Proteomic detection of proteins involved in perchlorate and chlorate metabolism

Reema Bansal · Lee A. Deobald · Ronald L. Crawford · Andrzej J. Paszczynski

Abstract Mass spectrometry and a time-course cell lysis method were used to study proteins involved in perchlorate and chlorate metabolism in pure bacterial cultures and environmental samples. The bacterial cultures used included *Dechlorosoma* sp. KJ, *Dechloromonas hortensis*, *Pseudomonas chloritidismutans* ASK-1, and *Pseudomonas stutzeri*. The environmental samples included an anaerobic sludge enrichment culture from a sewage treatment plant, a sample of a biomass-covered activated carbon matrix from a bioreactor used for treating perchlorate-contaminated drinking water, and a waste water effluent sample from a paper mill. The approach focused on detection of perchlorate (and chlorate) reductase and chlorite dismutase proteins, which are the two central enzymes in the perchlorate (or chlorate) reduction pathways. In addition, acetate-metabolizing enzymes in pure bacterial samples and housekeeping proteins from perchlorate (or chlorate)-reducing microorganisms in environmental samples were also identified.

Keywords Chlorine oxyanions ·
Perchlorate reductase · Chlorate reductase ·
Chlorite dismutase · Proteomics · Mass spectrometry

R. Bansal · L. A. Deobald · R. L. Crawford ·
A. J. Paszczynski (✉)
Environmental Biotechnology Institute, University
of Idaho, Moscow, ID 83844-1052, USA
e-mail: andrzej@uidaho.edu

Introduction

Compounds containing perchlorate have been used as a component of solid rocket and missile propellants and also have other industrial applications (Gu and Coates 2006). Perchlorate salts fully ionize in water, are very stable, non-volatile and nonreactive. Therefore, perchlorate spreads quickly throughout aqueous systems such as aquifers. Groundwater, surface water and soil in 35 U.S. states and Puerto Rico have been found to be contaminated with perchlorate (U.S. EPA 2005). The Government Accountability Office reported perchlorate contamination of drinking water supplies in 28 states, at concentrations ranging from 4 to 420 ppb (Houlihan et al. 2007; Government Accountability Office 2005). Recently the United States Food and Drug Administration reported that 75% of 300 food items and beverages, like lettuce, spinach, carrots, tomatoes, milk, fruit juices, and spring and bottled water tested positive for perchlorate (Murray et al. 2008; Wood 2008). The majority of perchlorate contamination is due to anthropogenic activities. Perchlorate can impair proper functioning of the thyroid glands at concentrations as low as 24 ppb (Wood 2008). It competitively inhibits iodine uptake by the thyroid glands, by blocking the sodium-iodide symporter protein on thyroid follicle cells (Sellers et al. 2007; National Research Council 2005). Biological reduction of perchlorate by microorganisms is the most widely used treatment technology for perchlorate contamination. The perchlorate (or

chlorate) reduction pathway in all microorganisms previously studied to date consists of two central enzymes: perchlorate (or chlorate) reductase and chlorite dismutase (CD) encoded by the *pcrABCD* (or *clrABCD*) operon and the *cld* gene, respectively (Gu and Coates 2006).

Proteomics is widely used to generate qualitative and quantitative information about proteins from pure cultures or complex biological mixtures (Aristoteli et al. 2007; Baldwin 2004). It can provide insight into the dynamics of biological systems. Moreover, it is known that more than one functional protein can result from a single gene. Post-translational modification can also contribute to protein complexity expressed by cells. Thus, it is important to study proteomics along with genomics and metabolomics (Singh 2006). Proteomics experiments can be applied to a variety of areas including drug target discovery, disease diagnosis, phenotypic trait analysis, and characterization of metabolic dynamics of complex environmental samples (Caprioli and Gross 2005). Environmental proteomics, also known as ecoproteomics, can be used to study natural microbial communities, their structure, metabolic activities, gene expression, genetic exchange and recombination patterns (Lo et al. 2007; Ram et al. 2005). Ecoproteomics can reveal signature proteins or peptides that are expressed by microorganisms in response to specific conditions. The expression of proteins involved in contaminant degradation pathways can be linked to presence of pollutants and processes mediating their natural attenuation. Proteomic analyses can provide protein profiles of a contaminated system prior to, during, and after the application of a remediation technology to gain insight into the degradation processes that are being investigated. Moreover, progress and success of a cleanup strategy can be monitored using proteomics-based information.

Mass spectrometry with electrospray ionization (ESI-MS/MS) in combination with ultra performance liquid chromatography is a sensitive and fast technique (Caprioli and Gross 2005). MS determines peptide amino acid sequences and peptide masses, which can then be used for identification of the proteins from which the peptides were derived. In this study we applied MS-based proteomics techniques to study perchlorate and chlorate reduction in bacterial cultures. Perchlorate (and chlorate) reductase and chlorite dismutase amino acid sequences from a number of

microbes are present in the publicly available databases Swiss-Prot and TrEMBL. TrEMBL (Translated EMBL nucleotide sequences) is a subdivision of Swiss-Prot. In addition, all the publicly available perchlorate (and chlorate) reductase and chlorite dismutase sequences were obtained. These sequences along with chlorite dismutase (*Dechlorosoma* sp. KJ, *Dechloromonas hortensis*, *Pseudomonas chloriti-dismutans* ASK-1 and *Pseudomonas stutzeri*) and perchlorate reductase (*Dechlorosoma* sp. KJ) sequences previously sequenced in our lab (Bansal 2009) were combined into a database that was searched using ProteinLynx Global Server 2.3 software (PLGS) (Waters, Milford, MA, USA).

Pure cultures used included *Dechlorosoma* sp. KJ, *Dechloromonas hortensis*, *Pseudomonas chloriti-dismutans* ASK-1, and *Pseudomonas stutzeri* (=*Pseudomonas chloritidismutans* AW-1). Mixed-culture-based environmental samples that were examined included an anaerobic sludge enrichment culture, a sample of a biomass-covered activated carbon matrix taken from a bioreactor used for treating perchlorate-contaminated drinking water, and a waste water effluent sample obtained from a paper mill. *Dechlorosoma* sp. KJ is a known perchlorate degrader. *Dechloromonas hortensis* can reduce both perchlorate and chlorate, while *Pseudomonas chloritidismutans* ASK-1 and *Pseudomonas stutzeri* can use chlorate as an electron acceptor for anaerobic respiration. The different environmental samples were chosen to determine if they have the potential to degrade perchlorate and might be used as sources for isolating novel perchlorate-reducing microbes. The approach used here focused on detection of perchlorate (and chlorate) reductase and chlorite dismutase proteins. In addition, acetate-metabolizing enzymes in pure bacterial samples were also detected as acetate was the sole electron donor used. For environmental samples, we focused on identification of housekeeping proteins previously observed in perchlorate (or chlorate)-reducing microorganisms. The presence of housekeeping proteins would further confirm the above results that the samples contain perchlorate (or chlorate)-reducing microorganisms. A novel time-course lysis method developed previously in our lab was used to extract whole cell proteins from the samples (Paidisetti 2007). This method is based on the fact that environmental samples contain a variety of microorganisms, some of which may be

more resistant than others to chemical or enzymatic lysis. Lysis in a step-wise fashion allows one to avoid overlooking proteins released from hard-to-lyse cells that might be masked by more abundant proteins released early from easily lysed cells. Results confirmed that proteomics-based methods are excellent tools for monitoring expression of perchlorate and chlorate-reducing enzyme systems in pure and mixed cultures, including environmental samples. Perchlorate and chlorate degradation in pure bacterial cultures and enrichment cultures was also monitored by measuring removal of chlorine oxyanions and by studying bacterial growth during the degradation period.

Materials and methods

Growth and maintenance of bacterial strains

Pure bacterial cultures

Bacterial strains were grown and maintained on the culture media recommended by the culture collections from which the cultures were obtained. *Dechlorosoma* sp. KJ (BAA 592) was grown in ATCC 2361 liquid medium under anaerobic conditions at 25°C with shaking at 200 rpm. *Dechloromonas hortensis* (DSMZ no. 15637) was grown on DSMZ 830a liquid medium under microaerophilic conditions in a jar with a GasPak (BD GasPak^TM EZ Campy container system) at room temperature ($\sim 25°C$). *Pseudomonas chloritidismutans* ASK-1 (BAA 775) was grown in tryptic soy broth under aerobic conditions at 25°C with shaking at 200 rpm. *Pseudomonas stutzeri* (DSM-No. 13592) (=*Pseudomonas chloritidismutans* AW-1) was grown on DSMZ 830-R2A liquid media under aerobic conditions at 30°C with shaking at 200 rpm. All the above media were prepared and sterilized as recommended by either ATCC (American Type Culture Collection) or DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, German Collection of Microorganisms and Cell Cultures). For studies of perchlorate or chlorate reduction, actively growing bacterial cells from log phase cultures in maintenance media were used to inoculate liquid media containing perchlorate or chlorate as the only electron acceptor. *Dechlorosoma* sp. KJ and *Dechloromonas hortensis* were

grown on ATCC 2106 medium supplemented with 500 ppm of ammonium perchlorate as electron acceptor and 20 mM sodium acetate as electron donor. *Pseudomonas chloritidismutans* ASK-1 and *Pseudomonas stutzeri* were grown on DSMZ 944 medium with 1,000 ppm of chlorate and 20 mM of acetate. The cultures (20 ml) were grown anaerobically in 25 ml stoppered tubes and incubated at room temperature without shaking.

Environmental samples

Mixed culture microbiological samples included an anaerobic sewage sludge enrichment culture prepared by inoculation of minimal ATCC 2106 medium supplemented with 500 ppm ammonium perchlorate (electron acceptor) and 20 mM lactate (electron donor) with a sludge sample obtained from a sewage treatment plant in the city of Moscow (Idaho, USA). Other undefined mixed cultures included an aliquot of a biomass-covered activated carbon matrix taken from a bioreactor being used to treat perchlorate contaminated drinking water (Brown et al. 2007) and a sample of waste water taken from a paper mill effluent treatment basin (Idaho, USA). The latter two cultures also were inoculated into ATCC 2106 media supplemented with 500 ppm ammonium perchlorate, but using 20 mM sodium acetate as the electron donor rather than lactate.

Growth curves and degradation of chlorine oxyanions

Dechlorosoma sp. KJ was grown in ATTC 2106 liquid media supplemented with 500 ppm ammonium perchlorate and 20 mM sodium acetate. *Pseudomonas chloritidismutans* ASK-1 and *Pseudomonas stutzeri* were grown in media DSMZ 944 media with 1,000 ppm of chlorate and 20 mM sodium acetate. An enrichment culture inoculated with anaerobic sludge obtained from a municipal sewage treatment plant (see above) was sub-cultured into ATTC 2106 media supplemented with 500 ppm ammonium perchlorate and 20 mM lactate. Growth was measured by monitoring absorbance at 600 nm (OD_{600}) and perchlorate/chlorate degradation was monitored at regular time intervals using ESI (electrospray ionization) mass spectrometry. A Micromass Quattro II

triple quadrupole mass spectrometer with electrospray ionization source (Waters Corporation, Milford, MA, USA) was used for sample analysis. The sample was delivered to the source using a syringe pump. The instrument was controlled by MassLynx V4.0 software (Waters Corporation, Milford, MA, USA). The following mass spectrometer settings were used: capillary voltage 2.80 kV, cone voltage 15 V, extractor voltage 5 V, source block and desolvation temperature 100°C. A total of 250 μl of the culture was removed at regular intervals and diluted with 250 μl of a water-acetonitrile (1:1 v/v) solution. The sample was centrifuged at $10,000 \times g$ for 10 min to remove cells. The supernatant fraction was further diluted ten times in water–acetonitrile (1:1 v/v) solution before injection into the MS. The water–acetonitrile (1:1 v/v) solution was used to increase the ES ionization efficiency of the chlorine oxyanions. Perchlorate and chlorate concentrations were determined by peak intensities in the total ion spectrum using standard curves (perchlorate and chlorate) prepared by analysis of a range of known concentrations of pure standards between 1 and 75 ppm.

Proteomics methods

Protein extraction using time-course lysis

Mid-log phase cells from pure cultures and enrichments were harvested by centrifugation at $10,000 \times g$ for 10 min. Each cell pellet was resuspended in 200 μl of 20% formic acid and boiled for 30 min in a water bath. During the 30 min incubation, samples were removed every 3 min for analysis. The samples were removed from the water bath, centrifuged ($10,000 \times g$ for 5 min), and the supernatant was transferred to a new tube (Fig. 1). The pellet was again suspended in fresh 20% formic acid and boiled for another 3 min, centrifuged, and separated into pellet and supernatant fractions. This procedure was repeated until the total lysis time was 30 min. Each of the ten supernatant fractions was dried (DNA Speed Vac, Savant, Ramsey, MN) and then re-dissolved in 100 mM ammonium bicarbonate buffer (pH 8.0). Disulfide bonds in extracted proteins were reduced by incubating with 10 mM dithiothreitol (DTT) at 50°C for 15 min; 10 mM iodoacetamide (IAM) was added and samples were incubated in the dark at room temperature for 15 min to alkylate sulfhydryl
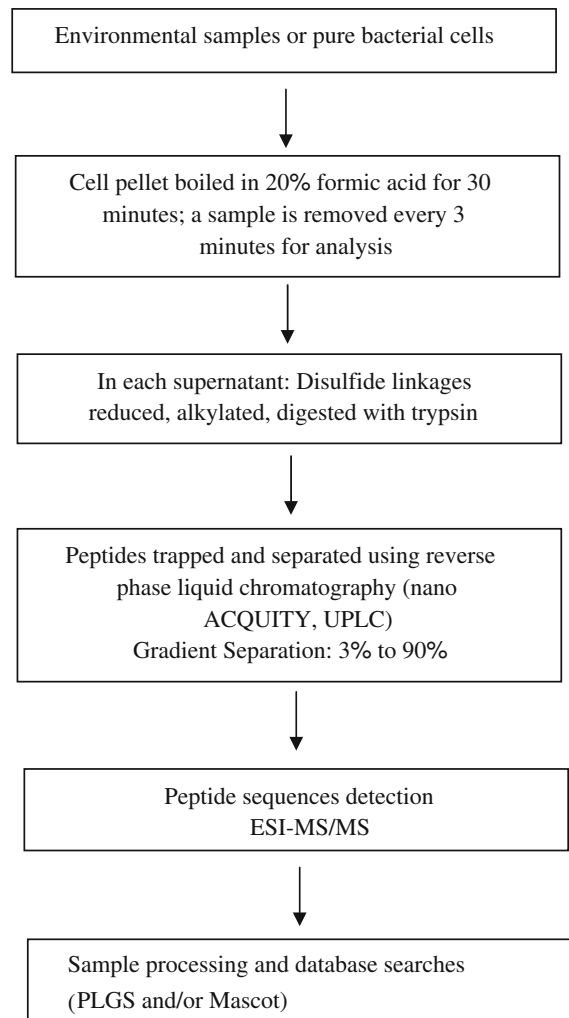
Fig. 1 Time-course lysis method (adapted from Paidisetti 2007)

groups. Lastly, derivatized proteins in the samples were digested by addition of trypsin (Seq. grade modified trypsin, porcine, Promega, Madison, WI, USA) (trypsin:protein ratio 1:50 weight/weight) and incubation at 37°C for 15 h. Digested protein samples were dried again in the Speed Vac. Dried samples from pure cultures were reconstituted in 20 μl and environmental samples in 30 μl of H_2O containing 5% acetonitrile and 0.1% formic acid. Samples were clarified by centrifugation at $10,000 \times g$ for 10 min just before mass spectral (MS) analysis. Peptides were then separated and analyzed by a LC-MS method (see below). The time-course lysis method,

adapted from Paidisetti 2007, is summarized in Fig. 1.

Analysis of peptides by mass spectrometry

The method for analysis of peptides is the one used by Paidisetti (2007) with some modifications as described here. Peptides in the sample were separated using reverse-phase liquid chromatography on a nanoACQUITY Ultra Performance Liquid Chromatograph (UPLC) (Waters Corporation, Milford, MA, USA) and analyzed using a Q-TOF Premier tandem mass spectrometry system. Two microliter of sample hydrolysate was injected and thereby loaded onto a 0.18 mm × 20 mm Symmetry C18 trap column (Waters Corp.) prior to injection onto the analytical column (0.075 mm × 200 mm) (I) BEH 130 C18 nanoACQUITY UPLC column (Waters Corp.). Solvents used were 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Peptides were trapped on the loading column using 100% solvent A at a flow rate of 5 µl min^{-1} for 3 min. Trapped peptides were then separated at a flow rate of 0.4 µl min^{-1} using the following conditions: (1) isocratic: 97% solvent A and 3% solvent B for 1 min; (2) gradient: 1 min after injection, concentration of solvent A was decreased to 50% and solvent B was increased to 50% over the next 90 min; (3) gradient: concentration of solvent A was decreased to 10% and solvent B was increased to 90% over the next 10 min; (4) isocratic: 10% solvent A and 90% solvent B for 10 min; and (5) gradient: concentration of solvent A was increased to 97% and solvent B was decreased to 3% over the next 10 min. The column effluent was delivered directly to the Q-TOF Premier quadrupole-time of flight mass spectrometer using a nanospray source (electrospray ionization) inlet. The nanosprayer was fitted with a 20 µm (i.d.) fused silica emitter tip. The following mass settings were used: 3.96 kV capillary voltage, 35 V cone voltage, 80°C source temperature, 0.45 bar sheath gas pressure, 2.3 V collision energy and 1,800 V detector voltage. The MS and UPLC were controlled by MassLynx 4.1 software (Waters Corporation, Milford, MA, USA). Data generation was performed using a data dependent analysis (DDA) MS method. This method allows the UPLC effluent to be monitored in MS survey mode. When multiply charged analytes having properties of peptides are detected, the instrument switches to MS/MS mode and increases the collision cell voltage to produce peptide fragmentation data suitable for sequencing. The following conditions were used for the MS survey: mass range setting of 400–2,000 Da and a scan time of 1 s/scan. MS/MS acquisition was limited to those multiple positively charged ions producing a signal over 20 cps. The MS/MS spectra were recorded over the range of 50–2,000 Da and had a scan time of 2 s. The collision voltages used for MS/MS ranged from 11 to 55 V, depending on the mass and charge state of the peptide. A reference peptide (or lockmass) was sprayed simultaneously with the LC effluent and was sampled for 1 sec every 30 s. The lockmass standard used was human [glu^1]-fibrinopeptide B (Sigma–Aldrich, St Louis, MO).

Mass spectrometric data analysis

Data were analyzed as described by Paidisetti 2007 with some modifications. ProteinLynx Global Server 2.3 (PLGS) (Waters, Milford, MA, USA) was used for mass spectra analysis, peptide sequencing and protein identification. This software uses both probability scoring (protein identification by peptide mass fingerprinting; http://www.waters.com) and a cross-correlation algorithm for protein identification by comparison of MS/MS data with database protein sequences (http://www.thermo.com). The raw data were converted into peak lists (*.pkl files) by PLGS using the following parameters: (a) smooth channels = 4, number of smooths = 2, smooth mode = Savitzky Golay, (b) percentage of peak height to calculate the centroid spectra = 80%, (c) no baseline subtraction was allowed, and (d) peptide tolerance of 100 ppm. Partial protein amino acid sequences were identified by searching the custom database created in PLGS 2.3 using perchlorate (and chlorate) reductase and chlorite dismutase sequences obtained in our lab and from public database (downloaded from ExPASy Proteomics Server, http://ca.expasy.org/). To verify data obtained with PLGS, Mascot software (TrEMBL protein sequences database) (htpp://www.matrixscience.com) was used. The following parameters were set for Mascot searches: (a) trypsin as the specific enzyme; (b) peptide window tolerance (error window on experimental peptide mass values) ±1.2 Da; (c) fragment mass tolerance of 0.6 Da; (d) number of missed

cleavage sites allowed was set to 1; (e) individual ion scores >50 indicated extensive homology ($P < 0.05$). Ion score is $-10*Log(P)$, where P is the probability that the observed match is a random event. During searches, "carbamidomethyl C" modification was the only amino acid modification allowed. Perchlorate (or chlorate) reductase and chlorite dismutase signature peptides were identified by using both custom PLGS 2.3 and TrEMBL databases. Peptides from enzymes involved in acetate metabolism and housekeeping proteins were identified only by using the TrEMBL database.

Results and discussion

Growth curves and degradation of chlorine oxyanions

Pure bacterial strains and mixed-culture-based environmental enrichments

Perchlorate or chlorate degradation by pure bacterial strains (*Dechlorosoma* sp. KJ, *Pseudomonas chloritidismutans* ASK-1 and *Pseudomonas stutzeri*) was monitored along with their growth. The decrease in the concentration of perchlorate and chlorate was measured using ESI-mass-spectrometry, and growth was determined by monitoring turbidity at 600 nm. As the growth (turbidity) increased, there was a corresponding decrease in the concentration of perchlorate or chlorate in the media. It was found that *Dechlorosoma* sp. KJ grown in a media with 500 ppm of ammonium perchlorate was able to reduce 85% of the perchlorate in 64 h. *Pseudomonas chloritidismutans* grown in a media with 1,000 ppm of chlorate was able to reduce 83% of the chlorate in 48 h. However, *Pseudomonas stutzeri* also grown in 1,000 ppm of chlorate could reduce only 25% of the available chlorate in 72 h. Figure 2a shows the results for *Dechlorosoma* sp. KJ; similar results were obtained for other pure cultures. Similarly, perchlorate degradation and growth was also monitored in an environmental sample (anaerobic sludge enrichment). It can be seen in Fig. 2b that the enrichment cultured in 500 ppm of ammonium perchlorate was able to reduce 68% of the available perchlorate in 88 h.
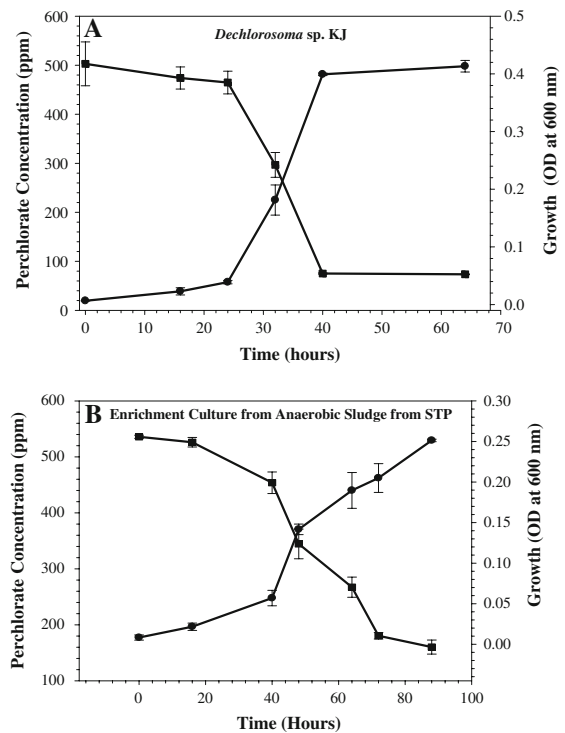
Fig. 2 Growth and concomitant degradation of perchlorate. **a** *Dechlorosoma* sp. KJ; **b** an enrichment culture from an anaerobic sludge sample from a domestic sewage treatment plant (STP); calculated using a standard curve linear between 5 and 50 ppm of perchlorate with a R^2 value of 0.99; *Error bars* show standard deviations for triplicate samples. (●) Growth, (■) perchlorate concentration

Proteomics results

Identification of peptides from pure bacterial cultures

The MS spectra obtained for the bacterial samples were searched against two databases, the TrEMBL protein sequences database using the Mascot program and the custom database created in ProteinLynx Global Server, Protein Identity 2.3. In addition to peptides from enzymes involved in perchlorate metabolism, sequences from enzymes involved in acetate metabolism were also compiled. Table 1 lists peptide sequences from perchlorate reductase (Pcr), chlorite dismutase (CD), and from enzymes involved in acetate metabolism identified from the hydrolysates of *Dechlorosoma* sp. KJ grown in a media with 500 ppm of ammonium perchlorate and 20 mM acetate.

Table 1 Peptides of chlorite dismutase, perchlorate reductase and peptides involved in acetate metabolism detected through "time-course lysis" in a late-log growth phase of *Dechlorosoma* sp. KJ culture

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Peptides of chlorite dismutase and perchlorate reductase detected using PLGS database		
Chlorite dismutase	K.NAEWWNMSPEER.L (56–68)[a, b, c, d, e, f, g]	3–9, 21
	K.SPGLNAGLSSATYSGPAPR.Y (51–79)[a, c, f]	3–9
	R.WGSPTTLGTIHSPEDVIK.A (31–63)[c, e]	3–9, 30
	K.DKSPGLNAGLSSATYSGPAPR.Y (45–66)[c]	3–9
	K.NADVFETLVGVTKPLNYISK.D (49–65)[a, b, c, d, e, f]	6–9, 21, 30
	K.EMEVHTTPTLAYLVNVK.R (70–77)[a, b, c, e, f]	6–9
	R.GLETNSDFFFR.I (59–63)[a, b, c, d, e, f, g]	6–9, 21, 30
	R.GTILTQPGVFGVFTMFK.L (65)[c, e]	6–9
	R.YVIVIPVK.K (60)[a, b, c, d, e, f, g, h, i]	6
	K.DNVLVDLYLTR.G (63–67)[a, b, c, d, e, f]	6–9
Perchlorate reductase alpha subunit fragment	K.EGVPYTPFQYFVVDK.K (39–62)[j, l]	3–9, 21, 30
	K.LAEDEAAAQYILDNAPQSK.G (60–68)[j, l]	6–9, 21, 30
	R.DSITTGQMPNMPEQGR.D (44–71)[j, l]	6–9, 21
	R.MDSTALYSDVVLPSAHWYEK.L (70–77)[j, k, l]	6–9, 30
	K.TDWQIFLALAK.L (60–70)[j, l]	6–9, 30
	R.DLSNLWNQMTMDGK.L (53–64)[j, k, l]	6–9, 21, 30
	K.GITIQMLR.E (56)[j, l]	9
	R.QQFYLDHDTFFDMGVELPTYK.A (44)[j, l]	9
	R.EADVVAGGSK.D (42)[j, k, l]	12
	K.EQTDLSYLVR.S (57–67)[k]	3–9, 21, 30
	R.YIILWGSNPTQTR.I (61–68)[k]	6–9, 21, 30
	K.DIAPMPNIPEYNPR.G (65–69)[k]	6–9, 21, 30
	R.ATWEEALDMIADK.C (65–69)[k]	6–9, 21, 30
	K.FYFWNAK.T (64)[k]	9
Perchlorate reductase beta subunit	R.IEMANGEPSTDPK.I (53)[m, n]	3
	K.LWTTGPGQDFMYWR.N (37)[m, n]	3
	K.IPPMIDYGIPFEFDYAGR.L (67–70)[m, n]	6–9, 30
	K.VALPLHPEFGTEPNVFYVPPVLGPR.I (48–53)[m, n]	6–9, 30
	R.NVETAPGLGYPR.N (72–77)[m, n]	6–9, 21, 30
	K.IPLAQLEGLFGK.Q (72–74)[m, n]	6–9, 21, 30
	R.DVLAILQSER.E (51–70)[m, n]	6–9, 30
	K.GLASDLMDVLIGR.R (67–70)[m, n]	6–9, 30
	R.QLTYVTDLNK.C (51–72)[m, n]	6–9, 30
	R.IEQGVAPACVAQCVGR.A (54–57)[m, n]	9, 21
	K.GKIPPMIDYGIPFEFDYAGR.L (44)[m, n]	30
Perchlorate reductase gamma subunit	K.APFTPFSPAVDSK.Y (52)[o]	3
Perchlorate reductase delta subunit	K.FAEADLQEILR.E (66–70)[p]	6–9, 21, 30
	R.GNELEDTLTAELEFMHFLTAK.Q (41–54)[p]	6–9

Table 1 continued

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Peptides involved in acetate metabolism detected using TrEMBL database		
Citrate synthase I	K.YGKGEPYMYPR.N (13)	9, 21
Dechloromonas aromatica RCB (Q47C41) (protein score 146; sequence coverage 8%)	K.MPTIVAMAYK.Y (73)	6–9, 21, 30
	R.IALEDPYFVEK.K (64)	6–9, 21, 30
	K.LYPNVDFYSGIVQK.A (62)	30
Aconitate hydratase 2	R.VPAGVDDAAK.V (36)	6
Dechloromonas aromatica RCB (Q47C19) (protein score 466; sequence coverage 16%)	K.SATNSVLWFTGEDIPFVPNKR.F (57)	6–9
	K.VTGETNTDDLSPAPDA WSRPDIPLHALAMLK.N (27)	6
	R.ELPSFISTR.G (34)	6–9, 21, 30
	R.ILEIEGLPDLK.V (55)	6–9, 21, 30
	R.DLVNAIPLYAIK.A (34)	6–9, 21, 30
	K.VEQAFELSDAAAER.S (68)	6–9, 21, 30
	K.MTTVGSQDTTGPMTR.D (84)	6–9, 21, 30
	R.LLLPDTVGTGGDSHTR.F (99)	6–9, 21, 30
	R.SAAACAIALNK.E (21)	9
Acetyl-CoA carboxylase	K.VIEEAPSPFVDPEMR.K (65)	6–9, 21
Dechloromonas aromatica RCB (Q478K1) (protein score 285; sequence coverage 11%)	R.AAELLALMPEK.Q (53)	6, 21, 30
	K.FLISPMPGLLR.E (44)	6–9, 21, 30
	K.AMGEQAVALAR.A (70)	9
	K.EFYFLEMNTR.L (19)	9
	R.LQVEHPVTELITGLDLVEQMIR.V (49)	9

[a] *Azospira oryzae* (Q673K5)—sequence coverage 61%; [b] *Pseudomonas* sp. PK (Q673K7)—sequence coverage 54%; [c] *Dechloromonas aromatica* RCB (Q47CXO) and *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 48%; [d] *Dechloromarinus chlorophilus* (Q673K6)—sequence coverage 42%; [e] *Pseudomonas chloritidismutans* (B1AAM3) and *Pseudomonas stutzeri* (B1AAM4)—sequence coverage 40%; [f] *Dechloromonas hortensis* (B1AAM5)—sequence coverage 36%; [g] Perchlorate reducing bacterium CR (Q673K3)—sequence coverage 25%; [h] *Dechlorospirillum* sp. DB (Q673K2)—sequence coverage 7%; [i] *Dechlorospirillum* sp. WD (Q673K4)—sequence coverage 6%; [j] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 38%; [k] *Dechloromonas* sp. PC1 (A7LI69) and *Dechlorosoma* sp. PCC (A7LI70)—sequence coverage 12.4%; [l] *Dechloromonas agitata* (Q84AD6)—sequence coverage 4.2%; [m] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 35%; [n] *Dechloromonas agitata* (Q4G1W2)—sequence coverage 4%; [o] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 6%; [p] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 14.3%

For *Dechlorosoma* sp. KJ, peptide sequences from chlorite dismutase and all the subunits of perchlorate reductase (*alpha*, *beta*, *gamma* and *delta*) were identified. Chlorite dismutase peptides detected in *Dechlorosoma* sp. KJ samples matched with the partial or complete chlorite dismutase peptide sequences previously observed from strains of *Azospira oryzae*, *Dechlorosoma* sp. KJ, *Dechloromonas aromatica* RCB, *Dechloromonas hortensis*, *Dechloromarinus chlorophilus*, *Pseudomonas* sp. PK, *Pseudomonas chloritidismutans*, *Pseudomonas stutzeri*, perchlorate-reducing bacterium strain CR, and

Dechlorospirillum sps. DB and WD. Peptides covering 61% of the amino acid sequence of chlorite dismutase were observed (Table 1). The number of peptide sequences detected corresponding to the CD protein increased during the period of cell hydrolysis between 3, 6 and 9 min. The peptide masses ranged from 454.3 to 969.4 Da. The chlorite dismutase signature peptides were detected at different time points in the time-course lysis method (3, 6, 9, 21 and 30 min), but no CD-derived peptides were detected at the 24 and 27 min time points. Signature CD peptide sequences such as LKEMEVHTTPTLAYLVNVK

and YVIVIPVKK appeared only at the 3 and 6 min time points, respectively, during the entire time-course lysis process. However, CD sequence YVI VIPVK was detectable from 3 to 6 min. The CD sequences GLETNSDFFFR and NADVFETLVGV TKPLNYISK were detected at both the 21 and 30 min time points. Perchlorate reductase *alpha* subunit peptide sequences matched to sequences previously observed from *Dechlorosoma* sp. KJ, *Dechloromonas* sp. PC1, *Dechloromonas* sp. PCC and *Dechloromonas agitata*, with sequence coverage of approximately 38%. These peptides were detected between 3 and 12 min and then later at 21 and 30 min. Their masses were in the range of 466.3 to 1,024 Da. Pcr *alpha* subunit signature peptide sequences GITIQMLR, QQFYLDHDTFFFDMGVEL PTYK and FYFWNAK were detected only at a single time point (9 min). Some Pcr sequences appeared twice during time-course lysis, including EQTDLSYLVR (3 and 30 min) and EGVPYTPFQY FVVDK (6 and 30 min), being detected once at an early time point and then at again at a later time point. Pcr signature sequence EADVVAGGSK, on the other hand, was found only at the early time points (9 and 12 min). Perchlorate reductase *beta* subunit peptide sequences were found at 3, 6, 9, 21 and 30 min time points with sequence coverages of 35% and masses ranging from 572.3 to 1,051 Da. They were similar to the previously described *β* subunits' sequences from *Dechlorosoma* sp. KJ and *Dechloromonas agitata*. The *beta* subunit Pcr peptide signature sequences IEMANGEPSTDPK, LWTTG PGQDFMYWR, and CIGCQTCTVACK were detected only at 3 min, whereas GKIPPMIDYGIPFE FDYAGR was only present at 30 min. For the *gamma* subunit of perchlorate reductase, only one peptide (APFTPFSPAVDSK) similar to that from *Dechloro-soma* sp. KJ was detected at 3 min with peptide sequence coverage of 6%. For the *delta* subunit, two signature peptides (FAEADLQEILR and GNELEDT LTAELEFMHFLTAK) matched with sequences observed previously from *Dechlorosoma* sp. KJ with peptide sequence coverage of approximately 14%. They were detected at 6, 9, 21 and 30 min with masses ranging from 652.9 to 803.7 Da.

Similar results were obtained with the other perchlorate reducer, *Dechloromonas hortensis*. Peptides of chlorite dismutase along with *alpha* and *delta* subunits of perchlorate reductase were identified.

However, no *beta* and *gamma* subunits of perchlorate reductase were detected. The CD and Pcr signature peptide sequences were detected only during the first 12 min of the time-course lysis, with peptide sequence coverage of approximately 44%.

Pseudomonas chloritidismutans and *Pseudomonas stutzeri* are chlorate-reducing microbes, so we should have been able to detect both chlorate reductase (Clr) and chlorite dismutase (CD) signature peptides. However, only chlorite dismutase signature peptides were detected using the time-course lysis technique. We were unable to detect signatures of any subunits (*alpha*, *beta*, *gamma* and *delta*) of chlorate reductase at any of the time points using both the databases. Chlorate reductase signature peptides probably were not detected in these microorganisms because of the differences in the Clr amino acid sequences between these organisms and the Clr peptide sequences in the databanks. There is also a paucity of available chlorate reductase sequences. Table 2 is a compilation of peptides from chlorite dismutase and acetate-metabolizing enzymes identified from the hydrolysis of *Pseudomonas stutzeri* grown in a media with 1,000 ppm of chlorate and 20 mM acetate, respectively. The CD peptides were detected throughout the time-course lysis, except between 24 and 27 min. The mass of these CD-derived peptides ranged from 529.9 to 1104.6 Da with a sequence coverage of 59%. The CD peptides obtained were found to be matching with the partial or complete chlorite dismutase peptide sequences previously observed from *Pseudomonas chloritidismutans*, *Pseudomonas stutzeri*, *Pseudomonas* sp. PK, *Azospira oryzae*, *Dechlorosoma* sp. KJ, *Dechloromonas aromatica* RCB, *Dechloromonas hortensis*, *Dechloromarinus chlorophilus*, perchlorate reducing bacterium strain CR, and *Dechlorospirillum* sp. DB and WD. Similar results were also obtained for *Pseudomonas chloritidismutans*. CD-derived peptides were detected from 3 to 15 min and then at the 21 and 30 min time points with sequence coverage of 59%.

In all the pure bacterial samples used, except *Pseudomonas chloritidismutans* ASK-1, peptides from the enzymes involved in the citric acid cycle were detected throughout the time-course lysis. In *Dechlorosoma* sp. KJ, peptides from citrate synthase and aconitase (aconitate hydratase), were found (Table 1). Peptide sequences from other enzymes like acetyl-CoA carboxylase were also found.

Table 2 Peptides of chlorite dismutase and peptides involved in acetate metabolism detected through "time-course lysis" in a late-log growth phase of *Pseudomonas stutzeri* culture

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Peptides of chlorite dismutase detected using PLGS database		
Chlorite dismutase	K.DKSPELNAGLTSATYSGPAPR.Y (43–70)[a]	6–21
	R.WGSPTTLGTIHSPEDVIK.A (56–61)[a, c]	9
	R.WGSPTTLGTIHSPEDVIKALAE.(–) (70)[a]	3
	R.GTILTQPGVFGVFTMFK.L (56–72)[a, c]	9–12
	R.GLETNSDFFFR.I (57–68)[a, b, d, e, f]	6–12, 18–21, 30
	R.YVIVIPVK.K (65–69)[a, b, c, d, e, f, g, h]	6–15
	K.NADVFETLVGVTKPLNYISK.D (50–68)[a, b, c, d, f]	6–21, 30
	K.NADVFETLVGVTKPLNYISK.D (69)[a, b, c, d, f]	6
	K.NAEWWNMSPEER.L (72)[a, b, c, d, e, f]	6–9
	K.EMEVHTTPTLAYLVNVK.R (65)[a, b, c, f]	9
	R.LKEMEVHTTPTLAYLVNVK.R (28)[a, b, c, f]	12
	K.LYHSTGLDDTDFITYFETDDLTAFNNVVLSLAQVK.E (62)[a]	9
	R.GLETNSDFFFR.I (57–68)[c]	6–21, 30
Peptides involved in acetate metabolism detected using the TrEMBL database		
Aconitate hydratase 2	K.GKMKPGITLR.D (14)	6–9
Dechloromonas aromatica RCB (Q47C19)	R.YLSFDQIAEFR.E (41)	6–12
(protein score 148; sequence coverage 9%)	K.HTLLMFDAFHDVAEK.A (46)	6
	R.AALGIVPQPLNAEQTAGLVELLK.N (51)	9
	R.AALGIVPQPLNAEQTAGLVELLK.N (66)	6–9
	K.VTGETNTDDLSPAPDAWSRPDIPLHALAMLK.M (83)	6
	K.TDVLLDEVR.A (52)	6, 12
	K.GQMQPGITLR.D (12)	6
	R.FKGQMQPGITLR.D (38)	6
	R.DLVHAIPYYAIQQGLLTVEK.K (73)	6, 12
	R.FPIGISFPAGSGLVAFAAATGVMPLDMPESVLVR.F (73)	6–9
	K.VTATKNGAVIAESQLK.T (23)	6–9
	R.DGIEPDVPGSVGPIK.K (30)	6–9
	R.MLLPDTVGTGGDSHTR.F (81)	9

[a] *Pseudomonas chloritidismutans* (B1AAM3) and *Pseudomonas stutzeri* (B1AAM4)—sequence coverage 57%; [b] *Azospira oryzae* (Q673K5) and *Pseudomonas* sp. PK (Q673K7)—sequence coverage 47.3%; [c] *Dechloromonas aromatica* RCB (Q47CXO) and *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 37%; [d] *Dechloromarinus chlorophilus* (Q673K6)—sequence coverage 36%; [e] perchlorate reducing bacterium CR (Q673K3)—sequence coverage 26.2%; [f] *Dechloromonas hortensis* (B1AAM5)—sequence coverage 25%; [g] *Dechlorospirillum* sp. DB (Q673K2)—sequence coverage 7.4%; [h] *Dechlorospirillum* sp. WD (Q673K4)—sequence coverage 6%

Acetyl-CoA carboxylase catalyses irreversible carboxylation of acetyl-CoA to form malonyl-CoA (Nelson and Cox 2005). In the case of *Dechloromonas hortensis*, citrate synthase, α-ketoglutarate dehydrogenase (2-oxoglutarate dehydrogenase) and succinate dehydrogenase signature peptides were detected. In the *Pseudomonas stutzeri* culture, only

aconitase (aconitate hydratase) peptides were detected (Table 2). From these results we can say that acetate is metabolized via the citric acid cycle in our bacterial samples. For *Pseudomonas chloritidismutans*, no peptides from enzymes involved in acetate metabolism were detected, which may be because of the differences in the amino acid

sequences between this organism and the peptide sequences available in the databanks.

Identification of peptides from mixed bacterial cultures derived from environmental samples

The signature peptides from environmental samples (an anaerobic sludge enrichment culture from a sewage treatment plant, a biomass-covered activated carbon matrix sample from a bioreactor used to treat perchlorate-contaminated drinking water, and a waste water effluent sample from a paper mill treatment basin) were identified by searching peptide sequences from these samples against the two above mentioned databases. We focused on perchlorate and chlorate-reducing enzyme systems, looking specifically to detect perchlorate and chlorate reductase and chlorite dismutase-derived signature peptides. This approach helped us to determine if the enrichments contained viable microbial populations that have the potential to degrade perchlorate or chlorate and might be used as sources for isolating novel perchlorate or chlorate reducing microbes. To strengthen the above results we also compiled lists of peptides from housekeeping proteins from perchlorate (or chlorate)-reducing microorganisms (data not shown). Tables 3, 4 and 5 list perchlorate reductase, chlorate reductase and chlorite dismutase peptides identified from the hydrolysis of bacterial cells in the anaerobic sludge enrichment sample, biomass-covered activated carbon matrix sample, and waste water effluent sample cultured in media with 500 ppm of ammonium perchlorate.

Results from an anaerobic sludge enrichment sample showed that chlorite dismutase peptide sequences were detectable only in the beginning of the time-course lysis in the 3 and 6 min samples; no CD signature peptides were detected after that (Table 3). The peptide masses ranged from 529.8 to 1,083.5 Da. Peptides corresponding to nearly 65% of amino acid sequence of the chlorite dismutase previously described in *Dechlorospirillum* sp. WD were detected. The CD peptides were also similar to partial or complete chlorite dismutase peptide sequences from *Dechlorospirillum* sp. DB, *Azospira oryzae*, *Dechlorosoma* sp. KJ, *Dechloromonas aromatica* RCB, *Dechloromonas hortensis*, *Dechloromarinus chlorophilus*, *Pseudomonas chloritidismutans*, *Pseudomonas stutzeri*, *Pseudomonas* sp. PK and perchlorate

reducing bacterium strain CR. This suggests that this enrichment may contain novel perchlorate/chlorate-reducing species. CD peptide sequences GLGAGSD YLLR, GLETNSDFFFR, DKSPGLNAGLSSATYS GPAPR and WGSPTTLGTIHSPEDVIK were the only sequences present at the 6 min time point. All other peptides were only detected at the 3 min time point. We also detected peptides corresponding to three subunits of perchlorate reductase (*alpha*, *beta* and *delta*). The *alpha* subunit peptides, like the CD peptides, were present in the first 6 min time-course samples. The only PcrA signature sequence that occurred in the 6 min time point was TDWQIFL ALAK; others were detectable at the 3 min time point. The *alpha* subunit peptides were found to be similar to those previously observed in *Dechloromonas* sp. KJ, *Dechloromonas agitata*, *Dechloromonas* sp. PC1 and *Dechlorosoma* sp. PCC with sequence coverage of 11.3% and peptide masses ranging from 571 to 894.9 Da. The peptides for the *beta* subunit of perchlorate reductase were detectable in 3–9 min samples and later at the 15 and 30 min time points. These sequences were found to be similar to PcrB peptide sequences from *Dechloromonas* sp. KJ and *Dechloromonas agitata* with 38% sequence coverages and masses from 572.3 to 915.8 Da. Pcr sequences VALPLHPEFGTEPNVFYVPPVLGPR and GNE LEDTLTAELEFMHFLTAK were the only PcrB peptides detected at the 15 and 30 min time points, respectively. The *delta* subunit signature peptides were similar to those previously observed in *Dechloromonas* sp. KJ with sequence coverage of approximately 21% and were detected in the 3–12 min time point samples. Pcr peptide FAEADLQEILR was detected only at the 12 min time point. Signature peptides derived from the *gamma* subunit of perchlorate reductase were not detected in this sample. One peptide (QALATLGGEMAK) similar to the *beta* subunit of chlorate reductase from *Ideonella dechloratans* was detected at the 3 min time point.

In the sample enriched from a bioreactor that had been used for treating perchlorate-contaminated drinking water, we detected signature peptides of chlorite dismutase and perchlorate reductase (*alpha*, *beta* and *gamma* subunits) (Table 4). Most of the CD peptides detected here were present only during the first 6 min of the time-course lysis. However, one CD peptide, TPITELVHRLP, was present at the 15 and 24 min time points. CD peptide sequences such as

Table 3 Peptides of chlorite dismutase, perchlorate reductase and chlorate reductase detected through "time-course lysis" using the PLGS database in a late-log phase enrichment culture prepared from anaerobic sludge from a domestic sewage treatment plant

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Chlorite dismutase	K.DKSPELNAGLSSATYSDSAPR.Y (60)[a]	3
	K.DKSPELNAGLSSATYSDSAPR.Y (65)[a]	3
	R.GLGAGSDYLLR.V (60–65)[a, b]	3–6
	K.LGMYSDVTENLVGITK.A (82)[a, c]	3
	K.DAAWWNMSDEQR.L (74)[a, b]	3
	R.YVIVIPVK.K (65)[a, b, c, d, e, f, g, h]	3
	K.VIVDTYLTR.G (73)[a]	3
	R.VHSTDMAATQAFLVDWR.A (42)[a, b]	3
	R.GLETNSDFFFR.I (65)[c, d, e, f, g, h]	3–6
	K.NADVFETLVGVTKPLNYISK.D (67–70)[c, d, e, f, g]	3
	K.DKSPGLNAGLSSATYSGPAPR.Y (50–70)[c, d, g]	3–6
	K.DKSPGLNAGLSSATYSGPAPR.Y (72)[c, d, g]	3
	R.WGSPTTLGTIHSPEDVIK.A (51–62)[d, f]	3–6
	R.WGSPTTLGTIHSPEDVIK.A (72)[d, f]	3
	R.GTILTQPGVFGVFTMFK.L (56)[d, f]	3
Perchlorate reductase alpha subunit fragment	K.EGVPYTPFQYFVVDK.K (58)[i]	3
	K.APIDADKYPFR.F (76)[i, k]	3
	K.TDWQIFLALAK.L (48–51)[i, j]	3–6
	R.IPDAHFLSEAQLNGAK.V (57)[j]	3
Perchlorate reductase beta subunit	K.VALPLHPEFGTEPNVFYVPPVLGPR.I (24–54)[l]	3–9, 15
	R.NVETAPGLGYPR.N (72)[l, m]	3
	K.IPLAQLEGLFGK.Q (75–77)[l]	3–6
	R.DVLAILQSER.E (59–72)[l]	3–9
	K.GLASDLMDVLIGR.R (65)[l]	3
	K.GKIPPMIDYGIPFEFDYAGR.L (63)[l]	3
	R.RQLTYVTDLNK.C (65)[l]	3
	K.GAQACVQSCPYAKPYFNPLTNK.A (48)[l]	3
	R.GNELEDTLTAELEFMHFLTAK.Q (46)[l]	30
Perchlorate reductase delta subunit	R.GNELEDTLTAELEFMHFLTAK.Q (52–66)[n]	3, 9–12
	K.VTTQFFVALTDLAEK.F (62–66)[n]	9–12
	K.FAEADLQEILR.E (64)[n]	12
Chlorate reductase beta subunit	K.QALATLGGEMAK.R (26)[o]	3

[a] *Dechlorospirillum* sp. WD (Q673K4)—sequence coverage 65%; [b] *Dechlorospirillum* sp. DB (Q673K2)—sequence coverage 53.3%; [c] *Azospira oryzae* (Q673K5)—sequence coverage 42%; [d] *Dechloromonas aromatica* RCB (Q47CXO) and *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 34%; [e] *Dechloromarinus chlorophilus* (Q673K6) and *Pseudomonas* sp. PK (Q673K7)—sequence coverage 27.4%; [f] *Pseudomonas chloritidismutans* (B1AAM3) and *Pseudomonas stutzeri* (B1AAM4)—sequence coverage 27%; [g] *Dechloromonas hortensis* (B1AAM5)—sequence coverage 22%; [h] perchlorate reducing bacterium CR (Q673K3)—sequence coverage 16.4%; [i] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 11.3%; [j] *Dechloromonas agitata* (Q84AD6)—sequence coverage 3%; [k] *Dechloromonas* sp. PC1 (A7LI69) and *Dechlorosoma* sp. PCC (A7LI70)—sequence coverage 1.5%; [l] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 38%; [m] *Dechloromonas agitata* (Q4G1W2)—sequence coverage 4%; [n] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 21%; [o] *Ideonella dechloratans* (P60069)—sequence coverage 4%

Table 4 Peptides of chlorite dismutase and perchlorate reductase detected through "time-course lysis" using the PLGS database in a late-log phase enrichment culture from bioreactor used for treating perchlorate-contaminated drinking water

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Chlorite dismutase	R.GLETNSDFFFR.I (62)[a, b, c, d, e, f]	6
	R.YVIVIPVK.K (65)[a, b, c, d, e, f, g, h]	6
	K.NADVFETLVGVTKPLNYISK.D (64–70)[a, b, c, d, e]	3–6
	K.NADVFETLVGVTKPLNYISK.D (76)[a, b, c, d, e]	6
	K.DKSPGLNAGLSSATYSGPAPR.Y (69)[a, b, e]	6
	R.WGSPTTLGTIHSPEDVIK.A (61–65)[b]	3
	R.WGSPTTLGTIHSPEDVIK.A (78)[b, d]	6
	R.GTILTQPGVFGVFTMFK.L (67)[b, d]	3
	R.GTILTQPGVFGVFTMFK.L (74–81)[b, d]	3–6
	R.WGSPTTLGTIHSPEDVIK.A (61–65)[d]	3–6
	R.TPITELVHRLP.(–)[i]	15, 24
Perchlorate reductase alpha subunit fragment	K.EGMPYTPFQNFVVDKKPWPTLTGR.Q (50)[j]	3
	R.ATWEEALDMIADK.C (65)[j]	3
	K.LAEDAAAAQYILDNAPHSK.G (60–82)[j]	6–12, 18
	R.YIILWGSNPTQTR.I (69–71)[j]	6–9
	K.IVSIAPDFNSSAIK.V (59–75)[j]	6–12
	K.GYIELGDLDPALEGNFK.I (73)[j]	6
	K.APLDADKYPFR.F (79)[j, k]	6
	R.MDSTALYSDVVLPSAHWYEK.L (68–74)[j, k, l]	6, 12
	K.DIAPMPNIPEYNPR.G (68–70)[j]	6–9, 15, 30
	K.EQTDLSYLVR.S (54–68)[j]	6–15, 21, 27
	R.ATWEEALDMIADK.C (65–79)[j]	6–12
	K.APPVAFMGR.N (45–65)[j, l]	6–12, 18
	K.EGMPYTPFQNFVVDK.K (55–56)[j]	9–15
	R.DLSNLWNQMTMDGK.L (62–63)[j, k]	9–15
	R.AMHLLTALTGSEGK.N (69–83)[j]	9–12
	R.IPDAHFLSEAQLNGTK.I (66)[j]	21
	R.EAISSGQMPNLPR.E (53–55)[j]	21–24
Perchlorate reductase beta subunit	K.LWTTGPGQDFMYWR.N (73)[m]	3
	K.VALPLHPEFGTEPNVFYVPPVLGPR.I (29–60)[m]	3–21, 27–30
	R.NVETAPGLGYPR.N (70–74)[m]	3–6
	K.IPLAQLEGLFGK.Q (55–77)[m]	3–30
	K.GLASDLMDVLIGRR.S (41)[m]	3
	K.GLASDLMDVLIGR.R (65–77)[m]	3–15
	R.QLTYVTDLNK.C (63–68)[m]	3–6
	R.IEQGVAPACVAQCVGR.A (74–60)[m]	3–6
	R.SAPNWDEDQGAGEYPNNSFFYLPR.M (63)[m]	3
	R.SAPNWDEDQGAGEYPNNSFFYLPR.M (65)[m]	3
	R.RQLTYVTDLNK.C (81)[m]	3
Perchlorate reductase beta subunit	R.NVETAPGLGYPR.N (70–74)[n]	3–6

Table 4 continued

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Perchlorate reductase gamma subunit	R.GVFKGDPVPAVR.A (68)[o]	3
	K.AFDSLKPNAK.S (67)[o]	3
	R.NLHGEASDR.L (41)[o]	3

[a] *Azospira oryzae* (Q673K5)—sequence coverage 42%; [b] *Dechloromonas aromatica* RCB (Q47CXO) and *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 34%; [c] *Pseudomonas* sp. PK (Q673K7) and *Dechloromarinus chlorophilus* (Q673K6)—sequence coverage 27.4%; [d] *Pseudomonas chloritidismutans* (B1AAM3) and *Pseudomonas stutzeri* (B1AAM4)—sequence coverage 27%; [e] *Dechloromonas hortensis* (B1AAM5)—sequence coverage 22%; [f] perchlorate reducing bacterium CR (Q673K3)—sequence coverage 16.4%; [g] *Dechlorospirillum* sp. DB (Q673K2)—sequence coverage 7.4%; [h] *Dechlorospirillum* sp. WD (Q673K4)—sequence coverage 6%; [i] *Nocardioides* sp. JS614 (A1SMY6)—sequence coverage 5%; [j] *Dechloromonas* sp. PC1 (A7LI69) and *Dechlorosoma* sp. PCC (A7LI70)—sequence coverage 19%; [k] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 10.4%; [l] *Dechloromonas agitata* (Q84AD6)—sequence coverage 3%; [m] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 38.4%; [n] *Dechloromonas agitata* (Q4G1W2)—sequence coverage 4%; [o] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 13%

GTILTQPGVFGVFTMFK, NADVFETLVGVTKPL NYISK, WGSPTTLGTIHSPEDVIK and CIGCQTC TVACK are detectable from 3 to 6 min. All other peptides were only found at the 6 min time point. The CD signature peptides obtained were similar to partial or complete chlorite dismutase peptide sequences from *Azospira oryzae*, *Dechlorosoma* sp. KJ, *Dechloromonas aromatica* RCB, *Dechloromonas agitata*, *Dechloromonas hortensis*, *Dechloromarinus chlorophilus*, *Pseudomonas chloritidismutans*, *Pseudomonas stutzeri*, *Pseudomonas* sp. PK, perchlorate reducing bacterium strain CR, *Dechlorospirillum* sp. DB, *Dechlorospirillum* sp. WD, and *Nocardioides* sp. JS614. The masses of the peptides were in the range of 529.9 to 1,104.6 Da with sequence coverages of 42%. Perchlorate reductase *alpha* subunit peptide fragments similar to PcrA peptides from *Dechloromonas* sp. PC1, *Dechlorosoma* sp. PCC *Dechloromonas* sp. KJ and *Dechloromonas agitata* were consistently present throughout the 30 min of the time-course lysis. The sequence coverages were found to be 19%, and peptide masses ranged from 473.3 to 926 Da. Some PcrA peptide fragments occurred only at a single time point: EGMPYT PFQNFVVDKKPWPTLTGR and ATWEEALDMI ADK at 3 min; GYIELGDLDPALEGNFK and APL DADKYPFR at 6 min; and IPDAHFLSEAQLNGTK at 21 min. PcrA peptide EAISSGQMPNLPR was detected at the 21 min time point and was not found after the 24 min time point. The perchlorate reductase *beta* subunit peptides were present from 3 to 30 min. These sequences were found to be similar to PcrB peptide sequences from *Dechloromonas* sp. KJ and

Dechloromonas agitata with approximately 38% sequence coverage and masses from 505.5 to 1,387.6 Da. PcrB sequences such as LWTTGPGQDF MYWR, GLASDLMDVLIGRR, SAPNWDEDQG AGEYPNNSFFYLPR, and RQLTYVTDLNK were detected only at the 3 min time point. On the other hand, PcrB peptide fragments VALPLHPEFGTEP NVFYVPPVLGPR and IPLAQLEGLFGK were present throughout the time-course. The *gamma* subunit peptides matched to *Dechloromonas* sp. KJ with sequence coverage of approximately 13% and peptide masses between 499.7 and 621.4 Da. These peptides were detected only at the 3 min time point. The *delta* subunit of perchlorate reductase was not detected in this sample.

For the waste water effluent enrichment sample from a paper mill effluent treatment basin, peptide sequences from chlorite dismutase and all the subunits of perchlorate reductase (*alpha*, *beta*, *gamma* and *delta*) were identified (Table 5). Chlorite dismutase peptides detected were found to be matching to CD peptide sequences from *Dechloromonas agitata* and *Ideonella dechloratans*. Peptides covering 39% of the amino acid sequence of chlorite dismutase were observed. The peptide masses ranged from 468.8 to 1,227.6 Da. The CD peptide fragments were detected at all the time points in the time-course lysis. CD sequence WGSPTVLGTIQSFDSVVNTLS MGR was present throughout the time-course; whereas, some other CD peptides were found only at one time point; YAFVIPVK, YAFVIPVKK, KGA AAEVVAVVEK, FGMNAEVTENLVGMTK, EM ETHTLPTLANLVNVK and LKEMETHTLPTLA

Table 5 Peptides of chlorite dismutase and perchlorate reductase detected through "time-course lysis" using the PLGS database in a late-log phase enrichment culture prepared from a wastewater effluent sample from a paper mill

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Chlorite dismutase	R.WGSPTVLGTIQSFDSVVNTLSMGR.(–) (33–74)[a]	3–9, 15–18, 27–30
	R.WGSPTVLGTIQSFDSVVNTLSMGR.(–) (72–75)[a]	3–6
	K.NADWWNLTDEQR.L (68–73)[a]	3
	R.LKEMETHTLPTLANLVNVK.R (35)[a]	6
	R.LKEMETHTLPTLANLVNVK.R (76)[a]	6
	K.EMETHTLPTLANLVNVK.R (60)[a]	6
	R.YAFVIPVK.N (78)[a]	6
	R.YAFVIPVKK.N (73)[a]	6
	R.FGMNAEVTENLVGMTK.D (60)[a]	6
	R.IHSYDMAATQAFLVDFR.A (43–57)[a]	6, 21–24
	K.GAAAEVVAVVEK.Y (45)[a]	6
	K.LYHSTGLDDTDFITYFETADLGAFNNLMLALAK.V (53)[a]	12
	R.GFEAQSDFFLR.I (64)[a]	21
	K.NADWWNLTDEQR.L (68–73)[b]	3–6
Perchlorate reductase alpha subunit fragment	K.GSWGDQPEQKAPPVAFMGR.N (68)[c]	3
	K.LQDGNTVEVRPVFEILK.S (66)[c]	3
	K.LAEDEAAAQYILDTAPHSK.G (75–84)[c]	6–12
	R.DLTNLWNQMTMDGK.L (73)[c]	6
	K.APVDADKFPFR.F (64)[c]	6–9
	K.EQTDLPYLIR.R (68)[c]	6
	R.ATWEEALDLISDK.I (68–69)[c]	6–9
	K.VVSISPDFNSSTIK.A (44–75)[c]	6–18
	K.APPVAFMGR.N (47)[c, d]	6
	K.YIILWGANPTQTR.I (64–65)[c]	6–9
	K.EGVPYTPFQNYIVDK.K (53)[c]	9
Perchlorate reductase beta subunit	K.GKIPPMIDYGVPFEFDYAGR.L (68)[e]	3
	K.GKIPPMIDYGVPFEFDYAGR.L (65)[e]	3
	R.FGVALPLHPEYGTEPNVFYVPPVLGPR.V (65)[e]	3
	K.ISMTQLEQLFGK.Q (74–77)[e]	3–6
	K.RQLAYVADLNK.C (71)[e]	3
	K.TLWTSGPGQDYMYWR.N (69)[e]	3
	R.NVETAPGLGYPR.N (64)[e, f]	3
	R.NVETAPGLGYPRNWQSK.G (49)[e]	3
	R.EQDGLVVIHQEK.C (74–80)[e]	3–6
	K.IKNQPSELMDILIGR.R (83)[e]	3
	R.EVLKTLQAER.E (67)[e]	3
	R.QLAYVADLNK.C (70)[e]	3
	K.NQPSELMDILIGR.R (65)[e]	6
	K.IPPMIDYGVPFEFDYAGR.L (73)[e]	6
Perchlorate reductase gamma subunit	R.GVFKGDPVPAVR.A (49)[g]	3

Table 5 continued

Protein name (accession ID)	Sequence (individual ion score range)	Cell lysis time points, occurrence of peptides in min
Perchlorate reductase delta subunit	K.GNELEDTLTAELDFMQFLALK.Q (52–57)[h]	12, 30
	K.GNELEDTLTAELDFMQFLALK.Q (39–65)[h]	9–15, 27–30
	K.VTTPFFVTLAEFAESFALANLR.E (56)[h]	18

[a] *Dechloromonas agitata* (Q8KP26)—sequence coverage 16%; [b] *Ideonella dechloratans* (Q9F437)—sequence coverage 4.2%; [c] *Dechloromonas agitata* (Q84AD6)—sequence coverage 11%; [d] *Dechloromonas* sp. PC1 (A7LI69) and *Dechlorosoma* sp. PCC (A7LI70)—sequence coverage 1.2%; [e] *Dechloromonas agitata* (Q4G1W2)—sequence coverage 42%; [f] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 3.6%; [g] *Dechlorosoma* sp. KJ (B2D1S8)—sequence coverage 5%; [h] *Dechloromonas agitata* (Q4G1W0)—sequence coverage 9.8%

NLVNVK were detectable at 6 min; LYHSTGLDD TDFITYFETADLGAFNNLMLALAK at 12 min; and GFEAQSDFFLR at 21 min. CD peptides like LKEMETHTLPTLANLVNVK and NADWWNLTD EQR occurred only during the early time points and were not detected after 6 min. Perchlorate reductase *alpha* subunit peptide sequences were found to be similar to the PcrA sequences previously observed from *Dechloromonas agitata*, *Dechloromonas* sp. PC1 and *Dechloromonas* sp. PCC with sequence coverage of approximately 11%. PcrA-derived peptides were detected from 3 to 18 min and their masses were in the range of 473.3 to 885.5 Da. The following PcrA peptide sequences were detected only at single time-point: LQDGNTVEVRPVFEILK and GSWGDQPEQKAPPVAFMGR at 3 min; APPV AFMGR, EQTDLPYLIR and DLTNLWNQMTM DGK at 6 min; and EGVPYTPFQNYIVDK at the 9 min time point. The PcrA-derived VVSISPDFN SSTIK fragment appeared from 6 to 18 min during the time-course lysis. Perchlorate reductase *beta* subunit peptide sequences were found at 3 and 6 min time points with sequence coverage of 48% and masses ranging from 567.8 to 1,044 Da. They were similar to the β subunits sequences previously observed from *Dechlorosoma* sp. KJ and *Dechloromonas agitata*. The PcrB peptide sequences NQPS ELMDILIGR and IPPMIDYGVPFEFDYAGR were detected only at 6 min, whereas ISMTQLEQLFGK, EQDGLVVIHQEK and CIGCQTCTVACK were present at the 3 and 6 min time points. All other PcrB peptide fragments were only detected at the 3 min time point. For the *gamma* subunit of perchlorate reductase, only one peptide (GVFKGDP VPAVR) similar to that previously observed from

Dechlorosoma sp. KJ was detected at 3 min with peptide sequence coverage of 5%. Finally, in the case of the *delta* subunit, three signature peptides (SDRPNLLLELK, VTTPFFVTLAEFAESFALAN LR and GNELEDTLTAELDFMQFLALK) were found and were similar to sequences previously seen from *Dechloromonas agitata* with peptide sequence coverage of approximately 14%. They were detected at 9–18, 27 and 30 min with masses ranging from 649.4 to 1,200 Da.

Various kinds of proteins involved in housekeeping metabolism were detected in all the samples throughout the time-course lysis. In the bioreactor enrichment sample, we found enzymes involved in the citric acid cycle such as citrate synthase, aconitate hydratase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. Other enzymes detected were acetyl-CoA carboxylase, phosphoenolpyruvate carboxykinase, malate synthase, acyl carrier protein, and glutamine synthetase. Proteins like 4Fe-4S ferredoxin and cytochrome c also were observed and are involved in the microbial electron transport chain. We also detected molybdopterin oxidoreductase, which was found to be similar to the PcrA subunit of perchlorate reductase. Chaperones (10 kDa), OmpA and signatures of exonucleases were also observed. For the waste water effluent sample, peptide sequences from citrate synthase, aconitate hydratase and 2-oxoglutarate dehydrogenase were detected. Along with these we also found peptides from acetyl-CoA carboxylase, phosphoenolpyruvate carboxykinase, acyl carrier protein, glutamine synthetase and phosphate acetyl transferase. Membrane-bound cytochrome *c* proteins were also found. Peptide sequences from cytochrome *c* oxidase, which

is an enzyme in the respiratory chain, were also detected. Presence of these proteins might substantiate the presence of metal-respiring organisms, some of which have the capacity for perchlorate metabolism. Other proteins like, pyrophosphatase, chaperones (10 kDa), OmpA, exonucleases, and an RNA polymerase subunit were also found. In the case of the anaerobic sludge enrichment we were able to detect molybdopterin oxidoreductase and other housekeeping proteins like a 4Fe-4S ferredoxin and a translation elongation factor.

Conclusion

We were able to monitor the expression of perchlorate or chlorate-reducing enzyme systems in pure bacterial cultures and environmental enrichments using a proteomics-based method. Proteomic analysis of the lysate from pure bacterial cultures, as well as enrichments, always showed proteins associated with perchlorate metabolism with high protein matching scores. Therefore, this method can be used in the future for monitoring the expression of enzymes involved in perchlorate metabolism, for example in the study of recombinant clones designed to produce these proteins in fermentors or the environment. The above method can also be used for identification, quantification of other degradative enzymes and for environmental characterization. Peptide sequences detected in environmental samples were found to be similar to sequences of enzymes from known perchlorate-reducing microbes as well as from some uncultured bacteria. This supports the idea that the environmental samples and mixed-culture enrichments can be used as the source for isolation of novel perchlorate-reducing microorganisms. The chlorite dismutase peptide sequences obtained from the pure bacterial cultures and environmental samples were found to match chlorite dismutases from previously described perchlorate and chlorate-reducing microorganisms like *Azospira oryzae*, *Dechloromonas aromatica* RCB, *Dechloromarinus chlorophilus*, *Pseudomonas* sp. PK, *Ideonella dechloratans*, perchlorate reducing bacterium strain CR, and *Dechlorospirillum* sps. DB and WD. This indicates and supports the previous work (O'Connor and Coates 2002; Bender et al. 2004) that chlorite dismutase enzyme is highly conserved in dissimilatory perchlorate-reducing organisms; therefore, it can be used to detect these microbes in environmental samples. To obtain complimentary results these peptides were identified by searching the MS spectra against two databases (TrEMBL and our custom database created in PLGS 2.3). Upon comparing the results from the searches in two databases, we observed more matches in our custom PLGS 2.3 database. The reason for this could be the different algorithms used in the two software; also the PLGS 2.3 database was small and contained only amino acid sequences of proteins for perchlorate catabolism.

Chlorite dismutase and perchlorate reductase signature peptides were detected in all the three environmental enrichments, as well as in cells of *Dechlorosoma* sp. KJ and *Dechloromonas hortensis*. In *Pseudomonas chloritidismutans* and *Pseudomonas stutzeri*, we could only detect the CD peptide fragments; no chlorate reductase peptides were detected. One chlorate reductase peptide was however detected in the anaerobic sewage sludge enrichment. The possible reason for the absence of other matching chlorate reductase peptides could be that there is a substantial difference in protein amino acid sequences between the chlorate-reducing microorganisms and the database sequences. Another reason could be the lack of chlorate reductase sequences in the databases we employed. Therefore, one short-coming of the above mentioned proteomics-based method is that it would not work well to identify divergent perchlorate and chlorate-reducing organisms. The chlorite dismutase and perchlorate reductase signature peptides appear at different time points during the time course lysis. The time of appearance may depend on their location and degree of association with cellular membranes. The variation in time of appearance may also reflect the sources of the enzymes; derived from easily lysed versus more difficult to lyse cells. Moreover, the combined effect of digestion of proteins during formic acid treatment and missed cleavages by trypsin might result in differently sized peptides which would appear at different times during UPLC chromatographic separation.

In all of the pure bacterial samples analyzed, except *Pseudomonas chloritidismutans*, we were able to detect enzymes involved in the citric acid cycle, which indicated that the Kreb's Cycle is particularly active due to acetate metabolism. Acetate was used as an electron donor in investigated pure cultures and

two of the enrichments. For environmental samples we found a variety of housekeeping proteins known to be employed by perchlorate (or chlorate)-reducing microbes, which again confirms the presence of perchlorate metabolism in these samples and supports the proteomics data.

These data were also very well supported by the growth rate and chlorine oxyanions degradation rates we determined. In this work we also discovered some advantages of time and cost savings by using mass spectrometry for detection of chlorine oxyanions as compared to the traditional use of ion chromatographic techniques. With mass spectrometry, we were able to monitor perchlorate, chlorate and chlorite together in a single run. The method is also fast and produces few interfering peaks.

References

Aristoteli LP, Molloy MP, Baker MS (2007) Evaluation of endogenous plasma peptide extraction methods for mass spectrometric biomarker discovery. J Proteome Res 6:571–581. doi:10.1021/pr0602996

Baldwin MA (2004) Protein identification by mass spectrometry: issues to be considered. Mol Cell Proteomics 3:1–9. doi:10.1074/mcp.R300012-MCP200

Bansal R (2009) Comparison and monitoring the expression of genes involved in perchlorate metabolism. Master's Thesis, Environmental Science Program, University of Idaho, Moscow

Bender KS, Rice MR, Fugate WH, Coates JD, Achenbach LA (2004) Metabolic primers for detection of per(chlorate)-reducing bacteria in the environment and phylogenetic analysis of *cld* gene sequences. Appl Environ Microbiol 70:5651–5658. doi:10.1128/AEM.70.9.5651-5658.2004

Brown J, Lauderdale C, Morgenroth E, Raskin L (2007) Direct fixed-bed biological perchlorate destruction demonstration. Poster ER-0544: SERDP and ESTCP's partners in environmental technology technical symposium and workshop. Washington, DC

Caprioli RM, Gross ML (2005) The encyclopedia of mass spectrometry. Biological applications part a: peptides and proteins. Elsevier, Oxford

Government Accountability Office (US GAO) (2005) Perchlorate: a system to track sampling and cleanup results is needed. Report to the Chairman, subcommittee of environment, and hazardous materials, committee on energy and commerce, house of representatives, GAO-05-462

Gu B, Coates J (2006) Perchlorate: environmental occurrence interactions and treatment. Springer Science and Business Media, Inc, New York

Houlihan J, Jacob A, Sharp R (2007) EPA caves to defense industry lobbyists: house committee taking action to protect children. Environmental working group. http://www.ewg.org/reports/perchlorateintoddlers

Lo I, Denef VJ, VerBerkmoes NC, Shah MB, Goltsman D, DiBartolo G, Tyson GW, Allen EE, Ram RJ, Detter JC, Richardson P, Thelen MP, Hettich RL, Banfield JF (2007) Strain-resolved community proteomics reveals recombining genomes of acidophilic bacteria. Nat Lett 446:537–541. doi:10.1038/nature05624

Murray CW, Egan SK, Kim H, Beru N, Bolger PM (2008) US food and drug administration's total diet study: dietary intake of perchlorate and iodine. J Expo Sci Environ Epidemiol 18:571–580. doi:10.1038/sj.jes.7500648

National Research Council of the National Academies (2005) Health implications of perchlorate ingestion. http://www.nap.edu/catalog.php?record_id=11202

Nelson DL, Cox MM (2005) Lehninger principles of biochemistry. W.H. Freeman and Company, New York

O'Connor SM, Coates JD (2002) Universal immunoprobe for (per)chlorate-reducing bacteria. Appl Environ Microbiol 68:3108–3113. doi:10.1128/AEM.68.6.3108-3113.2002

Paidisetti R (2007) Ecoproteomic and ecogenomic approaches to monitor microbial activity in a subsurface aquifer and effect of compost infusion enrichment on bacterial community structure of polycyclic aromatic hydrocarbon contaminated soils. Master's Thesis, Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow

Ram RJ, VerBerkmoes NC, Thelen MP, Tyson GW, Baker BJ, Blake RCII, Shah M, Hettich RL, Banfield JF (2005) Community proteomics of a natural microbial biofilm. Science 308:1915–1920. doi:10.1126/science.1109070

Sellers K, Alsop W, Clough S, Hoyt M, Pugh B, Robb J, Weeks K (2007) Perchlorate environmental problems and solutions. Taylor and Francis Group, New York

Singh OV (2006) Proteomics and metabolomics: the molecular make-up of toxic aromatic pollutant bioremediation. Preoteomics 6:5481–5492. doi:10.1002/pmic.200600200

US Environmental Protection Agency (2005) Known perchlorate releases in the US—March 25, 2005

Wood T (2008) Is toxic perchlorate in Utah's food. Environmental working group. http://www.ewg.org/node/2597