperature. The resultant solution was combined with the original digest supernatants and concentrated in a speedvac (Eppendorf Vacufuge) to <2 µl volume. The concentrate was brought up in 10 µl 0.1 % trifluoroacetic acid and sonicated for 30 min in a room-temperature water bath. Samples were further purified using ZipTips (C_{18} ; Millipore) following the manufacturer's protocol. The peptides were subsequently desalted and mixed with MALDI matrix (10 mg α -cyano-4-hydroxycinnamic acid in 1 ml acetonitrile and 1 ml 0.1 % trifluoroacetic acid). The peptides were identified using an Applied Biosystems 4700 Proteomics Analyser (MALDI-TOF-TOF) followed by a search using the Mascot search engine (Matrix Science) against the NCBI database using all taxonomies.

SYPRO Ruby-stained gel plugs were destained four times with 50 mM NH_4HCO_3 in 50 % acetonitrile for 20 min with vortexing. The plugs were washed and digested as described above with the exception that digested plugs were vortexed using 10 µl 50 % acetonitrile and 2 % acetic acid, instead of 2 % formic acid, and the digests were not purified with ZipTips. These protein digests were analysed using LC-MS-MS. The samples were injected onto a Waters Atlantis C18 reversed-phase column (2.1 × 50 mm and 5 µm particle size). Tryptic peptides were separated with a linear gradient using a Finnigan Surveyor MS pump (Thermo Electron) set to a flow rate of 200 µl min^{-1} . Mobile phases A and B were 0.1 % formic acid in water and acetonitrile with 0.1 % formic acid, respectively. The gradient used was as follows: 1 % B for 2 min, increasing to 33 % B over 58 min and increasing again to 80 % B over the next 5 min. The column effluent was directed into an LTQ ion trap MS (Thermo Electron) and MS-MS spectra were collected. Peptide MS-MS spectra were analysed against all protein sequences available for *B. vietnamiensis* G4 (<http://www.expasy.com>) using TurboSEQUEST (Thermo Electron). The following parameters were used during the database search: semi-tryptic cleavages allowed, variable deamination of the N terminus of the peptide, variable oxidation of methionine and variable carbamidomethylation of cysteine residues. Peptide identifications were considered to be correct for matches with Xcorr scores above 1.5, 2.5 and 3.0 for +1, +2 and +3 peptide ions, respectively, and for peptide probability scores below 0.1. Peptide identifications found above these thresholds were also verified manually.

Scanning electron microscopy (SEM). SEM was used to examine visually the influence of Ni and pH on PR1 morphology. Erlenmeyer flasks (125 ml) with Teflon-lined screw caps containing 4M (24.75 ml, pH 5, 6 or 7) amended with 0 or 3.41 mM Ni were inoculated with 250 µl microbial culture (Van Nostrand *et al.*, 2005). Cultures were shaken (200 r.p.m.) at room temperature (average

24 °C) for 30.5 h. A 500 µl aliquot of the culture was filtered through a 0.2 µm polycarbonate membrane (Isopore GTBP; Millipore) using a reusable syringe-filter holder. The cells were fixed with 2 % glutaraldehyde in MES buffer (100 mM MES, pH 6) for 2 h, rinsed three times with 100 mM MES buffer (1 ml MES, 10 min each rinse) and dehydrated with increasing concentrations of ethanol (25, 50, 75 and 100 %) for 10 min each. The membranes were stored in 100 % anhydrous ethanol, critical-point dried and carbon coated for imaging and elemental analysis using an LEO 982 field emission SEM.

Fatty acid analysis. PR1 cultures were grown and exposed to Ni or an equal volume of deionized water as described above. Cell pellets were transferred to hexane-rinsed glass culture tubes (8 ml) fitted with Teflon-lined screw caps. Fatty acids were saponified, methylated and extracted according to the Microbial Identification System protocol (Microbial ID) (Sasser, 2001; http://www.midi-inc.com/media/pdfs/TechNote_101.pdf). The resulting extract was analysed using an Agilent 6890N GC equipped with a flame-ionization detector and a 30 m Agilent HP-5 5 % phenyl methyl siloxane column (Agilent Technologies). Helium was used as the carrier gas. A 37-component fatty acid methyl ester (FAME) mixture (Supelco) was used as a standard and data were analysed with GC Chem Station (Agilent Technologies). Statistical analysis was performed using SAS version 9.1.3.

RESULTS

Examination of protein expression levels

Quantitative and statistical analysis of changes in protein expression in PR1 at pH 5 and 7 with and without 3.4 mM Ni revealed 27 unique spots on SYPRO Ruby-stained 2D gels with both a statistically significant ($P < 0.05$) and at least a twofold difference in level of expression between conditions (Table 1; see also Supplementary Tables S1 and S2). A representative gel is shown in Fig. 1(a) with these 27 spots indicated (replicate gels produced similar protein patterns; Supplementary Fig. S2). Seventeen of the spots were visible using Coomassie blue staining of the hand-poured gels, with 12 identified using MALDI-TOF-TOF (Supplementary Table S1). Proteins that were not visible by Coomassie blue staining ($n = 10$) or were not identified by MALDI-TOF-TOF ($n = 5$) were analysed by a complementary technique, LC-MS-MS. These 15 protein spots were excised from replicate SYPRO Ruby-stained gels. Spots from five or six gels were combined to increase the amount of protein for analysis and digested in-gel with trypsin. Six spots out of the 15 were identified by LC-MS-MS (Supplementary Table S1). Identified and unidentified proteins are listed in Table 1.

Two of the identified proteins and three unidentified proteins showed increased expression at pH 5, while 11 identified proteins and one unidentified protein were downregulated (Table 1; actual spot intensity values for each spot are presented in Supplementary Table S2). After Ni exposure, six identified and four unidentified proteins showed increased expression and three identified and two unidentified proteins were decreased. Several proteins showed different expression levels (increased or decreased)

after exposure to Ni at different pH values. For example, spot 6305 increased at pH 5 with Ni exposure but decreased with Ni exposure at pH 7.

Some of the protein spots resulted in more than one significant identification (Table 1). For example, the same protein spot identified as SOD was also identified as methane/phenol/toluene hydroxylase. This is not unusual, since there may be as many as six different proteins per spot on a 2D gel (Gygi *et al.*, 2000). In addition, in some cases, separate spots were identified as the same protein (Fig. 1b). Post-translational modification or modifications made during processing may result in a change in molecular mass or pI, causing the same protein to localize at different sites on the 2D gel (Gygi *et al.*, 2000). The estimated molecular masses for most of the protein spots were similar to the expected masses of the identified proteins (Supplementary Table S1), although most of the estimated pI values were greater than expected. This may be due to modifications made during processing (Gygi *et al.*, 2000) or to inexact estimates based on separation by the IEF strip.

Morphological and fatty acid composition changes in PR1

Because several of the identified proteins were involved in cell shape and membrane composition, SEM and FAME analysis were used to observe changes in morphology and fatty acid composition at pH 5, 6 and 7. SEM was used to observe qualitative changes in PR1 morphology after 30.5 h of growth (stationary phase) (Fig. 2). In the absence of Ni, PR1 grown at pH 5 and 6 are similar in appearance, with typical bacilli approximately 2.5 µm long; cells grown at pH 7 are visibly shorter (< 2 µm long). In the presence of 3.41 mM Ni, cells grown at pH 5 are similar in appearance to cells grown at the same pH without Ni, which correlates with similar growth under the same conditions. At pH 6, cells grown with 3.41 mM Ni are shorter (< 2 µm) than cells grown in the absence of Ni at the same pH (2.5 µm) and have a shrivelled and more rounded appearance. At pH 7, with 3.41 mM Ni, very few cells are visible, consistent with the lack of growth surmised from OD measurements (OD_{610} remained at 0.1 throughout the course of growth experiments) (Van Nostrand *et al.*, 2005; data not shown).

FAME analysis revealed statistically significant differences in the percentages of fatty acids in PR1 at pH 5, 6 and 7 (Fig. 3). Overall, there was a trend of increased percentages of long-chain fatty acids as the pH increased, while greater percentages of short-chain fatty acids were present as the pH decreased. This is illustrated by the statistically significant ($P < 0.05$) decrease in the C16:0 fatty acid from 22 to 17 % as the pH was increased from 5 to 7. In contrast, the C18:1n9t fatty acid increased ($P < 0.05$) from 27 to 31 % as the pH was increased from 5 to 7.

Table 1. Proteins that showed differential expression as a function of pH and Ni exposure

Relative protein differences are based on the mean intensities of protein spots as determined by image analysis (see Supplementary Table S2). * and † denote a statistically significant difference in intensity between two conditions.

Spot	Protein identification	Identification method	Function	Relative protein level			
				pH 5		pH 7	
				0 mM Ni	3.41 mM Ni	0 mM Ni	3.41 mM Ni
2205	Phasin	MS-TOF-TOF		+ †	+	+++*†	+/-*
2206	Not identified			+	+++	+++†	+†
2504	Methane/phenol/toluene hydroxylase	LC-MS-MS		+/-†	+	+++†	++
2605	Putative rod-shape determining protein; actin-like ATPase	MS-TOF-TOF	Shape determination/cell morphogenesis	+*†	+++†	+++*	+++
3202	Methane/phenol/toluene hydroxylase; Mn and Fe superoxide dismutase	LC-MS-MS	Stress response	+/-	++	+†	+++†
3703	Translation elongation factor EF-Tu; GTPase translation elongation factor	MS-TOF-TOF	Translation/stress response	+†	+++†	+++	+++
3806	F ₀ F ₁ -type ATP synthase, β subunit	MS-TOF-TOF	Transporter	+†	+++†	++	+++
4102	Hypothetical protein	LC-MS-MS		+++	++	+++†	+†
4403	Not identified			+	+++†	+	+/-†
4701	3-Oxoacyl-(acyl-carrier-protein) synthase	MS-TOF-TOF	Fatty acid synthesis	+/-†	+	+++†	+++
4705	Beta-ketoacyl synthase/3-oxoacyl-(ACP) synthase	LC-MS-MS	Fatty acid synthesis	+†	+	+++†	+++
4806	F ₀ F ₁ -type ATP synthase, α subunit	LC-MS-MS	Transporter	+/-	+/-†	+	+++†
5001	Not identified			++	+	+†	+++†
5202	GTPase translation elongation factor; translation elongation factor EF-Tu	MS-TOF-TOF	Translation	+++†	++	+†	+++
5305	Not identified			++	+++†	++	+†
5608	Phenylalanyl tRNA synthase, α subunit	MS-TOF-TOF	Translation	+†	+++†	++	++
5804	Betaine-aldehyde dehydrogenase; hypothetical protein; F ₀ F ₁ -type ATP synthase, α subunit	LC-MS-MS	Transporter	+/-*†	+++†	+++*	+++
6305	Acylhomoserine lactone synthase BviI	MS-TOF-TOF	Quorum sensing	++	+++†	+++*	+*†
6507	Lactylglutathione lyase; catechol 2,3-dioxygenase	MS-TOF-TOF		+†	++	+++†	+++
6606	Penicillin tolerance protein	MS-TOF-TOF	Shape determination	+†	++	+++†	+++
6806	Not identified			+++	+++	+/-†	+++†
7405	Not identified			+++†	+++	+†	+
7410	Esterase; hydrolase/acyltransferase	MS-TOF-TOF		+†	++	+++†	+++
7509	Not identified			+/-†	+++†	+/-	+++
7704	Hypothetical protein	MS-TOF-TOF		+†	++	+++*†	+
8502	Not identified			+	++	+†	+++†

Fig. 1. Location of identified protein spots on 2D gels. (a) Representative gel of protein extracts from PR1 grown at pH 7 and exposed to 3.41 mM Ni for 3 h. After exposure, proteins were separated on a pH 4–7 gradient. Marked protein spots displayed both a statistically significant and at least a twofold difference in level of expression between conditions as follows: pH 5, 0 mM Ni vs pH 5, 3.41 mM Ni (○); pH 5, 0 mM Ni vs pH 7, 0 mM Ni (△); pH 5, 3.41 mM Ni vs pH 7, 3.41 mM Ni (□); pH 7, 0 mM Ni vs pH 7, 3.41 mM Ni (◇). (b) pH 4–7 gradient 2D gel with identified protein spots indicated. Numbers along the vertical edges indicate molecular mass (kDa); those along the horizontal edge indicate pI.

DISCUSSION

Protein expression upregulated at pH 5 compared with pH 7 (without Ni)

At pH 5 in the absence of Ni, GTPase translation elongation factor and translation elongation factor EF-Tu (SSP 5202) showed increased levels of expression compared with pH 7 (Table 1). These proteins are involved in adding amino acids during translation of mRNA (Lodish *et al.*, 2000). This suggests that additional proteins are being synthesized under acid stress. *Streptococcus mutans* showed increased levels of EF-Tu at pH 5 compared with pH 7

when grown anaerobically (Len *et al.*, 2004). In addition to functioning as a translation elongation factor, EF-Tu has been shown to be involved in chaperone-like activities, assisting in folding and decreasing protein aggregation (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998). EF-Tu could be functioning in a similar manner in PR1, assisting in the folding or refolding of damaged or newly synthesized proteins in addition to its role in protein synthesis.

Proteins downregulated at pH 5 compared with pH 7 (without Ni)

According to our selection criteria, more proteins were downregulated than upregulated in PR1 at pH 5 than at pH 7 (Table 1). The protein identifications indicated that these downregulated proteins are involved in transport, cellular shape, fatty acid synthesis and carbon degradation. For example, an F_0F_1 -type ATP synthase (F-type ATPase; SSP 4806) was downregulated at pH 5. The F-type ATPases either produce ATP via loss of proton-motive force (i.e. an ATP molecule is created when protons are transported into the cell) or generate the proton-motive force by hydrolysing ATP (i.e. an ATP molecule is used to transport protons out of the cell). At pH 5, *Helicobacter pylori* showed a downregulation of ATPases, presumably to decrease proton entry into the cell (Bury-Moné *et al.*, 2004). Downregulation of ATPase in PR1 at pH 5 may similarly be a result of pH-regulatory functions in the cytoplasm.

Putative rod-shape determining protein (MreB) and actin-like ATPase (SSP 2605), involved in cell shape determination, were also downregulated at pH 5 (Table 1). Altered expression of these proteins suggests that cellular morphology is altered in acidic environments. The rod-shape determining protein (MreB, RodY) is an actin homologue (Thanbichler *et al.*, 2005). *E. coli* cells depleted of MreB had a shorter, more rounded appearance and eventually lysed, indicating that this protein is critical for cell survival (Kruse *et al.*, 2005). MreB also appears to be involved in cell-wall synthesis, regulating cell diameter (Formstone & Errington, 2005). Changes in cell shape may decrease the reactive surface area of the cell. A more rounded shape would decrease the surface area of the cell relative to cell volume (Dusenbery, 1998), thus potentially reducing the surface-to-volume ratio and limiting the diffusion of substances across the membrane. Based on these observations, it is somewhat surprising that PR1 cells were more elongated at pH 5 than at pH 7 (Fig. 2) even though the MreB homologue was downregulated. SEM images taken at 24 h in the absence of Ni did not show a difference in cell size between pH 5 and 7 (B. A. Neely, unpublished data). The observed decrease in MreB-like protein in PR1 may not have been sufficient to change the cell shape; it may serve a less important role in PR1 than in *E. coli* or the MreB-like protein may be active at an earlier growth phase, but not during stationary phase.

Penicillin tolerance protein (IspH/LytB) (SSP 6606), also downregulated at pH 5 (Table 1), has been shown to be

Fig. 2. SEM images of PR1 grown at pH 5, 6 or 7 at 0 or 3.41 mM Ni for 30.5 h. Bars, 5 μ m. Original magnification, $\times 3000$.

required for synthesis of an important intermediary in isoprenoid biosynthesis in *E. coli* via the 2-C-methylerythritol 4-phosphate pathway (Altincicek *et al.*, 2001). This pathway is also used by *Burkholderia* species (Putra *et al.*, 1998). Isoprenoids are ubiquitous in bacteria and perform a variety of biochemical functions including as membrane components, quinones in electron transport and pigments. Isoprenoids within the membrane affect membrane fluidity (Chamberlain *et al.*, 1991; Kannenberg & Poralla, 1999). For example, an increase in carotenoids in *Staphylococcus aureus* decreased membrane fluidity (Chamberlain *et al.*, 1991). The decrease of IspH/LytB in PR1 may alter membrane fluidity, which could, in turn, affect membrane permeability (discussed below), although the isoprenoids could be performing a different function.

Another protein that was downregulated at pH 5 was phasin (Table 1; SSP 2205), a low-molecular-mass protein associated with the production of polyhydroxyalkanoates (PHAs), which can be used to produce biodegradable

plastics (York *et al.*, 2001a). Phasin is only found in association with PHAs (Wieczorek *et al.*, 1995) and appears to be regulated by the amount of PHA in the cell; as the PHA level decreases, phasin decreases (York *et al.*, 2001b; Tian *et al.*, 2005). PHAs are thought to be carbon and energy storage polymers and are accumulated when carbon is in excess and when adverse conditions, such as nutrient or oxygen limitation, are present (Lee, 1996). As PR1 was grown to mid-exponential phase and then transferred to fresh medium for 3 h prior to collection, nutrient limitation would not be expected, especially since exponential growth would normally continue for an additional 3–4 h (Van Nostrand *et al.*, 2005; data not shown). Additionally, highest expression of phasin, and most likely PHAs, occurred at pH 7 in the absence of Ni, presumably the least stressful of the conditions examined in this study. This may be part of a natural cycle of increasing and decreasing levels of PHAs (and phasins) in bacterial cells, as suggested by Tian *et al.* (2005).

Fig. 3. Changes in fatty acid composition with change in pH. Results are presented as percentages of the total amount of fatty acids detected. Error bars indicate the standard deviation of triplicate samples. Different letters indicate statistically significant differences (analysis of variance followed by Tukey's test; $P < 0.05$) between pH values.

Another protein that was downregulated at pH 5 compared with pH 7 is 3-oxoacyl-[acyl carrier protein (ACP)] synthase (β -ketoacyl-ACP synthase) (Table 1; SSP 4701), a protein involved in fatty acid synthesis. Fatty acids in bacteria are primarily utilized as components of the cellular membrane. *Alcanivorax borkumensis* cells grown on hexadecane showed increased expression of 3-oxoacyl-ACP synthase compared with cells grown on pyruvate, presumably to maintain membrane structure and fluidity as the hexadecane was being degraded (Sabirowa *et al.*, 2006). The altered level of 3-oxoacyl-ACP synthase expression suggests that PR1 may be making changes to its membrane. Bacteria are known to modify their cellular membrane structure and composition at acid pH in order to protect the cell (Quivey *et al.*, 2000; Fozo & Quivey, 2004; Leaphart *et al.*, 2006). A more rigid membrane has been observed in acidic environments as a way to reduce proton permeability (Hornbaek *et al.*, 2004). A less rigid membrane allows more movement within the phospholipid bilayer, trapping water molecules within the membrane, which then provide a way for protons to enter the cell via transfer between the water molecules (Konings *et al.*, 2002). Examination of the cell-wall structure of *Shewanella putrefaciens* by atomic force microscopy demonstrated an increase in thickness from ~60 to ~140 nm as pH increased from 4 to 10 (Gaboriaud *et al.*, 2005). This increased thickness resulted in a less rigid membrane structure. At pH 8, *Bacillus licheniformis* showed increased transcription of genes involved in fatty acid and phospholipid synthesis (Hornbaek *et al.*, 2004). A majority of the proteins identified as having an increased level of expression at acid pH from *Vibrio cholerae* were involved in cell-envelope structure and function (Hommais *et al.*, 2002); however, 3-oxoacyl-ACP synthase was not detected. Similarly, *Shewanella oneidensis* has been shown to increase transcription of genes involved in cell-membrane structure and composition when exposed to acid stress (Leaphart *et al.*, 2006). PR1 may also alter the membrane structure at pH 5 in order to decrease membrane permeability.

FAME analysis of PR1 at pH 5, 6 and 7 showed that PR1 increased the relative concentration of long-chain fatty acids (C17:0, C18:3n6, C18:1n7, C20:4n6) as the pH

increased from 5 to 7, while the relative concentration of short-chain fatty acids (C10:0, C11:0, C12:0, C13:0) decreased (Fig. 3). In contrast, Fozo & Quivey (2004) observed a decrease in the amount of short-chain saturated fatty acids relative to long-chain monounsaturated fatty acids in *Streptococcus mutans* as the pH increased from 5 to 6.5. Cells that had shifted to long-chain fatty acids demonstrated increased survival at pH 2.5 compared with cells that were unable to make the shift (Fozo & Quivey, 2004). Additionally, the function of ATPase has been shown to increase as the membrane increases in fluidity (Storch & Schachter, 1984), and carrier molecules in the membrane can be inhibited by the decreasing fluidity of the membrane (Simkiss & Taylor, 1995). While the changes in relative abundance of fatty acids in PR1 over the pH range 5–7 may cause a change in membrane fluidity, the presence of shorter fatty acids may also allow for a more compact membrane at pH 5, which could reduce permeability.

Proteins upregulated by Ni

An increase in expression of proteins involved in translation when PR1 was exposed to Ni (Table 1) suggests that PR1 synthesized new proteins either to reduce toxicity or to replace damaged proteins, similar to that observed at pH 5. The EF-Tu elongation factor (SSP 3703) has been linked to Ni and Co resistance in *Serratia marcescens*, although its role in resistance is unclear (Marrero *et al.*, 2006). Resistance to Ni and Co was reduced by at least half in *S. marcescens* when EF-TuA was disrupted. As discussed above, MreB (SSP 2605) appears to be involved in cell-wall synthesis (Formstone & Errington, 2005). The presence of Ni has been shown to alter the cell morphology of some micro-organisms. Exposure of *Arthobacter marinus* to 0.4 mM Ni resulted in a shorter, more rounded morphology after 1.5 h (Cobet *et al.*, 1971). In addition, penicillin-binding protein, involved in peptidoglycan synthesis, is involved in Ni resistance based on metagenomic analysis of Ni-resistant microbial communities (Mirete *et al.*, 2007). Clones with penicillin-binding protein genes showed greater Ni resistance than the control. This finding suggests that cell size and shape may be important in Ni resistance. While expression of shape-determining proteins in PR1

was affected by Ni, PR1 cells grown in the presence of 3.4 mM Ni at pH 5 were similar in size and shape to cells grown at pH 5 in the absence of Ni (Fig. 2). Differences in cell size were noted, however, in the presence of Ni at pH 6. As stated previously, the level of MreB at pH 5 may not have been sufficient to produce a visible change in shape.

Interestingly, two F-type ATP synthases (SSP 3806 and 5804) were also upregulated by Ni at pH 5. The F-type ATPases are not known to transport metals, unlike P-type ATPases (Nies, 2003). Therefore, it is unlikely that the increase in F-type ATP synthase is due to expression of a Ni transporter. As such, it is unclear why this type of transporter would be upregulated in the presence of Ni, although they may be involved in a general stress response. Ni toxicity has been shown to limit the amount of ATP available to the cell (Guha & Mookerjee, 1979). Therefore, PR1 may be increasing the number of F-type ATP synthases in order to maintain a sufficient ATP pool within the cell.

SOD (SSP 3202) was upregulated in the presence of Ni at pH 7. This was not unexpected, as it has been previously shown that metals, including Ni, can cause oxidative damage through the formation of oxygen radicals (Sigler *et al.*, 1999). *E. coli* mutants deficient in SOD were unable to grow with 0.1 mM Ni, while wild-type cells were able to grow at this concentration of Ni (Geslin *et al.*, 2001). Exposure to aluminium citrate resulted in increased SOD activity in *Pseudomonas fluorescens* (Singh *et al.*, 2005). An increase in SOD also occurred in PR1 in the presence of Ni at pH 5, although this was not significant (Fig. 4a). The increase in SOD observed in PR1 is most likely to be the result of oxidative damage caused by Ni.

Proteins downregulated by Ni

Expression of an acylhomoserine lactone (AHL) synthase (SSP 6305), BviI, was downregulated at pH 7 in the presence of 3.4 mM Ni compared with the other conditions examined (Table 1; Fig. 4b). AHL synthases produce AHLs, molecules involved in quorum sensing that bind to a transcriptional activator, allowing targeted genes to be up- or downregulated (Miller & Bassler, 2001). The AHLs are freely diffusible across the cell membrane and are produced continuously at low levels. As the density of bacteria increases, the concentration of AHLs increases as well. Members of the *Burkholderia cepacia* complex are known to have two quorum-sensing systems, *cepIR* (Lewenza *et al.*, 1999) and *BviIR* (Dennis & Zylstra, 1998). The *cepIR* system is found widely throughout the *B. cepacia* complex, while the *BviIR* system is found only in *B. vietnamiensis* (Lutter *et al.*, 2001) and is the primary quorum-sensing system in *B. vietnamiensis* G4 (Conway & Greenberg, 2002). Little is known regarding the genes controlled by the *BviIR* system (Malott & Sokol, 2003, 2007); however, some antibiotic resistance may be controlled by the *BviIR* system, while siderophore production and toluene *ortho*-

Fig. 4. Expression level of BviI (AHL synthase) (a) and SOD (manganese and iron superoxide dismutase) (b). Protein expression was determined by signal intensity, expressed in counts by PDQuest software (Bio-Rad). Data represent the means and standard deviations of six replicate gels. Bars marked with * or + displayed both a statistically significant (Student's *t*-test, $P < 0.05$) and at least a twofold difference in expression when compared between two conditions.

monooxygenase are not (Park *et al.*, 2001). Although it is tempting to speculate that the *BviIR* system may control genes that alter the cell in ways that increase Ni resistance, the most likely explanation is that AHL synthase did not increase due to the lack of growth at pH 7 and 3.4 mM Ni (Van Nostrand *et al.*, 2005; data not shown).

Summary – changes in protein expression that lead to Ni tolerance

The protein expression changes in PR1 at pH 5 compared with pH 7, namely those involved in cell shape determination and membrane structure, appear key to the increased Ni tolerance observed in PR1 at pH 5 compared with pH 7. An increase in the percentage of long-chain fatty acids was observed using FAME analysis as the pH increased from pH 5 to 7. Similar changes in membrane composition have been shown to be involved in decreasing the permeability of the membrane to protons (Hornbaek *et al.*, 2004). These changes could also inhibit the function of transporters (Storch & Schachter, 1984; Simkiss & Taylor, 1995), including those involved in bringing Ni into the cell. In addition, porins, which are located in the outer membrane and allow small molecules to diffuse into the periplasm, have been shown to change from an open formation to a closed one as the environmental pH becomes more acid ($< \text{pH } 6$) (Todt *et al.*, 1992; Müller & Engel, 1999), further limiting the entry of Ni into the cell at

low pH. A decrease in internalized Ni could explain the decreased Ni toxicity observed in PR1 at pH 5 compared with pH 7. Since pH is a significant environmental parameter (Fierer & Jackson, 2006), a better understanding of its effect on micro-organisms is critical. In addition, since metal-contaminated sites are often acidic, a better understanding of how microbial responses to pH affect metal-tolerance mechanisms is needed to manage these sites effectively. A study of Ni-susceptible PR1 mutants is under way and should provide insights into the specific mechanism of Ni tolerance in PR1.

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